THE BIOLOGY OF PHOTODYNAMIC THERAPY IN THE BLADDER AND PROSTATE

Thesis submitted to the University of London for the degree of
Doctor of Philosophy

by

STANLEY SHI-CHUNG CHANG, MD

1996

The National Medical Laser Centre
Department of Surgery
University College London
London
ABSTRACT

The Biology of Photodynamic Therapy in the Bladder and Prostate

Photodynamic therapy (PDT) produces localised destruction of tissue with light after prior administration of a photosensitising agent. Connective tissue is relatively unaffected but attempts to treat the entire urothelium in the bladder using the photosensitiser Photofrin have led to detrusor muscle scarring and irritable, contracted bladders and obstructive uropathy.

This thesis studied PDT on the normal rat bladder using the photosensitising agent, 5-aminolaevulinic acid (ALA). ALA solution was given intravesically, and the kinetics of the active derivative, protoporphyrin IX (PpIX) followed with fluorescence microscopy. Peak urothelial levels were seen at 5 hours, when treatment with 50J red light gave uniform urothelial necrosis without muscle damage which healed by regeneration. The PDT effect was enhanced by giving the iron chelator, CP94.

Further studies looked at PDT on normal canine prostate using 3 photosensitisers: ALA, meso-tetra-(m-hydroxyphenyl)chlorin (mTHPC) and aluminium disulphonated phthalocyanine (AlS2Pc). Kinetic studies were undertaken on serial biopsies after sensitisation. Light was delivered interstitially and the animals killed up to 90 days later, revealing glandular necrosis (2.5cm diam. mTHPC, 1.2cm AlS2Pc, 0.2cm ALA) but little effect on connective tissues, and no change in the gland size and shape. Urethral damage sometimes caused urinary retention which resolved in a week. Deliberate treatment of the sphincter in rats caused incontinence in 25%.

Neoplastic areas of both prostate and bladder take up at least as much photosensitiser as adjacent normal tissue. Thus PDT using intravesical ALA is promising for carcinoma in situ of the bladder and preventing bladder tumour recurrence and PDT with mTHPC for prostate cancers localised to the gland, care being taken to avoid the sphincter. Both techniques are now ready for preliminary clinical trials.
PREFACE

The thesis studies biological changes of the bladder and prostate following photodynamic therapy. The aim of this project is to expand the dimensions of PDT in urology with well designed experiments and a few clinical trials, and with a comprehensive review of relevant literature.

Section A introduces the pathogenesis and the state-of-the-art therapeutic strategies for bladder and prostate cancer. This section also outlines the history and evolution of photodynamic therapy over the past half a century with comprehensive review of related articles. Current applications of high power laser for the treatment of BPH or other benign urological diseases are not discussed as the theme of this thesis is to look at the feasibility of photodynamic therapy in treating bladder and prostatic malignancies. However laser photocoagulation of bladder cancer which remains an acceptable therapeutic option is included as integral parts of laser treatment of cancer.

Section B comprises 5 chapters on experimental work and is stratified into 2 parts. Part 1 describes in detail the applicability of ALA based PDT for the bladder, and compares the bladder PDT effects with different routes of sensitisation. The results of oral iron chelator or light fractionation which was aimed to further enhancing photodynamic effects, are presented in an independent chapter. Part II involves experiments on the feasibility of PDT for the canine prostate. By evaluating 3 popular photosensitisers (ALA, AlS2Pc and mTHPC), the data obtained are presented in 2 chapters.

Section C gives conclusions on the applicability of PDT for the bladder and prostate based on the current experimental results. Future prospects as well as directions of research of PDT are outlined.
CONTENT

Title Page 1
Abstract 2
Preface 3
List of Contents 4
List of Tables and Illustrations 6
Dedication 14
Acknowledgements 15
Statement of Originality 16
Abbreviations 17

SECTION A: BACKGROUND AND INTRODUCTION 19
Chapter 1: Bladder Cancer 20
Chapter 2: Prostate Cancer 47
Chapter 3: Photodynamic Therapy:
  History, Principles and Nature 81
Chapter 4: Photodynamic Therapy in Urology: Now and Then 105

SECTION B: EXPERIMENTS AND RESULTS 129
Chapter 5: Biodistribution and Photodynamic Effects in the
  Rat Urinary Bladder with 5-Aminolaevulinic Acid
  Induced Protoporphyrin IX Sensitisation 130
Chapter 6: Quantitative Fluorescence Assessment of Protoporphyrin IX in the Bladder and Other Organs
  Following Administration of 5-Aminolaevulinic Acid:
  Comparison between Intravesical and Oral Routes 159
Chapter 7: Enhancement of Photodynamic Effects with Oral
  Iron Chelator and Light Fractionation after ALA Sensitisation 192
Chapter 8: Interstitial and Transurethral Photodynamic
  Therapy of the Canine Prostate Using Meso-Tetra-(m-Hydroxyphenyl)Chlorin 218
Chapter 9: Interstitial Photodynamic Therapy in the Canine Prostate with Disulphonated Aluminium

4
LISTS OF TABLES AND ILLUSTRATIONS

Chapter 1

Table 1.1. Staging systems for bladder cancer 29
Table 1.2: Doses (mg/m² body surface) and schedule for M-VAC 43
Table 1.3: Doses (mg/m² body surface) and schedule for CISCA 44
Table 1.4: Doses (mg/m² body surface) and schedule for CMV 44
Figure 1.1: The TNM and Jewett-Marshall classification for bladder cancer 29

Chapter 2

Table 2.1: Gleason score system for prostate cancer 52
Table 2.2: Whitmore-Jewett staging classification of prostate cancer 66
Table 2.3. TNM classification of prostate cancer 67

Chapter 3

Figure 3.1: Simplified diaphragm of photodynamic action. Type II (singlet oxygen) mediated pathway is the principal mechanism accounting for PDT tissue damage. 86
Figure 3.2: Chemical structure of haematoporphyrin derivative (HpD) 93
Figure 3.3: Simplified biosynthetic pathway for haem. Fluorescent and photoactive components are enclosed in rectangles. 96
Figure 3.4: Chemical structure of 5,10,15,20-tetra(m-hydroxyphenyl) chlorin 100
Figure 3.5: Chemical structure of chloroaluminium disulphonated phthalocyanine 102

Chapter 4

Table 4.1: Summary of focal photoradiation for bladder cancer with HpD or DHE 111
Figure 4.1: Setting up of fluorescence microscopy and CCD camera 119

Chapter 5
Table 5.1: Treatment parameters for the PDT study groups with BI ALA

Table 5.2: The colour change of 10% ALA solution at body and room temperature at different pH

Table 5.3: Macroscopic findings of the peritoneum and bladder 3 days after PDT

Table 5.4: Histological changes of the bladder wall 1, 2-3, 7 and 90 days after PDT in different treatment groups

Figure 5.1: PDT of the experimental animal

Figure 5.2: The emission spectrum detected on rat urinary bladder 3 hours after intravesical ALA instillation

Figure 5.3: (A) Computer processed fluorescence microscopy image of the bladder wall 5 hours after instillation of 10% ALA at pH 5.5

Figure 5.3: (B) Quantification of the tissue fluorescence intensity across the line on Figure 5.3 (A)

Figure 5.4: Plot of bladder wall fluorescence intensity against time after instillation of 10% ALA at pH 4

Figure 5.5: Plot of bladder wall fluorescence intensity against time after instillation of 10% ALA at pH 5.5

Figure 5.6: Plot of bladder wall fluorescence intensity against time after instillation of 1% ALA at pH 5.5

Figure 5.7: Comparison of fluorescence intensity in different layers of the bladder wall 5 hours after instillation of 10, 1 and 0.1% ALA solutions

Figure 5.8: Gross appearance of normal rat bladder 7 days after light illumination but without ALA sensitisation

Figure 5.9: Gross appearance of bladder 3 days after PDT with a light dose of 100J, intralipid 1%, 5 h after instillation of 10% ALA.

Figure 5.10: Bladder lesion showing urothelial damage of various degrees after PDT with a light dose of 100 J, 1% intralipid, 5 h after instillation of 10% ALA

Figure 5.11: Fibrinoid necrosis of the arteriole (arrows) 3 days after PDT with a light dose of 50J, Intralipid 1%, 5 h after instillation of 10% ALA

Figure 5.12: Patchy bladder lesions 3 days after PDT with a light dose of 50J, intralipid 1%, 5 h after instillation of 10% ALA
Figure 5.13: Homogenous urothelial lesion 3 days after PDT with a light dose of 50J, intralipid 10%, 5 h after instillation of 10% ALA

Figure 5.14: Regeneration of urothelium 7 days after PDT using a light dose of 50J, intralipid 10%, 5 h after instillation of 10% ALA

Figure 5.15: (A) Histology of normal untreated bladder and (B) increased collagen fibrils in the lamina propria 3 months after PDT with a light dose of 50J, 10% intralipid and 10% ALA

Chapter 6

Table 6.1: Histological changes of the bladder wall (2-3, 7 and 180 days) after PDT in different experiment groups

Figure 6.1: (A) CCD fluorescence microscopy of rat urinary bladder 5 hours after instillation of ALA, (B) histological matching of the above section

Figure 6.2: (A) CCD fluorescence microscopy of rat urinary bladder 5 hours after oral intake of ALA, (B) histological matching of the above section

Figure 6.3: (A) Plot of PpIX fluorescence intensity (counts/pixel) in the urothelium after ALA administration, (B) in the lamina propria

Figure 6.3: (C) Plot of fluorescence intensity (counts/pixel) in the muscularis propria of bladder oral and intravesical

Figure 6.4: Plot of liver PpIX fluorescence intensity after oral and intravesical ALA

Figure 6.5: CCD fluorescence microscopic picture of liver (A) 3 hours after oral ALA, (B) 5 hours after intravesical ALA

Figure 6.6: Plot of PpIX fluorescence intensity in the kidney after oral and intravesical ALA

Figure 6.7: CCD fluorescence microscopic picture of kidney 5 hours after ALA, (A) Renal cortex, (B) Medulla, oral; (C) Renal cortex, (D) Medulla, instillation

Figure 6.8: CCD fluorescence microscopic picture of abdominal muscle 5 hours after intravesical ALA.

Figure 6.9: Plot of PpIX fluorescence levels in abdominal muscle after oral and intravesical ALA
Figure 6.10: (A) CCD fluorescence microscopic picture of back skin 5 hours after oral ALA (200mg/kg)

Figure 6.10: CCD fluorescence microscopic picture of back skin (B) 3 hours after oral ALA (100mg/kg), (C) 3 hour after intravesical ALA

Figure 6.11: PpIX fluorescence intensity in the epidermis of back skin

Figure 6.12: PpIX fluorescence intensity in the dermis of back skin

Figure 6.13: Histology of normal bladder

Figure 6.14: Histology of urinary bladder 3 days after light illumination only without ALA

Figure 6.15: Microscopic picture of bladder 3 days after PDT (200mg/kg oral ALA, sensitising for 4 hours, Light: 100 mW for 500 seconds at 630 nm)

Figure 6.16: Microscopic picture of bladder 3 days after PDT (200mg/kg oral ALA, sensitising for 4 hours, Light: 100 mW for 250 seconds at 630 nm)

Figure 6.17: Microscopic picture of bladder 3 day after PDT (100mg/kg oral ALA, sensitising for 3 hours, Light: 100 mW for 500 seconds at 630 nm)

Figure 6.18: (A) Microscopic findings of the bladder section 7 days after PDT, (B) High power view of (A)

Figure 6.19: Microscopic finding of bladder 6 months after PDT oral ALA (A) H&E stain, (B) HVG stain

Figure 6.20: Histology of skin section 3 days after 50J light only but without ALA

Figure 6.21: Microscopic picture of skin 3 days after PDT with (A) oral ALA, (B) intravesical ALA

Chapter 7

Table 7.1: Histological findings of the bladder after PDT with ALA and iron chelator

Table 7.2: Histological findings of the bladder after PDT with ALA and light fractionation

Figure 7.1: The structure of 3-hydroxypyridin-4-ones (HPO)
Figure 7.2: CCD fluorescence microscopic picture of bladder 5 hours after intravesical ALA and CP94
(A) 10% ALA (B) 1% ALA

Figure 7.2: (C) CCD fluorescence microscopic picture with ALA and CP94, (D) matching histology of (C)

Figure 7.3: Plot of fluorescence intensity of bladder after intravesical 10% ALA with and without CP94

Figure 7.4: Plot of fluorescence intensity of bladder after intravesical 1% ALA with and without CP94

Figure 7.5: Plot of fluorescence ratios between the urothelium and muscularis propria after intravesical 1 and 10% ALA with and without CP94

Figure 7.6: Histological findings of bladder 2 days after PDT with 1% ALA but without CP94

Figure 7.7: Histological changes of bladder 7 days after PDT with 1% ALA and CP94

Figure 7.8: Microscopic findings of bladder 2 days after PDT with 1% ALA and CP94

Figure 7.9: (A) Microscopic findings of bladder 2 days after PDT with 10% ALA and CP94

Figure 7.9: (B) Close up view of Figure 7.9 (A)

Figure 7.10: Histologic findings of detrusor muscle degeneration 7 days after PDT as a result of inhomogeneous light distribution

Figure 7.11: Microscopic findings of bladder 3 days after PDT with 10% ALA and light fractionation (25/25J, dark interval 5 min)

Figure 7.12: Microscopic findings of bladder 3 days after PDT with 1% ALA and light fractionation (25/25J, dark interval 5 min)

Figure 7.13: Microscopic finding of bladder 3 days after PDT with 2.5% ALA and light fractionation (25/25J, dark interval 5 min)

Chapter 8

Table 8.1: Demographic data and macroscopic changes of the prostate (mTHPC study) of the experimental animals
Figure 8.1: Spring loaded automatic biopsy gun and needle

Figure 8.2: Illustration of PDT treatment of the canine prostate with transperineal approach

Figure 8.3: Interstitial PDT of the canine prostate under transrectal ultrasonography of the prostate, (A) Longitudinal scan, (B) Radial scan

Figure 8.4: Transrectal ultrasonography of the prostate for transurethral PDT, (B) 3-cm cylindrical diffuser fibre

Figure 8.5: (A) Plot of mTHPC concentration in the liver and prostate, (B) Plot of mTHPC in the prostate

Figure 8.6: (A) CCD fluorescence microscopic picture of the prostate 3 days after mTHPC sensitisation, (B) Matching histology

Figure 8.7: Fresh macroscopic appearance of the prostate capsule 3 days after PDT with mTHPC (4 treatment sites, 100 J each)

Figure 8.8: Fresh macroscopic appearance of the prostate 3 days after PDT with mTHPC

Figure 8.9: Macroscopic lesion 3 days after PDT with mTHPC (2 fibres, 20 mm apart, 100 J each)

Figure 8.10: Macroscopic lesions 3 days after PDT with 4 fibres

Figure 8.11: Macroscopic lesion 7 days PDT with 2 fibres

Figure 8.12: Macroscopic lesion 28 days after PDT with a single fibre

Figure 8.13: (A)(B)(C)(D) Serial macroscopic lesions 3 months after PDT with 4 fibres

Figure 8.14: Serial sections of a prostate 7 days after transurethral PDT

Figure 8.15: Gross specimen of the prostate and rectum ulceration

Figure 8.16: Gross prostate section 30 days after transurethral PDT

Figure 8.17: Haemorrhagic necrosis of the prostate 3 days after PDT

Figure 8.18: Urothelial and peri-urethral sloughing of the prostatic urethrae 3 days after PDT

Figure 8.19: Microscopic finding of the prostate 28 days after PDT
Figure 8.20: Microscopic finding of the prostatic urethrae 28 days after PDT, (A) regeneration of urothelium, (B) increased collagen deposition

Figure 8.21: Microscopic finding of the prostatic urethra 90 days after PDT

Figure 8.22: Microscopic finding of the prostate 90 days after PDT

Figure 8.23: Microscopic finding of the rectal mucosa 30 days after transurethral PDT

Chapter 9

Table 9.1: Treatment parameters and macroscopic results of the prostate of experimental animals (ALA and AlS2Pc)

Figure 9.1: Transrectal ultrasound probe

Figure 9.2: (A) A KTP laser, (B) pumped dye laser module 630

Figure 9.3: Plot of protoporphyrin IX fluorescence in prostate after ALA sensitisation

Figure 9.4: Plot of AlS2Pc fluorescence in the prostate after AlS2Pc

Figure 9.5: CCD fluorescence microscopic picture of the prostate 24 hours after sensitisation

Figure 9.6: Plot of ALA fluorescence in the liver and prostate after ALA sensitisation

Figure 9.7: Plot of AlS2Pc fluorescence in the liver and prostate after AlS2Pc sensitisation

Figure 9.8: Thermal effect of light in the canine prostate

Figure 9.9: Prostate lesions 3 days after PDT with ALA (100mg/kg)

Figure 9.10: Prostate section 7 days after PDT with ALA (100mg/kg)

Figure 9.11: Prostate lesion 3 days after PDT with AlS2Pc

Figure 9.12: Prostate lesion near urethra 7 days after PDT with AlS2Pc

Figure 9.13: Subcapsular prostate lesion 28 days after PDT with AlS2Pc
Figure 9.14: The junction between haemorrhagic necrosis and viable tissue in the prostate 3 days after PDT with AlS2Pc

Figure 9.15: Microscopic features of prostate 28 days after PDT with AlS2Pc

Figure 9.16: Microscopic features of periurethral tissue response to PDT effect
DEDICATION

To my parents and family, especially to Tina, with thanks for their love and
forbearance over many years
ACKNOWLEDGEMENTS

The work presented in this thesis was performed at the National Medical Laser Centre, Department of Surgery, University College London, under the supervision of Professor S G Bown, Director of the Laser Centre. I am extremely grateful to Professor Bown who with his profound interest in researches on lasers and relevant topics, has been a continuing source of encouragement, guidance and inspiration to my expansion of knowledge in Urology and Laser Science.

I would like to extend my gratitude to Professor A R Mundy, Director of Institute of Urology, for his moral support especially in the early stage when this proposal was under planning, and the continuing urological advice throughout. My thanks are also due to Miss Amanda Jones(Manager) and Lorraine Acheson(Secretary) of the Laser Unit, for their computer and administrative assistance.

I would particularly like to thank Dr A J MacRobert, Senior Lecturer at the Department of Surgery, UCL for his substantial help and input of knowledge with the CCD fluorescence microscopy. I extend my thanks also to Dr Giovanni Buonaccorsi and Mr Brian McIlory for assistance and advice on Lasers. I thank the staff of Biological Services for their help, especially Mr P Koder, Miss S Anthony and Janice Burrows for the great help with the canine experiments. My gratitutions are extending to the staff at the Histopathological Department of the Imperial Cancer Research Fund for their histological processing.

I am much obliged to the Compassion Relief of Tzu-Chi Foundation in Taiwan for the generous funding of this project. Several colleagues have provided considerable support and assistance throughout the three years of my project. Miss Alison Curnow whose arrangement to reallocate and maintain good running of the laser laboratories has made research works more efficient. I want to thank also Dr Kathy Fan, Mr Colin Hopper, Miss Sally Thorpe, Dr Glenn Spencer, Mr Johan Witt, Mr Alasdair Gordon, Mr Douglas Whitelaw and Dr Charles Millson.
STATEMENT OF ORIGINALITY

The work presented in this thesis has been carried out by myself and has not previously been entered for a higher degree or award of this or any other University. The principal content of this research involves concepts and observations of photodynamic therapy on the bladder and prostate in animal models. No similar works has been published by any other group to my knowledge. I hope that the results herein will contribute to the understanding and clinical practice of photodynamic therapy for bladder and prostate cancer.

The concept of intravesical ALA instillation (Chapter 5) and the use of oral iron chelator to enhance PDT effects of the bladder (Chapter 7) was initiated by Professor S G Bown and Dr A J MacRobert with a certain of adaptations and alterations made by myself. The comparison of PDT effects between oral and intravesical ALA administration (Chapter 6) was my own idea, but was planned with the help of Dr A J MacRobert, especially on CCD fluorescence microscopy. The idea of performing interstitial PDT on the canine prostate was originated by Professor S G Bown (Chapter 8 & 9) while the application of transurethral PDT (Chapter 8) was by me.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>5-aminolaevulinic acid</td>
</tr>
<tr>
<td>AIS2Pc</td>
<td>Aluminium disulphonated phthalocyanine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus-Calmette-Guerin</td>
</tr>
<tr>
<td>BI</td>
<td>Bladder instillation</td>
</tr>
<tr>
<td>BPD</td>
<td>Benzoporphyrin derivative mono acid</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge coupled device</td>
</tr>
<tr>
<td>CIS</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>CISCA</td>
<td>Cisplatin-cyclophosphamide-adriamycin</td>
</tr>
<tr>
<td>CMV</td>
<td>Cisplatin-methotrexate-vinblastin</td>
</tr>
<tr>
<td>CP</td>
<td>Coproporphyrin</td>
</tr>
<tr>
<td>CP20</td>
<td>1,2-dimethyl-3-hydroxypyridin-4-one</td>
</tr>
<tr>
<td>CP94</td>
<td>1,2-diethyl-3-hydroxypyridin-4-one</td>
</tr>
<tr>
<td>CT</td>
<td>Computerised tomography</td>
</tr>
<tr>
<td>DFO</td>
<td>Desferroxamine</td>
</tr>
<tr>
<td>DHE</td>
<td>Dihaematoporphyrin ester/ether</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital rectal examination</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FCM</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HpD</td>
<td>Haematoporphyrin derivative</td>
</tr>
<tr>
<td>Ho-YAG</td>
<td>Homium: yttrium aluminium garnet</td>
</tr>
<tr>
<td>HPO</td>
<td>Hydroxypyridinone</td>
</tr>
<tr>
<td>HVG</td>
<td>Haematoxylin and VanGieson</td>
</tr>
<tr>
<td>KTP</td>
<td>Potassium titanyl phosphate</td>
</tr>
<tr>
<td>Laser</td>
<td>Light amplification by the stimulated emission of radiation</td>
</tr>
<tr>
<td>LHRH</td>
<td>Leutenizing-hormone releasing hormone</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>MP</td>
<td>Muscularis propria</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance image</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mTHPC</td>
<td>meso-tetra-(meta-hydroxyphenyl)chlorin</td>
</tr>
<tr>
<td>M-VAC</td>
<td>Methotrexate-vinblastin-adriamycin-cisplatin</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>Neodymium:yttrium aluminium garnet</td>
</tr>
<tr>
<td>NPe6</td>
<td>Chlorin e6 aspartic acid</td>
</tr>
<tr>
<td>$^{1}\text{O}_2$</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>PBG</td>
<td>Porphobilinogen</td>
</tr>
<tr>
<td>Pc</td>
<td>Phthalocyanine</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
</tr>
<tr>
<td>PpIX</td>
<td>Protoporphyrin IX</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostatic specific antigen</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SnET2</td>
<td>Tin etiopurpurin dichloride</td>
</tr>
<tr>
<td>TCC</td>
<td>Transitional cell carcinoma</td>
</tr>
<tr>
<td>TRUS</td>
<td>Transrectal ultrasound</td>
</tr>
<tr>
<td>TUR</td>
<td>Transurethral resection</td>
</tr>
<tr>
<td>TURP</td>
<td>Transrectal ultrasound of the prostate</td>
</tr>
<tr>
<td>UP</td>
<td>Uroporphyrin</td>
</tr>
</tbody>
</table>
SECTION A: BACKGROUND AND INTRODUCTION

CHAPTER 1: Bladder Cancer

CHAPTER 2: Prostate Cancer

CHAPTER 3: Photodynamic Therapy: History, Principles and Nature

CHAPTER 4: Photodynamic Therapy In Urology: Now and Then
CHAPTER 1

BLADDER CANCER

1.1 Introduction ............................................................... 21

1.2 Cellular and Molecular Basis of Bladder Cancer
  1.2.1 Cytogenetic and molecular biology of bladder cancer 23
  1.2.2 Cellular biology of bladder cancer ....................... 24
    1.2.2.1 Grading ............................................. 24
    1.2.2.2 Urine cytology .................................. 25
    1.2.2.3 Flow cytometry (FCM) ................................ 26

1.3 Clinical Characteristics of Bladder Cancer
  1.3.1 Staging of bladder cancer .................................. 28
  1.3.2 Superficial papillary bladder cancer ............ 30
    1.3.2.1 Clinical assessment and staging procedures ....... 30
    1.3.2.2 Treatment and prognosis .................................. 31
  1.3.3 Carcinoma in situ ........................................... 32
    1.3.3.1 Clinical feature ..................................... 32
    1.3.3.2 Treatment and prognosis .................................. 33
  1.3.4 Muscle invasive and advanced bladder cancer ...... 33
    1.3.4.1 Clinical course ......................................... 34
    1.3.4.2 Treatment and prognosis .................................. 34

1.4 The Management of Bladder Cancer
  1.4.1 Endoscopic resection and fulguration .................. 36
  1.4.2 Intravesical therapy .............................................. 36
    1.4.2.1 Intravesical chemotherapy .................................. 36
    1.4.2.2 Intravesical BCG immunotherapy ..................... 37
  1.4.3 Surgery ........................................................... 38
    1.4.3.1 Partial cystectomy ..................................... 38
    1.4.3.2 Radical cystectomy and urinary diversion .......... 40
  1.4.4 Irradiation ..................................................... 41
  1.4.5 Systemic chemotherapy .......................................... 43
  1.4.6 Laser therapy .................................................... 45
1.1 INTRODUCTION

Bladder cancer is the second most common urological cancer with approximately 220,000 new cases worldwide each year which accounts for 3.5% of all cancers (Tomatis 1990). Most patients are diagnosed after the age of 60 and men outnumber women by a factor of 2-3. Common etiologic factors are occupational exposure to certain chemicals (aniline dyes and aromatic amines), cigarette smoking, analgesic abuse, previous pelvic irradiation, and chronic irritation or infection of the bladder (Catalona 1992). In western countries, the majority of bladder cancers (over 90%) are transitional cell carcinoma (TCC), whereas squamous cell carcinoma and adenocarcinoma account for 5 and 2%, respectively (Sternberg 1995). However, in other areas where schistosomiasis is endemic, squamous cell carcinoma may account for more than 75% of all bladder cancers (El-Bolkainy et al, 1981).

Painless haematuria, either gross or microscopic, is the most common presenting symptom occurring in 85% of cases (Varkarakis 1974). Other less frequent manifestations are bladder irritations, flank pain, pelvic mass or symptoms attributable to advanced or metastatic disease. Diagnosis is usually established by cystoscopy and biopsy, and sometimes, by urine cytology. Based on the degree of cellular and nuclear dysplasia, malignant transitional cells are stratified into three grades (well, moderately, and poorly differentiated).

Diagnostic and staging work-up of bladder cancer may require histology from biopsy, excretory urography, pelvic and abdominal CT scan, MRI, ultrasonography, and occasionally, pelvic lymph node dissection. Classically, bladder cancer is divided into two classes; superficial and invasive, because the natural history and treatment of the two forms are different. Overall, approximately 70-80% of bladder cancers are of low grade being superficial and confined either to the mucosa or the lamina propria at the time of diagnosis (Ro et al, 1992). Muscle invasive and metastatic disease, on the other hand, accounts for 20% and 3-5% of cases respectively (Silverberg et al, 1990).

Therapeutic strategy depends on tumour stage and clinical condition of the patient. Options currently available, either alone or in combination, are endoscopic resection, intravesical chemotherapy, immunotherapy, radical surgery, external beam irradiation, and systemic chemotherapy. After the initial treatment of superficial cancer with endoscopic resection, recurrence is found in 60-70% of cases, and 10-
20% will ultimately progress to invasive disease (Mellon et al, 1995). Adjuvant intravesical chemotherapy is an effective measure to reduce the frequency of recurrence, but has not been proven to lower the incidence of subsequent muscle invasion (Richie 1992). If the tumour involves the muscle layer, external beam irradiation can produce a 40%, 5-year survival rate whilst still preserving the bladder in the majority of cases (Bloom et al, 1982). In the United States in particular, radical cystectomy with continent urinary diversion is seen as an attractive alternative to irradiation (Skinner 1990). Systemic chemotherapy is potentially of palliative benefit for advanced cases, however, the development of optimum regimens is required (Scher 1992).

To further improve therapeutic effects, it is imperative to identify the patient whose tumour is at high risk for progression and metastasis. Molecular and cytogenetic studies, in conjunction with the tumour grade and stage, are likely to play central roles for prediction of the biological behaviour of the cancer. Flow cytometry, ABO blood group antigen, proto-oncogenes, suppressor genes, growth factor/receptors, and chromosomal analysis are all currently being enthusiastically investigated. The prognosis for low grade (G1), low stage (Ta, T1) bladder cancer is generally good, but is ominous for muscle infiltrative (T2, T3) and advanced (T4) diseases (Hendry, 1988). In general, the mortality rate from bladder cancer is about 20-30% of the incidence rate, indicating that most patients with bladder cancer die of other causes.
1.2 CELLULAR AND MOLECULAR BASIS OF BLADDER CANCER

1.2.1 Cytogenetic and molecular biology of bladder cancer

Recent advances in molecular biology have led to a rapid accumulation of knowledge on genes that may have a direct or indirect role in the biological behaviour of bladder cancer cells. It has been hypothesised that neoplastic transformation of the normal urothelium is the result of two processes - initiation and promotion. The first, involves the initiation of irreversible changes, either acquired or inherited, in the nuclear deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Subsequently, chromosomal alterations lead to a series of changes in the regulation of normal cell growth, proliferation and differentiation (Bishop & Varmus, 1982). The genes which are carried in the genomes of DNA or RNA viruses which are capable of causing cancer are termed oncogenes (r-onc), whilst genes in the normal somatic cells which are susceptible to cancerous transformation are called proto-oncogenes or cellular oncogenes (c-onc) (Bishop 1983). Currently, three major classes of these genes have been identified: (1) those involved in or causing transformation and proliferation; (2) those involved in suppressing normal regulatory function, and (3) those which do not induce transformation themselves but are able to modify the spread of neoplastic cells. To date, there is a general consensus that the structural or numerical alterations of proto-oncogenes may have an essential role in the pathogenesis of cancer (Slamon 1987). The absence or inactivation of genes (tumour suppressor) that regulate normal cell cycle (or proliferation) may be another cause for urothelial cancer (Strohmeyer & Slamon 1994).

About 10% of urothelial tumours contain mutant ras proto-oncogenes (Bos 1988). The c-Ha-ras point mutation of codons 12, 13, 61 and the resultant biochemical effects seem to be associated with the invasiveness and recurrence rate of urothelial cancer (Theodorescu et al, 1990; Stock et al, 1987). Studies of the epidermal growth factor (EGF) receptor by immunohistological methods in bladder TCC have demonstrated a strong correlation between the expression of this receptor and an increase in the stage and grade of the tumours (Neal et al, 1985; Berger et al, 1987). Current cytogenetic data also suggests the presence of potential tumour suppressor genes on the short arms of chromosomes 11 (11p) and 17 (17p) and the long arm of chromosome 9 (9q) and structural or numerical aberrations may contribute to the
development and progression of bladder cancer (Tsai et al, 1990; Sidransky et al, 1992). As a result, p53 and retinoblastoma (RB), the two most important tumour suppressor genes, have been investigated extensively. Altered RB gene expression was proved to be independent of other known prognostic variables, and its absence or heterogeneous expression had impacted a poorer survival in patients with locally advanced bladder cancer (Logothetis et al, 1992). Mutation of p53 genes were reported exclusively in grade 3 urothelial carcinomas, and might therefore be associated with a higher recurrence rate and a decreased survival in bladder cancer patients (Fujimoto et al, 1992). However, further studies are needed to clarify whether the detection of a mutated p53 gene or other cytogenetic change indicates an independent molecular prognostic parameter in urothelial cancer.

1.2.2 Cellular biology of bladder cancer

Although advances in molecular biology technology have refined the diagnosis of malignancy, histology and cytology remain the indispensable standard for the bladder cancer diagnosis. Currently, the grade and stage of a bladder tumour forms the basis for prognosis and management. Only with prompt histopathological examination and with appropriate treatment planning can optimal therapeutic results with the minimum of complications, be reasonably achieved. However, this is still not easily achievable at the present time. To identify the cancer cells having higher potential for invasion and metastasis, one has to rely not only on traditional histology, but also on a variety of contemporary histochemical and immunobiochemical techniques to increase the power of detection. Even so, the accuracy of these results remains an issue of great debate.

1.2.2.1 Grading

Despite the controversies and the lack of a uniformly accepted objective grading system, there is general consensus that the following are parameters for the assessment of anaplasia of TCC. They include morphologic irregularity with disturbance of cellular polarity, pleomorphism, increased cellularity, absence of differentiation throughout the urothelial layers, nuclear crowding, variation in nuclear shape and chromatin pattern, mitotic figures, and the presence of giant cells. Based on these criteria, a three-grade system was instituted by Mostofi et al (1973) for the World Health Organisation (WHO).
Grade 1 (G1) is a tumour which is well differentiated having a thin fibrovascular stalk and a thickened urothelium containing more than seven layers. The cells show only slight anaplasia and pleomorphism. The nucleus is slightly enlarged and its membrane may be prominent. The full thickness of urothelium shows little evidence of heterogenous maturation and mitosis is rare. Grade 2 (G2) depicts moderately differentiated tumours which have a broader fibrovascular core and a greater disturbance of the base-to-surface cellular maturation. The cells are irregular and have lost their polarity. The nuclear pleomorphism and chromatin pattern are more prominent. The mitotic figures are more frequent, and the nuclear-cytoplasmic ratio is higher than that of the G1 tumours. Grade 3 (G3) tumours are poorly differentiated demonstrating all the cellular and nuclear pleomorphism with rather constant mitotic figures (Friedell et al, 1980). Although this grading system has been criticised for being too subjective and without clearly established criteria for categorisation (Ooms et al, 1983; Abel et al, 1988), it has shown good correlation with subsequent tumour progression and prognosis (Friedell et al, 1976; Jordan et al, 1987). Modifications have been suggested by allocating (a) and (b) subgroups to the grade 2 classification which has the greatest degree of cellular heterogeneity to increase prognostic predictability of the current grading system (Pauwels et al, 1988; Carbin et al, 1991a,b).

7.2.2.2 Urine cytology

Urine cytology is a valuable adjunct to the evaluation of bladder cancer in spite being of limited usefulness in G1 tumours and having a suboptimal sensitivity, especially on void urine. Important features for the cytological diagnosis of malignancy are based on an increased nuclear-to-cytoplasmic (N/C) ratio, eccentric positioning of the nucleus, nuclear crowding, pleomorphism and irregularity, hyperchromatism and chromatin clumping, prominent nucleoli, lack of cytoplasmic vacuolisation, and loss of cellular cohesiveness (Murphy et al, 1984; Murphy 1990).

Since the distinction of G1 tumour from normal urothelium requires the demonstration of histological evidence of a fibrovascular stalk, increased epithelial thickness, and the presence of mitotic figures, it is difficult and sometimes even impossible, to differentiate them with urine cytology (Ro et al, 1992). The sensitivity of a single urinary specimen for grade 1 or 2, Ta tumours is approximately 20-40% (Curling et al, 1986; Badalament et al, 1987), but is improved to 60% if three specimens are analysed (Murphy et al, 1986). With meticulous attention to sample collection and processing, the detection rate of intermediate or high grade TCC can be
as high as 80-95% (Murphy & Soloway 1982; Wiener et al, 1993). False positive, which usually is the result of reactive or degenerative cell changes, remains at about 15% however (Geisse & Tweeddale 1978). The persistent yield of malignant cells in the presence of a low grade papillary tumour should raise the suspicion of having either carcinoma in situ or other lesions elsewhere which have not been discovered.

The advantages of urine cytology include convenience and a high sensitivity for high grade tumour and carcinoma in situ detection and follow-up. However, a high false negative rate (due to low cellular yield of the urine sample or subtle atypia of low grade tumour), and false positive results (from reactive atypia secondary to inflammatory processes on degenerative changes of cells following previous irradiation or intravesical chemotherapy, or as a result of suboptimal specimen collection) are all limitation of this technique (Ro et al, 1992).

1.2.2.3 Flow cytometry (FCM)
Flow cytometry (FCM) is the integrated application of knowledge and techniques developed independently in the fields of computer science, laser technology, immunology, cytochemistry, and fluorescence chemistry. The basic principle of FCM is the detection of nuclear chromatin changes which characterise neoplastic cells. The enlargement and hyperchromatism of nuclei may have significant implication on the biological behaviour of the cancer (Melamed 1984). This technology provides an objective and quantitative measurement of the DNA content (ploidy) and proliferative activity (S-phase proportion) of individual cancer cells. A freshly prepared single cell suspension is stained with a DNA or RNA binding dye, usually acridine orange or propidium iodide, before processing to the flow chamber. The narrow channel of the flow chamber (which is air pumped) forces each cell to propagate at a high speed through blue laser light (Argon gas, 488nm). This excites the fluorochrome in the nucleic acid to fluorescent light of specific wavelength for optical analysis (Wheeless & Kay 1985).

There is also a strong correlation between ploidy and the propensity for progression/metastasis of the bladder cancer (Shaaban et al, 1990). Low grade and low stage tumours tend to be diploid with a low proliferation rate (<10% S-phase) and these patients generally have a favourable prognosis. The same, however, is not true for aneuploid tumours which have a high S-phase proportion and carry a prognosis which is worse than that of the diploid or tetraploid analogues (Badalament et al, 1988; Shaaban et al, 1990; Wijkstorm & Tribukait 1990). Despite the usefulness of
identifying a subset of low grade tumours which have the potential for progression through this measurement of ploidy, it appears to be of little value as a prognosticator in patients with invasive tumours, which are mostly aneuploid, with a high proportion of cells in S-phase (Wijkstorm et al, 1984).

In addition to the measurement of DNA ploidy, FCM can be used to detect surface tumour makers with multiparameter analysis which will further improve the accuracy of prognosis assessment (Hijazi et al, 1989; Fradet et al, 1990). Using bladder wash as a flow cytometric specimen and adapting a cut-off value of over 16% of hyperdiploidy and/or the presence of aneuploidy as the criteria for diagnosing bladder cancer (Melamed 1984), a single FCM is as good or better than 3 consecutive sessions of urine cytology for the detection of bladder tumour (Badalament et al, 1987). The overall detection rates of CIS, T1 and Ta bladder tumours are 78%, 88% and 64%, respectively for urine cytology, whereas they are 88%, 94% and 83%, respectively for FCM (Badalament et al, 1988).

Despite the potential advantages of FCM; quantitative, requires no special trained pathologist, and seems to be more sensitive than urine cytology in the early detection of occult urothelial carcinoma (Devonec et al, 1981, 1982; Badalament et al, 1988), urine cytology remains the procedure of choice for detecting and monitoring urothelial carcinoma. This is probably as a result of the long experience and better understanding of this test which is widely available, along with its proven high specificity, noninvasiveness, and the possibility of detecting urothelial cancer of the whole urinary tract (Badalament et al, 1988).
1.3 THE CLINICOPATHOLOGICAL CHARACTERISTICS OF BLADDER CANCER

In bladder cancer, a malignant field change may be seen which involves the entire urothelium and possesses a variety of biological behaviours. This usually reflected clinically by the coexistence of different disease phases within the same patient, with varied chances of recurrence, invasion, progression, and metastasis (Limas & Lange 1988). The widespread field change may remain dormant or may express as papillary tumours and/or carcinoma in situ (CIS). The following sections look at bladder cancer under three separate categories.

1.3.1 Staging of bladder cancer

The evolution of bladder cancer staging has been derived from the observation of clinical course and outcome of different tumours in association with their depth of penetration through the bladder wall and with their spreading to adjacent or distant organs. An ideal staging system will allow the clinician to make a reasonable prediction of the response to various types of treatment and the prognosis (Skinner 1977).

The two most commonly used systems for staging bladder cancer are the Jewett-Strong system (1946) with Marshall modification (1952), and the TNM system which was developed by the Union of International Control of Cancer and the American Joint Committee on Cancer (UICC-AJCC) (Hermanek & Sobin 1987). The comparison of the two systems is shown in Table 1.1 and Figure 1.1. The current staging systems do not only provide a convenient "common language" for the surgeon and pathologists to communicate with, but also enable the clinician to stratify patients into various categories for treatment and thus compare the therapeutic effects.
Table 1.1. Staging systems for bladder cancer

<table>
<thead>
<tr>
<th>Features</th>
<th>Jewett-Marshall Stage</th>
<th>UICC-AJCC TNM Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>No evidence of primary tumour</td>
<td>0</td>
<td>T0</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>CIS</td>
<td>TIS</td>
</tr>
<tr>
<td>Tumour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Confined to the mucosa</td>
<td>0</td>
<td>Ta</td>
</tr>
<tr>
<td>Lamina propria invasion</td>
<td>A</td>
<td>T1</td>
</tr>
<tr>
<td>Superficial muscle invasion</td>
<td>B1</td>
<td>T2</td>
</tr>
<tr>
<td>Deep muscle invasion</td>
<td>B2</td>
<td>T3a</td>
</tr>
<tr>
<td>Perivesical fat invasion</td>
<td>C</td>
<td>T3b</td>
</tr>
<tr>
<td>Invasion to adjacent organs</td>
<td>D1</td>
<td>T4</td>
</tr>
<tr>
<td>Infiltration to prostate/uterus/vagina</td>
<td></td>
<td>T4a</td>
</tr>
<tr>
<td>Infiltration to pelvic wall</td>
<td></td>
<td>T4b</td>
</tr>
<tr>
<td>Regional lymph node</td>
<td>D1</td>
<td></td>
</tr>
<tr>
<td>Node(1), ≤ 2cm</td>
<td></td>
<td>N1</td>
</tr>
<tr>
<td>Node(1), 2-5 cm or node(&gt;1), ≤ 2cm</td>
<td></td>
<td>N2</td>
</tr>
<tr>
<td>Node(&gt;1), &gt; 2 cm</td>
<td></td>
<td>N3</td>
</tr>
<tr>
<td>Juxtaregional lymph node</td>
<td>D2</td>
<td>N4</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>D2</td>
<td>M1</td>
</tr>
</tbody>
</table>

Figure 1.1: The TNM and Jewett-Marshall classification for bladder cancers
1.3.2 Superficial papillary bladder cancer

Superficial bladder tumours are a group of largely grade 1 or 2 papillary transitional cell carcinomas that do not have muscle infiltration at the time of diagnosis. Only 10% of newly presenting patients with superficial disease have grade 3 cancer. This term therefore, indicates a spectrum of bladder cancers whose prognosis ranges from excellent in the G1Ta tumour to sometimes poor in the G3T1 tumour. It appears that one of the most critical factors reflecting the biological potential of bladder cancers is the distinction between those tumours that are confined to the bladder mucosa (stage Ta or 0) and those that have penetrated the basement membrane and extended into the lamina propria (stage T1 or A) (Smith et al, 1983). Despite the clinical heterogeneity and its variable biological potential, the term superficial bladder cancer, is acceptable to urologists world wide, and has been used for decades without posing particular difficulty in communication.

1.3.3.1 Clinical assessment and staging procedures

Superficial bladder cancers present as solitary (70%) or multiple (30%) lesions, and may or may not be associated with carcinoma in situ. Of these 70% are stage Ta, and 30% are T1 tumours (Heney et al, 1983). Sixty to 70% of T1 tumours are well (G1) or moderately differentiated (G2), while the rest are high grade (G3T1) lesions (Torti et al, 1987; Birch & Harland 1989). Except for the presence of irritative voiding symptoms (frequency, urgency and dysuria) which might be indicative of co-existing carcinoma in situ (and carry a worse prognosis than those who are asymptomatic), there is not correlation between the grade and stage of papillary disease and the clinical manifestations (Riddle et al, 1976). Although cystoscopy with biopsy is diagnostic, intravenous urography should be performed to exclude upper tract tumours which is positive in 2-4% of bladder cancer patients (Oldbring et al, 1989). Urine cytology can efficiently rule out the possibility of carcinoma in situ if the result is negative. Bimanual examination under sedation or anaesthesia is part of the staging work-up. Imaging studies (CT scan, MRI, and transurethral ultrasonography) are rarely helpful if the tumour is smaller than 5 mm, or is papillary on diagnostic cystoscopy (Cummings et al, 1992). For the small percentage of patients where there is suspicion of metastasis (<5%), recommended evaluations are chest X-ray, excretory urography, abdominopelvic CT scan, bone scan and liver function tests (Catalona 1992).
1.3.3.2 Treatment and prognosis

The standard treatment of superficial bladder neoplasms remains to be transurethral resection (TUR) (Soloway & Patel 1992). With TUR alone, the overall survival rate at 5 years is approximately 70% (Barnes et al, 1967). Laser is a possible energy source supplementary to the current electrocautery for endoscopic resection of bladder tumours. Photodynamic therapy (PDT) has shown to be promising as well, but remains investigational at the present time (Nseyo 1992). Intravesical therapy with various chemotherapeutic agents or BCG prove to be an effective adjuvant to the endoscopic resection (Newling 1990). Indications for adjuvant intravesical chemotherapy are a high risk of recurrence (already recurred, multiple lesions, tumour over 5cm in size), high grade tumours associated with urothelial atypia, or carcinoma in situ (Runnen et al, 1988). The overall recurrence rate with intravesical chemotherapy is reduced to 30-45% compared with about 70% in controls (Zincke et al, 1983). In the last 10 years, immunotherapy with local bacillus Calmette-Guerin (BCG) has emerged as one of the most effective modalities for treating superficial bladder cancer (Catalona & Ratliff 1990). Intravesical BCG is effective for prophylaxis against tumour recurrence (Lamm et al, 1980; Brosman 1982); for treatment of unresectable residual superficial cancer (Brosman 1984; Coplen et al, 1990), and for delaying cancer progression and invasion (Herr et al, 1988; Lamm 1992). Having had such a successful control of superficial bladder cancer with endoscopic resection and BCG, total cystectomy is rarely indicated as an initial treatment in patients with low grade cancer, but is reserved for those having diffuse unresectable papillary tumours or carcinoma in situ that does not respond to initial management, or repeated recurrent disease despite intravesical therapy (Matthews et al, 1984).

Recurrence, or more precisely, new occurrence is common even after seemingly successful local treatments. Heney et al (1983) reported recurrence rates of 50% for G1, 59% for G2, and 80% for G3 tumours at 3 years. Grade 3 cancer not only recurs more but also has a greater chance of tumour progression. The rate of tumour progression is 2%, 11%, and 45% respectively for G1, G2, and G3 superficial cancers. Only 4% of Ta tumours have progression at 3 years, in contrast to 30% of T1 tumours (Heney et al, 1983). Multiplicity of tumour is another risk factor for recurrence. Recurrence rates after solitary tumour are 18% and 33% respectively for Ta and T1 disease, whereas they are 43% and 46% for Ta and T1 multiple tumours (Lutzeyer et al, 1982). Similar results were reported by Fitzpatrick et al (1986). Although, multiplicity of papillary tumours at diagnosis is an important factor for
reoccurrence, it is not a significant determinant for disease progression (Kaubisch et al, 1991). The size of tumour at diagnosis has impact for subsequent progression. Larger tumours (> 5cm or >10gm) have higher rate for recurrence (Fitzpatrick et al, 1986; Heney 1992).

1.3.3 Carcinoma in situ (CIS)

Carcinoma in situ (CIS) is an intraepithelial cancer which was first described by Melicow as flattened areas of epithelial anaplasia, remote from visible tumour with a disordered pattern of growth and a potential for invasion (Melicow, 1952). It is a disease with considerable variation in biological behaviour. Two forms, an aggressive muscle invasive variant (diffuse form), and a more benign one (focal form) have been identified. The risk of subsequent invasion was reported as high as 78% in those with diffuse disease, but was only 8% in patients with focal CIS (Riddle et al, 1976). The majority (90%) of CIS is found in association with papillary or nodular bladder tumours, and only 10% develop as an isolated pathological finding (Hudson & Herr 1995). As most CIS usually co-exists with high grade bladder tumours, it is still suspected that CIS represents the earliest form of invasive cancer rather than an innocent bystander without potential for invasion or metastasis.

1.3.3.1 Clinical features

More than one quarter of carcinoma in situ patients may be asymptomatic, particularly those with the focal variant. Those with diffuse disease are more likely to have severe symptoms of urinary frequency, urgency and dysuria (Utz & Farrow, 1984; Hudson & Herr 1995). Varying degrees of suprapubic, lower abdominal, or lower back pain is also common. Haematuria is typically microscopic, and occurs in three quarters of all patients (Lemm 1992). Cystoscopic findings are non-specific and comprise mucosal erythema and oedema, suggesting the presence of profound submucosal inflammatory reaction and urothelial disarray (Utz & Farrow 1984). Diagnosis requires a high index of suspicion and liberal use of urine cytology. Due to reduced intracellular cohesiveness, CIS cells slough off easily, resulting in a high detection rate with urine cytology (Weinstein et al, 1980). The reported detection rate for diffuse CIS is as high as 90-95% with cytology (Farrow 1979; Zein & Milad 1991). Flow cytometry with DNA ploidy measurement is not only valuable for detection (Klein et al, 1982), but also shows promise as a prognostic indicator of the clinical course in patient with CIS (Norming et al, 1992).
Histologically, CIS appears as irregularly arranged malignant cells, almost always poorly differentiated, involving the entire thickness of the transitional cell layer without basement membrane penetration or intraluminal extension (Melicow, 1952). These cells are highly anaplastic with abundant mitotic figures and a loss of cellular cohesiveness. It can be found in any of the areas having urothelium including the renal pelvis, ureter, bladder, prostatic ducts, and anterior urethrae.

1.3.3.2 Treatment and prognosis
Being both poorly identifiable on cystoscopic inspection and of widespread nature, CIS treatment using transurethral resection (TUR) or fulguration has been very disappointing. The recurrence rate after TUR was 82% (Utz et al, 1970), with a rate of disease progression of 50-80% (Whitmore 1979), and a cure rate of only 8% (Melamed et al, 1993). Consequently, transurethral surgery is rarely considered a definitive treatment. Because CIS is associated with a high incidence for subsequent invasion (Whitmore, 1979; Lamm et al, 1992), early cystectomy has previously been recommended with 5-year survival rates of 71-83% (Tsujihashi et al, 1992). This strategy however, has been seriously questioned after the introduction of intravesical BCG immunotherapy (Lamm et al, 1991).

Currently, intravesical BCG is recommended as the treatment of choice for CIS and the overall complete response rate is about 70% (Hudson & Herr 1995). Persistence or recurrence of carcinoma in situ after the initial BCG therapy has a more ominous prognosis than previously untreated CIS (Herr et al, 1991a). In such cases, and in those whose CIS are accompanied with a high grade T1 or T2 papillary tumour, prompt cystectomy is often recommended. Recurrence frequently occurs after apparently successful treatment with intravesical BCG (Takashi et al, 1992; Nadler et al, 1994). A lifetime follow-up scheme is therefore strongly recommended.

1.3.4 Muscle invasive and advanced bladder cancer
This group accounts for 15-20% of patients presenting with bladder cancer and constitutes a variety of diatheses with differing biological potentials (Liu & Liotta 1992). Despite continuing controversies, some suggest that the more aggressive tumours, characterised as having a nodular architecture in histology and a solid appearance on cystoscopy, infiltrate deeper into the muscle and involve the bladder wall lymphatics and vasculature twice as often as the papillary types of muscle invasive cancers (Soto et al, 1977), whereas, the less aggressive papillary variants
proceed with a more benign course with 5-year survival of 60-80% (Pryor 1973). Fifty percent of patients who present with muscle-infiltrative cancer develop metastasis within 2 years of their initial diagnosis, despite radical therapy (Prout et al., 1979). Identifying factors influencing tumour invasion and metastasis in these high risk patients with molecular genetic techniques, although yet to be proven, is an important step in designing new therapies to prevent the spread of tumour and thus promoting survival (Liu & Liotta 1992).

### 1.3.4.1 Clinical evaluation

In addition to painless haematuria, there are no particular clinical manifestations attributable to the presence of muscle infiltration in bladder cancers. Occasionally, sign of extravesical extension may provide the first clue to the diagnosis of bladder cancer. Histological evaluation of the specimens from transurethral resection and biopsy remain currently the gold standard for local staging (See & Fuller 1992). Excretory urography has been used in an attempt to identify the presence of muscle invasion. Ureteric obstruction at the bladder level was seen in 92% of patients with muscle invasion (Hatch & Barry, 1986). Ultrasonography, in particular transurethral, has an average sensitivity of 93% (85-100%), and specificity of 79% (52-100%) in delineating muscle invasion (See & Fuller 1992). CT scan, MRI and lymphangiography are all useful investigations to evaluate extravesical extension or regional nodes. In less common cases where metastases are present at presentation, CXR, pulmonary CT, and bone or liver scans are indicated. Even with meticulous work-up, staging errors remain high.

For metastatic cancer, the accuracy of systemic staging is even worse than that of local and regional evaluation. Our inability to identify these patients preoperatively partly due to the existence of micrometastasis which with current imaging modalities are beyond detection.

### 1.3.4.2 Treatment and prognosis

The principal treatment for muscle invasive cancer confined to the bladder is either radical radiotherapy with bladder preservation or radical cystectomy in association with neobladder reconstruction (Montie 1990). Transurethral resection is certainly inadequate therapy for most muscle invasive tumours, but might have a position for appropriately selected patients (Herr 1987). Neoadjuvant chemotherapy, integrated preoperative irradiation with planned cystectomy, salvage cystectomy or systemic chemotherapy, alone or in combination are reserved only for more advanced bladder
cancers (Catalona 1992). Primary radical radiotherapy at a total dose of 5000-7000 cGy over 4-6 weeks reported 5-year survival rates of 40%, 30% and 10% respectively for T2, T3 and T4 (Rose & Shipley, 1988; Wesson 1992). The comparison of survival advantage between integrated preoperative irradiation (1000-4000 cGy) with planned cystectomy and that of radical radiotherapy is controversial (Prout 1976; Whitmore et al, 1977; Bloom et al, 1982; Montie et al, 1984). Outcome from radical cystectomy is poor (Lemer et al, 1992) and carries a high incidence of complication (Skinner & Lieskovsky 1988). Combination therapy with irradiation, systemic chemotherapy, and bladder preserving surgery is the focus of recent interest and the preliminary results seem promising (Prout et al, 1990). Systemic chemotherapy is likely to be the only chance to improve survival for more advanced bladder cancers which have pelvic lymph node involvement or distant metastases (Steinberg et al, 1992).
1.4 THE MANAGEMENT OF BLADDER CANCER

1.4.1 Endoscopic resection and fulguration

Despite the introduction of new therapies for bladder cancer, transurethral resection (TUR) or fulguration remains the mainstay for the treatment of superficial tumours (Soloway & Patel 1992). This technique is familiar to most urologists and is readily available at every urological service, making it the most popular option.

There are however, limitations for the TUR. Firstly, tumours inside of a diverticulum are not a good candidate for TUR because of the concern of perforation. Secondly, bleeding from the tumour surface may obscure the vision, making complete resection of all the tumour impossible. Thirdly, small tumours at the anterior bladder neck or high on the lateral wall are difficult to resect with a rigid resectoscope. Lastly, the sudden onset of bladder contraction as a result of electric stimulation to the obturator nerve during resection of tumour on the lateral wall may cause the bladder to be perforated inadvertently. Flexible cystoscopy which causes less discomfort to men, is convenient for diagnosis and follow-up, but is not practical for resection (Soloway & Patel 1992). Despite recurrence of superficial tumours in 40-80% of cases after TUR, (usually within 6-12 months) (Torti & Lum 1984), overall survival is good and a 70% 5-year survival is expected (Barnes et al, 1967).

1.4.2 Intravesical therapy

The development of intravesical therapy was prompted by the observation that a substantial number of superficial bladder cancer patients suffer from recurrence or progression after endoscopic resection. The current roles of intravesical therapy for superficial bladder cancer are: adjunctive, prophylactic, and therapeutic.

1.4.2.1 Intravesical chemotherapy

Intravesical chemotherapy was introduced as an option to reduce tumour recurrence after endoscopic resection in the 1960's (Jones & Swinney 1961). The rationale behind intravesical chemotherapy is the finding that low molecular weight chemotherapeutic agents readily penetrate the umbrellar cell layer of the mucosa by passive diffusion and this will lead to cellular toxicity by the inhibition of nucleic acid or protein synthesis (Richie 1992). Two principal factors, tumour biology (size,
multiplicity, grade, stage and ploidy) and regimen selection (agent, dose and therapeutic protocol) affect the initiation and outcome of intravesical chemotherapy (Badalament et al, 1992).

Popular agents for intravesical chemotherapy are thiotepa, epodyl, mitomycin C, epirubicin and doxorubicin (adriamycin). There is no clear evidence to demonstrate a significant advantage of any single agent over another (Lum & Torti 1988). Generally, for definitive treatment of superficial bladder cancer, complete responses occur in 30-50% of patients and partial responses occur in 20-40% (Catalona 1992; Hamdy et al, 1993). For prophylaxis of recurrence, an average reduction rate of 10-30% was reported (Richie 1992). Side effects, on the other hand, are dose and agent-specific. Thiotepa and epodyl are low molecular weight (198 & 262 daltons) alkylating agents. Systemic absorption of thiotepa, and to a much lesser degree, epodyl, will lead to myelosuppression in as many as one quarter of patients and irritative bladder symptoms in a high percentage (Hollister & Coleman 1980; Thrasher & Crawford 1992). Mitomycin C, an alkylating agent, and doxorubicin, an anthracycline antineoplastic antibiotic, are agents which have a higher molecular weight (334 & 580 daltons) than that of thiotepa and as a result, are less likely to be absorbed through the bladder mucosa to cause myelosuppression, although molecular weight is not the only determinant of absorption. However hypersensitive skin reactions, especially with mytomycin C, develop in 10-15% of cases and bladder irritative symptoms in 10-40%. A small percentage of patients (1-3%) eventually have bladder contraction requiring bladder augmentation or cystectomy. Newer agents which have higher therapeutic potential and are associated with fewer adverse effects, such as mitoxantrone, are under investigation (Thrasher & Crawford 1992).

1.4.2.2 Intravesical BCG immunotherapy

Bacillus Calmette Guerin (BCG) is an attenuated tuberculosis vaccine isolated from a virulent strain of Mycobacterium bovis (Crispen 1974). It was found to be a potent non-specific immune stimulant of the reticulo-endothelial system (Davies 1982). Despite the unquestionable efficacy of intravesical BCG, its mechanism of action remains largely undetermined. It has been suggested that multiple factors, including inflammatory reaction of the lamina propria, humoral and cell mediated immunity together with activation of lymphokines, are involved in the antitumour response from BCG (Lamm & Sosnowski, 1990).
The first clinical report on intravesical BCG therapy was published by Morales et al (1976) who demonstrated promising efficacy for the treatment of superficial neoplasms. Thereafter, rapidly accumulating research data have clearly shown that BCG is superior to thiotepa and doxorubicin in terms of superficial cancer treatment (Martinez-Pineiro et al, 1990; Lamm et al, 1991). BCG is also superior to other chemotherapeutic agents as an adjuvant therapy with TUR for prophylaxis of tumour recurrence (Martinez-Pineiro et al, 1990; Lamm et al, 1991). Although suffering from a possible bias on patient selection, most retrospective studies reveal that after intravesical therapy, the average tumour recurrence rates are 45%, 40%, 30% and 20% respectively for thiotepa, doxorubicin, mitomycin C and BCG (Herr 1991b; Brosman 1992).

The most important role of intravesical BCG immunotherapy is the treatment of high risk patients and carcinoma in situ (Herr 1992). Although highly controversial, recent data indicates that BCG is better than doxorubicin for prolonging survival, as it has a long-term complete response rate of 72% in contrast to 34% for doxorubicin (Lamm et al, 1991). It is also possible to maintain disease-free survival for 5-10 years in 54% of CIS patients receiving 1 or 2, 6-week BCG courses (Nadler et al, 1994). There is sufficient data suggesting BCG as the most effective conservative treatment for patients with G3T1 bladder cancer as long-term follow-up (mean 42, range 12-112 months) has shown a disease-free survival of 72% with low progression (12%) and recurrence rate (16%) (Pansadoro et al, 1995). With promising results from clinical experience, current opinion also favours the use of BCG immunotherapy for patients with aggressive superficial tumours and suggests that the development of other immunotherapeutic alternatives to BCG is likely to be rewarding and prospective (Lamm 1992).

1.4.3 Surgery

Surgery remains the mainstay for treating bladder cancer, especially in the United States. Partial cystectomy which was a time-honoured procedure for the treatment of bladder cancer before the era of modern anaesthesia, has been almost totally replaced by radical operation. The characteristics of the two procedures are discussed below.

1.4.3.1 Partial cystectomy

Partial cystectomy, with its relatively lower surgical morbidity (compared to that of total cystectomy) and the preservation of bladder function after operation, was one of
the main therapeutic options for muscle invasive and advanced bladder cancers before 1970. However, the disturbingly high incidence of tumour recurrence (Faysal & Freiha 1979) and the greatly reduced mortality rate for radical operations in the past 10 years, has rendered partial cystectomy a less important and relatively rare procedure.

Currently, the indication for partial cystectomy includes the treatment of localised muscle invasive tumour on patients who are otherwise of poor surgical risk, or have tumour in a diverticulum that is not suitable for endoscopic resection and also for operable adenocarcinoma or sarcoma of the bladder, where there is unlikely to be field change. Common contraindications of partial cystectomy include multiple tumours, carcinoma in situ, cellular atypia, tumour in the trigon, inability to resect with adequate margin (2cm) and an inability to maintain adequate bladder volume after the operation (Sweeney et al, 1992).

The overall complication rates of partial cystectomy range from 11% to 29%, although they are mostly minor (Brannan et al, 1978; Lindahl et al, 1984). The most serious complication is the implantation of tumour into the wound or suprapubic tract and this may occur as much as 18% (Novick & Steward 1976). Others less common complications are persistent vesicocutaneous fistula with or without later tumour recurrence, vesicovaginal fistula, and complications related to heart, pulmonary or other medical conditions. The principal drawback of partial cystectomy for bladder cancer is the occurrence of postoperative local recurrence which is as high as 38-78%. Half of those recurrences appeared in the first year and two thirds by 2 years (Resnick & O'Conor 1973). The overall 5-year survival is in the range of 35-60%. Stage by stage, 5-year survival rates for T2, T3a, T3b, and T4 diseases are 29-80%, 16-62%, 0-11% and 0, respectively (Sweeney et al, 1992). These results seem comparable to those in several contemporary series with radical cystectomy (Malkowicz et al, 1990; Brendler et al, 1990). Despite the seemingly good results on the survival following partial cystectomy, the completeness of local tumour control and the practicability remain controversial. Sumiyoshi and associates (1994) recently reported an overall 5-year disease free survival of 72% in those receiving neoadjuvant intra-arterial doxorubicin chemotherapy in combination with low dose (600 cGy) irradiation followed by partial cystectomy to treat T2-T4 Nx bladder cancer patients. There is an increasing possibility of preserving the urinary bladder without compromising survival, using a variety of combination of contemporary therapeutic approaches.
1.4.3.2 Radical cystectomy and urinary diversion

Over the past 40 years, the treatment of invasive bladder cancer has undergone many changes and modifications. Being discouraged by the results of the earlier series of total cystectomy and by the significant morbidity and mortality, which were as high as 35% and 20%, respectively prior to 1970 (Lerner et al, 1992), urologists started searching for another alternative, but found that radical surgery remains the treatment of choice for invasive bladder cancer. Fortunately, with advances in modern anaesthesia, comprehensive preoperative work-up and preparation, improved surgical technique and intensive postoperative care have significantly reduced the morbidity and mortality of radical cystectomy to 10% and less than 2%, respectively (Skinner 1988 & 1990; Freiha 1992). This procedure is also suitable for elderly patients, there being no definitely increased operative mortality in this group (Leibovitch et al, 1993). It is justified to state, that radical cystectomy, is the treatment of choice for patients with invasive bladder cancer in the Northern America (Catalona 1992). But in other parts of the world, where medical care is part of a National Health Service, equivalent results are being achieved with irradiation, especially in Europe where radical surgery is only one choice for the treatment of advanced bladder cancer.

Radical cystectomy, which includes total cystectomy and pelvic lymph node dissection, is the only available option for the complete sterilisation of locally advanced bladder cancer. In experienced hands, 5-year disease free survival rates were approximately 60-80%, 40-70% and 20-45% for node negative T2, T3a and T3b/T4 diseases, respectively (Skinner & Lieskovsky 1988; Freiha 1990; Pagano et al, 1991; Wishnow & Tenney 1991). It is also clear that local failure is highly correlated with distant failure. The 5-year survival rate being 77% in those having local control, compared with 29% for those with local failure was reported (Pollack et al, 1995). The prognosis is also ominous for node positive patients with an average 5-year survival rate of 25% (Skinner 1992). It seems firm that micrometastasis present in lymph nodes or in remote areas at the time of surgery is the most important factor determining prognosis. To eliminate these microscopic extensions, Whitmore et al (1977) suggested the integration of preoperative low dose irradiation with cystectomy in the hope of sterilising pre-existing microextension and preventing cancer cells spreading during the radical operation. However, the validity of the concept has been seriously questioned (Catalona 1980; Montie et al, 1984 ) and the survival advantage of this strategy remains controversial and non-conclusive (Prout 1976; Whitmore et al, 1977; Bloom et al, 1982; Crawford et al, 1987). Similarly, the combination of chemotherapy (neoadjuvant or adjuvant) with surgery has been studied in the hope of
prolonging tumour-free survival in advanced cancer patients. Unfortunately the results are conflicting (Skinner et al, 1991; Strenberg et al, 1993; Martinez-Pineiro et al, 1995) and further prospective trials with longer follow-up are needed to reach a conclusion. By combining TUR, chemotherapy, and irradiation, Kaufman et al (1993) were able to preserve the bladder and attain long-term tumour free survival in 50% of patients (T1-T4) at a median follow-up period of 48 months. They advocated that conservative combination treatment may be an acceptable alternative to immediate cystectomy on selective patients (Kaufman et al, 1993).

The three principal urinary diversions following a cystectomy are ileal conduit, continent stomal reservoir, and orthotopic neobladder (Catalona 1992). Even with the need for an external appliance for ileal conduit urinary collection, it remains the most standard method. A continent urinary reservoir can be constructed from ileum alone (Koch pouch), ileocecal segment (Mainz pouch), segment of colon with an ileal patch (Indiana pouch), or other modifications (Das 1990). In the male patient, the detubularised urinary reservoir can be brought down to the pelvic cavity and anastomosed with the urethral stump to create a continent neobladder for self controlled voiding with Valsalva manoeuvre (Ghoneim et al, 1987). These continent bladder substitutes are generally more acceptable than ileal conduit, but they are technically more demanding, and the risk of complication is higher (Skinner et al, 1987). Patients having orthotopic neobladder need regular follow-up of the urethra with washing cytology as recurrence of TCC remains as high as 35%, particularly in those primary tumours involving the bladder neck and prostate (Freeman et al, 1994). Recently, urinary diversion with orthotopic neobladder was also found satisfactory for women (Stein et al, 1995; Cancrini et al, 1995) with a low incidence of tumour recurrence in the urethra (Stenzl et al, 1995).

1.4.4 Irradiation

External beam irradiation is primarily an option for management of invasive bladder cancer, although a number of reports have been published treating non-invasive bladder cancer (Goffinet et al, 1975; Quilty & Duncan 1986). The employment of irradiation, despite being seriously challenged by radical cystectomy, and aggressive adjuvant and neoadjuvant chemotherapy regimens, remains an indispensable part of therapeutic armamentarium for invasive bladder cancer, although a modification of indication seems inevitable. In the United States where radical cystectomy has been the standard method of therapy for invasive bladder cancer, irradiation is usually
reserved for old, medically unfit patients and technically unresectable tumours. In contrast, in most European countries, irradiation is commonly used as the primary therapy for stage T2 or T3 cancer.

Review of the literature reveals that definitive irradiation at a dose of 5000 to 7000 cGy is able to achieve 5-year survival rates of 24-42% with T2, 15-35% with T3 and 7-11% with T4 diseases (Goffinet et al, 1975; Rose & Shipley, 1988; Wesson 1992). These unfavourable survival statistics, against those of radical cystectomy, are partly due to the bias of patient selection with a tendency to recruit older, physically unfit, or technically unresectable patients into the series for irradiation (Rose & Shipley, 1988). This bias is reflected in the observation that approximately 80% of patients with recurrent disease are unable to undergo salvage cystectomy because of advanced disease, age, or poor medical status (Parson & Million, 1990). For those having salvage cystectomy, the overall 5-year survival rate is 70%, if no residual is found in the bladder. Contrarily, if there is superficial residual tumour, the figure drops to 50%, and further down to 25%, if the residual tumour is muscle invasive (Blandy et al, 1980).

Preoperative irradiation with planned cystectomy (integrated irradiation and cystectomy) is likely to have some survival benefit over either radical cystectomy alone (40-56% versus 20-40%) (Whitmore et al, 1977; Anderstrom et al, 1983), or irradiation alone (34-46% versus 22-25%) (Miller 1977; Bloom et al, 1982), although the scientific implication and outcome of this integration have been questioned (Prout 1976; Montie et al, 1984). Postoperative irradiation is usually discouraging as an adjunct to radical surgery (Miller & Johnson 1973). Other less employed types of radiotherapy are interstitial or intracavitary brachytherapy. Interstitial radioactive seeds (Au-198 or Ta-182) implanted into the tumour was proven to be highly efficacious for the treatment of large solitary tumours. Ten-year survival rates are 69% with T2, and 59% with T3 invasive cancers (Van der Werf-Messing et al, 1983). Complications of external beam irradiation are closely related to the radiation doses delivered to normal tissue (which must be traversed before reaching the target tumour). Acute radiation bladder reactions which occur in 50-90% of patients and manifest as bladder irritation syndrome, usually subside 2-4 weeks after completion of the treatment (Goffinet et al, 1975). Subacute and chronic effects which are usually irreversible, include small bowel obstruction, radiation proctitis and bladder contracture (Miller 1977). Despite the aforementioned disadvantages, bladder
function is normal in 68% of those who survive more than 10 years after definitive irradiation (Goodman et al, 1981).

1.4.5 Systemic chemotherapy

The observation that 60% of patients who die of metastatic bladder cancer had their local disease controlled, suggests a high incidence of microscopic metastasis in the regional lymph nodes or distant organs at the time of initial diagnosis (Prout et al, 1979; Smith & Whitmore 1981). Radical surgery with meticulous pelvic lymph node dissection may be able to cure a small percentage of patients with limited lymph node involvement (Skinner 1982). However, for patients with extensive node metastasis, systemic chemotherapy is likely to be the only chance to improve survival (Steinberg et al, 1992).

Common criteria for response are as follows. Complete response is total disappearance of all measurable disease on physical examination and imaging, whereas a partial response depicts a 50% decrease in the product of two diameters of all measurable disease, which lasts for 4 weeks or longer and without the development of any new lesions (Stephens 1990). Using these criteria, single agent chemotherapy using cyclophosphamide, cisplatin, adriamycin, methotrexate, or bleomycin has been able to achieve overall response rates in the range of 10-30% (Steinberg et al, 1992).

There is a general consensus backed by clinical trials that combination chemotherapy, by integrating agents of different cytotoxic effect in a protocol, is more effective than a single agent (Loehrer et al, 1992). The most popular regimens (M-VAC, CISCA, CMV) are listed below.

Table 1.2: Doses (mg/m² body surface) and schedule for M-VAC

<table>
<thead>
<tr>
<th>Drug</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinblastin</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Repeat every 28 days
(Sternberg et al, J Úrol 1988; 139: 461-469)
Table 1.3: Doses (mg/m² body surface) and schedule for CISCA

<table>
<thead>
<tr>
<th>Drug</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td></td>
<td>650</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

Repeat every 21 days
(Sternberg et al, JAMA 1977; 238: 2282-2287)

Table 1.4: Doses (mg/m² body surface) and schedule for CMV

<table>
<thead>
<tr>
<th>Drug</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Vinblastin</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Repeat every 21 days
(Harker et al, J Clin Oncol 1985; 3: 1463-1470)

It seems that the efficacy and toxicity of CMV and M-VAC appear to be comparable, although there is evidence showing slight superiority in terms of response and survival of patients in favour of M-VAC (Loehrer et al, 1990). A result favouring M-VAC over CISCA was reported by Logothetis and associates (1990). However, some of these results are derived from non-randomised comparison, and may reflect differences from patient selection rather than effect of treatment (Young & Garnick, 1988). Neoadjuvant chemotherapy, in general, has an overall response rate in the range of 43% to 65% for T2-T4 cancer, however, its effect on survival remains uncertain. Based on this remission rate, the effect of downstage of TCC and probable long-term cancer control using M-VAC, attempts to preserve bladder function by combined-modality approaches including irradiation and surgery are under investigation (Jakse & Frommhold 1988; Prout et al, 1990; Sumiyoshi et al, 1994). It also may be possible to enhance response rate and disease free survival by administering chemotherapeutic agent intra-arterially (Naito et al, 1995). Although M-VAC is currently the best combination regimen and the standard of systemic chemotherapy for patients with advanced bladder cancer, there remains considerable room for improvement in efficacy and toxicity (Sternberg 1995).
1.4.6 Laser therapy

The thermal effects of laser energy have been used for the treatment of a variety of urological diseases for more than 15 years. Except for cutaneous and local treatment of genital disorders, urinary bladder is an organ suitable for laser manipulation by inserting the optical fibre through the endoscope to effect tumour coagulation. It is now generally accepted that laser surgery is an effective method for the management of superficial bladder cancer.

The most commonly used lasers for the treatment of bladder cancer are the KTP (potassium titanyl phosphate) and Nd:YAG (Neodymium:Yttrium Aluminium Garnet) lasers which emit light at the wavelengths of 532 and 1064 nm, respectively. At this wavelength, laser energy is poorly absorbed by water and tissue pigment and an average coagulation depth of 8-10 mm is obtainable without causing surface charring if laser is operated in a water medium. The optimum power for bladder tumour treatment is 30-40 W. Laser treatment for Ta or T1 superficial tumours is usually performed without anaesthesia and the procedure is well tolerated. Combined laparoscopic and cystoscopic laser coagulation might be a possible approach in the future to treat invasive bladder cancer penetrating through the full thickness of the bladder wall (Scaletscky et al, 1993). The most appropriate patients for laser treatment of invasive bladder cancer are those with superficial muscle layer infiltration (Smith 1986; Beisland & Sander 1990). Laser treatment of bladder cancer is used most often because of the observed decrease in patient morbidity and treatment related complications. Both prospective and retrospective clinical series have, in general, failed to support a favourable effect of laser treatment on recurrence rate of bladder cancer (Beisland & Seland 1986; Johnson 1994).

The advantages of laser treatment are lack of necessity for the anaesthesia for solitary superficial bladder tumour treatment, no immediate or delayed bleeding, no obturator nerve stimulation and hence no leg jerk so the risk of bladder perforation is much reduced, tumour in the diverticulum can be treated safely, and early initiation of BCG or chemotherapy is possible as a result of no bleeding postoperatively. Disadvantages include; laser manipulation being generally unfamiliar to the urologist and therefore special training being needed, lack of a surgical specimen for histological assessment, difficulty in eradicating a tumour of 2 cm or larger with laser alone and the depth of treatment cannot be monitored during lasering. This last disadvantage carries a risk of perforating an adjacent organ, especially the small bowel or colon which lies in close

45
vicinity to the peritoneal surface of the bladder. This complication occurs particularly when the laser power is in excess of 40W. It is possible for the bowel to be perforated with forward scattered light while the bladder remains intact as its muscle layer is thicker which makes perforation less likely. Of the 2000 consecutive treatments of superficial bladder cancer with laser, bowel perforation was observed in 3 (0.15%) (Sander & Beisland 1994).

Laser therapy is recognised as an effective treatment for superficial bladder cancer. The ability to eradicate existing visible tumours is comparable or, perhaps, superior to results obtained with electrocautery resection. Photodynamic therapy (PDT) is one of the applications of laser technology for cancer treatment. Instead of applying high power laser which induces tissue destruction by coagulation and vapourisation, PDT uses a low power laser, usually in the range of 100-250 mW to induce photochemical tissue destruction in conjunction with a photosensitiser given prior to the laser illumination. The principles and mechanism of PDT will be reviewed in section 3.3 of Chapter 3, with the overview of PDT on bladder cancer treatment being discussed in section 4.1 of Chapter 4.
# CHAPTER 2

PROSTATE CANCER

## 2.1 Introduction

- **Natural History**
- **Anatomical considerations**

## 2.2 The Cellular and Molecular Basis of Prostate Cancer

- **Histopathology and grading**
- **Prostate specific antigen (PSA)**
  - **Screening**
  - **Staging**
  - **Follow-up**
- **Flow cytometry (FCM) and DNA content analysis**
- **Cytogenetic and molecular biology of prostate cancer**

## 2.3 The Clinical Characteristics of Prostate Cancer

- **Staging of prostate cancer**
  - **Digital rectal examination (DRE)**
  - **Transrectal ultrasonography (TRUS)**
  - **Computed tomography (CT) and magnetic resonance imaging (MRI)**
  - **Bone scan**
- **Organ-confined prostate cancer**
- **Diagnostic workup and clinical presentations**
- **Treatment and prognosis**
- **Locally invasive and metastatic prostate cancers**
  - **Clinical course**
  - **Treatment and prognosis**

## 2.4 The Management of Prostate Cancer

- **Radical surgery**
- **Radiation therapy**
- **Hormonal therapy**
- **Other options**
2.1 INTRODUCTION

Prostate cancer is the most common male cancer and the second leading cause of cancer death in the United States. An estimated 200,000 new cases were expected in USA and 38000 men would die of prostate cancer in 1995 (Boring et al, 1994). In the countries of the European Community it accounts for more than 35000 deaths each year (Jenson et al, 1990). The incidence of prostate cancer is increasing steadily in many countries around the world, although the incidence varies in developed and developing countries. Afro-American's and the black population in the Caribbean and Africa have a higher incidence than Caucasians. The relative risk of prostate cancer in blacks is generally reported to be around a factor of 1.7 when compared to white American (Boyle et al, 1996). In Asian countries, the overall incidence rate is increasing in Japan and in Singapore, although the rate remains less than one-tenth those found in the USA (Coleman et al, 1993). However, Asian immigrants to the United States and their offspring show increases in clinical incidence that approach United States values (Akazaki & Stemmermann 1973). In view of the changes in incidence rate of prostate cancer after migration to the western countries, it has been proposed that factors responsible for the initiation of prostate cancer may be similar throughout the world but the clinical differences arise from differences in unidentified environmental promoting factors (Carter et al, 1990c).

A recent study has shown that the mortality rate in a population with prostate cancer is 2.25-fold that of an age-matched population without prostate cancer (Gann et al, 1995). Despite active research in identifying etiologic or predisposing factors for prostate cancer, no consistent evidence exist for substantial risk factors associated with diet, occupation, socioeconomic status, infectious disease history, sexual activity, or hormonal factors (Stamey & McNeal 1992).

2.1.1 Natural history

Natural history of a tumour may be defined as the evolution of the clinical and pathological manifestations of a neoplasm from inception until death in the untreated host. In contrast to other cancers, which end with fatality, the natural history of prostate cancer remains unpredictable as factors (host, tumour and environment) governing the natural history are poorly defined as yet (Whitmore 1984). For clinically detectable prostate cancer, 20-30% of men have distant metastases at the
time of diagnosis (Scardino 1989a), and the median duration of survival is less than 3 years, with more than 75% of them dying of cancer in 5 years (von Eschenbach 1981). Localised prostate cancer, on the other hand, accounts for approximately 55-60% of patients at the time of diagnosis (Schmidt et al, 1986). With appropriate treatment, 10-year survival as high as 90% can be achieved (Adolfsson et al, 1993). Currently, more than 80% of cases are diagnosed over 65 years of age, and fewer than 1% of patients with clinically detectable prostate cancer are less than 50 years old (Porter et al, 1995).

In an autopsy series, cumulative data demonstrated that 30% of 50 to 59 year old men and 67% of 80-89 year old men had microscopic evidence of prostate cancer (Fishman & deVere White 1994). A high prevalence of unsuspected cancer can also be found in the prostate removed surgically during cystoprostatectomy for bladder cancer (Kabalin et al, 1989). Previous data suggested that these latent cancer foci probably represented a distinct species of prostate cancer (Carter et al, 1990c), which was less aggressive in terms of malignant potential and had little chance to become clinically overt (Stamey & Kabalin 1989). Recent data however, suggested that with PSA blood testing, many clinically significant prostate cancer patients are being identified in whom the only clinical sign of their underlying disease is an elevated PSA level (Flanigan & Dougherty 1995). These authors advocated a more aggressive attitude toward treating these patients with curative measures. The central dilemma for screening or early detection of prostate cancer using prostate specific antigen (PSA) combined with digital rectal examination (DRE), and transrectal ultrasound (TRUS) and biopsy lies in the paradox of the high prevalence at autopsy and the low clinical incidence and mortality rate (Scardino 1989a). The controversy of screening prostate cancer with PSA will be discussed in section 2 of this chapter.

The natural history of prostate cancer suggests that while many more patients die with the disease than of it, a significant mortality does occur, and therefore attempting to control the disease has merit, particularly for those bearing a poorly differentiated variant. Unfortunately, the question of which patients to treat remains a therapeutic dilemma, as tumour-host interaction is playing a central role in determining the responsiveness of the tumour to treatment, it is insufficient to depend on tumour grade as a guide for therapeutic decision. Efforts to predict tumour biological activity for patients have focused on parameters including tumour grade, volume, DNA content, and molecular assessment in the hope for delineating guidelines for further reference.
2.1.2 Anatomical considerations

The new anatomical classification proposed by McNeal (1988) has drastically changed the classical concepts of prostate anatomy initially described by Lowsley and Franks. In this model, the prostate has been divided into 4 major regions: central, peripheral, transitional zone, and the periurethral gland region. The former two structures are compatible to the "outer prostate" as was defined by Franks and the latter to the "inner prostate". The peripheral zone, transitional zone, and periurethral gland region are histologically identical because of their common origin from the urogenital sinus. The central zone, in contrast however, is architecturally and cytologically distinctive having histological characteristics suggesting Wolffian duct origin.

The peripheral zone which comprises approximately 50-60% of the volume of the young adult prostate, is the site of origin of 70% of prostate cancer. This is also the region most accessible to rectal palpation of cancer. The transitional zone accounts for about 5 to 10% of the glandular prostate, together with the periurethral region (1% in total prostate volume) which is the site where benign prostatic hyperplasia develops (BPH). Approximately 20-30% of prostate cancers develop in this region. Cancer arising from transitional zone is usually associated with BPH, and is recognised clinically as incidental cancer in transurethral resection for BPH (Epstein et al., 1988; Christensen et al., 1990). The central zone, as a result of its distinctive structure, although it comprises 20-30% of the total prostate volume, is relatively resistant to malignant transformation and therefore gives rise to only 5-10% of all adenocarcinomas (McNeal et al., 1988).
2.2 THE CELLULAR AND MOLECULAR BASIS OF PROSTATE CANCER

Prostate cancer depicts a spectrum of malignancies arising from the prostate and its surrounding structures. It is no surprise to find cancers of rare histological type developing from the prostate. Adenocarcinoma and transitional cell carcinoma constitute 95% and 2-4%, respectively of all prostate cancers. Squamous cell carcinoma of the prostate is a rare variant that occurs as a result of prolonged or severe inflammation of the already transitional metaplastic epithelium to squamous phenotype, or as a result of chronic oestrogen administration to the duct-acinar cells (Stamey & McNeal 1992). Other rarer cancers are rhabdomyosarcoma, leiomyosarcoma, carcinoid tumours, or secondary cancers from direct extension or by metastasis. In the subsequent sections of this chapter, only adenocarcinoma will be discussed further.

2.2.1 Histopathology and grading

The normal prostate is composed of complex tubular alveolar glands within a fibromuscular stroma. The glandular epithelium is either of low cuboidal or tall columnar secretory type which is frequently arranged into papillary folds. Acini drain by individual intralobular ducts which unite into interlobular ducts. Ducts may be lined by cuboidal or columnar epithelium.

In common with other neoplasms prostatic adenocarcinomas demonstrate a varying degree of glandular differentiation and cellular anaplasia. However, the diversity of histological patterns commonly seen among different tumours and even within different areas of the same carcinoma in the prostate may be greater than that which is typical of cancer in any other organ (Stamey & McNeal 1992). It is this histological heterogeneity which makes assignment of grades of cellular dedifferentiation on cytological characteristics extremely difficult, although some systems have been proposed to assess morphological features of prostatic adenocarcinoma and hence derive a histological grade indicative of malignancy (Shelley et al, 1958; Utz & Farrow 1969). The histological diversity expressed by prostatic adenocarcinoma is mainly a function of its great variety of architectural patterns. Based on these findings, a number of different architectural grading systems have been devised (Gleason 1966; Mobley & Frank 1968; Mostofi 1975; Gaeta 1981). Among them
Gleason's grading system is most frequently utilised as its value of clinical correlation has been better established than others and criteria for assigning grade are clearly defined and relatively reproducible (Stamey & McNeal 1992).

The system derived by Gleason is based purely on glandular architectural criteria. The glands are stratified from grade 1 (very well differentiated) to grade 5 (anaplastic) according to the patterns of glandular differentiation. Since most tumours have more than one glandular pattern, Gleason assigned a "primary" grade to the predominant pattern, and a secondary grade to the pattern occupying the second largest area. Both grades are scored as digits which are summated to give a "Gleason score" from 2-10. The histological characteristics of Gleason grading system is tabulated as Table 2.1.

Table 2.1: Gleason score system for prostate cancer

<table>
<thead>
<tr>
<th>Grade</th>
<th>Differentiation</th>
<th>Glandular Architecture</th>
<th>Glandular Stromal Boundary</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Very well</td>
<td>Distinct glands; uniform shape and size; absent basal epithelial cells</td>
<td>Sharply delineated glands with no stromal infiltration</td>
</tr>
<tr>
<td>2</td>
<td>Well</td>
<td>Distinct glands; irregular shape and size; fairly defined margin; inter-glandular spaces</td>
<td>Less defined than grade 1; some infiltration along major stromal planes.</td>
</tr>
<tr>
<td>3*</td>
<td>Moderate</td>
<td>Fairly defined glands; increased irregularity in gland size and spacing</td>
<td>Ragged margins with some infiltration along major and minor stromal planes.</td>
</tr>
<tr>
<td>4#</td>
<td>Poor</td>
<td>Absence of gland pattern; masses of fused glands with clear cells</td>
<td>Poorly defined tumour boundaries with extensive infiltration throughout stroma</td>
</tr>
<tr>
<td>5</td>
<td>Anaplastic</td>
<td>Loss of glandular pattern; infiltrative trabeculation or non-glandular solid masses</td>
<td>Poorly defined boundaries to indistinct glands with diffuse and widespread infiltration</td>
</tr>
</tbody>
</table>

Gleason score = Primary grade + secondary grade

*A cribriform variant is assigned to grade 4 if the glandular architecture is completely lost
#Four variants can occasionally be identified. Cribriform pattern; fused gland pattern; cell nest pattern, and cell cord pattern.

The principal features distinguishing Gleason's system of grading from other morphological methods are: (1) emphasis on glandular differentiation and growth patterns of the neoplastic glands in relation to prostatic stroma; (2) assignment of a secondary grade to each tumour, thus accounting for morphological heterogeneity
found in the majority of prostate cancers and not relying on the most undifferentiated portion; and (3) specific exclusion of cytological characteristics of individual tumours.

The system adopted by the National Prostatic Cancer Treatment Group is the Gaeta's (1981) which is a four-grade system based on a combination of glandular and nuclear morphology of the tumour. It has been shown to be both objective and reproducible. The incorporation of nuclear parameters into the grading has demonstrated good clinical correlation between tumour grade and prognosis (Foster 1990). However, analysis relating progression free survival to Gleason and Gaeta grading systems has indicated the Gleason score to predict, more successfully than that of Gaeta, the probable biological behaviours of a prostatic adenocarcinoma especially when two or more glandular patterns are presented in the same tumour (Schroeder et al, 1985a, 1985b; Gardner et al, 1988).

Care must be taken when employing the grading system for specimens taken from prostatic biopsy. It has been the general experience that the biopsy grade of a tumour has not been as accurate a predictor of clinical course for the individual patient as that from a surgical specimen (Sagalowsky et al, 1982; Whitmore 1988). Limitations of grading to predict biological behaviour of a tumour lie on three considerations. First, biopsy samples are usually too tiny to express the genuine glandular architectural patterns of a particular tumour in which a great diversity of patterns have existed. Second, the correlation of tumour specific histological features to the biology of that cancer which underlies the fundamental concept for tumour grading is usually seriously challenged when the expression of a single parameter is applied to predict the behaviour of any single tumour arising within a particular patient. Third, giving a grading system the full strength to predict tumour behaviour, the fact that tumour biology within an individual is a dynamic process that evolves with time and tends to become less differentiated at the secondary deposit site (Fan & Peng 1983), the assumption of an unaltered morphological grade throughout the lifespan of a particular tumour seems inappropriate. It is very likely that identification of a variety of objective biological parameters at cellular and molecular levels, probably analysed in combination, will be required for more precise prediction of tumour behaviour in the future.
2.2.2 **Prostate specific antigen (PSA)**

Prostate specific antigen (PSA) was first called gamma-seminoprotein when it was identified in seminal plasma by Hara and associates (1971). Later it was isolated from the prostate tissue by Wang *et al.* (1979), and was named prostate specific antigen. Subsequently, this tissue protein was proved to be immunologically identical to that originally found in the seminal plasma (Wang *et al.*, 1982). Its three-dimensional antigenic domain has been characterised (Chu *et al.*, 1989) and a kallikrein-like gene sequence of 6 kilobases has been identified (Riegman *et al.*, 1989).

PSA is a single-chain glycoprotein of 240 amino acid residues, having a molecular weight of about 30,000. Except for urachal remnants (Golz & Schubert 1989) and some urethral glands in men (Iwakiri *et al.*, 1993). It is found in prostate epithelial cells and in the seminal fluid where it causes liquefaction of the seminal coagulum (Lee *et al.*, 1989b). The half-life of serum PSA is about 2.2 to 3.2 days (Stamey *et al.*, 1987; Oesterling *et al.*, 1989) and therefore it requires a minimum of 2-3 weeks for the serum level to reach its nadir after radical prostatectomy (Stamey *et al.*, 1987). It is important to note that BPH tissue also secretes PSA to the serum at an amount of 0.3±0.25 ng/ml per gram hyperplastic tissue by the polyclonal and 0.5±0.4 ng/ml by the monoclonal assay (Schellhammer & Wright 1993). Cancer tissue on the other hand, elevated serum PSA at an average of 3.5 ng/ml per gram (by Pros-check, polyclonal assay) (Stamey *et al.*, 1987). Serum PSA level is fairly constant throughout the day and shows no diurnal circadian changes (Dejter *et al.*, 1988). Factors that alter serum PSA levels are transurethral resection of BPH (Price *et al.*, 1991), prostate infection (Neal *et al.*, 1992) and prostate biopsy (Yuan *et al.*, 1992). Digital rectal examination of the prostate (Glenski *et al.*, 1992) and ejaculation (Netto *et al.*, 1996) rarely produce meaningful changes of serum PSA.

There are a few commercialised PSA test kits current available for clinical use worldwide, among them Hybritech (Tandem-R monoclonal) and Yang (Pros-check, polyclonal) assays are the most common in the United States. The cutoff value of serum PSA and the relevant strength of different assays remain suboptimally defined. However, most urologists now accept 4 ng/ml of Hybritech assay (equivalent to 7.3 ng/ml of Yang's) and 10 ng/ml (18.4 ng/ml of Yang's) as lower and upper normal cutoff values (Graves *et al.*, 1990).
In summary, PSA is organ specific for all practical purposes but is not cancer specific. It is produced by normal, hyperplastic and malignant prostatic epithelium. While PSA has offered clinicians a better tool for the detection of prostate cancer than mammography has for detecting breast cancer, the sensitivity and specificity of PSA are not yet sufficient to make it the perfect screening test for prostate cancer (Chodak 1993; Crawford & DeAntoni 1993; Parkes et al, 1995). Nevertheless, before the introduction of better tumour markers or the demonstration that early diagnosis using PSA will not decrease the prostate cancer specific mortality rate, PSA should be used to aid in early diagnosis and treatment planning for men with prostate cancer.

2.2.2.1 Screening

The use of serum PSA for early detection or screening of prostate cancer has been an issue of intense debate. Screening, in contrast to disease treatment, is to intrude in healthy people with an intervention that could have a net harmful effect, and therefore should be conducted with extreme care. For prostate cancer screening, the challenge is to distinguish clinically "innocent" cancers from potentially mortal ones. The possibility of overdiagnosis and overtreatment of nonprogressive cancers could outweigh any potential benefit from screening (Kramer et al, 1993). To justify screening of prostate cancer with serum PSA, one has to be certain that early detection is possible and its early treatment will lead to an increase in the survival rate and a reduction in the mortality rate from the disease. Unfortunately, most studies up to now have shown that the first two requirements can be fulfilled satisfactorily, the third, on the other hand remains unsolved (Chodak 1993). Other factors discouraging the use of PSA for prostate cancer screening are the uncertainty of the natural history of prostate cancer, low specificity (high false positive rate) of the PSA test, and the cost (Fishman & deVere White 1994).

Those who favour serum PSA for early detection argue that with current incidence and mortality rates, prostate cancer is expected to pose a significant public health problem in the United States in the future (Andriole & Catalona 1993). In the absence of further effective measures to reduce incidence of prostate cancer and to promote treatment, early detection and early treatment of cancers while they remain organ-confined seems to be a practical solution to reduce cancer specific death. An active PSA screening programme alone, identifies more organ-confined cancer than was detected with the traditional approach (62% vs 38%) (Catalona et al, 1993), and these cancers tend to be clinically and pathologically significant in contrast to those latent cancers discovered at autopsy (Humphrey et al, 1996). With an appropriate treatment,
the progression free survival rate in these clinically impalpable cancers which were detected by elevation of serum PSA (clinical T1c) and treated with radical prostatectomy was similar to that of T1a to T2a group (Lerner et al, 1996). A recent cost-effectiveness analysis suggested that because the costs of treating advanced prostate cancer are usually considerably greater than for early disease, the incremental costs of adding PSA to rectal examination for early detection are offset by lower treatment costs for localised disease (Catalona 1993). The National Cancer Institute is currently conducting a prospective, randomised trial to determine whether or not screening reduces the mortality rate, but the result will not be available for 16 years (Catalona 1993).

In a comparison of two large series screening patients referred for urological assessment (Cooner 1993) and men on a community basis (Hudson et al, 1991; Brawer et al, 1992), substantially more men in the referral group have abnormal DRE (33% vs 17%) and PSA levels higher than 4 ng/ml (35% vs 10-15%) who require further examination with transrectal ultrasound and biopsy. It seems reasonable to assume that serum PSA assays in the urologist's office would be a more cost-effective method for detecting cancer than using them to screen the general population (Stamey & McNeal 1992).

However, knowing that 40-45% of newly diagnosed prostate cancer is clinically advanced (stage C or D) and that the 2.5-year survival for patients with metastatic disease is only 50%, efforts to detect the disease while it is still localised to the prostate is logical and rational (Murphy et al, 1982). Based on the currently available data, the American Cancer Society and the American Urological Association now recommend a DRE and serum PSA determination on an annual basis for those seeking a cancer prevention check-up beginning at age 50 for early prostate cancer detection, and beginning at age 40 in high risk groups (Mettlin et al, 1993). On the contrary, given our incomplete understanding of its basic biology, prostate cancer detection through screening efforts could generate problems through overtreatment which could outweigh the benefit of early detection (Chodak 1993). It was on the basis of this rationale that the Canadian urologists recommended not to adapt PSA for population based screening (Feightner 1994). The decision whether or not to screen should be justified in the individual patient's interest, and only after thorough evaluation of patient age, current illness, social status and personal desire, should risk-benefit ratio of such test be reasonably reached.
2.2.2.2 Staging

The intention of using PSA to assist staging of prostate cancer was initiated by the discovery that PSA levels increased with advancing clinical stage and was proportional to the estimated volume of the tumour (Stamey et al, 1987). However, a strong correlation between serum PSA and tumour volume does not necessarily imply a strong relationship between serum PSA and stage of disease. As a result, PSA levels alone have a limited role in the preoperative staging of prostate cancer although they have been shown to increase with advancing clinical stage and are directly related to tumour volume (Hudson et al, 1989; Stamey & Kabalin 1989; Kleer & Oesterling 1993). Advocating serum PSA as a preoperative staging tumour marker (Stamey et al, 1989a) has been seriously questioned as there is considerable overlap of PSA values between clinical stages, making it impossible to distinguish clinical stage based exclusively on a single PSA measurement (Partin et al, 1990). This ambiguity is likely to be related to the unpredictable contribution from the BPH component of the gland (Weber et al, 1989) and the decreasing production of serum PSA by higher grade lesions with increasing tumour volume (Partin et al, 1990). Preoperative serum PSA levels have been reported to correlate with final pathological stage (Oesterling et al, 1988; Lange et al, 1989). Again serum PSA levels increase with advancing pathological stage, but overlap exists. No level of PSA can be established as an absolute cutoff for predicting organ-confined disease, even if it is less than 4ng/ml. In a recent series reported by Noldus and Stamey (1996), of 911 patients having radical prostatectomy, 187 (21%) had serum PSA levels equal to or less than 4 ng/ml. At this PSA level, surgical specimens showed organ-confined disease in 137 (73%) while 50 (27%) had capsular penetration. No patient had positive pelvic lymph node, and only 5 (2.7%) had seminal vesicle invasion. The ability of serum PSA to define pathological stage, however, can be enhanced by the combination of tumour grade (Gleason score), DNA ploidy and the clinical stage (Kleer et al, 1993). One clear benefit for preoperative PSA measurement may be in reducing the need of preoperative bone scans. Patients with a serum PSA level lower than 10ng/ml rarely have bone metastasis (Chybowski et al, 1991; Oesterling et al, 1993). Therefore, a staging bone scan does not seem to be necessary in an asymptomatic patient with untreated prostate cancer whose serum PSA level is low.

2.2.2.3 Follow-up

PSA has proved to be the most valuable option for monitoring the disease status of patients after definitive therapy for prostate cancer (Stamey et al, 1989b; Oesterling 1991; Stein et al, 1992; Ritter et al, 1992; Partin et al, 1994; Hancock et al, 1995).
This is especially true after radical prostatectomy and definitive radiotherapy. Numerous studies have shown that PSA is a sensitive marker for residual or recurrent cancer and serum PSA levels are predictive of eventual outcome after a variety of treatments (Ferguson & Oesterling 1994). An elevated serum PSA often precedes clinical recurrence by months or years (Lange et al, 1989; Frazier et al, 1993).

If surgery has removed the entire prostatic epithelium (benign and malignant), the postoperative serum PSA value, in theory, should be undetectable. However, owing to limitations on the precision of the assays at very low levels of PSA, an undetectable level at a true zero reading is impossible. The term "biological detection limit (BDL)" has been proposed as the lowest level of PSA measurable by the assay for which a high degree of confidence indicating PSA actually exists in the serum (Takayama et al, 1993). Despite the introduction of ultrasensitive assay which the BDL is as low as 0.008 ng/ml (Klee et al, 1994), there is general consensus that a BDL of 0.4 ng/ml or less using a Hybritech assay is an acceptable level for prognosis assessment (Ferguson & Oesterling 1994), although an ultrasensitive PSA assay with BDL of 0.1 ng/ml was reported to detect recurrent cancer 9-12 months earlier than the Tandem-R assay (Takayama et al, 1993). A detectable postoperative serum PSA level after radical prostatectomy almost uniformly implies incomplete surgical resection or recurrence of cancer. Several studies have investigated the significance of a detectable serum PSA within the first year following surgery (Hudson et al, 1989; Lange et al, 1989; Stein et al, 1991; Partin et al, 1993). Generally, disease progression, either in the form of local recurrence or distant metastasis was found in 50-100% of those having elevated serum PSA in the first follow-up year, and those whose preoperative PSA was greater than 10ng/ml have a substantial increase of risk of recurrence. Kaplan-Meier statistical analysis demonstrated an actuarial 5 and 10-year progression-free probability of 83% and 70% for those without postoperative serum PSA elevation, in contrast to 23% and 13% with elevation. An isolated elevation of serum PSA following radical surgery is clinically significant as it implies the presence of occult local recurrence or systemic dissemination (Partin et al, 1993).

The action of irradiation is supposed to be cytolytic or cytostatic affecting the dividing cell population (mostly cancer cells) at large. As a result, with possible presence of prostatic epithelium that remains after irradiation, the interpretation of serum PSA levels following definitive radiotherapy is extremely sophisticated. Serum PSA levels generally fall to low (< 4ng/ml) but often detectable ranges following definitive radiotherapy, and are dependent on several factors including pre-therapy
level, volume of normal prostate, quantity of hyperplastic tissue, degree of tumour differentiation, and dose and delivery of irradiation. The decline of serum PSA postirradiation is usually biphasic, with a sharp fall within the first 3 months and a more gradual fall over the following 9 months (Zagars et al, 1991). PSA levels declined in 82% of patients during the first year, but only in 8% thereafter (Stamey et al, 1989c). The time interval following radiotherapy at which the nadir PSA level can be expected has not been strictly defined, however, most investigators basically agree that the PSA level reaches a nadir value within 12 months (Pisansky et al, 1993), and approximately 80% of patients demonstrate a decrease to the normal range within 6-12 months (Zagars et al, 1991). In the first 3 years after irradiation, 40%-70% of men have stable baseline serum PSA but then begin to experience elevation. The sources contributing to elevation of serum PSA include residual radio-resistant tumour, growth of new tumour in the prostate, continuous growing BPH and distant metastasis (Schellhammer et al, 1991).

Regardless of the source of elevation of PSA after irradiation, increase above baseline portend disease recurrence and progression as subsequent clinical evidence of relapse has been demonstrated in 70% of patients who had serum PSA above normal values when followed at 3 years (Kaplan et al, 1993). The trend of serum PSA levels was suggested to be an even more significant indicator than absolute PSA value in predicting relapse of cancer following irradiation (Zagars 1993). However, studies of PSA trends after irradiation have generally indicated that biochemical evidence for persistent and recurrent prostate cancer are more common than expected on the basis of clinical findings, and precede clinical relapse by a variable period of time (Ritter et al, 1992; D'Amico & Hanks 1993). The rising PSA trend and the persistent elevation of serum PSA (biochemical recurrence) with or without tissue proof constitutes a difficult clinical problem as no data is currently available concerning the optimal management of patients with early recurrence. Generally, patients are either allocated to hormonal treatment or rarely to salvage radical prostatectomy for further control (Rogers et al, 1995). The longterm results of serum PSA after definitive irradiation were reported by Hancock et al (1995) who depicted a complete biochemical control of serum PSA levels (<4ng/ml) in 38% (42/11) of patients followed-up averaging 12.4 years and remained clinically disease-free. They also reported that PSA doubling times were significantly shorter among cases measured after relapse and with further hormonal therapy, and short PSA doubling times were associated with distant metastasis rather than with local recurrence.
Hormonal therapy remains the mainstay of treatment for patients with stage D2 disease and for those failing definitive irradiation, as prostate cancer are composed of a heterogeneous population of cells that differs in androgen dependency. Tumours that are composed of predominantly androgen dependent and androgen sensitive cells will show a better response to androgen deprivation therapy in the form of a decline in serum PSA than those composed largely of androgen independent cells. Also the production and secretion of PSA are androgen dependent and decreases in PSA levels do not always indicate cure of cancer (Leo et al, 1991). Experimental and clinical studies have confirmed that the decline in serum PSA levels after androgen withdrawal is attributable predominantly to reduction of both number of prostatic epithelial cells and PSA production capacity on a per-cell basis (Grignon & Troster 1985; Weber et al, 1989). Thus, lower serum PSA levels in men who have received hormonal therapy should be interpreted with caution.

A number of PSA parameters (pretreatment PSA levels, absolute PSA levels at a specific time point, time to nadir, nadir value, and trend of PSA decrease) have been advocated as indicators predicting treatment outcome and survivals, however, the results are conflicting (Patros & Andriole 1993). Although significantly longer progression-free survival in patients whose serum PSA declines more than 80% in one month compared with those with lesser declines in PSA was reported (Leo et al, 1991). Some patients (15%) will have their cancer progress despite a normal PSA level (Dupont et al, 1991). In summary, pretreatment PSA levels generally correlate with the extent of disease at presentation but cannot be used to predict which patients will have better prognosis in terms of prolonged disease-free interval or survival. In patients who respond to hormonal manipulation, there is a steady fall in serum PSA in the early treatment period; nadir levels are frequently attained by 2 months and nearly always by 6 months, but never to undetectable levels (Patros & Andriole 1993). Patients who respond with an early (within 6 months) normalisation of PSA levels are likely to have a survival advantage. The large majority of patients who have disease progression while on endocrine therapy will have rising serum PSA, and it precedes by 6 to 12 months before the appearance of clinical or radiological sign of progressive disease (Cooper et al 1990). A rising PSA in the hormonally treated patients, even if the levels are within the normal range, signals impending clinical progression who should be considered for possible alternative therapies, as a theoretically favourable window of opportunity to retard disease progression may exist when the PSA begins to rise.
2.2.3 Flow cytometry (FCM) and DNA content analysis

The basic principles underlying the design and development of flow cytometry (FCM) have been discussed in section 1.1.2 of Chapter 1 and this section is dealing with updated knowledge with regards to the application and usefulness of FCM for prostate cancer.

Adenocarcinoma of the prostate is characterised by great variation in its biological behaviour, often with an unpredictable course. The histological heterogeneity of prostate cancer is projected on to the complexity of Gleason grading system which has to combine primary (major) with a secondary (minor) gland pattern to depict a full spectrum of degree of malignancy. If the Gleason grade in itself is of sufficient precision to predict cellular malignant potential, there should be no further prognostic value to be gained by testing DNA content of the cancer. However, it is because of this deficiency in morphological criteria, FCM is adding valuable information to prognostic evaluation of patients with prostate cancer. It has been shown that early prostate cancers are almost invariably diploid in DNA (McIntire et al, 1988). Nevertheless, most patients who die from advanced prostate cancer have aneuploid DNA content. It was suggested that prostate cancer undergoes an evolution from diploid to tetraploid and finally to aneuploid at an annual conversion rate of 9% to 15% (Adolfsson & Tribukait 1990). Since prostate cancers are not "ploidy" stable, it might be helpful to use FCM in identifying cases whose DNA remain diploid as the chance of effective control is greater with surgery or irradiation before they evolve to aneuploid (Lieber 1995).

There is general consensus that patients with DNA diploid tumours, irrespective of clinical stages, are more likely to have favourable responses in terms of lymph node involvement, disease progression, disease-free survival, and distant metastasis to radical prostatectomy, irradiation and to hormonal therapy than patients with nondiploid tumours (Shankey et al, 1993). The majority of patients (60%-70%) with pathologically organ-confined prostate cancers (stage T2) have diploid tumours, and almost without exception, these patients have a superior prognosis when treated by radical prostatectomy (Montgomery et al, 1990). The addition of FCM DNA ploidy checkup enables us to identify a small group of patients (about 5%) who have high grade tumour with aneuploid DNA content and although they remain organ-confined at the time of assessment, they are likely to have rapid systemic progression and early cancer death (Montgomery et al, 1990). With future availability of more effective
measures for systemic treatment in conjunction with radical surgery, identification of
this subgroup of patients at high risk for progression will further enhance actuarial
survival. For stage T3 tumours, FCM of DNA ploidy is particular useful to help to
differentiate a group of patients for further prognosis evaluation whose Gleason
scores are between 5 and 7, and therefore are potentially progressive if the ploidy is
aneuploid (Lee et al., 1988; Lieber 1990). For more advanced prostate cancers, DNA
ploidy has been confirmed to be an accurate indicator which predicts tumour response
to hormonal therapy, with excellent response for diploid DNA content, and ominous
prognosis for aneuploidy (Tribukait 1991; Miller et al., 1991). It was estimated that
about 40% of patients with stage D1 disease have diploid tumours (Winkler et al.,
1988) and are likely to benefit particularly from early initiation of hormonal therapy
(Myers et al., 1992; Zincke et al., 1992) and to have a prolonged response.

2.2.4 Cytogenetic and molecular biology of prostate cancer

Cytogenetic and molecular biology studies of prostate cancer are complicated by the
profound heterogeneity of the tissues being examined. DNA, RNA and proteins
isolated from tissue specimens, usually represent mixed extract from benign cells and
cells of differing malignant grade. Methods having direct detection of molecular
targets at the cellular level, such as in situ hybridisation, are rarely applied in prostate
cancer.

Several cancer associated genes have been studied in prostate cancer, including the
ras family of proto-oncogenes, c-myc, c-sis, c-fos and epidermal growth factor
receptor (EGFR). The demonstration of an oncoprotein (p21) in the ras family has
been of particular interest recently. Its level is highest in undifferentiated prostate
tumour but is absent in normal and hyperplastic tissue (Viola et al., 1986). It was
detected in all bony metastatic tumours but only in 20% of the subpopulation of the
primary tumour, suggesting the possibility of being a metastatic indicator in the future
(Fan 1988). However, despite the increased expression of ras oncoproteins at the
mesenger RNA level, it is generally accepted that structural or numerical alterations
of ras genes at the DNA level are rare in prostate cancer, suggesting that the increased
p21 expression is resulting from alterations in transcription half-life, promoter or
enhancer elements (Carter et al., 1990a; Moul et al., 1992).

Another proto-oncogene of current interest is c-myc in which messenger RNA has
been shown to be highly expressed in prostate cancer of various histological grades
(Buttyan et al, 1987; Eaton et al, 1988). Amplification of c-myc has been found in cell lines but not in primary tumours (Peehl et al, 1987), and was reported to be associated with poor differentiation and increasing malignancy (Fukumoto et al, 1986; Nag & Smith 1989). However, the clinical role and application of c-myc RNA expression for tumour grading remains to be defined (Fleming et al, 1986). C-sis is another proto-oncogene which has been extensively studied in prostate cancer in vitro. It is detectable in normal and hyperplastic protein tissue, and is highly expressed in prostate cancer cell lines (Rijnders et al, 1985; Smith & Nag 1987; Sitaras et al, 1988). It has been suggested that androgen administration results in increased expression of c-sis which then leads to malignant transformation of the prostate cell via down regulation of platelet derived growth factor that affects the messenger RNA expression of c-fos and c-myc (Smith & Nag 1987; Sitaras et al, 1988). Androgen deprivation results in decreased c-fos messenger RNA expression by 90% (Rijnders et al, 1985). The expression of c-myc, c-sis and c-fos in prostate cancer are only detectable at the RNA and protein levels, suggesting altered regulation at the transcription level in cells over expressing the protein (Peehl et al, 1987). The data on EGFR and Her2/neu expression in prostate cancer are highly controversial, and the pathogenetic relevance of their expression to malignant potential remains to be defined (Eaton et al, 1988; Mellon et al, 1992; Sadasivan et al, 1993).

Studies of tumour suppressor genes have demonstrated that frequent deletions of genetic material have been found in chromosome 8, 10, and 16 for colon, breast, and prostate cancers (Carter et al, 1990b; Collins et al, 1991). However, the identification of a locus, specific for prostate cancer, remained ill-defined. In an in vitro study, the tumourigenicity of the prostate cell line DU 145 could be abolished by the introduction of a functionally active tumour suppressor retinoblastoma (RB) gene which was reconstituted after retrovirus-mediated gene transfer (Bookstein et al, 1990). The expression of the tumour suppressor gene p53 has been shown to be altered in prostate cancer cell lines at messenger RNA and protein levels by Northern and Western blotting, respectively (Rubin et al, 1991). The p53 gene mutation or oncoprotein accumulation have been correlated with higher malignant potential (Mellon et al, 1992; Macoska et al, 1992; Visakorpi et al, 1992).

In conclusion, several chromosomal and gene alterations have been reported in prostate cancer. The expression of messenger RNA and protein products in relation to the aggressiveness of tumour biology are still both controversial and confusing. It
requires additional careful molecular biological studies at multiple levels on a large number of tumours to clarify the usefulness of molecular biological indicators for diagnosis and thus the management of patients with prostate cancer.
2.3 THE CLINICAL CHARACTERISTICS OF PROSTATE CANCER

In this section, only the most important and clinically relevant topics, such as staging, diagnosis, and the basic principles for treatment of prostate cancer are going to be discussed. The current techniques for diagnosis and staging such as, transrectal ultrasound, computed tomography and MRI will be mentioned briefly.

2.3.1 Staging of prostate cancer

With the increasing number of prostate cancers which are expected to be diagnosed in the future, there has been an immense effort to improve upon the accuracy of non-invasive staging modalities. This attempts to minimise the morbidity associated with the evaluation of these patients as well as to direct therapy more appropriately at those patients most likely to benefit from intervention. Despite all the technical improvements in staging modalities, the fundamental problems remain centred around the controversies of prostate cancer staging (Catalona & Whitmore 1989). Staging is particularly important in the daily management of cancer, as the selection of treatment and prediction of prognosis are largely based on the extent of disease.

There are two principal staging systems that are currently used by urologists worldwide; Whitmore-Jewett and the UICC/AJCC TNM system. There is no absolute superiority of one system over the other as to the clinical applicability, but some evidence does suggest that the TNM system seems better in terms of prognosis assessment (Ohori et al, 1994; Zagars et al, 1994). Regardless of the system used, characterisation of the local lesion remains the mainstay for staging, although it is largely subjective, arbitrary, and sometimes not well correlated clinically. With the introduction of pelvic CT scan, transrectal ultrasound, MRI and the increasingly popular uses of PSA for early detection of prostate cancer, minor modifications of the two systems have been adopted with the intention to allow flexibility, so that refinements may be incorporated for subgroups to include information from new technology (Montie 1993).

The Whitmore-Jewett staging system (Table 2.2) which was originally proposed by Whitmore (1956) and later modified by Jewett (1975) has been used widely because
its stage stratifications are relatively simple, non-controversial and there are no obligatory evaluation requirements for the various stage classification.

Table 2.2: Whitmore-Jewett staging classification of prostate cancer

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>Clinically unrecognised (Tumour an incidental finding at TURP)</td>
</tr>
<tr>
<td>A1</td>
<td>&lt; 3 chips of prostatic tissue low to medium grade</td>
</tr>
<tr>
<td>A2</td>
<td>≥ 3 chips of prostatic tissue neoplastic, any grade, or &lt; 3 chips of prostatic tissue neoplastic, all high grade</td>
</tr>
</tbody>
</table>

**Stage B** Clinically intracapsular

- B0 Nonpalpable tumour identified because of elevation of PSA level
- B1 Tumour involves one half of a lobe or less
- B2 Tumour involves more than one half of a lobe but not both lobes
- B3 Tumour involves both lobes

**Stage C** Clinically extracapsular, localised to periprostatic area

- C1 Extracapsular extension
- C2 Tumour invades one or both seminal vesicles
- C3 Tumour invades other pelvic organs

**Stage D** Metastatic disease

- D1 Pelvic lymph node metastases, or ureteral obstruction causing hydronephrosis
- D2 Distant metastasis

The UICC/AJCC proposed a TNM staging system based on data from local tumour (T), nodal status (N), and metastasis (M) for categorising prostate cancer (Wallace et al., 1975). This system is more complicated than the Whitmore-Jewett's but is thought to be more specific for comparison among various categories of patients. The TNM system is tabulated as Table 2.3.
Table 2.3. TNM classification of prostate cancer

<table>
<thead>
<tr>
<th>T Category -- Primary tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T</strong></td>
</tr>
<tr>
<td>Tx</td>
</tr>
<tr>
<td>T0</td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>T1a</td>
</tr>
<tr>
<td>T1b</td>
</tr>
<tr>
<td>T1c</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>T2a</td>
</tr>
<tr>
<td>T2b</td>
</tr>
<tr>
<td>T2c</td>
</tr>
<tr>
<td>T3</td>
</tr>
<tr>
<td>T3a</td>
</tr>
<tr>
<td>T3b</td>
</tr>
<tr>
<td>T3c</td>
</tr>
<tr>
<td>T4</td>
</tr>
<tr>
<td>T4a</td>
</tr>
<tr>
<td>T4b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N Category -- Nodal involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
</tr>
<tr>
<td>Nx</td>
</tr>
<tr>
<td>N0</td>
</tr>
<tr>
<td>N1</td>
</tr>
<tr>
<td>N2</td>
</tr>
<tr>
<td>N3</td>
</tr>
<tr>
<td>N4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M Category -- Distant metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M</strong></td>
</tr>
<tr>
<td>Mx</td>
</tr>
<tr>
<td>M0</td>
</tr>
<tr>
<td>M1</td>
</tr>
<tr>
<td>Specify sites according to the following notations</td>
</tr>
<tr>
<td>PUL (pulmonary)</td>
</tr>
<tr>
<td>OSS (osseous)</td>
</tr>
<tr>
<td>HEP (hepatic)</td>
</tr>
<tr>
<td>BRA (brain)</td>
</tr>
<tr>
<td>LYM (lymph nodes)</td>
</tr>
</tbody>
</table>

Despite the complexity of the TNM system in allocating tumour stages, it seems destined for widespread use, not only because it permits an abbreviated but definitive
characterisation of the tumour relative to local extent, regional spread, and distant dissemination, but also because specification of the minimal requirements for these various categories reduces one potentially significant variable. The latter feature is often lacking in other staging systems (eg. Whitmore-Jewett). Nevertheless, both systems will continue to suffer from the uncertainties created by continuously evolving changes in clinical staging techniques and criteria, also the inevitable technical and interpretational differences which occur with the same clinical staging techniques performed at different institutions (Sogani & Whitmore 1987).

2.3.1.1 Digital rectal examination (DRE)
Over the past decades, index finger with digitally guided biopsy has been the most powerful tool for prostate cancer diagnosis. However, its limitations are an experience-dependent, low detection rate (0.8% to 1.7%), high false-negative rate (30% to 40%) and a low specificity (50% to 65%) (Chodak et al, 1989; Scardino 1989a). Furthermore, upstaging of DRE assessed lesions was reported as high as 40% to 60% (Thompson et al, 1987).

2.3.1.2 Transrectal ultrasonography (TRUS)
The advent of transrectal ultrasound (TRUS) has markedly enhanced the efficacy of prostate cancer detection and diagnosis (Lee et al, 1985). The commonest ultrasonic feature of prostate cancer is a hypoechoic lesion in the peripheral zone, but hyperechoic cancer can also be found occasionally. Hypotexture of the peripheral zone is a non-specific finding for malignancy and biopsy of such lesions is therefore necessary. About 40% of hypoechoic lesions in the peripheral zone are shown to be malignant (Lee et al, 1989a). Even with the improved resolution of modern transducers operating at 7 MHz, as many as 25% of cancers are still isoechoic and this remains a major challenge to imaging of the prostate (Dahnert et al, 1986). The addition of colour Doppler imaging to TRUS appears to offer little advantage over conventional grey-scale imaging for diagnosis of cancer (Rafkin et al, 1993). As a result of substantial variation in cancer echogeneity, the sensitivity and specificity of TRUS in staging prostate cancer is 60-70%, and 40-50%, respectively (Rifkin et al, 1990).

Recently investigations have indicated that TRUS may have merit in guiding seminal vesicle biopsy for cancer staging (Terris et al, 1993), but is of limited value in evaluation of patients after radical prostatectomy (Wasserman et al, 1992). Nevertheless, in the absence of a more convenient and reliable technique for
diagnosis of cancer, TRUS-guided biopsy will probably remain the imaging modality of choice for the diagnosis of prostate cancer until the 21st century.

2.3.1.3 Computed tomography (CT) and magnetic resonance imaging (MRI)

Computed tomography (CT) scan is a popular imaging modality for assessment of various internal organs. However, CT imaging lacks the sensitivity necessary to discern the internal architecture of the prostate gland and therefore cannot differentiate neoplastic tissue from benign hyperplastic tissue (van Engelshoven & Kreel 1979). Its role in delineating fine structures of the prostate capsule or seminal vesicles has been disappointing (Denis et al, 1980). Finally, the inability of CT scans to differentiate a cancerous pelvic lymph node from an inflammatory lymph node and the cost renders it a modality of very limited value in the patient with prostate cancer.

After the introduction of magnetic resonance imaging (MRI) in 1980s, it has become one of the essential imaging modalities for detailed study of the prostate before surgery (Hricak et al, 1987). The advantages of MRI over conventional radiological techniques are (a) better soft tissue contrast resolution; (b) direct multiplanar imaging without loss of resolution; (c) no ionising radiation is used; (d) lack of signal in blood flowing allowing precise depiction of vascular and perivascular anatomy; and (e) negligible artefact from bone or nonferrous surgical clip (Bretan & Williams 1987). While studies utilising MRI have indicated that both the body surface and endorectal coils provide superior resolution of the zonal anatomy of the prostate compared to TRUS, MRI cannot reliably differentiate benign from malignant lesions within the gland, nor can MRI accurately stage prostate cancer when it is present (Kahn et al, 1989; Bryan et al, 1986). Nevertheless, the reported sensitivity and specificity for staging prostate cancer are 65% and 69%, respectively using a body coil, and 87% and 85% using an endorectal coil (Schiebler et al, 1993). The value of MRI for assessing pelvic lymph nodes, like that of CT scan, is limited due to lack of specificity in discriminating cancerous from inflammatory nodes. MRI has better sensitivity than TRUS (77% vs 66%) for staging of prostate cancer (Rifkin et al, 1990).

2.3.1.4 Bone scan

Bone scans are probably the most popular nuclear medicine technique used as a method of screening for bone metastases from various primary cancers (Citrin et al, 1977). It is a sensitive and convenient method. It is better than a whole body bone survey to assess the skeleton in those suspicious of bony metastases. Before the
discovery of PSA, bone scans are an integral part of workup for staging of prostate cancer, and for monitoring status of disease progression after treatment (Stone et al, 1980). Current state-of-the-art preoperative PSA measurement is aimed at eliminating the need for bone scans if PSA levels are elevated above a certain level. In a comprehensive study comparing serum PSA levels and findings on a bone scan, Chybowski et al (1991) showed that the false-negative rate of serum PSA level over 20ng/ml for a positive bone scan was 0.3%. Based on the probability plot of the above study, a patient with a serum PSA of 10ng/ml has a 1.4% probability of having a positive bone scan (Oesterling 1993). It is now a general consensus that serum PSA is an accurate and reliable predictor of the bone scan findings for newly diagnosed prostate cancer patients. For those with no skeletal symptom and a serum PSA level less than 10ng/ml, a bone scan is not necessary as it provides no further staging information.

2.3.2 Organ-confined prostate cancer

This group of patients inevitably include those whose prostate cancer are incidentally discovered (T1, stage A) and those confined to the prostate gland (T2, stage B). A historical review of a series reporting incidentally discovered prostate cancer is found in about 10% of cases performed for benign hyperplasia (Sheldon et al, 1980), but this statistic clearly depends on the completeness of the specimen being reviewed (Denton et al, 1965; Newman et al, 1982). Recently, other than the two stratified subgroups of T1 (T1a and T1b), a third group of patients was included (T1c) in the T1 category whose prostate cancer are discovered by the suspicion of an elevation of PSA level following tissue proof from biopsy but not surgically removed specimens (Epstein et al, 1994). Stage T2 (B) cancer is clinically evident on digital examination but apparently confined within the limit of the prostatic capsule. Before 1990 only about 50% of the cancers detected belonged to this category (Murphy et al, 1982). Today, 80-90% of cancer detected in screened populations are organ-confined (Mettlin et al, 1993). However, not all clinically confined diseases are pathologically confined. Local upstaging (T3) is found in 26% of patients who were previously diagnosed as T2c disease or lower (Paulson et al, 1994). The incidence of positive nodal involvement is 2% for clinical stage T1a, 26% for stage T1b, 10% for stage T2a, 25% for stage T2b, and 46% for stage T3 (Donohue et al, 1982).
2.3.2.1 Diagnostic workup and clinical presentations

With growing public awareness of the prevalence of prostate cancer in elderly men, an increasing percentage of patients diagnosed having localised prostate cancer are identified by the clinic based screening programme with DRE, PSA checkup, and TRUS. Most of patients with organ-confined prostate cancer are asymptomatic or have symptoms related to bladder outlet obstruction caused by BPH. Patients may also present with severe irritative bladder symptoms in the absence of infection, but isolated haematuria or haematospermia is rare. There are no special symptoms/signs attributable to the presence of prostate cancer in the lower urinary tract.

Since pelvic lymph nodes are typically the first site of metastatic spread in prostate cancer, it is necessary to evaluate the status of pelvic lymph nodes before definitive therapy. Lymphangiography was once a time-honoured option for staging prostate cancer, it is now considered of limited value because of high false-positive and false-negative rates (Paulson 1979). Staging pelvic lymph nodes by dissection is the warranted procedure nowadays. However, the value of extended lymphadenectomy with therapeutic intention, remains undefined (Golimbu et al, 1979). Bone scan can be eliminated if the serum PSA levels are below 10ng/ml. A chest X-ray is needed to rule out the possibility of pulmonary metastasis.

2.3.2.2 Treatment and prognosis

There is no general agreement regarding the optimal therapy of localised prostate cancer. Stage T1a (A1) cancers generally are low-volume, low-grade malignancies, and have sufficiently low progression (14%) and mortality rates (6%) in men over the age of 70. These patients should not be considered for definitive treatment and a simple expectant management is justified to achieve a 10-year disease specific survival of 87% (Johansson et al, 1992). A similar result was reported by Chodak et al (1994) who suggested that watchful waiting is a reasonable option for men with grade 1 and 2, stage T1 prostate cancer especially if their life expectancy is 10 years or less, but not for those with grade 3 cancer because the rate of metastasis is high and survival low. In younger men (aged 50-65), there is no convincing data regarding prognosis for any management strategy; however, the demonstration of 25% of cases had residual cancer foci in the peripheral zone raises serious concerns for disease progression in those whose projected life span is more than 10 years (Paulson & Robertson 1991; Bahnson 1993). Thus, younger healthy men should be offered definitive therapy, either radical prostatectomy or irradiation, for the best chance for survival.
Currently, modern treatment, especially with radical prostatectomy, is able to eradicate the disease completely in more than two thirds of patients overall, and in 90% of patients if the cancer is confined within the prostate pathologically (Dillioglugil et al, 1995). While radical prostatectomy carries greater risks of serious complications than irradiation, it offers the only chance for complete eradication of cancer. The cancer-specific survival rates range from 82% to 90% at 15 years and 90-93% at 10 years (Walsh et al, 1994; Paulson 1994; Zincke et al, 1994).

Irradiation offers another definitive therapy for treating localised prostate cancer although its ability to control and possibly cure prostate cancer in the long term remains uncertain (Byrne & Neal 1996). Reported 10-year cancer-specific survival after radiotherapy appears to be comparable to other forms of management (Bagshaw et al, 1994). The incidence of positive postirradiation biopsy range from 15-35% at 2 years (Forman et al, 1993; Schellhammer et al, 1987), and do not predict disease-free survival or overall survival at 10 years (Leach et al, 1982).

Options for recurrent prostate cancer following definitive therapy are salvage prostatectomy (Rogers et al, 1995), cryosurgery (Onik et al, 1993), laser therapy (Sander & Beisland 1984), and hormonal therapy. These procedures although showing evidence of local control or delaying local recurrence on a short term basis, have not been shown to prolong life.
2.3.3 Locally invasive and metastatic prostate cancers

Locally invasive prostate cancer contains disease of several clinical and pathological categories. These include clinically localised disease (T1 or T2) that upgrades to either pathological T3 (stage C) after radical prostatectomy or stage T3-4N+ (C or D1 disease) at the time of surgery, as well as clinical stage T3 and T4N+ (D1 disease). Unlike that of organ-confined disease, 40-50% of these locally advanced cancers have evidence of regional lymph node involvement or distant metastasis (Donohue et al, 1982; Zincke et al, 1986). With the concern for systemic dissemination, there is no strict rule applicable to the treatment of any particular stage of disease. The potential benefit of a proposed therapy should be weighed carefully against the overall cost of that therapy to each patient.

2.3.3.1 Clinical course

Even with advanced local disease, the most common clinical manifestation remains lower urinary tract obstruction. Haematuria is rare but may be profuse in occasional cases. Local spread of cancer may cause bilateral obstructive uropathy leading to uraemia. Advanced pelvic lymphadenopathy with cancer cells infiltration can result in lymph oedema of the lower limbs. Direct rectal invasion, although rare, is possible and in most advanced stages, may cause intestinal obstruction.

In about 10% of cases, the first presenting symptoms are those attributable to distant metastases. Pelvic bone and vertebral involvement will cause lower back pain or even pathological fracture, causing paraplegia. Haemoptysis may be secondary to pulmonary metastasis whilst neurological signs may be due to brain metastases.

2.3.3.2 Treatment and prognosis

Two options, local irradiation and hormonal therapy, are available for managing patients whose cancer has upstaged to pathological T3 after radical prostatectomy; however, their effects for prolonging survival remain unclear. Adjuvant irradiation seems to reduce the risk of local recurrence to 5-10% (Gibbons et al, 1986). For clinical stage T3 disease, hormonal therapy, radical prostatectomy alone or with hormonal therapy, and irradiation (either external or interstitial) are the treatment options. The issue of early versus late initiation of hormonal therapy remains controversial. Evidence from current studies suggests that early hormonal therapy, even though there is a need for a definitive randomised study to justify it, may increase patient survival in comparison to delayed hormonal therapy (Stamey &
McNeal 1992). The comparison of effectiveness of radical prostatectomy with or without hormonal therapy on disease specific survival is not straightforward as patient selection, surgical skill and grade of cancer all play roles in determining the final outcome after treatment. Irradiation has been the most popular option for treating stage T3 and T4 (stage C) prostate cancer. However, as a result of inadequate staging and the extremely heterogenous sample population in this group, reported local failure rates after radical irradiation range between 27% to 58% at 12 months (Kiesling et al, 1980; Schmidt et al, 1986). Survival rates are in the range of 60-70% at 5 years, and 35-45% at 10 years (Bagshaw et al, 1987; Zagars et al, 1987). The treatment for patients with pelvic lymph node involvement (stage D1 or T1-4N+) includes combined radical prostatectomy with early hormonal therapy and hormonal therapy alone. The former seems more favourable than the latter in terms of survival (Myers et al, 1983). The treatment for stage D2 disease (TNM+) is palliative and available modalities are hormonal therapy, palliative irradiation, and cytotoxic chemotherapy. Hormonal manipulation is the mainstay of therapy which attains a median survival of 24 to 30 months (Brendler 1988).
### 2.4 THE MANAGEMENT OF PROSTATE CANCER

#### 2.4.1 Radical prostatectomy

Radical surgery for prostate cancer implies the en bloc extirpation of the prostate, ejaculatory ducts, seminal vesicles, and the investing Denonvilliers' fascia together with pelvic lymph node dissection. The two principal approaches for radical surgery of the prostate are retropubic and perineal prostatectomy. The detail of surgical procedures is not the main issue of this thesis, and therefore will not be discussed. Nevertheless, this section will highlight the impact of radical treatment on the welfare of patients with special emphasis on complications and quality of life after operation.

The advantages of radical retropubic over perineal approach are the feasibility of simultaneous pelvic lymphadenectomy, less complicated anatomy which is more familiar to most surgeons, fewer rectal injuries, less urinary incontinence, and most importantly, much less impotence (Walsh 1992). However, the perineal approach provides a relatively avascular field, good exposure for vesicourethral reconstruction, and, a dependent postoperative drainage (Paulson 1992). With the introduction of nerve sparing techniques for preservation of the neurovascular bundle during dissection of the prostate (Walsh & Donker 1982), potency preserving retropubic prostatectomy is becoming the gold standard for surgical treatment of prostate cancer.

Radical prostatectomy now is the preferred option for the treatment of patients with stage T1 and T2 diseases. Applying this option to treat T3 and T4 (stage C) patients remains controversial, although it has been advocated to be of great value in prolonging survival with or without adjuvant hormonal therapy (Tomlinson et al, 1977; Zincke et al, 1984; Zincke et al, 1986). There is a trend toward removing these locally advanced prostate cancers with surgery and then adjuvant with hormonal therapy or irradiation to gain control over the pelvic region (Stamey & McNeal 1992). The surgical treatment of stage T1-4N+ (D1) patients is equally controversial as that for T3-4 diseases, however, the major issue of debate is the time of initiation of hormonal therapy after radical prostatectomy of local disease (Zincke et al, 1989; deKernion et al, 1990; Steinberg et al, 1990).

Complications of radical prostatectomy include severe haemorrhage, pulmonary embolism, thrombophlebitis, bladder neck contracture, incontinence and impotence.
The last 2 complications have been markedly reduced with the anatomical approach to radical retropubic prostatectomy. Recovery of erections sufficient for intercourse can be as high as 40-60%, and urinary continence preserved in 94% of cases (Steiner et al, 1991; Catalona & Basler 1993). However, from the patient point of view, the rate of impotence and incontinence is higher, being 51% and 34%, respectively (Jonler et al, 1994). Quality of life assessment based on questionnaires sent to patients who had undergone radical prostatectomy disclosed that despite the presence of complications, 92% of the responders showed affirmative attitude toward having the same procedure if needed (Lim et al, 1995).

2.4.2 Radiation therapy

The two principal radiation techniques used for treating prostate cancer are external-beam irradiation and interstitial brachytherapy. External-beam irradiation is accomplished by delivering megavoltage radiation generated by cobalt-60 or linear accelerator to the pelvic area. This therapy has been used to treat prostate cancer for over 30 years with acceptable side effects (Bagshaw et al, 1990). State-of-the-art irradiation protocol involves the delivery of 7000 cGy to the prostate gland while pelvic lymph nodes at the same time absorb up to 5000 cGy over a period of 6-7 weeks (Goffinet & Bagshaw 1992). Interstitial brachytherapy on the other hand, involves implantation of radiation seeds into the prostate by a retropubic or percutaneous approach (Holm et al, 1983).

Radiotherapy is reserved for those patients presenting without evidence of distant metastasis. The decision to offer radiation therapy is based on a judgement of the balance between the life expectancy of an individual patient and the chance of disease progression during that period of time. As a rule, most evidence suggest that external beam irradiation provides excellent palliation in patients with T3-4 (stage C) disease or for T1-2 (stage A, B) who otherwise are not suitable for radical operation (Zagars et al, 1987; Scardino 1989b). Disease specific survivals at 5 and 10 years for clinically organ-confined prostate cancer range 73-75% and 55-62%, respectively (Perez et al, 1988; Bagshaw et al, 1990). There is evidence that once this disease has spread to pelvic lymph nodes, distant metastases can be expected in 80% of cases within 5 years (Hanks et al, 1987). As staging pelvic lymph node dissection is usually not done for patients undertaking irradiation, there is the possibility that a number of locally advanced cases have been included in the organ-confined group for comparison, which partly accounts for the slightly inferior survival curves compared
to those receiving radical prostatectomy. Recent study, however, indicated that there were no significant differences among the disease specific survival rates of patients with stage A and B tumours who were treated with radical prostatectomy or irradiation, but the all cause survival rates favour the surgery group (Fowler et al, 1995). It seems fair to say that patients having external beam irradiation generally do well for substantial periods of time of 5-8 years, but the long-term survival rates are generally inferior to those with radical prostatectomy (Walther 1994). Despite numerous reports of the apparent efficacy of irradiation for organ-confined disease, radiotherapy alone for T3 prostate cancer may have serious limitations as local failure rate may be as high as 53-58% 12 months after radiation therapy and with substantial morbidity (Kiesling et al, 1980; Holzman et al, 1989).

Interstitial brachytherapy with $^{125}$I seems more appropriate for patients with localised disease and whose cancers are well- or moderately differentiated as local failure is high for high grade disease (Schellhammer et al, 1985). Short term follow-up also supports this therapy as an appropriate option for low grade smaller tumours, whereas combined brachytherapy and low dose external irradiation is suitable for larger tumours of moderate differentiation (Kaye et al, 1995). Long term survival with interstitial radioactive seeds implantation is disappointing (Kuban et al, 1989).

Complications of irradiation include rectosigmoid sequelae (rectovesical fistula, rectal ulcer and radiation proctitis leading to bleeding and strictures), urinary sequelae (urethral stricture, haemorrhagic cystitis and fistular formation). Retrospective study showed that erectile dysfunction could occur in 14-50% of patients depending on age and technique of irradiation, however, prospective, well-documented quantitative assessment of this sequelae is lacking (Zinreich et al, 1990). With the advent of three-dimensional treatment planning and conformal radiation therapy, it is feasible to deliver higher tumour doses to selected target volumes, thus improving tumour control probability without increasing treatment morbidity (Perez et al, 1993).

2.4.3 Hormonal therapy

Approximately 40-45% of prostate cancers are locally advanced or metastatic at the time of diagnosis (Murphy et al, 1982), and cure of these patients is impossible with current therapeutic modality. Hormonal therapy, therefore is palliative for those who have pelvic lymph node involvement or distant metastasis. However, as prostate cancer is a biologically heterogeneous tumour composed of androgen-dependent,
androgen-sensitive and androgen-independent populations of cells, response rate after hormonal therapy is 70-80% only, and 20-30% persistently will not respond clinically (Graversen & Iversen 1996).

The major circulating androgen in men is testosterone which production is regulated by the hypothalamic-pituitary-testicular axis. This axis is responsible for 95% of circulating androgens; 5% of circulating androgens are produced by the adrenal gland. There are 3 different ways to accomplish hormonal therapy in prostate cancer patients: interruption of the hypothalamic-pituitary-testicular axis, inhibition of intracellular binding of testosterone, and direct inhibition of androgen synthesis.

Interruption of the hypothalamic-pituitary-testicular axis can be achieved by bilateral orchidectomy, administration of oestrogen and related compounds, and luteinizing hormone-releasing hormone (LHRH) agonists. Orchidectomy is a reliable and durable method and can effectively reduce 95% of circulating testosterone (Maatman et al., 1985), but psychological impact after removal of testicles is a major concern. Oestrogen and related compounds competitively inhibit the binding of testosterone to its receptor in the hypothalamus and inhibit the release of LHRH. This was popular before the 1980, but is rarely used today as a result of associated cardiovascular complications (Byar 1973). Administration of LHRH agonists causes an initial surge of serum testosterone (flare period) for 1 week. The major disadvantages of LHRH agonists are the flare period, hot flush, impotence, and the need for parenteral or nasal spray administration. The effectiveness of LHRH agonist administration was reported to be similar to orchidectomy in terms of suppression of serum testosterone level, tumour response and relapse rates (Koutsilieris et al., 1986).

Inhibition of intracellular binding of testosterone or dihydrotestosterone (DHT) can be achieved by the administration of antiandrogen. Antiandrogens are defined as substances which compete for the androgen receptor, thereby inhibiting the action of androgens at their target sites. There are two types of antiandrogens, steroidal and non-steroidal. Steroidal antiandrogens have a dual mechanism of action. They compete with testosterone and DHT for the androgen receptor but also possess progesterone-like antigonadotrophic activity, leading to loss of sexual function and gynecomastia. Non-steroidal antiandrogens interact with the androgen receptor and block the intracellular effects of testosterone and DHT but without causing impotence (Graversen & Iversen 1996). Response rates in patients treated with antiandrogen
alone are comparable to orchidectomy in clinical trials (Goldenberg & Bruchovsky 1991; Geller 1991).

Inhibition of steroid synthesis can be accomplished by blocking enzymes which are responsible for testosterone synthesis. Monotherapy with these agents, although theoretically possible, is not popular because of the risk of adrenal insufficiency. It is reserved only for patients with disease progression or with hormone-refractory diseases (Crawford et al, 1987).

2.4.4 Other options

Other modalities available for controlling localised prostate cancer are cryosurgery, laser ablation of the prostate, and photodynamic therapy. In this section only the former two options will be reviewed whilst the application of PDT for prostate cancer is subject to comprehensive discussion in Chapter 4, 8, 9, and 10 of this thesis.

Cryotherapy was introduced by Gondor et al (1966) as a possible new approach for the treatment of prostate cancer on account of its minimal invasiveness and the ability to cause cell death by creating a hyperosmotic state within the cell, protein denaturation and membrane disruption. Possible advantages of cryosurgery include minimal blood loss, applicability for high risk patients, and less operation associated morbidity. Traditional cryosurgery needs perineal incision and carries some risks of urethrocystaneous and rectourethral fistula formation (14%), bladder neck obstruction (7%) and stress urinary incontinence (7%) (Flocks et al, 1972; Bonney et al, 1982). Although a favourable survival rate over that of radical prostatectomy was reported in patients treated with cryosurgery (Bonney et al, 1982), it has never gained wide acceptance in urological practice owing to the high complication rate.

Recent advances in imaging and cryoprobe technology has lead to a resurgence of interest. Onik et al (1993) reported a high negative biopsy rate (82.6%) in 55 patients evaluated 3 months after cryosurgery under transrectal ultrasound guidance. Complications included impotence (65%), rectourethral fistula (2.9%), perineal ecchymosis, penile oedema, and temporary ileus. Urinary incontinence was not observed in their series. Coogan and McKiel (1995) followed 87 patients receiving 95 cryosurgical procedures and reported that the median PSA level at 12 months was 0.55ng/ml (mean 1.73) with a 17% positive biopsy rate at 3 months. However, impotence was seen in 50% and urethral sloughing in 9%. The authors concluded that
cryoablation of the prostate appeared to be a useful technique for eradication of localised disease. The low percentage of positive biopsies after treatment was encouraging but the significance of the persistent elevation of PSA levels remained uncertain.

In contrast, Cox and Crawford (1995) found that over 50% of patients had at least one significant complication following cryosurgery of the prostate and claimed that it could be associated with substantial morbidity and should only be performed under careful controlled circumstances. Clearly, cryosurgery, like PDT, is an investigational procedure for which therapeutic effectiveness, durability and long term complications remain uncertain requiring further stringent assessment.

Thermal laser treatment of prostate cancer was initiated by the Norwegian group (Sander et al., 1982). In the initial 5 patients who had been treated with high power YAG laser (7,000-13,000 J) 4-6 weeks after extended TUR, no sexual dysfunction, perforation or other major complications were found. The authors, however, did not report the therapeutic effect of these combinations. With more patients recruited, the same group (Sander & Beisland 1984) followed-up 16 patients for 3-22 months after treatment. Using DRE as an indicator, only one failed laser therapy whilst the other 15 had no sign of disease. With encouraging short term results and the lack of complications, they advocated that laser treatment combined with transrectal resection was a possible approach that deserved further attention. Their most updated report in a series of 63 patients (T1: 36, T2: 27) showed that the actuarial disease-free survival was 98% at one year and 80% in the 2-4 year period. There were no instances of impotence or incontinence (Beisland & Sander 1986). Using a similar technique, McNicholas et al. (1988) reported a disease free rate of 88.2% and with a minimum associated side effect in 17 patients followed up for 1-14 months (mean 6). Although the concept of combining high power laser with the traditional TUR for treating localised prostate cancer is attractive and theoretically possible, the extent and tissue response of heat dissipation on the prostate and adjacent structures, the completeness of glandular destruction, the durability of treatment effect, and the safety of curative intention with laser ablation remain unsettled.
# Chapter 3

## Photodynamic Therapy: History and Nature

<table>
<thead>
<tr>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3.1</strong> Introduction</td>
</tr>
<tr>
<td><strong>3.2</strong> History of Photodynamic Therapy</td>
</tr>
<tr>
<td><strong>3.3</strong> Principles of Photodynamic Therapy</td>
</tr>
<tr>
<td><strong>3.3.1</strong> Mechanism of action</td>
</tr>
<tr>
<td><strong>3.3.1.1</strong> In vitro effect</td>
</tr>
<tr>
<td>(a) Membrane damage</td>
</tr>
<tr>
<td>(b) Mitochondria damage</td>
</tr>
<tr>
<td>(c) DNA damage</td>
</tr>
<tr>
<td>(d) Apoptosis</td>
</tr>
<tr>
<td><strong>3.3.1.2</strong> In vivo vascular damage</td>
</tr>
<tr>
<td><strong>3.3.2</strong> Lasers and light delivery systems</td>
</tr>
<tr>
<td><strong>3.3.2.1</strong> Basic principles</td>
</tr>
<tr>
<td><strong>3.3.2.2</strong> Lasers for photodynamic therapy</td>
</tr>
<tr>
<td><strong>3.3.2.3</strong> Light coupling system</td>
</tr>
<tr>
<td><strong>3.3.3</strong> Photosensitisers</td>
</tr>
<tr>
<td><strong>3.3.3.1</strong> Haematoporphyrin derivative (HpD) and Dihematoxylin ether (DHE, Photofrin)</td>
</tr>
<tr>
<td><strong>3.3.3.2</strong> 5-aminolaevulinic acid (ALA)</td>
</tr>
<tr>
<td><strong>3.3.3.3</strong> Tetra-hydroxyphenyl-chlorin (THPC)</td>
</tr>
<tr>
<td><strong>3.3.3.4</strong> Aluminium sulphonated phthaloxyanine (AlSPc)</td>
</tr>
</tbody>
</table>
3.1 INTRODUCTION

Photodynamic therapy (PDT) is a technique based on the interaction of a dye (photosensitiser) with the incident light of appropriate wavelength in the presence of tissue oxygen (Foote 1976). The cytotoxicity of PDT is attributable to photochemical reaction, a process involving the formation of singlet oxygen (${}^{1}\text{O}_2$) or, to a lesser degree, generation of free radicals that finally leads to tissue destruction by direct cell killing or indirect effects. Dihaematoporphyrin ester/ether (DHE or Photofrin) is the most popular photosensitiser currently being used for most clinical trials, and is the only agent approved by the Canadian government for clinical use (Larsen 1993). In December 1995, Photofrin was also approved by the FDA in the USA for advanced oesophageal cancer and by the Japanese government for early oesophageal and lung cancers. Over the past 20 years, PDT based on HpD and Photofrin, although remaining far from being satisfactory photosensitisers, has been tested to treat almost every type of malignancy. Some of the treatment outcomes look promising although most are anecdotal. The position of Photofrin based PDT in oncology remains to be verified with well designed prospective controlled clinical trials. Recent advances in medical physics, photochemistry and photobiology technology, have lead to the discovery of a series of new photosensitisers, and the development of more efficient laser instruments which all together, make PDT an ever better prospective option for future clinical applications.

This chapter will start with an overview of previous PDT history, followed by a comprehensive review of the principle of PDT, including the mechanism, the drug and the laser systems. Fluorescence imaging of tissue photosensitiser, although is becoming an integral part for most PDT related studies will not be discussed in the chapter. Instead, the basic principles of fluorescence microscopy and its attached charge-coupled device (CCD) camera will be reviewed in section 4.1.6 of Chapter 4.
3.2 HISTORY OF PHOTODYNAMIC THERAPY

The history of photodynamic therapy can be traced back to the turn of this century when Raab used acridine dye to kill paramecia in the presence of light (Raab 1990). Shortly thereafter, von Tappeiner and Jesionek (1903) applied this concept to treat skin cancer with topical application of eosin followed by sunlight exposure, and achieved some improvement in most cases. Subsequently, they also suggested that the photodynamic effects relied on the presence of tissue oxygen, although they were not able to prove it with a model. However, as a result of their outstanding studies, they are generally regarded as the pioneers leading PDT to the field of oncology. Shortly thereafter, with the enthusiasm of some scientists, a wide range of compounds were shown to have photochemotoxicity. Hausman (1911) persistently having focused on a substance derived from dry haem, had conducted a series of animal studies some results of which were encouraging, and had made substantial contributions to the eventual discovery of porphyrin-based photosensitisers. The initial crude extract from certain human and animal tumours, which emitted red fluorescence on woods lamp exposure was found to be a group of porphyrin compounds called haematoporphyrin (Hp) which was the transformed product of haem by a haemolytic bacterial infection associated with the tumours (Policard 1924). This agent was later reported to have selective or preferential retention in neoplastic tissue (Auler and Banzer 1942).

The discovery of Haematoporphyrin derivative (HpD) was partially incidental. In the 1950's when Schwartz and associates (1955) began to study porphyrin uptake by mouse tumours, they found that all commercial Hp preparations were very impure, and only 30-65% of the porphyrin included was actually haematoporphyrin. With partial purification, the haematoporphyrin-rich fractions were among the poorest localiser in tested tumours. On the contrary, they found that porphyrins in the normally discarded HCl residue, localised in tumours much better than the crystallised Hp (Dougherty et al, 1992). After a series of chemical purification processes, Lipson et al (1961) discovered an acetic-sulphuric acid porphyrin which later came to be named as HpD.

HpD was the most commonly used photosensitiser for experiments and clinical trials until it was replaced by a further purified compound which was thought to contain a combination of haematoporphyrin dimers linked with either ester or ether bonds: an agent now known as dihaematoporphyrin ether/ester (DHE) or Photofrin (Dougherty...
Since the first cancer patient who was treated with HpD by Diamond et al (1972) until Photofrin was approved for clinical use by the Canadian Government (Larsen 1993), there were thousands of patients treated with HpD or Photofrin, and a similar amount of publications relating to PDT including almost all types of solid cancers in man (Dougherty 1986). While the final analyses of the phase III trials are still pending, some preliminary reports on early malignancies have been published with favourable responses (Dougherty 1993). In the meantime, several second generation photosensitisers which are pure compounds and, are more potent, in terms of photochemical properties, became potential contenders of Photofrin. Among them aminolaevulinic acid (ALA), benzoporphyrin derivative mono acid (BPD), chlorin e6 aspartic acid (NPe6), meso-tetra-\( m \)-hydroxyphenyl)chlorin (mTHPC), and tin(II) etiopurpurin dichloride (SnET2) are the most promising, and have entered phase I/II clinical trials for a variety of malignancies. It is hoped that by the turn of this century, 100 years from the first observed PDT effect on paramecia (Raab 1900), PDT will stand a chance to develop into a standardised therapeutic option for a few malignancies.
3.3 PRINCIPLES OF PHOTODYNAMIC THERAPY

Photodynamic therapy requires the combined interactions of a photosensitiser, light of an appropriate wavelength, tissue oxygen and a tissue substrate that retains an adequate level of the photosensitiser. When the photosensitiser in the tissue absorbs light, some molecules are activated to a state having a higher energy (singlet state), which is very unstable and short-lived (lifetime usually in the order of nanosecond). The excited molecules may decay by any of three ways, by releasing heat, by emitting light, or by conversion to an intermediate energy level (triplet state) before resuming a more stable ground state. The photosensitiser molecules in the metastable triplet state react with tissue elements, either by producing hydroxyl and superoxide radicals (type I phototoxicity), or by transferring energy to molecular tissue oxygen to form singlet oxygen ($^{1}\text{O}_2$) via a type II phototoxicity mechanism which in turn, causes cellular destruction through the effect of functional or structural alterations of cellular components by photo-oxidation (Dougherty et al, 1983). The basic principle of PDT are illustrated in Figure 3.1.

3.3.1 Mechanism of Action

The role of singlet oxygen ($^{1}\text{O}_2$ type II reaction) in the pathogenesis of phototoxicity has been firmly established, although the type I photo-oxidation may also play a small role (Weishaupt et al, 1976). The measurement of singlet oxygen ($^{1}\text{O}_2$) in vivo however, as a result of its rapid physical quenching and chemical reactions with the tissue, is currently unsuccessful. Nevertheless, there is indirect evidence supporting tissue $^{1}\text{O}_2$ as the most important species for cytotoxicity (Firey et al, 1988; Patterson et al, 1990). It is clear that nearly all cellular constituents are able to react with singlet oxygen ($^{1}\text{O}_2$). The three main subcellular constituents susceptible for photodamage following PDT are proteins, lipids and nucleic acids. Structural change of the constituent can lead to secondary cross-link formation or other biochemical reactions amongst various components, causing functional or structural breakdown of cellular organelles and thus resulting in cell death. The PDT induced in vitro and in vivo effects on cellular or subcellular ultrastructures, are described in the following sections.
Figure 3.1: Simplified diagram of photodynamic action. Type II (singlet oxygen) mediated pathway is the principal mechanism accounting for PDT tissue damage.

3.3.1.1 In vitro effect
(a) Membrane damage
The plasma membrane is the primary site of action of most photosensitisers. The main constituents of the cell membrane are phospholipids and proteins. However, there is sufficient evidence that suggests interruption of the integrity of the membrane by photo-oxidation is mostly mediated by protein damage rather than by lipid peroxidation (Ceckler et al, 1986). The amino acids tryptophan, cysteine and histidine in the membrane are very sensitive to photosensitisation with porphyrins, chlorins, purpurins or methylene blue. Following light illumination, impaired amino acid transportation, increased permeability and formation of multiple blebs were characteristic of plasma membrane dysfunction which preceded cell lysis (Moan et al, 1979; Kessel 1986b). Other subcellular membranes, including those of the nucleus, mitochondria, lysosomes, Golgi apparatus, and endoplasmic reticulum, in addition to the plasma membrane, may be at risk for photodynamic damage (Pass 1993).
(b) Mitochondria damage

Despite the focus on the plasma membrane as the principal target for porphyrin induced photodestruction, mitochondrial damage, not relating to disruption of mitochondrial membrane, is another possible mechanism of cell killing for most photosensitisers (Rosenthal & Ben-Hur 1989). Specific mitochondrial damage after PDT includes inhibition of oxidative phosphorylation with diminished transmembrane electron transport enzyme cytochrome c oxidase activity (Hilf et al., 1984; Moan 1986), and a reduction in cellular adenosine triphosphate (ATP) levels (Hilf et al., 1986). This mechanism of action is thought to be of secondary significance with porphyrin, chlorin and phthalocyanine induced in vitro effects, but is of vital importance for ALA induced PpIX photosensitivity as the mitochondria seem to be the principal PDT target, at least in the early stage after sensitisation (Inunuma et al., 1994).

(c) DNA damage

Nuclear localisation of photosensitiser is not common in cells. However, in view of the close vicinity of DNA loops to the nuclear membrane, which contains most of photosensitiser, DNA damage is a reasonable expectation following photodynamic therapy. The synthesis of DNA is very sensitive to photodynamic therapy. Low doses of porphyrins and light, which have a sublethal cellular effect, may be able however, to reduce the DNA synthetic capability to almost zero (Moan et al., 1983).

HpD based PDT is generally regarded as non-mutagenic even though the production of DNA strand breaks (Fiel et al., 1981), and chromosome aberrations (Evensen & Moan 1982) have been observed in the presence of minute amount of porphyrins in the nucleus. However, DNA-DNA cross-links have never been found (Dubbelman et al., 1983). In comparison with radiation effects, HpD-PDT induced DNA and chromosome damages are significantly less (Moan et al., 1980).

Phthalocyanine, on the other hand, has been reported to result in single strand breaks and protein-DNA cross-linkage in vitro after light illumination, their importance relative to other damage in the mechanism of cell killing remains to be defined however (Ben-Hur et al., 1987a; Ramakrishnan et al., 1988). The observation of mutagenic effects in a few cell lines (Evans et al., 1989) has raised great concerns for its safety for treatment. However, the mutagenic potential of phthalocyanines, as evaluated by resistance to 6-thioguanine and to ouabain, is small (Rosenthal 1991).
Apoptosis, a physiological mode of cell death related to internucleosomal degradation of DNA, occurring during normal tissue modelling, metamorphosis, and other developmental changes, is another possible mechanism for PDT induced cell destruction (Agarwal et al., 1991). This programmed cell death process is characterised by cytoplasmic condensation, dilatation of the endoplasmic reticulum, cell shrinkage, perinuclear membrane chromatin condensation, and endonucleolysis of DNA between nucleosomes. The dying cell implodes into membrane-delineated fragments called apoptotic bodies which are phagocytosed by macrophages or neighbouring cells. Typically, apoptotic cells do not induce an inflammatory reaction, even when present in large numbers, due to the maintenance of the osmotic gradient of the cells which avoids spillage of intracellular contents (Wyllie et al., 1980; Arends et al., 1990). Apoptosis requires the active participation of the dying cell, is controlled by growth factor levels, and depends on the expression of regulatory genes. This mode of cell death is observed with HpD, Photofrin and a series of new phthalocyanines in LY-R lymphoma cells (He et al., 1994) and other cell lines (Oleinick et al., 1992; Nyamekye et al., 1994), and in in vivo tumour models (Oseroff 1993; Zaidi et al., 1993).

Although many details of the cell death mechanism of apoptosis remain obscure, it is proposed that nuclear changes are brought about by activation of an endogenous calcium-magnesium sensitive endonuclease, and this results in the cleavage of chromatin between nucleosomes, reducing the DNA of apoptotic cells to a series of fragments containing multiples of 180-200 base pairs (Arends et al., 1990). It is now clear that the induction of apoptosis by PDT is dependent on the cell types, possible growth conditions, the photosensitiser, and the overall PDT dose. The absence of inflammatory responses of apoptosis induced cell death makes it a possible measure to prevent intimal hyperplasia after angioplasty (LaMuraglia et al., 1994), and may have important implications for other clinical applications.

3.3.1.2 In vivo vascular damage
There is convincing evidence that vascular damage is one of the principal mechanisms accounting for porphyrin-based PDT effects in vivo (Selman et al., 1984; Star et al., 1986; Reed et al., 1989), although ALA might be a possible exception which has more direct cytotoxic than vascular-mediated effect (Kennedy & Pottier 1992). Since most tumour vessels are derived from venous structure, and are not supported adequately by the interstitium, any structural changes of the lining
endothelium are likely to result in total stasis of blood flow and tumour destruction. The endothelia which are thin layered in arrangement, are particularly vulnerable to deposition of photosensitiser, and hence may be a prime target for PDT. Early blood flow stasis in both arterioles and venules occurs with arteriolar vasoconstriction, venule thrombosis, and perivascular interstitial oedema. Clotting factors, vasoactive intermediates, and prostaglandins (PGE2, PGI2, and thromboxane) release are later events contributing to further shutdown of the tumour vasculature (Fingar et al, 1990; Ben-Hur & Orenstein 1991). Nelson et al (1988) suggested that destruction of the subendothelial collagens and other connective elements in the tumour capillary wall might be the cause leading to rapid tumour necrosis with porphyrin, chlorin, and phthalocyanine. Hilf (1992) questioned the role of vascular shutdown as the primary cause of PDT induced cell death by pointing out that the improved tumour destruction following light irradiation at lower fluence rates over a longer exposure time was likely the result of diminished early vascular damage which provided continuing oxygenation of tissues for singlet oxygen ($^1\text{O}_2$) production. It seems reasonable to suggest, even at this pro vascular effect stage, that both direct cellular and indirect vascular damage are essential components for tumour destruction with PDT. The contribution of each of these mechanisms to tumour cell demise, on the basis of individual photosensitisers, remains to be quantitated.

3.3.2 Lasers and light delivery systems

Lasers are a contemporary technology which was proposed by Einstein in 1917, developed as an extension of the quantum theory. Nearly 40 years after Einsteins' "thought experiments", Townes and his colleagues at Columbia University built the first device to exploit this phenomenon. This device, then called a "Maser", which is an acronym for microwave amplification by stimulated emission of radiation, emitted energy at microwave level (Gordon et al, 1955). The first genuine laser, however, was produced by Maiman (1960) who used a cylinder of pink ruby as the active medium to emit deep red visible light at 694 nm. Immediately after this invention, ophthalmologists claimed satisfactory results treating retinal detachment with ruby laser (Kapany et al, 1963). These preliminary results mark the beginning, in both physical and medical fields, of a Laser Era of the 20th Century.

3.3.2.1 Basic principles

Light is an essential part of photodynamic therapy which requires the excitation of a specific wavelength depending on the absorption spectrum of the photosensitiser
used. Although beam from an incandescent light source is applicable for some cases, state-of-the-art PDT uses a laser as the source of light. Laser is the acronym of Light Amplification by the Stimulated Emission of Radiation. When an atom or molecule absorbs energy, electrons move into higher orbits, but soon return to their resting energy level (ground state) by emitting a photon. The energy of the photon is simply the difference in energy between the two levels involved. Energy determines the wavelength, which is the colour of the light. If the decay of different atoms occur at random, many energy levels are involved and the light is emitted out of phase (incoherent) and in different directions (uncollimated). This process is called "spontaneous emission". If, however, after energy supply the photon from an initial spontaneous decay is controlled to strike an already excited atom in its path to emit an identical photon, in phase (coherent), of the same colour (monochromatic), and travelling in the same direction (collimation), this is known as "stimulated emission" which constitutes the fundamental principle underlying the production of laser.

Theoretically, a substance has the potential to become a lasering medium if it can be made to have more atoms or molecules in a high energy state than in its resting state. Different media emit characteristic colours of light at different pumping requirements. Common lasering mediums include solid-state materials, gases, liquids (dye), and semiconductors (Svelto 1989). Lasers of particular medical importance are carbon dioxide (CO2), argon, Nd-YAG, krypton, Ho-YAG, alexandrite, metal vapour pumped dye, and semiconductors. Since high power lasers, the purpose of which is to coagulate or vaporise tissue (thermal effect), or to disintegrate urinary stones (photomechanical effect), are not the main topic of this thesis, I discuss here only those used for photodynamic therapy (photochemical effect).

3.3.2.2 Lasers for photodynamic therapy
All photosensitisers with the potential for clinical application have an absorption spectrum reaching into the red or near infrared part (600-750 nm) of the visible spectrum as tissue penetration of light in this range is much better than in others (Moan 1990). To cover this range of wavelengths, the most currently available medical lasers which emit light at wavelengths longer than 750 nm (Nd-YAG, Ho-YAG and CO2) are not applicable as a light source. Dye lasers use various liquid organic dyes as the lasering medium, if pumped by another light source (or laser), can output light of the desired wavelength. In theory, any laser can be used as the pumping source as long as it produces an output wavelength shorter than that desired from the dye, though the efficiency of power coupling is greatly reduced if the two
are too disimilar. In recent years, argon-pumped dye lasers have become widely used as a convenient light source for HpD based PDT. Continuous coherent light at the wavelength of 630 nm, and at a power of 1-5 W can be obtained from an argon laser pumping rhodamine dye laser. Another alternative is the copper vapour laser pumped rhodamine B dye laser which wavelength is tuneable over a range of 30-50 nm (620-650 nm), and which has been used extensively in PDT related research. Such a pulsed laser with a pulse repetition rate of 10kHz, and pulse duration of 30-50 nanoseconds, was shown to be equivalent to a continuous wave laser in terms of PDT biological responses (Barr et al, 1989). Copper vapour pumped dye lasers are more efficient, have a higher power of output, and are more reliable than argon-pumped dye lasers. Despite the fixed wavelength of 628 nm produced by a pulsed gold vapour laser, it was another popular choice for HpD or Photofrin mediated PDT in clinical practice.

More recently, the frequency doubled potassium titanyl phosphate (KTP) Nd:YAG laser is becoming an important and attractive light system for PDT. Similar to that of a copper vapour laser, the 532 nm light produced by the KTP is an even more reliable and efficient source than the former for pumping the dye laser. The dye module can be easily changed to fit the absorption spectrum of different photosensitisers, making it a cost-effective laser system which provides two different wavelengths for interdisciplinary thermal use and an wavelength for PDT. Another possible light source with prospects are diode lasers which use semiconductor crystals to emit light with high power (for coagulation and vaporisation) at wavelength of 800 and 980 nm respectively. The compact size, lower price, and high laser output efficiency offer significant ergonomic and economic advantages, making them very attractive for office-based laser procedures. Recently, diode laser at a wavelength of 652nm and with enough power is available for PDT. However, the new trend of diode lasers with shorter wavelengths needs to be continued and the ability to remain optical stability is necessary if they are to be used for future PDT application with various photosensitisers.

3.3.2.3 Light coupling system
Regardless of the type of laser used, light generated by a laser is usually coupled to an optical fibre, permitting access to body surface or viscera through the working channel of endoscopy. Most optical fibres used in PDT are made from fused silica glass or quartz, and are 200-1200μm in diameter. The transmission efficiency within the length of the fibre is excellent, and the energy loss is generally less than 1%. However, substantial energy loss occurs in coupling systems, which is up to 25% in
most instances (Milam 1994). Light distribution can be adapted to the target tissue configuration by modification of the optic fibre (Benson 1985; Carruth & McKenzie 1986), use of multiple fibres (Bolin et al, 1987), or with various light-scattering devices and diffusion techniques (Baghdassarian et al, 1985; Marijnissen et al, 1989). These types of modification allow both external and interstitial treatment of surface, intraluminal, and intracavitary lesions where contours can be quite diverse, causing difficulty for direct light irradiation to some target areas.

### 3.3.3 Photosensitisers

The most important common feature of all photosensitisers is the ability that these compounds can transform absorbed light energy (photo-excitation) into chemical energy. In general, an ideal photosensitiser must have the following theoretical characteristics in order to be maximally useful: it must be non-toxic, biochemically stable for long periods of time, and retained selectively in the target tissue relative to normal surrounding organs. Besides, it should be efficient photochemically (high triplet quantum yield), and absorb in the red or near infrared portion of the spectrum (Moan 1990). So far, no such ideal photosensitiser has yet existed.

Several classes of agents have shown promise as clinically useful photosensitisers. These include 5-aminolaevulinic acid (ALA), phthalocyanine and its derivatives, chlorins, purpurins, and porphyrin-based compounds. Each has advantages and disadvantages. ALA is promising for superficial lesions, but seems less useful for larger tumours (Chapter 9). Phthalocyanines can be prepared easily in pure form and in large quantity, and have fulfilled the most criteria of being an ideal photosensitiser (MacRobert et al, 1989). There are a few members in the chlorin family currently undergoing extensive research. They can be easily synthesised from chlorophyll or by reduction from porphyrins. In general, the shift of absorption peak to over 650 nm in the red band, and the reduced skin photosensitivity are major advantages over Photofrin as tissue penetration is greatly improved (Nelson et al, 1987). Higher singlet oxygen ($^{1}O_2$) quantum yield than that of HpD is another advantage (Spikes 1990). Purpurins are modified chlorins having many of the sensitising and localising properties of porphyrins, and are readily synthesised in pure form although in limited quantities (Morgan et al, 1988). In this section, only the photosensitiser which related to the experimental projects of this thesis will be discussed.
3.3.3.1 **Haematoporphyrin derivative (HpD) and Dihaematoporphyrin ester/ether (DHE, Photofrin)**

Although a wide range of substances, including a number of porphyrins have been investigated as photosensitisers, only HpD and, more recently, DHE (Photofrin), have been proven to be suitable for clinical use, although it is still far from an ideal photosensitiser. In fact, Photofrin is the only photosensitising agent so far approved by a government for clinical application for the prophylaxis of recurrence of bladder papillary tumour (Larsen 1993).

HpD is a complex mixture of porphyrin monomers and oligomers, the composition of which is sensitive to the conditions of its preparation, and therefore, it is difficult to establish a reliable dose-response relationship (Bonnett & Berenbaum 1989). Further purification of HpD with alkaline, produces a fraction containing dimers (DHE) or oligomers with ether, ester, or carbon-carbon linkages, this fraction are the most active components of the HpD preparation, which is now commercially available under the name of Photofrin. There remain some arguments as to the exact structure of DHE, some favour the ether structure (Dougherty 1984); others favour the ester structure (Kessel *et al.*, 1986a), but most agree that the active components of HpD are aggregates of some kind in aqueous solution, and a time related conversion from an ester to an ether is possible (Berenbaum *et al.*, 1982; Byrne *et al.*, 1987). The proposed structure of HpD is illustrated in Figure 3.2.

![Chemical structure of haematoporphyrin derivative (HpD)](image)

**Figure 3.2:** Chemical structure of haematoporphyrin derivative (HpD)

Pharmacokinetic studies and tissue distribution with $^{14}$C-radiolabeled Photofrin in murine demonstrate that 1% of the total Photofrin remains in the circulation 24 hours
after injection and 65% is excreted in the faeces by 192 hours (Bellnier et al, 1989). Nevertheless, traces of the photosensitiser (0.04%) remain detectable 75 days after injection. Once in the circulation, porphyrins concentrate in normal as well as neoplastic tissue. There is a predilection for accumulation within tissue containing significant reticuloendothelial components (Henderson & Dougherty 1992). Peak porphyrin tissue levels usually occur 24-48 hours after administration. Differences in porphyrin uptake between transplantable, subcutaneous tumours in mice and tumour-surrounding skin are small, usually in the ratio of 1-2:1. Prolonged retention of Photofrin in tissues is not unusual. Elevated porphyrin levels in the liver and kidney may result from metabolism rather than retention of Photofrin (Gomer & Dougherty 1979). Although the exact localisation of porphyrins in the stroma is unclear, Bugelski et al (1981) found that more porphyrins, in the ratio of 5 to 1, were distributed in the stroma rather than in the parenchymal tissue 24 hours after sensitisation. The factors that govern Photofrin distribution among the various tissue components have not yet been fully elucidated, but the binding capacity of the photosensitiser to plasma protein may be an important determinant (Jori 1984).

Since porphyrin-sensitised photodynamic tissue destruction is thought to be mediated by singlet oxygen (\(^{1}\text{O}_2\)), sensitive targets in the cells are most commonly those containing high concentration of porphyrins (Moan 1986). Detection of distribution of porphyrins in the subcellular structures can be achieved by cell homogenisation followed by differential ultracentrifugation, electron microscopic evaluation, or, fluorescence microscopy (Moan et al, 1989). Cellular uptake of the components of HpD and Photofrin increases with increasing lipophilicity of the components, and as a result, plasma membrane and mitochondria are damaged at an early stage (Moan 1984). After the initial binding with the plasma membrane, porphyrins progressed in time by migration to internal cellular components (Kessel 1986b). Besides the membranes of the various organelles which are particularly vulnerable to photodynamic damages induced by Photofrin, other subcellular structures such as enzymes, lysosomes and DNA are possible targets (Dubbelman et al, 1992; Hilf 1992).

A review of the literature revealed that porphyrin based photodynamic therapy has been used to treat almost every type of malignancy. As HpD and Photofrin have been the only photosensitisers available for clinical trials in the past 15 years, a comprehensive review of clinical studies on various cancer or precancerous diseases with HpD or Photofrin based PDT is discussed in section 4.3 of Chapter 4.
3.3.3.2 5-Aminolaevulinic Acid

A recent advance in photochemistry has been the development and extensive pharmacokinetic studies of the compound called 5-aminolaevulinic acid (ALA). This is a natural occurring and endogenous intermediate substance in the biosynthetic pathway for haem in living cells (Kennedy & Pottier 1992). In most mammalian cells, ALA is synthesised from glycine and succinyl Co-A under the catalyse of ALA synthetase which is located mostly in the matrix mitochondria (Ades 1990). In the chain reactions, the synthesis of ALA is the first committed step which in turn controls the activity of ALA synthetase through negatively feedback (Hindmarsh 1986). Since ALA is the intermediate substance immediately after a rate-limiting step, endogenous or exogenous over saturation of the pathway with ALA results in overproduction of substrate(s) prior to the second rate limiting step, which in the case of haem biosynthesis, is the step converting protoporphyrin IX (PpIX) to haem. An excess of exogenous ALA therefore leads to intracellular accumulation of protoporphyrin IX which is both fluorescent and a potent photosensitiser (Pottier et al, 1986) (Figure 3.3). Other than PpIX, there are 4 photosensitising intermediates produced in the chain reactions. Uroporphyrin I and III are very soluble in water, have a short half-lifes in the body, and are excreted primarily via the kidney. Coproporphyrins are less water soluble and have a longer half-lifes than uroporphyrins, are readily excreted via the bile to faeces, and to a lesser extent, via the urinary tract (Kennedy et al, 1992; Kennedy & Pottier 1992). PpIX is slightly water soluble, has a high affinity for membrane lipids, and consequently is expected to be retained by most tissues for photosensitisation.
**Figure 3.3:** Simplified biosynthetic pathway for haem. Fluorescent and photoactive components are enclosed in rectangles.
Although, in theory all nucleated cells are capable of synthesising PpIX, there are great variations in the amount of PpIX formed in various tissue structures when ALA is given in excess. Divaris and associates demonstrated that following administration of ALA to mice the sebaceous glands and hair follicles had higher PpIX fluorescence than that of epidermis and dermis of the skin (Divaris et al, 1990). The same group also showed in vivo a greater PpIX build-up in the urothelium and endometrium than in the underlying muscle layer, and suggested that cells of ectodermal origin produced more PpIX than those of mesodermal origin (Kennedy & Pottier 1992). Since haem containing enzymes are directly involved in the process of oxidative phosphorylation in the mitochondria, the varied tissue specificity for the biosynthesis of PpIX, may suggest that cells with rapid turnover, as a result of a greater energy requirement, produced more PpIX (Rebeiz et al, 1992). However, Inuma et al (1994) failed to demonstrate a perfect correlation between cell doubling time and the intensity of PpIX accumulation in cell lines. They concluded that cellular PpIX build-up is a dynamic process that is determined by both the efflux of PpIX from the cells and enzyme activities in the haem biosynthesis pathway. In addition, the degree of cellular PpIX content does not seem to correlate to the degree of differentiation of the tumour from which the cell lines were derived (Steinbach et al, 1995).

Despite the fact that PpIX levels are not always in parallel with tissue destruction after light illumination, a higher photosensitiser concentration in the cells usually gives a better chance for effective cytotoxicity. The cytotoxicity induced by ALA based PDT was proposed to be mediated by destruction of mitochondria (Kennedy & Pottier 1992; Inuma et al, 1994). Plasma membranes were also suggested as a potential cytotoxic target 24 hours after ALA incubation in vitro (Szeimies et al, 1995). It is reasonable to assume that PpIX is originally produced in the mitochondria matrix, diffusing to the mitochondrial membrane over the next few hours before further migration to cytoplasm and the plasma membrane by 24 hours. Attempts have been made to increase intracellular conversion of ALA to PpIX as a means for facilitating PDT effect after light exposure. One possible mechanism to achieve this is the administration of iron chelator at the same time of exogenous ALA to remove tissue iron which is essential for the conversion of PpIX to haem. By retarding the conversion of PpIX to haem which is catalysed by ferrochelatase in the presence of iron, tissue PpIX is efficiently elevated and photosensitisation process accelerated with desferrioxamine (Ortel et al, 1992; Inuma et al, 1994), dimethylsulfoxide-ethylenediaminetetraacetic acid (Peng et al, 1995), and with oral iron chelators (Chapter 7).
In animal studies of PpIX biodistribution after ALA sensitisation, there is usually a greater PpIX buildup in the epithelial lining of skin (Divaris et al, 1990), larynx (Kleemann et al, 1996), stomach (Loh et al, 1993b), uterine endothelium (Yang et al, 1993), bladder (Chapter 5), and colon (Bedwell et al, 1992). The ALA induced PDT effect in the normal tissue is usually of the depth of 1-2 mm (Bedwell et al, 1992; Chapter 5), although exceptions which might be as deep as 8-10mm are possible (Regula et al, 1994; Johnson et al, 1995; Lofgren et al, 1995). Factors influencing the depth of PDT lesions produced by ALA induced PpIX photosensitisation include wavelength of incident light (630 or 635 nm), route of ALA administration (intravenous, oral, or intraluminal), mode of administration (bolus or fractionation), species variation (mice, hamster, rat, or rabbit), organ specificity (skin, bladder, prostate, and pancreas), and biological specificity (normal or neoplastic).

Clinically ALA has been proved to be a useful agent for photodynamic treatment of a variety of malignant and precancerous lesions (Kennedy et al, 1990; Cairnduff et al, 1994). Kennedy et al (1990) reported total response rates of 98% in 80 basal cell carcinoma lesions treated by PDT with topical ALA; whereas Cairnduff and associates (1994) found an overall response rate of 89% for treating Bowen's disease, basal cell carcinoma and metastatic skin secondaries, although the metastatic nodules responded poorly. Other researchers find ALA induced PpIX a promising photosensitiser for gastrointestinal diseases (Regula et al, 1995, Mlkvy et al, 1995), oral cancers (Grant et al, 1993; Hopper et al, 1994), and for the diagnosis of bladder cancer (Kriegmair et al, 1996). For oral cancer treatment the result was a little disappointing as lesions are shallow (0.15-1.8mm, average 0.86mm) and confined to the epithelium. There are however, several advantages to using ALA induced PpIX photosensitisation. Firstly, ALA, PpIX, and other intermediates are rapidly eliminated from the body and hence the risk of skin photosensitivity is limited to day or two days (Kennedy & Pottier 1992; Grant et al, 1993). Secondly, after administrating ALA, PpIX is preferentially accumulated in the epithelial lining of the gastrointestinal or urinary tract and to a much lesser extent in the lamina propria and muscle layers (Loh et al, 1993a; Chapter 5, 6), and the ratio between the mucosa and muscle can be as high as 10:1 (Bedwell et al, 1992; Chapter 5). Thirdly, ALA seems to offer better selectivity in terms of uptake between tumour and normal tissue (Bedwell et al, 1992). Lastly, ALA can be given orally, providing an convenient route of administration for patients (Grant et al, 1993; Regula et al, 1995).
In conclusion, ALA is a promising new generation photosensitiser precursor which biosynthetic product, PpIX is a potent photosensitiser, and is rapidly eliminated from the body in 24-48 hours. It can be delivered via intravenous, oral or intravesical route without posing serious side effects. Although ALA mediated PDT lesions are usually superficial in the range of 1-2 mm in depth, this actually is an advantage if a shallow cytotoxic effect is the goal of treatment such as for carcinoma in situ or precancerous lesions of the gastrointestinal, or urinary tract and the oral cavity. Another potential advantage of ALA is the possibility of repeating PDT treatments on the same lesion in a short period of time without jeopardising the adjacent and underlying normal structures, or causing troublesome skin photosensitivity.

3.3.3.3 *meso-Tetra-(m-Hydroxyphenyl)Chlorin* (*mTHPC*)
Chlorin is a substance derived from the reduction of porphyrins. This minute structural modification results in a shift of absorption peak to the longer wavelength in the red spectrum. Further more, with addition of phenolic substitutions at the *meso* position of a porphyrin, the effects of photocytotoxic and tumour selectivity are enhanced. Hydroxylation of the phenyl substitution, either at the *ortho*, *para* or *meta* position, alters the hydrophilicity of the compound (Bonnett & Djelal 1993). Biological assay has demonstrated that the spatial configuration of the hydroxyl group at the *meta* position can give rise to an optimum cost-benefit effect (defined by tumour necrosis /muscle oedema) of the tissue following PDT with the various compounds being derived from HpD (Bonnett & Berenbaum 1989). It is a pure chemical agent with an absorption peak at 650 nm allowing deeper tissue penetration than the red light at 630nm used with Photofrin, and is a potent photosensitiser that has been reported as having better tumour selectivity, and higher triplet state quantum yield (Berenbaum *et al.*, 1993). The chemical structure of mTHPC is illustrated in Figure 3.4.
Figure 3.4: Chemical structure of 5,10,15,20-tetra(m-hydroxyphenyl)chlorin

*In vitro* pharmacokinetic study with HT29 human colon adenocarcinoma cell line revealed that the cellular uptake kinetics of mTHPC reached a plateau 12 hours after incubation. Fluorescence microscopy showed mTHPC diffusely distributed in the cytoplasm, and to a lesser extent (50%), in the nuclear area. Following light illumination at 650 nm, the maximum photodynamic cytotoxicity was observed at 48 hours. On a molar basis, mTHPC is 50-time more active than HpD (Rezzoug *et al*, 1995). Plasma pharmacokinetics in the human and three other animal models demonstrated that mTHPC drug concentration was directly proportional to the injected dose and decayed single exponentially to levels close to the baseline after 8 days regardless of the injected dose. The half-life of mTHPC in the human was twice as long as in the rabbit, and the plasma drug concentration correlated closely to that of skin, suggesting a potential predictor role of the former in defining a light dose that would be deemed safe for healthy tissue. It is suggested that the more extensive network of capillaries in man can retain a higher level of mTHPC than would be expected from simple weight or surface area considerations (Ronn *et al*, 1995).

Preliminary studies on the selectivity of mTHPC between malignant mesothelioma and normal tissues showed that this photosensitiser had a preferential uptake in
tumour tissue with up to a ratio of 15 to 1 (Braichotte et al, 1992). At a light dose of 10J/cm², and 48 hours after sensitisation of 0.3 mg/kg mTHPC, tumour infarction as deep as 10 mm was induced in 4 patients with malignant mesothelioma of the pleura (Ris et al, 1991). Skin photosensitivity was mild, dose dependent and occurred 3-10 days after administration of mTHPC. The same group claimed an even better PDT effect on nude mice bearing human malignant mesothelioma xenografts with a drug-light interval of 72 hours (Ris et al, 1993). Based on the available animal experiments and preliminary clinical data, phase I trials on the treatment of bronchial, prostate, skin, laryngeal, and nasopharyngeal cancers was initiated in Sweden (Abramson et al, 1994). The University College London Hospital has been approved by the Ethical Committee of the UCL to treat prostate cancer patients who had failed their definitive irradiation with mTHPC based PDT as an alternative remedy for controlling locally advanced prostate cancer. Meso-5,10,15,20-tetra-(m-hydroxyphenyl)chlorin, is a potent drug which is becoming a rising star among the second generation photosensitisers for future clinical applications.

3.3.3.4 Aluminium sulphonated phthalocyanine (AlSPc)
Observations that neoplastic tissues have affinity for phthalocyanines (Pc) were reported more than 30 years ago (Wrenn et al, 1951). However, the increase of interest using Pc as a photosensitiser for PDT was not started until 1985 when its efficiency and advantages over HpD was reported (Ben-Hur & Rosenthal 1985). Phthalocyanines are synthetic azaporphyrins containing a ring system made up of 4 isoindoles linked by nitrogen atoms (Figure 3.5). The Pc macrocycle is a weak photosensitiser if it doesn't have a metal ion incorporated into the central ring (Chan et al, 1987). Change of the chemical structure by inserting a metal ion in the central Pc skeleton and by substituting rings of the isoindole units with various chemical groups, will result in profound change of its photobiologic efficiency.
The singlet oxygen (\(^1\text{O}_2\)) quantum yield and lifetime of the excited triplet state, which are essential for photobiology, depend on the nature of the central metal ion. With a diamagnetic metal (Al, Zn, Ga) in the centre, the lifetime of triplet state is longer than an analogue compound containing a paramagnetic metal (Cu, Fe, Co, Cr) (Rosenthal & Ben-Hur 1989). Thus the triplet lifetime of the sulphonated derivatives of ZnSPc, AlSPc, CuSPc, and CrSPc is 245, 500, 0.065, and 0.02 \(\mu\)sec respectively (Darwent et al, 1982). Triplet energies for Pc range from 1.21 to 1.31 eV, sufficiently enough to produce singlet oxygen (\(^1\text{O}_2\)), which has an energy of 1 eV. Estimated \(^1\text{O}_2\) quantum yield of Pc with diamagnetic metal ion is in the range of 0.18-0.62, whereas it is undetectable in those with paramagnetic ions (Rosenthal et al, 1986).

Another important photochemical property of the Pc is the tendency to aggregate in aqueous solution which is strongly influenced by the type and degree of substitution in the isoindole rings. Aggregation is reflected spectrophotochemically by a shift of the Q band absorption peak to shorter wavelengths. The dimerised aggregate is photochemically inactive because of greatly enhanced rate of singlet state deactivation (Darwent et al, 1982). Disaggregation can be accomplished by added detergents or organic solvent. Alternatively, introduction of sulphonate groups in the ring substitution will render Pc more soluble in water and reduce aggregation. Decreasing the number of sulphonate substituents result in increased aggregation, lipophilicity, and cellular uptake (van Lier & Spikes 1989). In biological system, aluminium mono- or di-sulphonated Pc (AlSPc) despite a tendency to aggregate,
shows a uniform distribution of fluorescence in the cytoplasm, and with an increased phototoxicity in vitro (Paquette et al, 1988).

Phthalocyanines tend to accumulate in membrane fractions and following photodynamic treatment, morphological changes in membrane organelles have been observed. This leads to the suggestion that the plasma membrane, microsomes and mitochondria are the major sites of photodynamic action on cells in vitro (Ben-Hur et al, 1987b; Rosenthal 1991). The molecular basis for the breakdown of membrane structure after PDT is thought to be relevant to oxidation of some amino acid (Deuticke et al, 1989), or inhibition of normal enzyme activity (Robinson et al, 1987). However, there is also evidence that hydrophilic dyes, such as tri- or tetra-sulphonated Pc, appeared to have a tendency to localise in lysosomes; whereas lipophilic or hydrophobic preparations like mono- or di-sulphonated Pc, tend to accumulate in the membrane structure of the organelles (Moan et al, 1992). In view of the ambiguity of the experimental results, it is probable that the actual targets and mechanisms of Pc-mediated cell destruction may be cell type and photosensitiser specific, and involve a series of reactions rather than a single biochemical alteration. Further investigations on the molecular and biochemical basis are warranted to define the nature of cell killing.

Although phthalocyanines are not seen to localise in the nucleus as shown by fluorescence microscopy study, the concern of DNA damage is raised as a result of observed DNA-protein cross-links (Ramakrishnan et al, 1988). In one strain of mouse lymphoma cells, the photodynamic action of chloroaluminium Pc was proved mutagenic at the thymidine kinase locus (Evans et al, 1989). Moreover, Zinc-Pc was demonstrated to be slightly mutagenic in herpes virus (Lytle et al, 1989). However, the discovery of DNA changes and probable mutagenicity in vitro after PDT with some derivatives of Pc can not be readily interpreted into the fact that AlSPc is potentially mutagenic in vivo and whether this mutagenic potential can be expressed in transforming normal cells into cancer has yet to be studied.

In the tumour model, except for the malignant glioma, aluminium sulphonated phthalocyanine (AlSPc) generally retains selectively in the cancerous cell than the normal tissue with a ratio of 2-3 to 1 (Tralau et al, 1987a). In the bladder, AlSPc accumulates preferentially in the mucosa 24 hours after intravenous sensitisation. However the window for selective destruction of urothelium but without damaging the underlying structures, as with that of HpD, is fairly narrow (Pope & Bown 1991).
Photodynamic efficacy and the physico-chemical characteristics of di- and tetra-
sulphonated phthalocyanines were studied by Chatlani et al (1991). They found that
the lipophilic disulphonated derivative peaked and cleared up more rapidly than the
more hydrophilic tetrakisulphonated compound in the rat colon. AlS2Pc is also a more
effective photosensitiser than HpD for producing photodynamic necrosis in the liver
(Bown et al, 1986) and in a subcutaneous fibrosarcoma tumour model (Tralau et al,
1987b). The mechanism accounting for in vivo tissue destruction, like HpD, is
believed to be mediated through shutdown of microcirculation as vascular collapse
with decreased blood flow and stasis have been observed in tumour models (Selman
et al, 1986; Stern et al, 1990). Shortened skin photosensitivity caused by AlSPc,
particular the di-sulphonated derivative which lasts for 2 weeks, is an apparent
advantage over skin photosensitivity with HpD which persists for 2 months (Tralau et
al, 1989).

In this chapter I have reviewed the history, principle, and the mechanism of action of
photodynamic therapy. The basic principles of light and lasers are briefly discussed as
well. Lastly, the chemo-physical and biological properties of the most popular
photosensitisers, such as HpD, ALA, AlSPc, and THPC, are discussed in great detail.
It seems from the above review that the second generation photosensitisers are
becoming more popular and are more likely, than HpD and Photofrin, to be used as
sensitisers in the future. However, since HpD has been the only agent approved by the
FDA and other drug controlling Bureaus, the clinical experiences of HpD on a variety
of urological malignancies should not be ignored, and will be further discussed in
Chapter 4.
CHAPTER 4

PHOTODYNAMIC THERAPY IN UROLOGY: NOW AND THEN

4.1 An Over View of PDT for Bladder Cancer 106
4.1.1 Basic research 106
4.1.2 Route of photosensitiser administration 108
4.1.3 Clinical trials 109
  4.1.3.1 Focal photoradiation 110
  4.1.3.2 Integral and whole bladder photoradiation 112
  4.1.3.3 Prophylaxis of recurrence 114
4.1.4 Complications 114
4.1.5 Light distribution techniques 115
4.1.6 Methods for detecting fluorescence 116
  4.1.6.1 Fibreoptic in vivo detection with spectroscopy 117
  4.1.6.2 Ex vivo or in vitro fluorescence detection with CCD camera 117
  4.1.6.3 Fluorescence detection of bladder cancers 120
4.2 An oOer View of PDT for the Prostate 122
4.2.1 Basic research 122
4.2.2 clinical trials 125
4.3 Current Status of PDT in Urology and Aims of This Project 126
4.1 AN OVERVIEW OF PDT FOR BLADDER CANCER

Since 1976 when Kelly and Snell (1976) reported treating bladder cancer with PDT using HpD as photosensitiser, more than 5000 patients with various malignant lesions have been treated by this modality (Marcus & Dugan 1992). Most PDT treatments have been tried to treat malignancy or precancerous lesion in one of the 3 hollow organs: bladder, gastrointestinal tract, and the airways. So far, there are more than 20 English articles published presenting clinical treatment effects of PDT for papillary bladder cancer or carcinoma in situ in the past 20 years. Despite the steady clinical applications of PDT in several medical units, basic research on bladder cancer remained relatively scarce until 1990 when clinicians found it necessary to go back to the bench to solve the many problems encountered in clinical treatment. Basic as well as clinical literature on this aspect will be reviewed.

4.1.1 Basic research

A review of articles on basic studies of PDT for bladder cancer elicited scarcely enough fundamental research to justify the start of clinical trial on bladder cancer treatment with PDT. In the 1980's, most interest and enthusiasm for PDT of the bladder with HpD seems to have been derived from a single clinical paper which showed some evidence of tumour destruction in a papillary lesion in one patient after photodynamic therapy using poorly defined drug and light dosimetry in 1976 (Kelly & Snell). No further basic information concerning in vitro or in vivo bladder cancer studies was available before the Japanese and the United States groups published their clinical reports in 1983 (Tsuchiya et al; Hisazumi et al; Benson et al).

Before 1990, except for the research undertaken by Selman et al (1984; 1985a) and Reed et al (1989) to look at microcirculation in a bladder tumour model and the normal bladder after PDT, all other studies using experimental bladder or bladder tumour models were designed to evaluate photosensitiser pharmacokinetic or light dose efficacy unrelated to urology (Haas et al, 1986; Williams & Runge 1987; Morgan et al, 1990). Thereafter, some research papers appeared sporadically on various aspects of urological PDT. Pope and Bown (1991) evaluated biodistribution and PDT induced morphological and functional changes in rat bladder following phthalocyanine sensitisation. They concluded that 24 hours after intravenous injection of aluminium sulphonated phthalocyanine(AlSPc), bladder mucosa retained 4 times
as much fluorescence as the underlying muscle layer. PDT at a higher AlSPc dose (1.5mg/kg) usually resulted in irreversible bladder functional changes whereas a lower dose (0.5mg/kg) did not do so. Intravesical AlSPc did not seem to be a promising route of administration to optimise the PDT effect. In a similar study Stewart et al (1992) evaluated functional and histological changes of the mice bladder following Photofrin sensitisation. They suggested that the optimum light dose for mice bladder PDT was between 7.5 and 11.25 J/cm² at which the histological changes were reversible and functional disturbances kept to a minimum. In a separate study, Stewart and Oussoren (1993) noted that the bladder damage was Photofrin dose dependent when the light dose was fixed, and there was no difference in the urinary frequency index of mice in which drug-light intervals had been tested between 1 and 7 days. Damaged urothelium regenerated with re-epithelialisation in 4 weeks after PDT, and mild submucosal fibrosis was the only notable pathology in most of bladders examined at 10-52 weeks. The authors claimed that whole bladder PDT should be undertaken carefully, and particular attention should be paid to the total fluence which may be 3-4 times the incident dose due to internal reflection within the bladder. To further enhance the therapeutic effect, combination therapy of PDT in association with intravesical immunochemotherapy was found to retard tumour cell growth more efficiently than either of them alone treating C3H/He mice bearing MBT-2 tumours cell in the bladder (Cho et al, 1992). However, its effect on human bladder cancer remains to be defined.

Second generation photosensitisers have been a source of continuing interest for researchers to test their biological activities in living tissue. Photodynamic characteristics of Chlorin e6 (Bachor et al, 1991), copper benzochlorin (Selman et al, 1993), 9-acetoxy-tetra-n-propylporphycene (Aicher et al, 1994), and methylene blue (Keane et al, 1994) have been defined using bladder tumour cells in vitro or in vivo. Purpurins are a group of agents having a higher singlet oxygen quantum yield and a higher absorption peak in the red region of the visible spectrum than that of HpD. In rats bearing FANFT induced urothelial tumours (AY-27), tin(II) etiopurpurin (SnET2) was found to be most potent for inducing tumour necrosis (Morgan et al, 1988; 1990). With supporting data from these studies, SnET2 is currently the target of interest of the purpurins, and is being evaluated in phase I/II trials for several cancers.

The biodistribution of ALA induced PpIX and its photosensitisation effects have been studied in vitro (Linuma et al, 1994; Steinbach et al, 1995), in vivo (Kriegmair et al, 1994b; Linuma et al, 1995) in bladder tumour models, and in normal bladder (Loh et
It is a general consensus that the bladder tissue PpIX buildup after ALA sensitisation is, in decreasing order, bladder cancer model > urothelium > underlying tissues, and at ratios up to 7-10 to 1 between structures. These ratios are subject to change by differing the route of ALA administration (intravenous, oral or intravesical), the method of PpIX quantification (chemical extraction or fluorescence microscopy), the sensitivity of the fluorescence detection device, and the biological characteristics of tissues.

4.1.2 Route of photosensitiser administration

Prolonged skin photosensitivity is one of the major adverse effects following PDT with HpD or Photofrin. In order to circumvent this problem, routes of administration other than systemic were extensively studied. Amano and associates investigated the possibility of intratumor injection as an alternative for HpD sensitisation before PDT (Amano et al, 1988). In a subcutaneously implanted mouse tumour model, they showed that 3 to 98 hours after sensitisation, tumour porphyrin levels were 3-15 times higher by intratumour injection than by intraperitoneal injection. At the same time, porphyrin concentrations in skin and other tissue were 1.3 to 10 times lower with intratumour injection. This study clearly demonstrated that intratumour injection lead to higher porphyrin levels in tumour and lower in normal tissues, when less skin photosensitivity, systemic toxicity and possibly greater tumour photodestruction might be expected. The result from such an in vivo model is of scientific interest but has limited clinical value as this procedure is possible only for tumours growing on the skin and is not readily applicable for bladder cancer as intratumour injection is technically difficult if not impossible. Also local injection makes it very difficult to be sure drug has been given to all parts of a tumour.

Bladder instillation is another alternative to systemic administration. This route is particularly familiar to urologists because chemotherapeutic agents or BCG for adjuvant intravesical therapy of bladder cancer are usually given this way. However, not all photosensitisers are suitable for intravesical instillation. Larger molecules diffuse into the cytoplasm of the transitional cells less easily. Other factors influencing topical absorption of compounds instilled in the bladder include lipophilicity, water solubility, and the dissociation coefficient of the particular agent. Several photosensitisers have been tested for the applicability of intravesical sensitisation. Chloro-aluminum sulphonated phthalocyanine(CaSPc), a water soluble, largely tetrasulphonated phthalocyanine with traces of the mono-, di-, and tri-
sulphonated derivatives, has been tested in rat urinary bladder containing AY-27 transplantable bladder tumour for the feasibility of bladder instillation for sensitisation. The concentration of CaSPc in bladder and tumour tissue after intravenous injection and intravesical instillation was similar. The ratio of photosensitiser uptake between tumour and normal bladder after either administration was approximately 2. The pattern of dye distribution in the tumour was diffuse and perivascular after intravenous injection, in contrast to surface accumulation after intravesical instillation. A comparison of the photodynamic effect showed that tumour destruction after either method was similar but there were less side effects to the normal bladder wall after intravesical instillation. The authors concluded that intravesical instillation of CaSPc may be a viable alternative to systemic administration (Bachor et al, 1992). In a similar experimental design but using ALA as a photosensitiser precursor, the same group found that ALA induced PpIX, either given intravesically or intravenously was equally effective for destroying bladder tumours (Linuma et al, 1995). A favourable photodynamic effect on the rat bladder tumour model was also demonstrated with intravesical ALA by Kriegmair et al (1994b).

Intravesical instillation of AlSPc (predominantly the sulphonated derivative) was tried by Pope & Bown (1991) who found that at concentrations ranging from 0.02-0.4mg/ml, the uptake of AlSPc was patchy. In some areas high levels of fluorescence extending through the full thickness of the bladder wall were seen adjacent to areas in which there was no uptake. Using a more lipophilic disulphonated preparation of AlSPc for instillation, the distribution of sensitisier was more uniform, but was still not as even as that seen after intravenous administration. The authors did not explain the reason for unsatisfactory fluorescence distribution after instillation even with the more lipophilic derivative. However, it might be attributable to the low concentration and the short retention time of the instillant, resulting in insufficient buildup of AlSPc throughout the bladder wall.

4.1.3 Clinical trials

PDT of bladder tumours was one of the most exciting prospects in urology in the early 1980's. Kelly and Snell (1976) deserved to be credited as pioneers in the field of urological PDT. They reported the first clinical experience using haematoporphyrin derivative as photosensitiser and followed by light illumination of the bladder with a high pressure mercury vapour lamp for the diagnosis and treatment of bladder
cancers. Using *in vivo* fluoresescoscope and *ex vivo* fluorescence microscopy to define the presence of tissue HpD, they claimed that papillary tumours and carcinoma in situ in particular, retained more HpD than the normal bladder mucosa and selective destruction of bladder tumours was possible if sufficient illumination could be delivered into the bladder. This report called to the attention of some urologists the possibility of fluorescence detection and photodynamic treatment of bladder cancer.

Clinical trials aiming at treating bladder cancers with PDT can be stratified into 2 categories. Focal photoradiation means illumination of visible papillary tumours after sensitisation, and is a means of local treatment designated to ablate tumour but without causing damage to the normal tissue immediately adjacent to the tumour. Another category is the treatment of multifocal papillary bladder tumours or carcinoma in situ with whole bladder PDT. The scientific rationale behind that of whole bladder PDT is the observation of preferential accumulation of photosensitiser in the cancer cells *in vitro*. By projecting this finding into clinical treatment, it would mean that cancer cells will accumulate more photosensitiser and will be more easily eradicated following light illumination than the surrounding normal tissue that retains less dye. Based on this assumption, and without solid *in vivo* or clinical data to support its reality, a number of clinical trials were attempted to treat bladder carcinoma in situ or multifocal tumours with integral PDT.

### 4.1.3.1 Focal photoradiation

After the first report of Kelly and Snell (1976), a few reports on the effect of PDT for papillary bladder cancer treatment have been published (Tsuchiya *et al*, 1983; Hisazumi *et al*, 1983; Benson *et al*, 1983; Prout *et al*, 1987); however they all suffered from major deficits in their methodology and statistical analyses of treatment results. Small case numbers, inadequate staging of local lesions, short followup spans, inconsistency in experimental protocol, and overly speculative conclusions were common. Tsuchiya *et al* (1983) had complete remission in 6 of 8 patients with solitary or multiple Ta to T1 tumours followed by biopsy 6-18 months after PDT. They however, concluded that the result of treatment of Ta to T2 lesions have been satisfactory. Hisazumi *et al* (1983) treated 46 superficial lesions (Ta and T1) in 9 patients with HpD based PDT. The treatment effects were evaluated 3 weeks after PDT with transurethral ultrasonography. They found that 36 tumours showed complete remission and these were all smaller than 1 cm in diameter. No complete remission was found if tumours were greater than 2 cm. Skin photosensitivity was found in 4 cases but this disappeared in 5 to 10 days.
Benson et al (1983) treated 4 cases of refractory bladder carcinoma in situ with focal PDT. Multiple treatment sessions were needed to achieve temporary remission as virtually all cases recurred with new carcinoma in situ lesions a few months after PDT treatment. No major side effects were found in the 4 patients treated at light dose of 150J/cm². In view of the apparently favourable treatment results, the authors did call attention to the importance of whole bladder photoradiation if multifocal carcinoma in situ was to be effectively treated with PDT. They suggested that in the hope of altering the biological course of the tumour, a more extensive light illumination to cover the whole bladder mucosa may be essential in treating diffuse multicentric disease such as carcinoma in situ to reduce subsequent recurrence. In a slightly larger series of 19 patients with stage Ta (17) and T1 (2) diseases, Prout et al (1987) were able to achieve a 47% complete response rate when patients were evaluated 3 months after PDT. The overall tumour response rate was 74%. Bladder irritative symptoms were very common occurring in 18 patients and lasted for 1 week after focal treatment. The authors did not imply that these results were anything superior to intravesical chemotherapy, but did highlight its usefulness in some area where medical resources are limited. The treatment results of the above 4 series are tabulated as Table 4.1.

Table 4.1: Summary of focal photoradiation for bladder cancer with HpD or DHE

<table>
<thead>
<tr>
<th>Author</th>
<th>Pt No(*)</th>
<th>PS</th>
<th>Dose¹</th>
<th>D-L Int²</th>
<th>Light Dose³</th>
<th>F/U⁴ (mean)</th>
<th>CR</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tsuchiya</td>
<td>8</td>
<td>HpD 2.5</td>
<td>48-72h</td>
<td>120-360</td>
<td>6-18(--)</td>
<td>6/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hisazumi</td>
<td>9 (46)</td>
<td>HpD 2-3.2</td>
<td>48-72h</td>
<td>150-300</td>
<td>3 wks</td>
<td>36/46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benson</td>
<td>4</td>
<td>HpD 2.5</td>
<td>3-48h</td>
<td>150</td>
<td>1-9</td>
<td>4/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prout</td>
<td>20 (50)</td>
<td>DHE 2.0</td>
<td>48h</td>
<td>100-200</td>
<td>3</td>
<td>9/19</td>
<td>9/19</td>
<td></td>
</tr>
</tbody>
</table>

* Number of lesion; ¹ mg/kg; ² Drug-Light interval; ³ J/cm²; ⁴ months

In conclusion, focal ablation of superficial papillary cancers is possible using a variety of optical fibres and light diffusion devices to facilitate PDT effects. Generally, at light doses of 100-200 J/cm², overall short-term (3 weeks -12 months) response rates varied from 70-95% were observed in tumours less than 5 mm in size. Response rate are 50% or less, if tumours are larger than 2 cm. Recurrence of tumour, or more appropriately, new occurrences, are the most common cause of failure when one is dealing with a field change disease such as multifocal papillary tumours or
carcinoma in situ. Despite fluorescence evidence of preferential accumulation of HpD in the cancer tissue, none has ever demonstrated any selectivity between tumour and normal tissue destruction in the focal photoradiation series. Moreover, the results of PDT for focal lesions as was shown above are not superior to traditional electrocautery or intravesical chemotherapy of low stage bladder cancer which has an overall response rate of 30-50% with a followup period of 6-12 months. Bladder irritation and skin photosensitivity following PDT treatment have not being regarded as major side effects, but are rather bothersome for some weeks and put unnecessary pressures on patients who otherwise are good candidates for alternative treatments which are equivalent in efficacy but without those side effects.

4.1.3.2 Integral and whole bladder photoradiation

The first attempt at whole bladder photoradiation with HpD was performed by Hisazumi et al (1984) who used a motor driven system to achieve homogeneous light distribution through-out the bladder mucosa to treat 2 patients with carcinoma in situ. The light dose (10J/cm²) employed was much lower than that for focal treatment(100-200J/cm²). Both patients were cytologically and cystoscopically negative for 4 months after whole bladder PDT. Bladder irritative symptoms were more pronounced and lasted for 3-4 weeks. Reduction of bladder capacity was noted temporarily but recovered to the original volume 2-3 months after treatment. There was no evidence of obstructive uropathy on intravenous urography. As a result of post-photoradiation inflammatory changes of the urothelium seen in the urine samples, false positive cytology report were likely in the first month after whole bladder photoradiation.

Using a similar treatment protocol, Benson (1985) treated 10 patients having diffuse, resistant transitional cell carcinoma of the bladder with whole bladder PDT. He introduced a spherical bulb tip laser fibre to enhance light distribution. At a PDT light dose of 25-45J/cm² and posttreatment evaluation undertaken at 3 months, a complete response was found in 6 cases who had multifocal carcinoma in situ alone. Partial responses were found in 4 who had focal papillary Ta or T2 tumours in addition to their carcinoma in situ. Of the 6 with complete responses, 4 remained cytologically negative when followed for 5-10 months. The author also claimed that adverse effects of whole bladder PDT were minimal and the subjective bladder capacity returned to its pretreatment levels by 3 months. In view of frequent recurrences after focal photoradiation of multifocal bladder tumours, Benson (1986) suggested an integral strategy by combining focal with whole bladder photoradiation to achieve more satisfactory treatment outcome in those who had both diffuse carcinoma in situ and
papillary lesions. He also suggested reducing the light dose to 20J/cm² for whole bladder PDT to prevent side effects if it was integrated with focal treatment.

Nseyo et al (1987) reported PDT treatment results in 23 patients who had carcinoma in situ (CIS) refractory to intravesical chemotherapy or BCG immunotherapy. Nineteen had complete responses for a follow-up of 2 to 12 months. Although the authors took special care not to overdistend the bladder by keeping its volume at around 150-200ml under 30cm H₂O hydrostatic pressure, bladder shrinkage was found in 4 patients (17%). A similar response rate was also reported in a subsequent series (Nseyo 1992). Shumaker and Hetzel (1987) also achieved a complete response rate of 68% in 16 patients treated with Photofrin who had been followed for 6-36 months. They assumed the presence of unknown local immune responses developed after PDT as possible mechanisms for long-term efficacy of PDT effect. Naito et al (1991) reviewed their experience of integral PDT of the bladder for 35 patients who had refractory bladder tumours or carcinoma in situ. Of the 24 patients having complete response (68.6%) at 3 months, 14 had recurrence when followed up to 60 months. Despite the disappointing long-term results, they concluded that PDT may be a useful modality for the treatment of carcinoma in situ.

A similar short-term response rate of (73.5%) was found in 34 patients with refractory carcinoma in situ who had been treated with PDT. Despite a high incidence of recurrence (77.8%) within 2 years of treatment, most recurrences were superficial and low grade papillary tumours amenable to transurethral resection and preservation of the bladder (Uchibayashi et al, 1995). This downstaging effect is also observed by D'Hallewin & Baert (1995) who showed a response rate of 100% at 3 months and 60% at 3 years. Kriegmair et al (1995) attained a complete response rate of 57% with a mean disease-free interval of 18 months in a total of 21 patients who had been treated with integral PDT for their difficult multiple superficial tumours. Only one patient was found to have bladder shrinkage combined with incontinence. They concluded that PDT was an effective measure for bladder preservation in patients in whom transurethral resection and intravesical therapy had failed. In another series of 11 patients receiving PDT for their carcinoma in situ or invasive bladder tumour, a complete response rate of 80% was reported with a mean follow-up of 12 months. Deterioration of bladder capacity following PDT was prominent in all the 4 patients who had previous radiotherapy for controlling bladder cancer as only one out of 7 experienced such irreversible changes (Windahl & Lofgren 1993).
Despite the small number of patients and a limited span of follow-up, the promising therapeutic outcomes and the generally acceptable rates of complication with careful in situ light dosimetry in the recent series of retrospective clinical trials, a phase II study has been designed to evaluate the possibility of using PDT as an alternative to cystectomy for controlling CIS of the bladder (Marcus 1992). It is also justified to start phase III studies on primary therapy of carcinoma in situ and for prophylaxis of recurrences.

4.1.3.3  Prophylaxis of recurrence

Prophylaxis of tumour recurrence has been attempted in a small series of patients and with a short followup (Nseyo 1992). In the 11 patients with low grade and low stage papillary bladder tumour (Ta and T1), PDT was used for prevention of recurrence after TUR of visible tumours. After follow-ups ranging from 6 to 50 (mean 19.4) months, only 2 developed recurrences. The preliminary results look promising, although it is not a controlled trial.

4.1.4  Complications

Skin photosensitivity is the most frequently mentioned and annoying side effect which may persist for 4-6 weeks after PDT treatment of the bladder with HpD or Photofrin. However, as a result of taking special precautions and proper patient counselling for its prevention, no serious skin complications were reported in all the aforementioned clinical trials. Common manifestations of skin side effect include erythema, slight oedema, and sunburn in those areas exposed to direct sunlight. Although troublesome and unavoidable with porphyrin sensitisation, skin phototoxicity occurs only in patients who fail to follow doctors' instruction (Benson 1986). The second generation photosensitisers: BPD, NPe6, AlSPc, and ALA all have a shorter half-life than Photofrin which probably leads to a shorter span of skin photosensitivity after administration. This improvement has made PDT a more acceptable modality for clinical use.

Urological side effects following porphyrin based PDT are not uncommon, and range from reversible irritative bladder symptoms (frequency, urgency, dysuria, haematuria, bladder spasm, or suprapubic pain) to contracted bladder with reflux uropathy requiring cystectomy (Nseyo et al, 1987; Harty et al, 1989; D'Hallewin & Baert 1995). Depending on the light dose delivered to the whole bladder during PDT, lower urinary tract irritation usually appears in all patients at 24-48 hours and persists for 1-
2 weeks at a lower total light dose, and for 4-6 weeks at higher doses (Nseyo et al., 1987). A transient reduction of bladder capacity is observed in most patients receiving whole bladder PDT, but the capacity returns to the pretreatment levels in most by 3 months (Benson 1985; Naito et al., 1991). Permanent shrinkage of the bladder occurs in 15-57% of cases in the earlier series using high light doses under suboptimal control (Nseyo et al., 1987; Harty et al., 1989; Nseyo 1992). This serious side effect has not been a significant problem in patients who have been treated under current standardised protocols with careful in situ light dosimetry (D'Hallewin & Baert 1995), or with prolonging of time interval between bladder biopsy and PDT treatment as the mucosa denuding caused by deep bladder biopsy was thought to be a possible cause for muscle damage after PDT (Naito et al., 1991; Uchibayashi et al 1995).

4.1.5 Light diffusion techniques

Following early clinical bladder PDT reports from the USA and Japan, methods have been sought to facilitate light illumination in the bladder, as an obvious obstacle to satisfactory treatment of bladder cancer with haematoporphyrin derivative-photodynamic therapy was nonuniform distribution of light. To enhance isotropic light illumination during PDT, a Japanese urologist designed a lasering device consisting of a built-in cone-shaped reflecting mirror with an incorporated core quartz fibre to reflect the laser light perpendicular to the incident light. This device was connected to a motor driven system and withdrawn slowly at a speed of 40-160mm/h. The resultant light distribution seemed satisfactory (Hisazumi et al., 1984).

The idea of modifying the tip of quartz laser fibres into a bulbous configuration so as to facilitate spherical light distribution was initiated by Benson (1985). The optical guide used was a medical-grade quartz optical fibre with a core diameter of 400 μm. A clear polycarbonate spherical bulb was attached to the end of the fibre. A reasonable light distribution on bladder urothelium was achievable judged from the isotropic fluence curve shown in his study (Benson 1985).

The use of intralipid as a light dispersing medium inside the bladder was first suggested by Jocham et al (1984). He used 100J/cm² of light at a wavelength of 630nm to illuminate tumours in the rabbit bladder. However, he did not explore the light scattering properties of this medium which would permit easier dosimetry for clinical use. Intralipid is an emulsion of soybean lipid particles in an aqueous suspension used for hyperalimentation. The particles are in the form of a solid micelle
with a diameter of 0.5 \mu m. It has the following properties: very low light absorption coefficient; adjustable scattering coefficient simply by dilution; cheap and readily available, and stable and safe in a biological environment. Phantom models using glass flasks to simulate hollow organs revealed that 1:100 dilution of intralipid was the optimal concentration for light scattering in the human bladder as the power loss was minimum and degree of light distribution maximum at a volume larger than 100ml. Light distribution in the flask with small volume (< 50ml) was heterogenous in spite of the presence of intralipid. (Baghdassarian et al, 1985). Others suggested that 0.25% intralipid be used as a light dispersing medium for clinical PDT (Allardice et al, 1992). The use of intralipid for integral whole bladder PDT for multifocal bladder carcinoma (Jocham et al, 1986), was questioned by Star and colleagues (1987) who showed in a phantom and canine model that in the presence of a light scattering medium, when the light source was moved away from the centre, a substantial variation of fluence rate was detected on the bladder wall. However, it remains to be determined which concentration is optimum clinically at a volume where there is flatting bladder mucosa folding is expanded flat, but without overstretching to cause thinning of the bladder wall.

Recently, a new design for better illumination of hollow organs has been developed. It is based on the application of a thin layer of highly backscattering material placed next to the wall of the bladder (Beyer et al, 1990). The reflectivity of the layer is about 90%, which exceeds the backscattering property of the tissue(40-50%). As a result of the low light absorption of the layer, light is transmitted with an efficiency of more than 90%. Thus, the demands on the correct positioning of the light emitter are considerably reduced in comparison to the use of light-scattering medium or a bulb-tip microlens fibre. Another major advantage is that it can be applied in organs with a cylindrical, spherical, or an irregular shape. The light application device, however needs further clinical assessment to verify its usefulness.

4.1.6 Methods for detecting fluorescence

The measurement of fluorescence offers a rapid non-invasive means of assaying the structure and status of a tissue. Spectroscopy is a method for fluorescence detection in living tissue in vivo by placing an optic fibre on the tissue surface to allow delivery of the excitation light and local collection of the emitted fluorescence. Transmittance fluorescence microscopy on the other hand, is a technique applied mostly for
fluorescence study of biological systems in vitro. Details of the two methods are the main topics of the following section.

4.1.6.1 Fibreoptic in vivo detection with spectroscopy
Tissue fluorescence detection is an integral part of studies involving photosensitiser pharmacokinetics or tissue biodistribution of fluorophore after sensitisation. With in vivo spectrophotofluorometry, the nature and chemical purity of compounds accounting for fluorescence can be characterised. The technique of obtaining in vivo fluorescence excitation and emission spectra was introduced by Pettier et al in 1986, and offers a number of advantages over other visual fluoromicroscopic methods that have been used. It is highly sensitive, and it can record complete fluorescence emission spectra so rapid that it is now feasible to obtain a series of such spectra in non-anaesthetised animals (Pettier et al, 1986).

The detection of tissue fluorescence is a 2-step process involving absorption of excitation light by photosensitiser, and followed by emission of fluorescence from photosensitiser. The medium or organ containing fluorophore is excited with light of various wavelengths, while the detection of emitted light is tuned to a fixed range of wavelength. The resultant plot is an absorption spectrum of a particular photosensitiser or its metabolite fluorophore in the biological system. It is valuable for the identification of a photosensitising agent which is yet to be determined in a tissue. The emission spectrum, on the other hand, is a technique for measuring fluorescence wavelengths or intensity from a tissue which has been illuminated with light of a fixed wavelength. For fluorescence detection of a pure fluorophore with a known absorption peak in vivo, light of a single wavelength corresponding to its peak is used, however, if there are more than one fluorescing compounds presented in a biological system, multiple emission spectra should be obtained for each components.

4.1.6.2 Ex vivo or in vitro fluorescence detection with CCD camera
Microscopic optical imaging with high performance cooled CCD(charge-coupled device) cameras, is becoming an increasingly important technique in a wide range of biological experiments. As all photosensitisers will fluoresce when illuminated with light of a wavelength corresponding to an absorption peak, it is possible to measure the level of fluorescence emitted from the biological systems(in vivo or in vitro), at cellular or even molecular level, using microscopic imaging. Fluorescence microscopy is a quantitative technique designated for measurements of photosensitiser distributions in sections of tissue and cultured cells. The emission
spectrum of tissue fluorescence can be recorded with negligible interference from sensitiser photodegradation, using Helium Neon laser excitation in many cases.

The set up for fluorescence microscopy is shown in Figure 3.5. For fluorescence imaging and quantification of PpIX levels in the bladder sections, an inverted phase pixels) slow-scan charge coupled device (CCD) camera (model 1, Wright Instruments Ltd, Cambridge, UK) was utilized. The set-up of this highly sensitive photometric system is described as follows (Pottier et al, 1986). In brief, an 8 mW helium neon laser (632.8 nm) was used to excite the tissue PpIX and the emitted fluorescence was detected between 665 and 710 nm (maximum response at 690 nm), using a combination of bandpass and longpass filters. The fluorescence signal was processed by an IBM PC clone into a falsely colour-coded image depicting the signal in counts per pixel. The image processing software enabled the fluorescence intensity in each tissue layer to be quantified digitally by averaging over specified areas.
The use of a slow-scan CCD system with a fixed internal gain together with constant power laser excitation permitted highly reproducible signal calibration and satisfactory correlation has been found previously between microfluorometric measurements obtained with this system and chemical extraction measurements in normal rat stomach and colon (Loh et al, 1993b). Moreover, the advantages of using a CCD camera over video imaging system are higher sensitivity, direct digital image integration, and a higher dynamic range. The high sensitivity allows low-power excitation and short integration times which prevents the occurrence of photosensitiser bleaching that may distort the fluorescence image.
Fluorescence excitation may be achieved using either the ultraviolet (UV) or red wavelength absorption bands, but generally, excitation at longer wavelengths is preferred because the tissue autofluorescence is reduced. The advantages of fluorescence microscopy over chemical extraction for measuring sensitisier concentration and distribution is the feasibility for microscopic assessment of intracellular fluorophore distribution. However, its major limitation is the inadequacy for tissue sampling as only a minute amount of tissue is examined. Recent advances in optico-biological techniques have expanded the application of CCD arrays into the field of molecular genetics for high resolution gene mapping with fluorescence imaging of hybridised DNA, monitoring nerve cell activity by calcium-ion imaging, and three-dimensional reconstruction microscopy (Spring & Smith 1987; Gray et al, 1994).

4.1.6.3 Fluorescence detection of bladder cancer
The investigation of tumour fluorescence was pioneered by Lipson and associates (1961), who found that salmon-red fluorescence could be detected in a transplantable rat tumour model after low dose sensitisation of the rat with HpD. Before its first application for detection of bladder carcinoma in situ, HpD induced fluorescence was used to study a variety of human malignant tissues, and indeed 76.3% of them took up more HpD than adjacent normal tissue with a false negative rate of 10.5% (Gregorie et al, 1968; Leonard & Beck 1971). The first in vivo fluorescence detection of bladder cancer was initiated by Kelly and Snell (1976) who described the most intense fluorescence in macroscopically normal mucosa, which proved histologically to be frank carcinoma, carcinoma in situ or premalignant lesions, and therefore suggested HpD fluorescence could be a useful diagnostic aid in some patients. Benson et al (1982) using an ex vivo photography model an in vivo fluorescence modulated auditory system, further confirmed that fluorescence emission from the bladder mucosa 2 hours after HpD administration was a sensitive method to detect dysplastic or neoplastic urothelium which was not seen with a routine cystoscopy. A high degree of correlation was found between the fluorescing tissue and the presence of bladder neoplasms or carcinoma in situ (Benson et al, 1982; Tsuchiya et al, 1983), whereas no fluorescence was detected from the normal urothelium (Benson et al, 1982). However, it has been shown that HpD induced fluorescence is not specific for neoplasia as inflammatory and regenerating tissues also fluoresce (Selman et al, 1985b).
Advances in fibreoptic technology made possible the building of an \textit{in situ} point-monitoring fluorescence detection system based on a low-energy pulsed laser, fibre transmission optics, and an optical multichannel analyser (OMA) for endoscopic diagnosis of patients with cancer. Multispectral fluorescence imaging (MSFI) is a technique combining simultaneous measurement of four different fluorescence images with computer assisted analysis of fluorescence to create an artificial real-time image. These optical spectroscopic techniques using either Photofrin or ALA have been used for detection of gastrointestinal neoplasias (Messmann \textit{et al}, 1994), head and neck (Svanberg \textit{et al}, 1994) and bronchial cancer (Gamarraet \textit{et al}, 1994). Lower sensitiser doses or a shorter sensitisation time generally leads to better tissue discrimination, but there is a substantial individual variation. In a study of 800 fluorescence spectra analysis and 300 corresponding biopsies from 24 patients with bladder cancer who were sensitised with low dose Photofrin (0.35-0.5mg/kg) 48 hours before fluorescence diagnosis, Baert \textit{et al} (1993) claimed that differentiation between normal and neoplastic tissue was possible although false results did occur. Using a violet laser light incorporated fluorescence cystoscopy and intravesical ALA instillation for \textit{in vivo} cancer detection, Kriegmair \textit{et al} (1994a) were able to identify malignant or precancerous lesions of the bladder in 68 patients at a sensitivity of 100\%, and specificity 68.5\%. They claimed that the fluorescence intensity between bladder tumour and the surrounding tissue reached ratios more than 10, and there was no correlation between the time of ALA exposure and when the fluorescence was measured. The same series was expanded to include 106 patient with similar results (Kriegmair \textit{et al}, 1996). It is also likely that fluorescence cystoscopy, besides its diagnostic value, might be useful to guide transurethral resection of bladder tumours with a higher degree of precision.
4.2 AN OVER VIEW OF PDT FOR THE PROSTATE

The application of PDT for treating prostate disease, although relatively primitive at this moment in time, is gaining more and more attention from urologists. In the predictable future, cancer of the prostate, especially localised lesions which are detected by an elevated PSA, and confirmed by biopsy, will become one of the major parts of urological practice. Methods which are effective and tumour specific, and are convenient, both to the urologists and patients, for treating this "popular disease", will be extremely welcome as they may save lives as well as time of urologist. To date, however, such a tumour-specific chemotherapeutic agent has not been found. PDT is potentially a modality that could fulfil some of the requirements for such selective therapy.

4.2.1 Basic research

The first PDT report on the prostate in the English literature, in contrast to most basic research, was an in vivo rather than an in vitro study. McPhee and associates (1984) in an effort to determine the effect of a diffusing fibre, applied light interstitially into 2 prostate tumour models growing on the skin of Fischer-Copenhagen rats. They found that multiple fibre insertions were needed to cure R3327-AT prostate cancers, but the concern of possible thermal effects could not be overlooked. A light power of 200 mW was enough to induce significant temperature changes even with a single fibre. One year later, Camps Jr. and colleagues (1985) conducted the first in vitro study by sensitising Dunning R3327-AT1 anaplastic prostatic cancer cells with HpD, and followed by light exposure at 630 nm to determine cell survival in relation to light and drug doses. Maximum cytoplasmic fluorescence was observed using fluorescence microscopy in cells incubated with HpD at a concentration of 100 μg/ml for 2 hours. Cytotoxicity was a function of light exposure time and not a function of total energy delivery as cell survival decreased with increasing time of exposure to red light. The authors concluded that PDT may be applicable to the treatment of locally invasive prostate cancer. Gonzalez et al (1986), colleague of McPhee, reported that the addition of misonidazole, a hypoxic cell radioenhancer prior to or after PDT with HpD, prolonged growth delay (2-fold) of tumours and lead to significant increase of percentage (20-70%) of local cancer cure on Dunning 3327-AT cells growing on Fischer-Copenhagen rats. This paper highlighted the possibility of treating solid
prostate cancer by interstitial PDT with diffuser fibres in the absence of thermal effects.

Light distribution in the human prostate was studied ex vivo firstly by the research group in Manchester. Pantelides and associates (1990) calculated optical constants on 3 prostates obtained from men dying of non-urological causes and found that the mean absorption and scattering coefficients were $0.07 \pm 0.02$/mm and $0.86 \pm 0.05$/mm respectively. Light propagation in the liver, as a result of blood pooling, was 4 times poorer than in the prostate. This finding prompted them to the conclusion that light was predominantly scattered rather than absorbed in the prostate, a factor contributing additional advantage to PDT treatment of human prostate, making it feasible to treat the entire gland with a relatively small number of fiber sites. The same team (Whitehurst et al, 1994) employing a synchronised delivery/detector fibre system to measure fractional fluence of light in patients suspicious of prostate cancer, found that in agreement with the results from their previous report (Whitehurst et al, 1989), the mean attenuation coefficients for benign and malignant prostate tissue were $0.35 \pm 0.02$/mm and $0.36 \pm 0.02$/mm, respectively, indicating similar optical properties. Patients with bilateral lobe involvement showed little interlobular variation in attenuation coefficient, although interpatient variation did exist, reflecting biological differences. Their results further enhance the likelihood of interstitial PDT for prostate cancer treatment as the light distribution pattern seems reasonably predictable, and the volume of tumour destruction can be simulated in a computer model with reasonable accuracy (Whitehurst et al, 1993). Further to light propagation studies, this group also investigated the distribution of HpD in the prostate and adjacent tissues (Pantelides et al, 1993). In contrast to the reticuloendothelial system (liver & spleen) which retained high levels of porphyrin 24 hours after sensitisation, prostate porphyrins were relatively low, and were similar to those in the rectum, urethra and the skin. There was no particular affinity of zinc incorporated HpD to the prostate despite a high avidity of prostate tissue for zinc.

In the same period, a comprehensive study on human prostate specimens ex vivo and canine prostate in vivo was done by Chen et al (1993) who measured fluence attenuation at various sites remote from the incident light source in 5 excised human cancerous prostates. The data were plotted as iso-fluence distributions for evaluating the homogeneity of light in relation to the location of cancer foci in the sample after histological examination. They concluded that the cancer tissue had similar light attenuation properties to non-cancerous prostate. However, in the light of their
inevitable deficiency using an *ex vivo* model, they pointed out that *in vivo* study was needed to verify this result as different microvasculature distribution between the two tissues may change the pattern of light transmittance. They also demonstrated the feasibility of inducing Photofrin based PDT lesions as large as 16 mm in diameter using a 2-cm cylindrical diffuser fibre at a total light dose of 500J. However, there was inconsistency of the lesion size with the measured effective penetration depth of light *in vivo*. The authors suggested that it might be caused by inter-animal variation of photosensitiser uptake and optical properties of the prostate (Chen *et al*, 1993). Putting all available experimental data together, it seems clear that the prostate is a good, if not ideal, organ for interstitial PDT because the incident light is mostly scattered but not absorbed. The penetration depth of light at a wavelength of 633 nm is 4.3 times greater than in the liver. There is no major difference between cancerous and normal tissue in terms of light properties. It therefore is theoretically possible to destroy a large amount of prostate tissue, whether cancerous or not, if the photosensitiser is evenly distributed in the prostate. Also, this treatment is technically feasible. Although there are questions remaining to be answered, a step forward into clinical trials seems feasible to further assess its usefulness.

With the surge of newer photosensitisers, studies on the canine prostate with sensitisers other than HpD or Photofrin have begun to appear. Selman and Keck (1994) studied a new chlorin derived photosensitiser tin (II) etiopurpurin (SnET2) on the canine prostate. Tissue pharmacokinetics of SnET2 were not evaluated; instead they selected 24 hours as a time point to sacrifice mongrel dogs and harvested tissues for chemical extraction of SnET2. Mean tissue concentration of SnET2 in the 3 animals were arranged, in decreasing order liver> kidney> prostate> spleen> bladder= urethra= muscle. With 660 nm wavelength and at a light dose of 285J/cm (285mW/cm×1000sec), transurethral illumination of the prostate was undertaken with a 2-cm cylindrical diffuser fibre placed in the prostatic fossa under transrectal ultrasound guidance. Animals were sacrificed either 48 hours or 3 weeks after PDT. Circumferential periurethral necrosis as large as 1 cm from the urethral wall was found 48 hours after treatment. Glandular atrophy induced by PDT remained evident 3 weeks following light illumination. The authors concluded that transurethral PDT with SnET2 was feasible, and the use of this treatment for benign prostatic hyperplasia warranted further investigation (Selman & Keck 1994). I consider this was an inappropriate conclusion, as discussed later in this thesis (Chapter 10).
4.2.2 Clinical trials

The only available clinical data on PDT of the prostate appeared in 1990. It was a case report describing the result in controlling localised prostate cancer with HpD and Photofrin based PDT (Windahl et al., 1990). Two patients having localised prostate cancer were first treated by radical transurethral resection to remove as much prostate tissue as possible. Six weeks after resection the patients were injected one with HpD (1.5 mg/kg body weight) and the other with Photofrin (2.5mg/kg body weight), and followed by illumination of the prostate cavity 48-72 hours later with laser light (628nm) through a quartz fibre with a spherical tip at a light dose of 15J/cm². At 3 months follow-up, random biopsy of the prostate bed elicited no evidence of malignancy. PSA values decreased from 10 and 6μg/l preoperatively to 2.5μg/l and 0.2μg/l, respectively 5 months after PDT. One patient who died of lung cancer 6 months later, had no sign of residual cancer on histological examination of the prostate. There was no frequency or urgency observed in the 2 patients following prostate PDT.
4.3 CURRENT STATUS OF PDT IN UROLOGY AND AIMS OF THIS PROJECT

Like other medical specialities, most urologists understand the concept of PDT for various cancers, however, their attitude has become more conservative because of the fear of inducing significant side effects with this treatment. Generally, they are looking for an "ideal" photosensitiser to "cure" bladder or other cancer. It seems reasonable for them to consider that with current technique such as BCG intravesical therapy or radical surgery with continent urinary diversion, most troublesome bladder cancers can be treated rather satisfactorily with acceptable rates of complications. Nevertheless, there is a small group of urologists showing continued enthusiasm for treating bladder cancers with PDT. Not surprisingly, they take special precaution to prevent the occurrence of complications by careful selection of patients, choosing more refined photosensitisers, and employing more restricted light doses. With these modifications, therapeutic results are promising with a much reduced incidence of side effects but there is a long way to go before PDT is likely to become routine. More experience will come from the recent release of DHE by the Canadian government for preventing recurrence of papillary bladder tumours.

When I was reviewing the history of PDT in urology, in particular, the use of focal or whole bladder PDT for cancer treatment, I came to realise how little basic research on animal model was done before clinician attempted human trials. The lesson of substantial side effects following whole bladder PDT may be largely attributed to an inadequate understanding of biological responses of the bladder tissue following photodynamic tissue destruction. Based on my personal interest and enthusiasm for urological applications of PDT, I started the projects of this thesis. Although full of optimism for the future of PDT in urology, it is my belief that only after a more generalised and solid foundation is laid can human trials be confidently and safely initiated. The aim of this thesis is to study the biology of photodynamic therapy on the bladder and prostate as a preparatory step for clinical trials. The experiments were designed to answer those questions. The aims of each experimental chapter will be presented separately, but, I would like to summarise the key aims of my projects as follows.

For the work on bladder PDT, the aim is to achieve complete urothelial destruction with a new photosensitiser precursor, aminolaevulinic acid (ALA) without damaging
the underlying muscle. The selectivity of tissue destruction between the urothelium and muscle layer is much more important than any selectivity between tumour and normal urothelium as the most important complication is irritability and contraction of the bladder due to detrusor muscle damage. Regeneration of the bladder urothelium is generally rapid and complete, hence its destruction causes no serious problems in the long run. It is because of this that I did not choose a tumour model for my experiments. Also important is the cutaneous photosensitivity which may be devastating if skin is inadvertently exposed to strong light. To reduce this side effect, which is associated with systemic absorption of photosensitisers, this project will evaluate the possibility of intravesical instillation as a route of drug administration.

The feasibility of bladder instillation of ALA is the key aim of Chapter 5. The aim was to show that more of the active derivative of ALA, protoporphyrin (PpIX) can be retained in the urothelium than in muscle and this differential accumulation leads to selective destruction of the urothelium after light illumination. The comparison of oral and intravesical administration of ALA on the systemic and bladder PpIX buildup is the main aim of Chapter 6. The demonstration of generally higher PpIX buildup in the liver, kidney, muscle, and skin depicts the possibility that more systemic reactions are likely to result if ALA is given orally (systemic) than given intravesically (local). I also try to prove with histological evidence that more skin damage is likely with the oral route. The main theme of Chapter 7 is to increase the efficiency of ALA mediated PDT effects by adding an iron chelator to retard conversion of PpIX to haem or to fractionate the light delivered to the bladder. If these methods are proved worthwhile, then the light as well as drug doses used clinically may be reduced.

For the second part of my thesis, the aim is to test the feasibility of PDT in the canine prostate model. Before the start of this project no relevant peer reviewed paper had been published in the English literature. The key aim of Chapter 8 is to observe the prostate tissue responses and general tolerance of the experimental animals after PDT with meso-tetra-m-hydroxyphenyl-chlorin (mTHPC). I also want to compare the differences of tissue effect between interstitial and transurethral routes of light illumination. In Chapter 9, I test the efficacy of ALA and aluminium disulphonated phthalocyanine (AlS2Pc) as photosensitisers in the prostate in an attempt to achieve smaller lesions which may be more valuable for future application in men who have small cancer foci located near the prostate capsule. Safety of PDT treatment is another
major concern of this study particularly for treatment of peripheral or apical areas of
the prostate.

General conclusions of these experiments together with discussion of future prospects
for PDT for urological applications will be made in Chapter 10.
SECTION B: EXPERIMENTS AND RESULTS

CHAPTER 5: Biodistribution and Photodynamic Effects in the Rat Urinary Bladder with 5-Aminolaevulinic Acid Induced Protoporphyrin IX Sensitisation

CHAPTER 6: Quantitative Fluorescence Assessment of Protoporphyrin IX in the Bladder and Other Organs Following Administration of 5-Aminolaevulinic Acid: Comparison between Intravesical and Oral Routes

CHAPTER 7: Enhancement of Photodynamic Effects with Oral Iron Chelator and Light Fractionation after ALA Sensitisation

CHAPTER 8: Interstitial and Transurethral Photodynamic Therapy of the Canine Prostate Using Meso-Tetra-(M-Hydroxyphenyl)Chlorin

CHAPTER 9: Interstitial Photodynamic Therapy in the Canine Prostate with Disulphonated Aluminium Phthalocyanine and 5-Aminolaevulinic Acid Induced Protoporphyrin IX
CHAPTER 5

BIODISTRIBUTION AND PHOTODYNAMIC EFFECTS IN THE RAT URINARY BLADDER WITH 5-AMINOLAEVULINIC ACID INDUCED PROTOPORPHYRIN IX SENSITISATION

5.1 Background ................................................................. 131
5.2 Aims ........................................................................... 132
5.3 Materials and Methods
   5.3.1 Preparation of ALA solutions ................................. 133
   5.3.2 Intravesical instillation of ALA and systemic anaesthesia 133
   5.3.3 Fluorescence microscopy
       5.3.3.1 In vivo studies .................................................. 134
       5.3.3.2 Studies on tissue sections ................................. 134
   5.3.4 ALA sensitisation for PDT ................................. 134
   5.3.5 Light dosimetry and diffusion medium ................. 135
5.4 Results
   5.4.1 Stability of ALA solution ....................................... 137
   5.4.2 Fluorescence imaging
       5.4.2.1 In vivo studies .................................................. 138
       5.4.2.2 Studies on tissue sections ................................. 138
   5.4.3 Macroscopic findings ............................................ 142
   5.4.4 Histological findings ............................................ 145
5.5 Discussion
   5.5.1 Advantages of ALA instillation for bladder PDT ........ 151
   5.5.2 Biochemical characteristics for ALA instillation .......... 152
   5.5.3 The importance of pH for ALA instillation ............... 153
   5.5.4 Fluorescence selectivity after ALA instillation ............. 153
   5.5.5 ALA induced PDT effect on the urothelium ............... 154
   5.5.6 ALA induced PDT effect for urothelial tumour ............ 155
   5.5.7 Site of action of ALA induced PpIX in vivo ............... 156
   5.5.8 Optimal concentration for light scattering ............... 156
   5.5.9 Potential for bladder cancer treatment with PDT ........ 157
5.6 Conclusions ............................................................... 158
5.1 BACKGROUND

Photodynamic therapy (PDT) is a modality receiving increasing attention in many medical specialities for the treatment of early malignancies (Jin et al, 1989; Corti et al, 1989; Zhao et al, 1991). The mechanism of PDT which was firstly described by Foote (1976) has been comprehensively reviewed in Chapter 3. Despite the potential for preferential retention of sensitisers in tumour tissue followed by selective destruction after light illumination, photodynamic therapy has so far remained of limited clinical value. Previous PDT studies using HpD or its purified ester/ether component (Photofrin) for treating bladder cancers showed promising results (Tsuchiya et al, 1983; Benson 1986) but complications such as scarring of the detrusor muscle and reflux uropathy which at times requiring cystectomy to control deterioration of renal function could not be ignored (Harty et al, 1989). This has prompted the search for more selective photosensitisers which can efficiently control tumour as well as diseased urothelium but without causing damage to the underlying lamina propria and detrusor layer.

One of the most interesting photosensitising agents is 5-aminolaevulinic acid (ALA). The use of ALA indiced protoporphyrin IX (PpIX) based PDT for both experimental and clinical trials, has been extensively reviewed in Chapter 3. It has been shown that ALA induced PpIX for PDT of dysplastic lesions in gastrointestinal hollow organs has attracted much interest since the risk of significant systemic side effects should be minimal (Mlkvy et al, 1994; Régula et al, 1995). Since the PDT lesions mediated by ALA are usually superficial and in the range of 1-2mm in depth (Bedwell et al, 1992; Mlkvy et al, 1994; Régula et al, 1995), it makes ALA an appropriate photosensitiser for whole bladder PDT if the urothelium, whether diseased or normal, is the target of treatment. The concern of detrusor muscle damage may be less if the depth of necrosis is confined to the superficial layers of the bladder wall. However, to further reduce skin photosensitisation caused by systemic administration, it may be possible to deliver ALA intravesically. This chapter is the first part of a series of experiments aiming at exploring new applications of ALA based PDT in urology.
5.2 AIMS

Despite the potential for treating tumours of the bladder with PDT, its clinical applicability has been hampered by the risk of detrusor muscle damage and prolonged skin photosensitivity. In the management of carcinoma-in-situ or multifocal papillary tumours of the bladder, the challenge is to achieve generalised destruction of the transitional epithelium without damaging the underlying layers. Although systemic administration of ALA has been shown to give selective sensitisation of the urothelium (Loh et al, 1993a), this is also likely to cause more skin and systemic sensitisation, even if this only lasts for 24 hours and only induced transient elevation of liver enzyme (Regula et al, 1995). In the present study I evaluated the stability of ALA solution at various pH, and investigated the feasibility of local administration of ALA using the intravesical route. This study is the first stage of a programme to assess the applicability and future potential of ALA induced PpIX for the treatment of bladder cancer. I hope to prove that intravesical instillation of ALA is able to produce selective urothelial damage but without the problems of needing to administer the photosensitiser systemically. As ALA solution discolours rapidly following preparation, this study also looks into the optimum concentration and pH of ALA solution for instillation. After photodynamic therapy with various drug and light doses, both immediate(within 7 days) and long-term(3 and 6 months) bladder specimens will be assessed by macroscopic and microscopic changes. The optimal concentration of intralipid which in this study, is a light diffusion medium, will be determined.
5.3 MATERIALS AND METHODS

5.3.1 Preparation of ALA solutions

Purified ALA powder (ALA.HCl 98%, DUSA Pharmaceuticals Inc., New York, USA) was dissolved in normal saline and titrated with saturated sodium bicarbonate solution to a final pH of 2.1, 4.0, 5.5 or 7, at a concentration of 10% (100mg/ml). The solutions were stored in the dark at 37 °C and in dim room light at 25 °C for 72 hours for assessment of chemical stability. Stability was evaluated by the severity of colour changes during the observation period.

5.3.2 Intravesical instillation of ALA and systemic anaesthesia

Based on the results of section 5.3.1, ALA solutions with pH 4 and 5.5 at concentrations of 10%, 1% and 0.1% were selected for the instillation studies using female Wistar rats weighing 100-180 grams. Under general anaesthesia with Hypnorm (Fentanyl and fluanisone, Jansen Pharmaceuticals Ltd.), transurethral catheterization was carried out with an 18.5 gauge Teflon cannula after a small episiotomy (1-2 mm) was made in the vestibule to expose the urethral meatus. The ALA solution was administered intravesically at a volume of 0.2 to 0.32 ml (0.2cc/100 gm body weight) and retained in the bladder for 2-2.5 h. After that, the rats were allowed to wake up and void freely. The small episiotomy over the vestibule facilitated the insertion of transurethral cannula but without posing additional distress to the experimental animals. I found that, catheterization with a blind method although technically feasible in most of cases, was traumatic to the urethral meatus and sometimes resulted in false passage of the cannula to the retroperitoneal region. It was from this observation that I performed episiotomy routinely in each case. Throughout this chapter, the times from instillation given are the times from the initial infusion of ALA into the bladder. With the exception of the animals killed one hour after ALA, all animals had ALA solution in the bladder for at least 2 hours. The exact time the solution was in the bladder varied and depended on when the animal voided after it was allowed to recover from the general anesthesia. For each ALA solution of different concentration and pH, 2-5 rats were tested. The control fluorescence group consisted of 2 rats receiving no treatment and 2 others with bladder instillation just of 0.3 ml normal saline.
5.3.3 Fluorescence microscopy

5.2.3.1 In vivo studies
The fluorescence emission spectra of ALA induced porphyrins were measured in vivo in 5 rats; two controls without photosensitisation, one examined 2 hours after 200mg/kg ALA given intravenously and two examined 3 hours after intravesical administration of a 10% solution of ALA at pH 5.5. In vivo fluorescence spectroscopy was carried out using a Perkin-Elmer LS50B spectrofluorimeter with a bifurcated fibre-optic bundle probe. At laparotomy, the probe was placed 3 mm above the exposed bladder, which had been rinsed once and then filled with normal saline. The emission spectrum was recorded from 600-750 nm using an excitation wavelength of 410nm.

5.3.3.2 Studies on tissue sections.
Sensitised rats were killed and the urinary bladders removed 1, 2, 3, 4, 5, 7 and 24 hours after the time of ALA instillation. Before removing the bladder, it was rinsed transurethrally 3 times with normal saline and then filled with 0.3ml OCT embedding medium (Tissue-Tek, Miles Inc, Elkhart, IN, USA) to keep the lumen distended. The samples were kept in liquid nitrogen prior to sectioning (10 micrometer) for fluorescence imaging. The details of quantitative fluorescence microscopy are discussed in section 4.1 of Chapter 4.

5.3.4 ALA sensitisation for PDT
After determining the optimum time for ALA sensitisation from the fluorescence studies which is 5 hours after initial instillation, purified ALA powder (ALA.HCl 98%) was titrated to a final pH of 4.0 or 5.5, and at a concentration of 0.1, 1 and 10%. The solution was delivered via a urethral catheter into the bladder of female Wistar rats(100-180 gm) at a volume of 0.2 ml/100 gm body weight. It was retained in the bladder for two hours with the animals under general anaesthesia, after which they were allowed to recover consciousness and void at will. Laser treatment was applied 3 hours later(5 hours after initial instillation). As 5 hours was the time of maximum PpIX selectivity between urothelium and muscle demonstrated in the fluorescence study, I chose it as the standard sensitisation time through out these intravesical experiments.
5.3.5 Light dosimetry and diffusion medium

A copper vapour pumped dye laser (Oxford Lasers Ltd. Oxford, UK) emitting red light at 630±2 nm and delivered through a 200μm silicon coated quartz fibre was used. The copper vapour laser was switched on to warm up 2 hours before starting pumping rhodamine dye through the dye module of the system and the wavelength was then tuned to the required value by an experienced physicist. Emulsified soybean solution for injection (Intralipid) was purchased from Abbot Pharmaceuticals Inc., which is ready to use by simply diluting the original 20% solution to 10 or 1% by adding normal saline at a volume/volume ratio. Of the six rats in the control group, 2 received ALA alone, 2 received laser alone and 2 had neither. In the experimental groups, the exact treatment parameters used are listed in Table 5.1 while the procedure of PDT treatment is shown in Figure 5.1.

Table 5.1: Treatment parameters for the PDT study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Rat</th>
<th>pH</th>
<th>Sensitiser [ALA] [dose]</th>
<th>Laser Time(sec) Energy(J)</th>
<th>Intralipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>5.5</td>
<td>10%</td>
<td>200</td>
<td>100 J</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>5.5</td>
<td>10%</td>
<td>200</td>
<td>50 J</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>5.5</td>
<td>1%</td>
<td>200</td>
<td>50 J</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>5.5</td>
<td>1%</td>
<td>20</td>
<td>50 J</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>5.5</td>
<td>0.1%</td>
<td>2</td>
<td>50 J</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>4</td>
<td>10%</td>
<td>200</td>
<td>50 J</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>4</td>
<td>1%</td>
<td>20</td>
<td>50 J</td>
</tr>
</tbody>
</table>

[ALA], [Intralipid]: Concentration of ALA, and intralipid, [dose]: ALA dose in mg/kg body weight
In all animals, ALA was instilled for 2 hours and treatment delivered 5 hours after initial instillation. The laser power was set at 100 mW.
At 5 hours after initial ALA instillation, a small laparotomy was made under a second general anaesthetic to expose the urinary bladder. The bladder was emptied of urine and filled through a urethral cannula with 0.3 ml of 10% or 1% intralipid solution to enhance isotropic light distribution. To optimise the laser fibre position, the fibre tip was pushed through the dome and placed 3-5 mm from the top of the bladder, so the light distribution through the bladder looked uniform. The fibre was so thin that the bladder sealed around it without causing any visible leakage during and after treatment. The power from the fibre tip was calibrated to 100 mW before each treatment, which was for 500 or 1,000 seconds (50 or 100 J) per rat. After light exposure, the abdomen was closed and the animals allowed to recover consciousness. The rats were killed 1, 2, 3 or 7 days after laser treatment (at least 2 for each set of treatment variables) and the bladders removed and distended by instillation of 0.3 ml formalin. Four rats treated with 50J were killed at 3 and 6 months, respectively after PDT. Specimens were sectioned and stained with haematoxylin and eosin and Van Gieson stains for histological assessment.
5.4 RESULTS

5.4.1 Stability of ALA solution

Colour changes of the freshly prepared 10% ALA solutions at different pH and at body and room temperature were used as a simple measure of the stability of the solution. This method offers a qualitative estimation of the stability of ALA in the aqueous solution by observing serial colour changes at the room or body temperature in various pH preparations. At both 37 and 25 °C, the solution turned light yellow a few seconds after titration to pH 7 with saturated sodium bicarbonate solution (pH=8.06) and became light brown at 5 hours, so no attempt was made to use the preparation at this pH for animal experiments. At pH 5.5, the solution was relatively stable in colour for the first 5 hours although it became brown after 24-48 hours whether stored at body or room temperature. This was considered acceptable if the solution could be used within a few hours of preparation. At pH 4, the solution remained colourless over the first 24 hours, staining to light yellow through the following 2 days. Only the untitrated ALA solution with a pH of 2.1 exhibited no colour change during the 72 hour observation period. The preparations stored at 37°C tended to discolor earlier than those at 25°C. The qualitative changes of colour in the solutions are tabulated in Table 5.2.

Table 5.2: The colour change of 10% ALA solution at body and room temperature at different pH

<table>
<thead>
<tr>
<th>No</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc(mg/ml)</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>pH</td>
<td>2.1</td>
<td>4.0</td>
<td>5.5</td>
<td>7.0</td>
<td>2.1</td>
<td>4.0</td>
<td>5.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Temp( °C)</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Grade of colour* upon preparation</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>10min</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1h</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2h</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3h</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4h</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>5h</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>6h</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>24h</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>48h</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>72h</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

* 0: colourless, 1: faint yellow, 2: light yellow, 3: golden yellow, 4: light brown, 5: amber
5.4.2 Fluorescence imaging

5.4.2.1 In vivo studies
The fluorescence emission spectrum of the 2 rats having no ALA showed only background activity whereas the other rats, one with intravenous ALA, and two with intravesical instillation of 10% ALA at pH 5.5, all exhibited emission spectra which were characteristic of protoporphyrin IX with maxima clearly observed at 635 nm and 710 nm (Pettier et al, 1986) (Figure 5.2).

![Fluorescence emission spectrum](image)

Figure 5.2: The emission spectrum detected from rat urinary bladder 3 hours after intravesical ALA instillation showing the peak at 635nm characteristic of PpIX.

5.4.2.2 Studies on tissue sections
The fluorescence microscopy image of a bladder after intravesical instillation of pH 5.5 ALA together with the quantitative fluorescence profile across the wall is shown in Figure 5.3. Fluorescence measurements for each specific tissue layer (urothelium, lamina propria and muscle) were averaged over more than 20 representative areas (minimum 10x10 pixels) taken from 2-3 rat bladders for each ALA solution pH and concentration, with correction for background autofluorescence. Following instillation of 10% ALA solution, total dose 200 mg/kg body weight at pH 4, PpIX fluorescence in the urothelium was variable and unpredictable with a peak 4-7 hours after administration (Figure 5.4).
Figure 5.3: (A) Computer processed fluorescence microscopy image of the bladder wall 5 hours after instillation of 10% ALA at pH 5.5. The colour scale depicts the signal in counts per pixel (white is high fluorescence, black low).

Figure 5.3: (B) Quantification of the tissue fluorescence intensity across the line on (A).
This acidic preparation seemed uncomfortable for the animals as most of them moved around much less after recovery from anesthesia than those given ALA at a higher pH. With 10% ALA solution at pH 5.5, fluorescence signals were first detectable 1 hour after administration and the peak urothelial intensity was again reached between 4 and 7 hours. By 24 hours, the signal had fallen to near background levels (Figure 5.5). Using a lower concentration (1%) at pH 5.5, the PpIX biotransformation curve was similar in configuration to that from 10% ALA but the amplitude was lower and there was a small shift to the left (Figure 5.6). At all pH values, the fluorescence in the lamina propria and muscle layers was much less than in the urothelium. With the 10% ALA solution, a peak urothelium to muscle layer fluorescence ratio of 5.5 and urothelium to lamina propria ratio of 7.0 was achieved 5 hours after instillation. Although the absolute urothelial fluorescence at this time was not the maximum, it was the time showing the best selectivity between the urothelium and other layers.
Figure 5.5: Plot of bladder wall fluorescence intensity against time after instillation of 10% ALA at pH 5.5. Each value is an average of measurements from 20-30 areas in 2-3 rats.

Figure 5.6: Plot of bladder wall fluorescence intensity against time after instillation of 1% ALA at pH 5.5. Each value is an average of measurements from 20-30 areas in 2-3 rats.
PpIX fluorescence against ALA concentration 5 hours after instillation at the pH of 5.5 is illustrated as Figure 5.7. As the concentration declined from 10 to 1 and to 0.1%, the change of fluorescence intensity was not linear. However, the urothelium remained the layer with the highest level and ratios as high as 11 : 1 (urothelium/muscle) and 9 : 1 (urothelium/lamina propria) could be obtained with the 1% solution.

**Figure 5.7:** Comparison of fluorescence intensity in different layers of the bladder wall 5 hours after instillation of 10, 1 and 0.1% ALA solutions.

### 5.4.3 Macroscopic findings

In the 6 control rats receiving ALA alone, laser illumination alone or neither, the bladders were pinkish and easily dissectable from other surrounding tissues. Macroscopically, the bladder looked pale and smooth without evidence of ulceration (Figure 5.8).
Figure 5.8: Gross appearance of rat bladder 7 days following light illumination but without ALA sensitisation. The appearances are normal.

The macroscopic treatment results are summarised in Table 5.3. Group 1 consisted of 10 rats sensitised with 10% ALA and treated with a light dose of 100J (100mW x 1,000 sec) using 1% intralipid as the light scattering medium. This was the highest light dose used, and most rats were unwell after treatment. There were 2 deaths in the first 2 days. On autopsy in these two, the peritoneal cavity was filled with fluid and the bladder showed evidence of leakage. This could have been the result of inserting the laser fibre through the bladder wall, although this was not seen in any of the other groups. It is more likely that it was related to treatment in some way. There was no macroscopic evidence of abscesses in the kidneys or elsewhere to suggest serious local infection. No other rat died thereafter, and the others were killed at the planned time. One day after treatment, all the bladders in this group were grossly puffy and pale. On the second and third days, areas of petechial haemorrhage became prominent (Figure 5.9).
On dissection, the bladders were covered by intraperitoneal fat and there was serosanginous exudate between the bladder wall and the covering fat. In groups 2 and 3, the light dose was reduced to 50J, with intralipid concentrations of 1% and 10% respectively. The macroscopic findings were milder in severity in these 2 groups and there was negligible intraperitoneal fluid accumulation compared with that seen in group one. There were no PDT related deaths in these groups. The 20 rats in Groups 4 and 5 treated at lower ALA concentrations had no identifiable abnormalities on gross inspection of the bladder at any time after treatment. Adhesion of peritoneal fat to the dome of bladder, oedema of the bladder wall and the presence of intraperitoneal fluid were found only in animals treated with 10% ALA.

Table 5.3: Macroscopic findings of the peritoneum and bladder 3 days after PDT

<table>
<thead>
<tr>
<th>Group</th>
<th>[ALA]</th>
<th>[Light]</th>
<th>[Intralipid]</th>
<th>pH</th>
<th>No perioperative death</th>
<th>intraperitoneal exudate adhesion</th>
<th>bladder petechiae</th>
<th>bladder leakage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10%</td>
<td>100J</td>
<td>1%</td>
<td>5.5</td>
<td>10</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>10%</td>
<td>50J</td>
<td>1%</td>
<td>5.5</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>10%</td>
<td>50J</td>
<td>10%</td>
<td>5.5</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>1%</td>
<td>50J</td>
<td>1%</td>
<td>5.5</td>
<td>10</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>0.1%</td>
<td>50J</td>
<td>1%</td>
<td>5.5</td>
<td>10</td>
<td>+</td>
<td>±</td>
<td>--</td>
</tr>
<tr>
<td>6</td>
<td>10%</td>
<td>50J</td>
<td>1%</td>
<td>4</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>7</td>
<td>1%</td>
<td>50J</td>
<td>1%</td>
<td>4</td>
<td>5</td>
<td>+</td>
<td>±</td>
<td>--</td>
</tr>
</tbody>
</table>

+++: severe, ++: moderate, +: mild, ±: negligible
5.4.4 Histological findings

On microscopic examination in control animals, the urothelium was composed of 3-6 tightly opposed layers of transitional epithelial cells. Deep to the urothelium, the lamina propria consisted of loose connective tissue with scanty cellular components and a few vascular structures, extending to the muscularis propria. In the bladders which had been treated with laser light through a laparotomy but without prior ALA sensitisation, mild inflammatory cell infiltration in the serosa was a common finding 2-3 days after operation (presumably due to handling) but had cleared by the 7th day. No abnormalities were seen in any other layers of the bladder wall.

The most prominent findings in the bladder 1-3 days after PDT in group 1 (100 J) were diffuse swelling of the lamina propria and focal sloughing of tissue which in some cases, extended from the urothelium through the muscularis propria to the serosa. The urothelial damage was patchy with areas of complete urothelial destruction and seemingly normal tissue in between (Figure 5.10).

Figure 5.10: Bladder lesion showing area of urothelial damage (small arrows), extensive tissue destruction (open arrow), and nearly undamaged urothelium (bold arrow) after PDT with a light dose of 100 J, 1% intralipid, 5 h after instillation of 10% ALA (X40, H&E stain).
Acute inflammatory cell infiltration, mostly with mononucleated round cells, was seen throughout all tissue layers. Fibrinoid degeneration of small arterioles was evident by 3 days after PDT (Figure 5.11). Regions of full thickness necrosis and patches of superficial ulceration confined to the lamina propria were most striking 2 days after treatment. Extensive full thickness bladder damage was seen in areas facing the laser probe, where the light dose per cm² was greatest, indicating that the concentration of the intralipid used as light scatterer was too low to achieve isotropic light distribution. Treatment at the lower light dose of 50J (group 2) produced similar focal lesions which were patchy in distribution with varying degrees of tissue destruction, but there was much less full thickness necrosis than in group 1 (Figure 5.12). With the higher concentration of intralipid used in group 3 (10%), the changes in the mucosa were much more uniform and essentially limited to the urothelium and lamina propria (Figure 5.13).

Figure 5.11: Fibrinoid necrosis of arterioles (arrows) 3 days after PDT with a light dose of 50J, intralipid 1%, 5 h after instillation of 10% ALA (X100, H&E stain).
Figure 5.12: Patchy bladder lesions 3 days after PDT with a light dose of 50 J, intralipid 1%, 5 h after instillation of 10% ALA. Suboptimal light distribution resulted in some areas of undamaged urothelium (small arrows) and others of full thickness damage (large arrows) (X40, H&E stain).

Figure 5.13: Histology of bladder 3 days after PDT with a light dose of 50 J, 5 h after instillation of 10% ALA, and 10% intralipid. There is now much more uniform urothelial necrosis (X40, H&E stain).
The microscopic changes in group 4 and 5 were even milder and ranged from small areas of urothelial sloughing to no damage at all. The most prominent histological change of the bladder in group 6 was patchy urothelial sloughing, indicating heterogenous tissue PpIX and light distribution. In all groups, oedema of the lamina propria was the most constant histological finding in the first few days after PDT, although the severity varied markedly with the treatment parameters used. By the 7th day after treatment, although the necrosed mucosa had healed with regeneration of healthy urothelium in all groups, there was persistent evidence of ongoing tissue repair processes such as increased mitotic figures and prominent hypochromatic nuclei in the basal cell layer. Healing in the lamina propria was also essentially complete by 7 days (Figure 5.14).

**Figure 5.14:** Bladder histology 7 days after PDT using a light dose of 50 J, intralipid 10%, 5 h after instillation of 10% ALA. Regeneration of urothelium is evident (X100, H&E stain).

In group 1, there was scarring in the muscularis propria at 7 days, but in the other groups, there was nothing more than very minimal changes in the muscle layer at this time in sections stained with H & E or Van Gieson's stain. The principal finding of the bladders examined 3 months after PDT (Group 2, 50J) was mild collagen fibre
deposits with an increased fibroblast activity in the lamina propria compared with that of normal bladder. The collagen fibre formation in the muscularis propria was negligible (Figure 5.15). The main histological findings are summarised in Table 5.4.

Table 5.4. Histological changes of the bladder wall 1, 2-3, 7 and 90 days after PDT in different treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Loss of Oedema</th>
<th>Necrosis of Urothelium and L.P.</th>
<th>Muscle Necrosis</th>
<th>Inflamm. cell Infiltration</th>
<th>Fibrosis L.P. M.P. Serosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (10% ALA, 100J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>patchy ++ + + + ± + + + -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>patchy ++ ++ ++ ++ +++ +++ ++ + -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>-- -- -- -- -- -- + + + + + ++ --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (10% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>patchy + + -- -- + ± + -- -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>patchy ++ + + + ++ ++ ++ -- -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>-- -- -- -- -- -- + ± -- ± ± --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90d</td>
<td>-- -- -- -- -- -- -- -- -- + ± --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (10% ALA, 50J, 10% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>diffuse + + + + -- + + + -- -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>near uniform ++ + ++ + ++ ++ + -- -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>-- -- -- -- -- -- + ± -- ± ± --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 (1% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>rare + ± -- -- + + + -- -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>occasional ++ ± -- -- ++ + -- -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>-- -- -- -- -- -- -- -- -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (0.1% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>rare + + -- -- + + + ± -- -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>rare ++ + -- -- + + + + -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>-- ± -- -- -- -- -- ± -- -- --</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Treatment parameters: ALA concentration, light dose, concentration of intralipid)
Changes within layers: --: none, ±: minimal, +: mild; ++: moderate; +++: severe
L.P.: Lamina propria, M.P.: Muscularis propria
Figure 5.15: (A) Normal untreated bladder showing scanty collagen fibrils (red) in the lamina propria. (B) Moderate increase in collagen fibrils in the lamina propria 3 months after PDT. The treatment parameters were 50 J light dose, 10% intralipid, 5 h after instillation of 10% ALA (X100, HGV stain).
5.5 DISCUSSION

The major attraction of PDT in the management of bladder disease is the possibility of ablating extended areas of abnormal urothelium without damage to the underlying muscle and doing so in such a way that the treated areas heal with regeneration of normal urothelium. Previous clinical reports of PDT for severe dysplasia and carcinoma in situ of the bladder have shown effective ablation of the abnormal areas, but also showed a high incidence of permanent damage to the bladder, sometimes severe enough to warrant total cystectomy in spite of the eradication of the neoplastic disease (Harty et al., 1989). The purpose of the present work has been to assess whether it is possible to use intravesical ALA to produce selective urothelial accumulation of PpIX in the bladder and to develop a "window" of effective doses as was achieved with intravenous aluminium sulphonated phthalocyanine (AlSPc) (Pope & Bown 1991).

5.5.1 Advantages of ALA instillation for bladder PDT

Skin photosensitivity is always a major concern with systemic administration of photosensitisers. Intravesical instillation is an attractive alternative as it should avoid systemic photosensitisation and could give better urothelial selectivity. As the rat urinary bladder is small and difficult to dissect, assessment of PpIX content in different tissue layers by biochemical extraction would be technically difficult and inaccurate. However satisfactory correlation (94%) has been found previously in normal rat stomach and colon between microfluorometric measurements obtained with this system and chemical extraction measurements (Loh et al., 1993b). This study using quantitative fluorescence microscopy indicates that absorption of small molecules like ALA through the tight barrier of the transitional epithelium is possible and a higher concentration of PpIX has accumulated in the urothelium than in the underlying layers. Nevertheless, the rat bladder is very thin, so one would anticipate rapid diffusion across all layers, whereas the wall of the human bladder is much thicker, so diffusion to the muscle layer may be less which could lead to even better selectivity. This hypothesis, however, awaits confirmation. The distribution of PpIX through each layer of the bladder wall was much more uniform than had previously been reported with intravesical AlSPc (Pope & Bown 1991). Possible reasons for the difference observed between ALA and AlSPc are the dose of photosensitiser, time of bladder retention (time of instillation plus post-instillation interval) and chemical
properties of the specific drugs. In this study, the instilled dose of ALA was similar to that used in previous studies for systemic administration whereas in the previous AlSPc study, the dose used intravesically was much lower than that used systemically. The time that the photosensitisers stay in the bladder is another factor that is likely to influence whether adequate and uniform urothelial buildup of PpIX is achieved. In this study, it was 2 hours whereas it was only 30-60 min in the AlSPc work. A third factor for the poorer absorption of AlSPc through the bladder wall might be the molecular weight which is higher for AlSPc than for ALA (770 vs 168). Although PpIX was virtually undetectable 24 hours after intravenous injection (Bedwell et al, 1992; Loh et al, 1992; Loh et al, 1993a), the intravesical route is likely to be an effective means of reducing skin photosensitivity during this 24 hour period as the fluorescence signals from rat skin (epidermis and dermis) using the intravesical route are only 25-50% of those seen with oral administration of the same dose of ALA (unpublished data).

5.5.2 Biochemical characteristics for ALA instillation

The urinary bladder is a specially designed reservoir for urinary storage and evacuation. Its inner surface is covered with urothelium which constitutes an unique and integral barrier to urine. For any substance to be absorbed efficiently from the urothelium, it should have a low molecular weight (<200), be water soluble and yet be sufficiently lipophilic for binding with plasma membranes (Bridges et al, 1979). The biochemical properties of ALA (low molecular weight, high water solubility and possibly high lipophilicity) fulfil these criteria. Despite the lack of direct evidence supporting the existence of a mechanism for transportation of small molecules across the urothelium, a concentration gradient from the mucosa to serosal surfaces has been demonstrated after instillation of amino acids (Morris & Bryan 1966). The finding of increased accumulation of PpIX in the urothelium after instilling the ALA solution is good evidence to support the permeability of the urothelium to ALA. After penetrating the superficial umbrella cells, it may take some time to diffuse into deeper transitional cell layers and to convert to PpIX. The relative speeds of absorption of ALA into the urothelium and conversion of ALA to PpIX once in the urothelium and other layers of the bladder wall are not known, but it would seem likely that the longer the ALA remains in the bladder, the more that will be absorbed, so the time of retention is an important factor influencing the PpIX levels in the layers of the bladder wall.
5.5.3 The importance of pH for ALA instillation

The pH of an agent delivered intravesically is also important. As urinary pH depends on the body acid-base balance, the urothelium is well adapted to substantial changes of pH although under normal conditions, it rarely gets below 5.4. The unbuffered ALA solution has a pH of 2.1 which is unphysiological to the bladder and was not used in this study. An absorption spectroscopic study on the stability of aqueous ALA solution indicated that, upon titrating to neutral pH, dihydropyrazine and pyrazine were formed as a result of condensation of ALA molecules (Rodriguez et al, 1995), the former being the agent responsible for the yellow colour change observed in this study. The authors concluded that the optimum ALA preparation for instillation is a solution at pH 5 and at a concentration of less than 5% (Rodriguez et al, 1995). Results from this study showed higher levels of PpIX in vivo with pH 5.5 than with pH 4.0 solution. The fluorescence intensity of PpIX in the urothelium with pH 5.5 solution retained for 4 to 7 hours was 30% higher than that using ALA at pH 4 retained for the same time. It is probable that at the lower pH, although the ALA is more stable chemically, some of the transitional epithelium might be damaged as a result of prolonged contact with the strong acid. The optimal pH for ALA instillation that achieves a balance between PpIX buildup and bioadaptability of the urothelium seems to lie in the range 5-6, and it is with this rationale that I selected pH 5.5 for this study. The poor PpIX fluorescence detection after topical administration of ALA reported by Leveckis et al (1994) could be the result of instilling an ALA solution which was so acidic (pH=2.1) that it reduced the ability of transitional cells to absorb ALA, and damage intracellular enzymes.

5.5.4 Fluorescence selectivity after ALA instillation

Following intravesical administration of 10% ALA solution, PpIX buildup in the bladder urothelium peaked at 4 to 7 hours which is slightly later than after either intravenous (3-4 hours) or oral administration (4-6 hours) (Loh et al, 1993a). The absolute fluorescence levels in the urothelium are similar using the three different routes and comparable total administered doses. The selectivity of PpIX accumulation between the urothelium and muscle layer is also similar (5:1 with the intravenous route, 4:1 with the oral route (Loh et al, 1993a) and 5.5:1 with the intravesical route). The ratio of PpIX concentration between the urothelium and muscle layer is one of the best indicators for safe PDT treatment of the bladder, so the greater the ratio, the better the chance to selectively destroy the urothelium without harming the lamina
propria and the detrusor muscle layer. Using the intravesical route has only shown a marginal benefit from this point of view, but it does avoid the transient liver damage which can occur after oral administration (Regula et al., 1995). Although ALA has rarely been tested using the intravesical route (Kriegmair et al., 1994b; Leveckis et al., 1994), like other photosensitisers (AlSPc), the results from this route of administration seem conflicting (Pope & Bown 1991; Banchor et al., 1992; Kriegmair et al., 1994b; Leveckis et al., 1994). It is probable that different types of photosensitiser are taken up by different mechanisms which will lead to different tissue concentrations and distribution patterns after different routes of administration. Even for closely related agents, the chemical structure, spatial configuration, number of side chains and molecular weight are all determinants of the final distribution. This experiment suggests that intravesical instillation of ALA might be an attractive alternative to other routes because it leads to less skin photosensitisation but retains considerable PpIX selectivity between the urothelium and other layers.

5.5.5 ALA induced PDT effect on the urothelium

Comparable to previous results with AlSPc given intravenously (Pope & Bown 1991), I have achieved selective urothelial necrosis with ALA given by the intravesical route, and with a much shorter time interval between drug and light (5 hours instead of 24 hours). With inappropriate dosimetry, muscle damage is still inevitable. Nevertheless, histological data showed that the PDT effect was mostly superficial and predominantly confined to the urothelium with a total light dose of 50J. I have not found a dose of ALA at which I could get completely uniform urothelial ablation but no muscle damage, even with larger light doses, but from my results, it is possible that such a dose exists between 1% and 10% (20mg/kg and 200mg/kg). With any sensitiser given intravenously, the ratio of sensitiser concentration between urothelium and muscle is likely to be the same in rats and patients, but with the intravesical route, the need for drug to diffuse across a greater thickness of urothelium and lamina propria may give better selectivity in patients. Also, the red light used for PDT is attenuated rapidly as it goes through the bladder wall, so the relative intensity of light in muscle in the human bladder compared to that in the overlying urothelium is less than in rat bladder muscle compared to the overlying urothelium. This may further improve selectivity in clinical use. However, care must be taken with this aspect as clinical trials have shown that scarring is easily produced in muscle using Photofrin as the photosensitiser with red light at the same wavelength as is used for ALA (Harty et al., 1989).
5.5.6 ALA induced PDT effect for urothelial tumour

Using a similar instillation technique and ALA dosimetry (pH 5.5, 20% ALA retained for 3.5-4 hours), Kriegmair et al. (1994b) achieved selective damage to chemically induced bladder tumours sparing the normal urothelium after laser treatment of rat bladder at various light doses (15-100 J/cm²), although tumour destruction was more constant at higher light doses. It is possible that during bladder illumination in their experiments, the laser beam was aimed mainly at the tumour, and as a result, less light fell on the surrounding normal urothelium. My experiments did not study bladder tumours, but showed selectivity between the layers of the normal bladder, particularly between urothelium and muscle, using treatment parameters comparable to those of Kriegmair et al. (1994b). I feel it is important to emphasise the need to destroy all the transitional cell lining as a crucial step in the management of bladder cancer. With current urological techniques, papillary tumours which can be visually identified under cystoscopy pose no particular therapeutic problem as they can be eliminated with laser coagulation or electrocautery. However, intractable problems remain with dysplastic or pre-cancerous foci that can be distributed throughout the urothelium which are not easily detected macroscopically and which may develop into invasive cancers. If the diseased urothelium can be eradicated completely with PDT and the bladder lining regenerates subsequently with normal urothelium and without causing underlying muscle damage, then PDT might be the most attractive modality for prevention of bladder cancer recurrence. It is with this rationale that I focused on selectivity of damage between normal bladder layers rather than on the difference between normal and neoplastic urothelium.
5.5.7 Site of action of ALA induced PpIX in vivo

Previous PDT studies using HpD and Photofrin have shown that the cytotoxic effect of those photosensitisers was due mainly to shut down of the tumour and normal tissue microvasculature (Selman et al, 1984; Reed et al, 1989). The site of ALA associated cell killing has been shown to be the mitochondria (Malik & Lugaci 1987), however, the possibility of damage to other cellular structures at a later time when most of the PpIX has accumulated in the stroma cannot be ruled out (Reed et al, 1989). The presence of arteriolar fibrinoid lesions raises the possibility that the photodynamic threshold (the product of tissue photosensitiser concentration and the light dose) for necrosis in the arteriolar smooth muscle is lower than for the adjacent venules. It is also possible that the lesions are caused by disruption of the microcirculation, although this mechanism has not yet been clearly shown using ALA. It remains to be established what the importance of vascular effects is in the destruction of tissue, whether it is normal or neoplastic.

5.5.8 Optimal concentration for light scattering

I also demonstrated that the better concentration of intralipid for light scattering in the rat bladder with a volume of 0.2-0.3 ml was 10%. At this concentration, uniform urothelial sloughing with oedema of the lamina propria was observed. With the same treatment parameters but reducing the intralipid to 1%, urothelial damage was patchy and fibrinoid change became more common in the arterioles of the lamina propria. This finding was in accord with the prediction by Baghdassarian and associates who tested the uniformity of light distribution at different intralipid concentrations in flasks of various volumes (Baghdassarian et al, 1985). In a sphere with a volume of 50ml or less, light tended to focus on the site opposite to the tip of the laser fibre (180°) and uniform distribution of light was not possible in the presence of 1% intralipid. Higher concentrations of scatterer lead to more uniform light distribution but at the expense of more energy loss. In the clinical situation, as the capacity of the human bladder is usually in the range of 300-450 ml, although it is not an ideal sphere, a 1% or even 0.02% intralipid would likely to be the concentration of choice to obtain the maximum uniformity of light distribution with the minimum amount of light being absorbed in the scattering medium (Manyak 1994). The causes of recurrence of bladder tumours following PDT which might be the result of (a) field dysplastic changes of the urothelium that had already existed in the urinary tract not amenable to the current treatment, or (b) of inhomogenous light distribution during
PDT of the bladder, rendering areas of suboptimally treated urothelium the focus of tumour regrowth. although remain to be defined, it is imperative that more studies, in particular those of basic light fluence measurements in biological system are needed to verify the optimal light scattering concentration which will lead to satisfactory urothelial destruction with or without the assistance of optic fibre modification as this is a technical problem with potential to be solved recently.

5.5.9 Potential for bladder cancer treatment with PDT

Before adopting PDT for the treatment of multifocal carcinoma in situ or diffuse low grade papillary bladder tumours, it is worth emphasising that for any therapeutic modalities to be clinically applicable, the ability to achieve uniform urothelial necrosis is crucial as complete removal of the urothelial lining is the only reliable proof of cure of this type of bladder malignancy. As I have demonstrated the possibility of achieving selective urothelial destruction with appropriate ALA concentrations and light dosimetry in the rat bladder, it may be feasible to extend PDT to treat patients with recurrent bladder cancer who have failed intravesical chemotherapy or BCG instillation. One advantage of ALA over other more potent photosensitisers is the feasibility of multiple treatments as is current practice with chemotherapeutic agents as skin photosensitivity only lasts 1-2 days. However, care must be taken in applying in vivo data from rodents to clinical trials as the bioavailability of ALA and PpIX and PDT treatment parameters may differ from species to species.
In this in vivo study using ALA as a photosensitiser precursor, I conclude that intravesical instillation of ALA solution at an appropriate pH and concentration, may be a clinically feasible route to achieve selective accumulation of PpIX in the urothelium with reduced systemic photosensitisation. Furthermore, I have illustrated histologically that, with careful light dosimetry, a diffuse urothelial sloughing without damaging the underlying muscle layer can be achieved using ALA. ALA also has an advantage over AlSPc as urothelial regeneration is essentially complete by the 7th day whereas this takes 14 days with AlSPc (Pope & Bown 1991). Although the underlying mechanism for the rapid healing process is yet to be defined, it could be that only the urothelium rather than the microvasculature in the lamina propria is damaged. Better preservation of the lamina propria is likely to lead to faster urothelial regeneration, and to less residual histological changes below the urothelium which might be of clinical significance. The results of this work justify further studies comparing oral versus bladder instillation on the systemic buildup of PpIX, in particular, the skin where the concern of photosensitivity is the greatest. Only when instillation of ALA produces PDT effect as good as that of systemic administration and with a reduced risk of skin and systemic photosensitivity, can pilot clinical studies be justified to instill ALA into the human bladder for PDT treatment of resistant carcinoma in situ or frequent and multiple recurrence of bladder cancer.
CHAPTER 6

QUANTITATIVE FLUORESCENCE ASSESSMENT OF PROTOPORPHYRIN IX IN THE BLADDER AND OTHER ORGANS FOLLOWING ADMINISTRATION OF 5-AMINOLAEVULINIC ACID: COMPARISON BETWEEN INTRAVESICAL AND ORAL ROUTES

6.1 Background ---------------------------------------------------------- 160
6.2 Aims ------------------------------------------------------------------- 161
6.3 Materials and Methods
  6.3.1 Animals and anaesthesia ------------------------------------------- 162
  6.3.2 Photosensitiser -------------------------------------------------------- 162
  6.3.3 Routes of administration
    6.3.3.1 Bladder instillation ----------------------------------------- 162
    6.3.3.2 Oral administration ---------------------------------------- 162
  6.3.4 Fluorescence imaging microscopy --------------------------------- 163
  6.3.5 Photodynamic therapy --------------------------------------------- 163
6.4 Results
  6.4.1 Fluorescence microscopic imaging
    6.4.1.1 Bladder -------------------------------------------------------- 164
    6.4.1.2 Liver ----------------------------------------------------------- 169
    6.4.1.3 Kidney --------------------------------------------------------- 171
    6.4.1.4 Muscle --------------------------------------------------------- 173
    6.4.1.5 Skin ------------------------------------------------------------ 174
  6.4.2 Photodynamic effects
    6.4.2.1 Macroscopic findings --------------------------------------- 177
    6.4.2.2 Microscopic findings ---------------------------------------- 177
6.5 Discussion
  6.5.1 Which route is better for bladder PDT? --------------------------- 186
  6.5.2 Mechanism of ALA-PDT effect, systemic versus local ------ 186
  6.5.3 Systemic distribution of ALA induced PpIX --------------------- 187
  6.5.4 Fluorescence microscopy vs chemical extraction ---------------- 188
  6.5.5 Skin photosensitivity ------------------------------------------ 189
  6.5.6 Advantages of intravesical instillation ------------------------ 189
6.6 Conclusions ------------------------------------------------------------- 191
In a survey conducted by Spinelli (1996) from 46 medical centres worldwide on the use of PDT, it was revealed that of the total of 3193 patients who had been treated previously, HpD or Photofrin was the photosensitiser employed in more than 95% of cases. However, despite its wide acceptance, the notable prolonged cutaneous photosensitisation which lasts for 4-6 weeks is an obvious disadvantage. The manifestation of skin photosensitivity ranges from mild erythema to plaster formation on areas exposed to strong sun light. This side effect can be largely avoided if patients avoid going outdoors, although this poses an unnecessary inconvenience to patients, and sometimes is not always possible. Research aimed at developing newer photosensitisers with better tissue selectivity is being widely pursued, but the problem of cutaneous photosensitivity remains. B-carotene although effective, takes up to 2-3 months to reach therapeutic levels and has the concern of protecting the tumour as well as the skin from the effects of light (Mathews-Roth 1984). Hydroxychloroquine or anti-histamine agents (cimetidine, hydroxyzine), have been shown to reduced incidence of photosensitivity in association with Photofrin administration in mice (Manyak et al, 1988). Activated charcoal was proved to be futile in preventing skin photosensitivity (Lowdell et al, 1992). An alternative approach for this is to apply photosensitisers locally, e.g. intratumoural injection or intravesical administration, which was shown to be possible (Amano et al, 1988; Kennedy & Pottier 1992; Chapter 5).

In previous chapter I have demonstrated that intravesical instillation of ALA was able to ablate the urothelium extensively and diffusely, with only negligible damage to the underlying lamina propria and muscle layer, if the pH and concentration of ALA solution, light scatterer concentrations, and light dosimetry can be carefully designed. Although the levels of bladder PpIX after instillation of ALA have been determined successfully with fluorescence microscopic measurement, the biodistribution of PpIX in a variety of vital organs following ALA instillation remains largely undefined. Previously PpIX fluorescence in human skin (Pottier et al, 1986) and in rodent organs after systemic administration of ALA has been evaluated (Kennedy & Pottier 1992; Loh et al 1993a). However, no attempts has been made to compare PpIX intensities in the bladder, liver, kidney, muscle and skin after oral and instillation of ALA and to define the severity of skin photosensitivity with these two routes of administration.
6.2 AIMS

Further to the previous ALA study on the urinary bladder (Chapter 5), I started with examining PpIX fluorescence in the liver and kidney following oral and intravesical instillation of various concentrations of ALA. Since liver and kidney are the 2 major organs responsible for metabolism and excretion of ALA and its subsequent metabolite porphyrins, the PpIX levels in which would be a useful index to assess the systemic biodistribution of PpIX after topical and oral application of ALA, and to define the concentration that had minimal systemic effect. I also tried to obtain a PpIX level induced by oral ALA in the urothelium which is similar to that achieved by bladder instillation of 200mg/kg, and compared skin fluorescence as well as PDT effects with the two drug regimens. If the same degree of urothelial ablation is achievable by intravesical instillation at an ALA concentration that causes less skin photosensitivity, then instillation is the preferred route for bladder PDT with ALA. In this study, I aimed to answer these questions and to find out which route will result in less systemic effects following administration of ALA.
6.3 MATERIALS AND METHODS

6.3.1 Animals and anaesthesia

Female Wistar rats weighing 100 to 180 gram were used in this study. The ALA solution was administered by a gastric gavage to rats anaesthetised with a mixture of Halothane and O2 (volume ratio 1: 2). However, for longer procedures such as transurethral instillation of ALA and laser treatment, general anaesthesia with intramuscular injection of Hypnorm (fentanyl and fluanisone, Jansen Pharmaceuticals Ltd.) and diazepam was used.

6.3.2 Photosensitiser

The preparation of ALA solution is the same as in section 5.3.1. In this study however, only ALA solution at pH 5.5 was used.

6.3.3 Routes of administration

6.3.3.1 Bladder instillation
Bladder instillation (BI) of ALA was accomplished by infusing a 10% solution at the dose of 200 mg/kg (0.2 ml/100 gm body weight) through an 18.5 gauge Teflon catheter which had been inserted transurethrally and was kept in the bladder for 2 hours while the rats remained under general anaesthesia. The procedure of urethral catheterization was described in section 5.3.2. The definition of instillation is the same as in section 5.3.2.

6.3.3.2 Oral administration
Oral administration (Oral) of ALA was undertaken by delivering ALA solutions at doses of 100, 200 and 400 mg/kg through a bulb tip gavage needle into the stomach under inhalation anaesthesia. As rats are incapable of vomiting or regurgitation, all animals ingested the full delivered dose of ALA. All the rats resumed normal activity within 5 minutes of termination of inhalation anaesthesia. The oral dose used was that found in preliminary studies to give urothelial levels of PpIX comparable to those found in my previous study using intravesical ALA.
6.3.4 Fluorescence imaging microscopy

For each ALA dose and route of administration, 2-3 rats were tested. The control group consisted of 2 rats receiving nothing and 2 others with bladder instillation of 0.9% normal saline (0.24-0.3 ml). Specimens from the urinary bladder, liver, kidney and back skin were collected 1, 2, 3, 4, 5, 7, 9 and 24 hours after administration of ALA. The tissues harvested were initially placed in a precooled isopentane medium (BDH Chemicals Ltd., Poole UK) and then transferred to liquid nitrogen before being processed to thin frozen section (10 μm) for fluorescence imaging.

6.3.5 Photodynamic therapy

The control group consisted of 3 rats receiving laser illumination without ALA sensitisation. The experimental groups were stratified into Group A: Intravesical ALA (200mg/kg), and 50J light; Group B: Oral ALA (100mg/kg) and 50J light; Group C: Oral ALA (200mg/kg) and 25J light; Group D: Oral ALA (200mg/kg) and 50J light, and Group E: Oral ALA (400mg/kg) with 50J light dose. The light source used in this study was a copper vapour pumped dye laser (Oxford Lasers Ltd.) tuned to 630 nm which was delivered through a 200 μm silicon coated quartz fibre. At the optimal time after sensitisation (5 hours with intravesical, 4 hours with 200 and 400 mg/kg oral, and 3 hours with 100mg/kg oral ALA), the bladder was exposed for PDT treatment. The details of laparotomy and lasering procedures have been described in section 5.3.5. Power output from the tip was set at 100mW for 250 or 500 seconds giving a total light dose of 25 or 50J, respectively per treatment. Following PDT, animals were killed at 2, 3 and 7 days, and the bladder sectioned and stained for histological assessment. Four rats, 2 with intravesical (200mg) and 2 with oral ALA (400mg) were treated at a light dose of 50J, and were kept alive for 6 months after PDT for long term comparison. These specimens were processed in the same manner as in Chapter 5.

Eight rats (2 without drug, 2 with intravesical ALA, 2 with 200mg/kg, and 2 with 100mg/kg oral ALA) were investigated for the skin photosensitisation study. All the sensitised rats were treated 3 hours after giving ALA. The fur overlying the lower abdomen was shaved and the exposed skin was illuminated with red light (630 nm) through a 200 micron laser fibre placed 1 cm above, at a power of 100 mW (Power density 100mW/cm²) for 500 seconds (50 J). The animals were sacrificed 3 days after light illumination and the specimens stained with H&E for histological evaluation.
6.4 RESULTS

6.4.1 Fluorescence microscopic imaging and photometry

6.4.1.1 Bladder

Figure 6.1 shows the fluorescence false colour image of a bladder cross-section with its histological matching section which demonstrates that the highest levels of PpIX fluorescence were present in the urothelium after intravesical ALA. The photometric fluorescence intensity of a specific tissue layer (urothelium, lamina propria, muscularis propria, skin epidermis, dermis and hair follicle) was the average measurement from more than 20 representative blocks (minimum 10x10 pixels) with correction for background autofluorescence. However, for sections of solid organs such as liver, kidney and abdominal muscle, the fluorescence quantification profile was the average of 10-12 representative blocks (minimum 350x500 pixels) taken from various areas.

The CCD fluorescence microscopic picture of the bladder after oral ALA is shown as Figure 6.2, whereas figure 6.3(A) shows PpIX fluorescence intensity in the urothelium after giving ALA via bladder instillation (BI) or by gastric gavage (oral). The buildup of urothelial PpIX was faster following oral administration of ALA. At the higher oral doses (200 and 400 mg/kg), fluorescence in the urothelium peaked at 6-7 hours, but at 100mg/kg, it was at 3 hours. With bladder instillation of 200mg/kg, the optimal urothelium PpIX level occurred between 4 and 7 hours at which time it was similar to that found 3 hours after 100mg/kg orally. It was notable that bladder instillation gave a more sustained level of PpIX in the urothelium, whereas the level fluctuated more after oral administration. Although the peak urothelial PpIX intensity was generally higher in the oral groups with the doses used, the ratio between the urothelium and the underlying layers remained similar as the fluorescence signals in lamina propria and muscle were also higher with oral administration (Figure 6.3(B)(C)).
Figure 6.1: (A) Fluorescence microscopy of rat urinary bladder 5 hours after instillation of 10% ALA at pH 5.5 showing higher PpIX (White) intensity in urothelium than in the underlying layers (Red). The intensity of PpIX is indicated with the colour bar at the top of the photo. (B) Histological matching of the above fluorescence microscopy with H&E stain (X 150).
Figure 6.2: (A) Fluorescence microscopy of rat urinary bladder 5 hours after oral intake of 200mg/kg ALA showing higher PpIX (White) intensity in urothelium than in the underlying layers (Red), (B) Histological matching of the above fluorescence microscopy with H&E stain (X 150).
Figure 6.3: (A) Plot of PpIX fluorescence intensity (counts/pixel) in the urothelium after ALA administration, (B) in the lamina propria.
Figure 6.3: (C) Plot of fluorescence intensity (counts/pixel) in the muscularis propria of bladder after giving ALA via oral or intravesical route.
6.4.1.2 Liver

The results of porphyrin fluorescence in the liver are shown in Figure 6.4. At the early stage following oral ALA, porphyrin fluorescence in the liver was much higher than that after bladder instillation. The signal observed most probably corresponds to an average of protoporphyrin, uroporphyrin and coproporphyrin (see discussion). However at longer times, the differential declined and was virtually abolished after 24 hours. Figure 6.5 demonstrates the CCD camera fluorescence microscopic pictures of porphyrins in the liver tissue following either oral or intravesical administration of ALA.

![Fluorescence intensity vs time graph]

**Figure 6.4:** Plot of liver PpIX fluorescence intensity after giving ALA via oral or intravesical route.
Figure 6.5: (A) CCD picture of liver tissue 3 hours after oral administration of 100mg/kg ALA showing accumulation of porphyrins. (B) 5 hours after bladder instillation of 200mg/kg ALA.
6.4.1.3 Kidney

The fluorescence activity of porphyrins in the renal cortex resembles that of liver, but wide fluctuations in the readings obscured any clear pattern being identified (Figure 6.6). The fluorescence microscopic images of the renal cortex as well as tubular area are demonstrated in Figure 6.7. The PpIX intensity of the renal cortex was always higher than that of the collecting tubular tissue. It should be noted that readings of fluorescence level at 0 hour, which indicated the baseline porphyrin activity in the liver and kidneys without ALA administration, were much higher than in other organs measured, presumably owing to the presence of endogenous porphyrins. Despite the lack of a clear pattern in terms of porphyrin pharmacokinetics, renal fluorescence signals resumed baseline level 24 hours after ALA administration. As the data of PpIX levels in the kidney after 100mg/kg ALA are not available, the comparison of renal PpIX intensity between 100mg/kg oral, and 200mg/kg intravesical ALA which give rise to similar urothelial PpIX signals is not possible.

![Figure 6.6](image-url)

**Figure 6.6:** Plot of PpIX fluorescence intensity in the kidney showing fluctuating pattern of PpIX buildup following ALA administration via different routes. There were no data for oral ALA at 100mg/kg.
Figure 6.7: CCD fluorescence microscopic picture of kidney 5 hours after oral and intravesical ALA. (A) Renal cortex, (B) Medulla, 400mg/kg ALA orally; (C) Renal cortex, (D) Medulla, 200mg/kg ALA bladder instillation.
6.4.1.4 Muscle

The PpIX fluorescence signals in the abdominal muscle were relatively low as compared with that in the liver and kidney (Figure 6.8). Following instillation of ALA, the muscle PpIX level remained very low throughout the time except at 6 hours. With oral ALA, peak intensity was observed at 2 hours (200mg/kg) and 3 hours (400mg/kg), respectively (Figure 6.9). Again in this part, data on 100mg/kg oral ALA are not available.

Figure 6.8: CCD fluorescence microscopic picture of abdominal muscle 5 hours after bladder instillation of 200mg/kg ALA.

Figure 6.9: Plot of PpIX fluorescence levels in abdominal muscle after instillation and oral administration of ALA. No data were available for oral ALA at 100mg/kg.
6.4.1.5 Skin

CCD fluorescence microscopic images of skin after various doses and routes of ALA are shown in Figure 6.10(A)(B)(C). The PpIX fluorescence levels in epidermal and dermal layers of the skin on the back are plotted as Figures 6.11 and 6.12. Comparing the fluorescence intensity in each layer, signals from the oral groups were generally higher than those from BI group. At an oral dose of 100 mg/kg which was able to produce a peak urothelial PpIX level similar to that from 200mg/kg instillation, PpIX in the epidermis was twice as higher in the oral as the instillation group. At higher oral doses(200 & 400mg/kg), 3 to 7 hours after administration, PpIX intensity in the epidermis surged 3-10 times higher than that after BI ALA. Intravesical instillation also resulted in less PpIX formation in the hair follicles.

![Figure 6.10: (A) CCD fluorescence microscopic picture of back skin 5 hours after oral administration of 200mg/kg ALA.](image)
Figure 6.10: CCD fluorescence microscopic picture of back skin (B) 3 hours after oral ALA 100mg/kg. (C) 3 hours after bladder instillation of 200mg/kg ALA.
Figure 6.11: PpIX fluorescence intensity in the epidermis of back skin.

Figure 6.12: PpIX fluorescence intensity in the dermis of back skin.
6.4.2 Photodynamic effect

6.4.2.1 Macroscopic findings
Control bladders which underwent light illumination only showed no evidence of tissue injury. Under the most severe conditions (Group E, 400 mg/kg oral ALA and 50J light) 3 of the 12 rats died after 48 hours. Others in the group which survived the early stages, were physically unwell until 7 days after PDT. The perioperative findings of rats in Group D (200 mg/kg oral ALA and 50J light) were similar to Group E, and 2 died within 48 hours of PDT. However, with a reduced light dose: Group C (200mg/kg oral ALA and 25J light), or reduced ALA dose: Group B (100mg/kg oral ALA and 50 J light), no perioperative mortality was seen in either group. Similarly, for intravesically sensitised rats in Group A, no PDT associated death or perioperative distress was observed. On necropsy of the rats which died within 48 hours of PDT (Group D and E), the most probable cause of death was extravasation of urine with possible peritonitis as accumulation of turbid fluid in the abdomen was a consistent finding. The cause of urinary extravasation remains uncertain, although there is the possibility of leakage through the site of fibre puncture which could have become an established perforation after PDT.

Whatever the dose of oral ALA, 48 hours after treatment the bladder looked inflamed, puffy and oedematous. Only the 5 animals discussed above showed evidence of perforation, although a perivesical fatty tissue reaction was prominent in most. The bladders receiving 50J of light contained some tissue debris, and were particularly thin and flaccid on filling with formalin. The lower light dose of 25J provoked less tissue reaction. By the seventh day, perivesical fatty inflammation was subsiding, although was not completely resolved. Animals with intravesical ALA (Group A), on the other hand, demonstrated much milder changes macroscopically at these times after PDT. Six months following PDT, the appearance of bladders and the surrounding tissue in animals that had been sensitised with oral or intravesical ALA were macroscopically indistinguishable from that of control rats.

6.4.2.2 Microscopic findings
The microscopic section of normal rat bladder is shown in Figure 6.13. Histologically, no evidence of urothelium damage was discovered in rats receiving only light illumination. However, mild inflammatory cell infiltration in the serosa and outer layer of muscularis propria was seen (Figure 6.14). Except for 100mg/kg oral ALA group, the bladder lesions 2-3 days after PDT were much severer in the oral
ALA treated group than in with the instillation group, and showed full thickness tissue destruction and fibrinoid necrosis of arterioles in most part of the bladder if the light dose was 50J (Figure 6.15). At 25J, the urothelium was successfully ablated with preservation of part of underlying structures. However, muscle damage of various degrees seemed inevitable (Figure 6.16). Interestingly, only negligible degrees of urothelial destruction was observed in Group B (Figure 6.17), although the fluorescence intensity at 3 hours was as high as that of 5 hours in Group A. Despite nearly complete regeneration of damaged urothelium in 7 days after PDT, subepithelial fibroblast infiltration became salient, especially in the lamina propria.

Figure 6.13: Histology of normal bladder showing 3-4 layers of urothelium overlying the loosely arranged lamina propria and muscularis propria. No evidence of inflammatory cell infiltration in the bladder wall (X 100, H&E stain).
Figure 6.14: Histology of urinary bladder 3 days after light illumination only without ALA. Scanty inflammatory cells in the serosal layer (X100, H&E).

Figure 6.15: Microscopic picture 72 hours after PDT treatment showing full thickness bladder damage with prominent inflammatory cell infiltration. No evidence of tissue selectivity is seen (X100, H&E stain) (200 mg/kg ALA, oral, sensitising for 4 hours. Light: 100 mW for 500 seconds at 630 nm).
Figure 6.16: Microscopic picture 72 hours after PDT treatment showing full thickness bladder damage with fibrinoid necrosis of vessels and prominent inflammatory cell infiltration. No evidence of tissue selectivity is seen (X60, H&E stain) (200 mg/kg ALA, oral, sensitising for 4 hours. Light: 100 mW for 250 seconds at 630 nm).

Figure 6.17: Microscopic picture of bladder 72 hours after PDT showing extensive damage to the lamina propria and muscle layers. The urothelium remains intact (X60, H&E stain) (100 mg/kg ALA, oral, sensitising for 3 hours. Light: 100 mW for 500 seconds at 630 nm).
In contrast, the histological changes found with intravesical ALA sensitised rats showed less prominent but more selective urothelium ablation without lamina propria or muscle damage (See Figure 5.13) even though they were treated with 50J. By the 7th day, bladder epithelium regenerated and fibroblast infiltration was scarcely presented in both lamina propria and muscle layer (Figure 6.18). The findings of the bladder 6 months following PDT revealed significantly more collagen fibrils deposit in both the lamina propria and muscularis propria of the orally treated than with intravesically treated rats (Figure 6.19). The microscopic findings of the experiment groups are summarised in Table 6.1.

**Figure 6.18:** (A) Microscopic findings of the bladder section 7 days after PDT showing regeneration of urothelium and fibroblast infiltration (X60), (B) High power view of (A) showing increased fibroblast activity in the lamina propria (X400, H&E stain).
Figure 6.19: Microscopic finding of bladder 6 months after PDT with 400mg/kg oral ALA. (A) Normal appearing urothelium with thickened lamina propria (X100, H&E stain), (B) Marked collagen deposition in the lamina propria and muscularis propria (X100, HVG stain).
Table 6.1. Histological changes of the bladder wall (2-3, 7 and 180 days) after PDT in different experiment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Urothelium damage</th>
<th>Oedema</th>
<th>Necrosis of Urothelium and L.P</th>
<th>Necrosis of Muscle L.P</th>
<th>Inflamm. cell L.P</th>
<th>M.P</th>
<th>Serosa L.P</th>
<th>M.P</th>
<th>Fibroblast L.P</th>
<th>M.P</th>
<th>Serosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Light illumination only)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>A (BI 200mg/kg, 50J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d diffuse</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>--</td>
</tr>
<tr>
<td>180d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B (Oral 100mg/kg, 50J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d patchy</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C (Oral 200mg/kg, 25J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d extensive</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>±</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>D (Oral 200mg/kg, 50J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d extensive</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7d patchy</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E (Oral 400mg/kg, 50J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d extensive</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7d patchy</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>180d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

(Treatment parameters: ALA concentration, light dose)
BI: Bladder instillation
Grade of histological changes: --: none, ±: minimal, +: mild; ++: moderate; +++: severe
L.P.: Lamina propria, M.P.: Muscularis propria
After surface illumination of abdominal skin, there was no histologically detectable lesion of any kind in the control rats receiving only light treatment (Figure 6.20); however, the epidermal and dermal layers of orally sensitised rats, both with 100 and 200mg/kg ALA, showed much more damage than that after intravesical ALA (Figure 6.21).

**Figure 6.20:** Histology of skin section with light only (50J) but without ALA shows no detectable cutaneous lesion 3 days after PDT (X100, H&E stain).
Figure 6.21: Microscopic picture of abdominal skin 3 days after PDT. (A) Extensive epidermis and dermis destruction after oral sensitisation with 100mg/kg ALA (X60). (B) Mild epidermis sloughing with keratolysis after intravesical sensitisation with 200 mg/kg ALA (X60, H&E stain).
6.5 DISCUSSION

6.5.1 Which route is better for bladder PDT?

Although PDT has the potential for treating bladder tumours, and some of the preliminary results using HpD as a sensitiser seemed encouraging (Hisazumi et al, 1983; Benson et al, 1983; D'Hallewin et al, 1992), its universal acceptance is unlikely unless the concerns of detrusor muscle damage (Harty et al, 1989) and prolonged skin photosensitisation (Lowdell et al, 1992) can be satisfactorily resolved. In my previous study using 10% ALA solution at pH 5.5 administered intravesically and treating the bladder at light dose of 50J (630 nm), I was able to induce homogenous urothelial ablation with only negligible damage to the underlying layers (Chapter 5). The selective necrosis of the transitional cell layer caused by ALA induced PpIX is therefore relatively mild and well tolerated. Although the risk of skin photosensitisation caused by ALA is short and mild and lasts for only 1-2 days (Regula et al, 1995), measures leading to further reduction of its severity or occurrence are warranted. It is clear from this study that for ALA based PDT of the bladder, intravesical administration is likely to be the preferred route of sensitisation as the chances of skin photosensitisation and full thickness bladder damages were significantly less in comparison to those sensitised with oral ALA at the same light dose and similar PpIX levels.

6.5.2 Mechanism of ALA-PDT effect, systemic versus local

It is also important to point out that the mechanism accounting for urothelial necrosis might be different if ALA is administered through different routes. Urothelial PpIX levels as measured on fluorescence microscopy after oral ALA(100mg/kg) which were comparable to those with 200mg/kg instillation showed more lamina propria and muscle damage and less uniform urothelial ablation than that from instillation. In some extreme cases giving oral ALA at 100mg/kg, only the lamina and muscularis propria were damaged whereas the urothelium was scarcely necrosed. It might indicate that bladder instillation provokes more direct cytotoxic effects than the systemic route which affect cell killing through interruption of microcirculation. If it is the case, intravesical instillation of ALA for bladder PDT is an even more valuable route than it was originally thought of as it further reduces chances of lamina propria and muscle damage by avoiding tissue destruction mediated by systemic PDT effect.
ALA molecules after penetrating the superficial umbrella cells, may take some time to diffuse into deeper transitional cell layers and to convert to PpIX. The time of bladder instillation is important as it enables continuous PpIX generation in the cells, allowing a concentration gradient of PpIX in various tissue layers to be built-up through the constant contact of the urothelium with ALA. The relatively persistent urothelial PpIX intensity over 4-7 hours after bladder retention is an advantage over oral administration which usually manifests phasic concentration patterns making photodynamic effect of the bladder less uniform and unpredictable. However, systemic administration, on the other hand, readily provides substantial amount of substrate (ALA) for PpIX biosynthesis through the vasculature, is therefore likely to buildup more PpIX in the urothelium and lamina propria where blood supply is abundant, rendering PpIX tissue selectivity between the two layers less distinct than that from bladder instillation. In accordance with a previous report from my colleague (Loh et al, 1993b), peak PpIX levels in the epithelium came up earlier at lower (100mg) than at higher ALA doses(200 & 400mg) (3h vs 7-8h). It is also noteworthy that 400mg oral ALA produced no higher peak PpIX in the urothelium than that of 200mg. It is probable that above the dose of 200mg, the available ALA substrate in the urothelium has already oversaturated which made further increase of PpIX by increasing ALA doses impossible.

6.5.3 Systemic distribution of ALA induced PpIX

The substantially higher porphyrin levels (mostly PpIX, but possibly with smaller amounts of uroporphyrins and coproporphyrins (Kennedy & Pottier 1992)) detected in the liver soon after oral ALA are likely to be due to rapid absorption and first pass conversion in the liver. The levels after oral and intravesical sensitisation do not become comparable until 7 hours after delivery, although they are still relatively high at this time. Intravesical administration would appear to slow down rather than eliminate systemic absorption as there are still substantial amounts of PpIX detected in the liver, but if the delay in absorption is comparable to the time for PpIX to be metabolised to haem, then the levels in tissues other than the bladder are likely to stay low at all times, reducing the risks of systemic phototoxicity. From the clinical studies by my colleagues, transient elevation of serum aspartate aminotransferases was found in one third (6/18) of patients receiving 60 mg/kg oral ALA (Regula et al, 1995). I did not carry out any enzyme assays in this study, but it seems unlikely that rises would be seen after intravesical administration although this warrants further studies.
With the kidney, the situation is different. With bladder instillation, in addition to systemic absorption, there is also the possibility of ureteric reflux, which might explain my rather variable results as seen in Figure 6.6. Clinically, prevention of vesico-ureteral reflux can be achieved by reduction of the intravesical pressure during instillation by reducing the volume of fluid used. I have no evidence at present that increased levels of PpIX in the kidney, both at cellular and subcellular levels, do any harm in the absence of light, but this does need further investigation. Definitive answers can only be provided by examining changes in subcellular structure or the presence of tissue specific substances (such as Tamm Horsfall protein) in the renal tubule or the glomerulus after prolonged contact with ALA and PpIX.

6.5.4 Fluorescence microscopy vs chemical extraction

Applying fluorescence microscopy for quantitative study of photosensitise tissue biodistribution, there is always concern that the results might not be as accurate as a chemical extraction method as specimens used for fluorescence study are thin sections (10 μm), in contrast to a small blocks of tissue for the latter. The advantage of using microscopic fluorescence photometry instead of chemical extraction is that fluorescence levels (and relative changes) in the various tissue layers of the bladder and skin can be resolved whereas a gross extraction technique only provides an averaged measurement (albeit absolute) of the tissue level. It could be argued that fluorescence quenching effects may differ between each layer but previous studies with ALA on rat colon and stomach would not support this contention since microscopic fluorimetry showed good correspondence with absolute levels measured in the mucosal and muscle layers which could be physically separated from one another (Loh et al, 1993b); a similar study on the bladder would be infeasible owing to the small thickness of the urothelium. Nevertheless, it remains impractical to compare the absolute porphyrin levels in different organs solely on the basis of fluorescence microscopy, especially if several different porphyrin species are present. Using a chemical extraction method, Schoenecker Jr. et al (1994) found that the concentration of liver and kidney porphyrins 4 hours after intraperitoneal injection of 200 mg/kg ALA were 35.8±10.1 and 20.1±11.3μg/mg, respectively. The result of liver/kidney ratio of porphyrins was 1.8 (range 1-5.2) in their study, which is roughly compatible to my data of 2.6 using fluorescence microscopic technique. It therefore, appears reasonable to assume that fluorescence microscopy is a convenient and reasonably reliable method for study of ALA induced porphyrins biodistribution in various organs, especially in the hollow viscuses where detailed PpIX level in
different tissue layers is essential for accurate assessment of photosensitise
distribution. Whereas for solid organs such as liver and kidney, information from
fluorescence microscopic studies of biopsies may serve as a useful reference for
relative PpIX levels among various tissues which will be important in planning
therapeutic schemes to minimise systemic and cutaneous effects. Care must be taken,
however, to minimise technical error resulting from tissue sampling. A probable
solution is to obtain biopsy specimens from different area of the organ and, to take as
many blocks as possible for fluorescence quantification.

6.5.5 Skin photosensitivity

Despite a severer cutaneous photodynamic response after oral ALA than after
intravesical ALA as found in this study, it is also noteworthy that a similar degree of
skin damage is not likely if the experimental animals were placed under solar light
rather than receiving a full dose of monochromatic laser exposure. However, the
results on rat skin, both from biodistribution and therapeutic points of view, clearly
indicate that the PpIX intensity in the epidermis is less after intravesical instillation.
At the same ALA dose but via different routes, less PpIX was formed in the
epidermis, and with laser illumination, only negligible cutaneous lesions were
induced if ALA was given intravesically. Since previous studies showed evidence of
cutaneous photosensitisation in 1/3 of patients taking oral ALA for photodynamic
therapy of their oesophageal or duodenal lesions (Regula et al, 1995), I believe the
incidence of skin photosensitivity can be further reduced if the result of this
experiment can be applied to clinical trials.

6.5.6 Advantages of intravesical instillation

Other than the apparent advantage of less skin photosensitivity with instillation of
ALA, the other obvious advantage of intravesical ALA for photodynamic
management of urinary bladder cancer, particularly for field change disease, is the
better selectivity achievable between the urothelium and underlying layers. The
resultant subepithelial fibroblast infiltration 7 days after PDT is apparently more after
oral ALA sensitisation. Furthermore, with a special stain, collagen fibrils were found
to deposit more in the lamina propria 6 months after PDT after oral ALA
sensitisation. Although the actual role of collagen fibre in the bladder is yet to be
defined, less collagen deposit in the lamina propria is always welcome since it poses
less chance for the bladder to become functionally impaired as a result of altered
elasticity. However, further studies are needed to correlate the amount of collagen fibrils with the function of the bladder. Also noteworthy is the finding of relative PDT sparing of the urothelium with oral ALA although the PpIX fluorescence at the treatment point (oral 100mg/kg, 3h sensitisation vs BI 200mg/kg, 5h sensitisation) is similar in intensity. It is probable that the governing mechanisms of PDT mediated by ALA induced PpIX are different with respect to routes of administration. With bladder instillation, as a result of gradient diffusion of ALA into the urothelium, more direct cytotoxic effect is anticipated. However, with oral delivery, microcirculatory shutdown which is the proposed mechanism for HpD mediated PDT (Nelson et al., 1988), might more likely be the cause for tissue destruction particularly in the lamina propria and muscle layer which are rich in blood supply and are vulnerable to vascular collapse.

The algorithm for the management of bladder cancer, unlike that for other malignancies, is focused primarily on the prevention of recurrence which is estimated as high as 40-60% at 12 months after initial treatment of papillary tumours (Hall et al., 1994). To reduce subsequent recurrence, measures such as intravesical chemotherapy with antineoplastic agents (Nseyo & Lamm 1994) and Bacillus Calmette-Guerin (BCG) (Herr et al., 1988) have been tried with some success. However, for effective prevention of recurrence, the only reliable option is to replaced the diseased urothelium, whether visible or not, with healthy transitional cell lining, a prospect which is technically difficult at this moment. PDT of the bladder offers a new hope for this by destroying urothelium selectively and repairing damaged tissue with regeneration of normal tissue.
6.6 CONCLUSIONS

In this chapter I compared the systemic bisdistribution of PpIX following either oral or intravesical administration of ALA. Although urothelial PpIX fluorescence was generally higher after oral administration of ALA at the same dose as instillation, selectivity of PpIX between the urothelium and underlying muscle was no better than that after bladder instillation at the same drug dose. Oral ALA at 100mg/kg was able to induce a similar level of PpIX in the urothelium as was achieved by 200mg/kg ALA given intravesically. Skin photosensitisation was, however, more prominent after oral ALA as the epidermal PpIX intensity was 2-3 times higher than after intravesical delivery of ALA when urothelial PpIX was at similar levels. Photodynamic effects on the bladder varied with different routes of administration and light doses. At the same drug and light dose but with different route of administration, pathological changes of the bladder, particularly the lamina propria and muscularis propria were generally more prominent in the orally sensitised rats. At a therapeutic light dose, the epidermis and dermis of back skin showed prominent photodynamic necrosis in those sensitised with oral ALA (400, 200 and 100mg/kg) but not with intravesical ALA. Based on the above findings, I conclude that intravesical instillation of ALA is a preferred route for PDT of rat bladder, and its usefulness for human bladder cancer management seems very likely, however further case controlled clinical trials are warranted.

Despite the demonstration of less skin side effects with intravesical instillation of ALA, I am seeking methods which can further widen PpIX selectivity between the urothelium and the underlying layers of the bladder to reduce the risk of muscle damage after PDT. The following chapter looks at other ways in which this might be done.
CHAPTER 7

ENHANCEMENT OF PHOTODYNAMIC EFFECTS WITH ORAL IRON CHELATOR AND LIGHT FRACTIONATION AFTER ALA SENSITISATION

7.1 Background ............................................................. 193
7.2 Aims ........................................................................... 195
7.3 Materials and Methods
   7.3.1 Chemicals .............................................................. 196
   7.3.2 Animals and technique for drugs administration ............. 196
   7.3.3 Fluorescence microscopy with CCD camera ................. 196
   7.3.4 Photodynamic therapy
      7.3.4.1 Assessment of iron chelator .................................. 197
      7.3.4.2 Assessment of light fractionation ......................... 197
7.4 Results
   7.4.1 Tissue fluorescence quantification .............................. 199
   7.4.2 Photodynamic effects
      7.4.2.1 CP94 in vivo effect .......................................... 203
      7.4.2.2 Light fractionation effect ................................. 209
7.5 Discussion
   7.5.1 Importance of tissue selectivity for PDT ...................... 212
   7.5.2 Mechanism of action of hydroxypyridinones (HPOs) ....... 212
   7.5.3 CP94 and CP20 versus desferroxamine ....................... 213
   7.5.4 Optimal ALA concentration for instillation with CP94 .... 214
   7.5.5 Enhancement of PDT effects and selectivity with CP94 ..... 214
   7.5.6 The implication of PDT for bladder cancer management ... 215
   7.5.7 The effect of light fractionation on the bladder .............. 215
7.6 Conclusions .............................................................. 217
7.1 BACKGROUND

Attempts to increase PpIX tissue selectivity have been tried with some success. Using PpIX directly as a photosensitiser is possible in vitro because of its excellent transmembrane absorption, despite its poor water solubility and tendency to aggregate in aqueous solvents. However, PpIX is a poor tumour localiser in vivo (Kongsbang et al, 1989). Modifying the delivery vehicle of PpIX results in better cellular absorption but changes its pharmacokinetic properties which may reduce its photosensitising activity (Allison et al, 1990). Inhibiting ferrochelatase, which catalyses the conversion of PpIX to haem, by chelating the ferric ions is a simple way to increase the tissue level of PpIX. Ethylene-diamine-tetraacetic acid (EDTA) has been used to do this (Hanania & Malik 1992), but its clinical use is limited due to its nonselective chelation of all metallic cations and the resultant complications. The widely used iron chelator Desferrioxamine (DFO) has been shown to enhance photosensitisation with ALA (Ortel et al, 1993), but gains access to intracellular iron pools relatively slowly (Hoyes & Porter 1993). The hydroxypyridinones (HPOs) are group of orally active iron chelators which are highly selective for iron(III) and which by virtue of their neutral charge and low molecular weight, gain access to intracellular iron rapidly (Figure 7.1) (Hoyes & Porter 1993) and would therefore be expected to enhance porphyrin levels more rapidly than DFO.

![Figure 7.1: The structure of 3-hydroxypyridin-4-ones. With different substitutions at R¹ and R², different CP's are formulated.](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R¹</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP20</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>CP21</td>
<td>CH₃</td>
<td>CH₂CH₃</td>
</tr>
<tr>
<td>CP51</td>
<td>CH₃</td>
<td>(CH₂)₂OCH₃</td>
</tr>
<tr>
<td>CP93</td>
<td>CH₂CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>CP94</td>
<td>CH₂CH₃</td>
<td>CH₂CH₃</td>
</tr>
</tbody>
</table>
Recent work suggests that HPOs may increase intracellular PpIX levels (Smith et al., 1994). These same properties also result in rapid interaction with intracellular iron pools thereby resulting in the inhibition of iron containing enzymes such as ribonucleotide reductase significantly more rapidly than Desferrioxamine (Porter et al., 1993a). This may account for some unwanted long term effects on the bone marrow (Porter et al., 1991; Hoyes et al., 1993). However the short term administration of two hydroxypyridinones namely, the 1,2-dimethyl derivative(CP20) and the 1,2-diethyl derivative(CP94) to iron overloaded humans has been shown to achieve rapid and effective iron mobilisation without significant toxicity (Brittenham 1992). It seems very likely that HPOs may have the potential to increase cellular PpIX in vivo after intravesical ALA administration. The first part of this chapter was designed to look at this issue on the bladder of Wistar rats.

Another approach to enhancing PDT effects is light fractionation. The idea of fractionating X-irradiation doses to achieve better tumour cell killing is not new (Hawkes et al., 1968; van Putten & Kallman 1968). Applying the same principle for Photofrin mediated PDT on an implanted mammary tumour, Foster et al (1991) demonstrated that at a fixed total light dose, splitting delivering light into 30 seconds on and 30 seconds off fractions (dark interval) resulted in a 2-fold increase of tumour volume doubling time compared to that without light fractionation. Similar results were reported with ALA (van der Veen et al., 1994) and HpD (Pe et al., 1994). A more recent study using ALA induced PpIX as a sensitisier showed that by a single short interruption (150 seconds) in the light irradiation, the lesion size in the normal colon mucosa was greatly enhanced, from 13 mm² to 94 mm² with the same total energy (Messmann et al., 1995). This finding prompted us to design a study to assess whether the same enhancement effect could be induced in the rat urinary bladder.
7.2 AIMS

As an extension of my previous ALA studies the first part of this chapter was looking at the build-up of PpIX in rat bladders after intravesical administration of ALA with and without an iron chelator. CP94 was selected for this study, being more lipophilic than CP20 and in principle likely to produce a more rapid enhancement of PpIX level. After determining the optimum drug-light interval of ALA with the addition of iron chelator, photodynamic therapy of the bladder was conducted at the time of maximum PpIX levels and the histopathological results studied.

The second part of this study was designed to compare the photodynamic effects of the bladder between those receiving light fractionation (dark interval of 5 minutes in the middle) and those without interruption of illumination at a same total light dose. My purpose was to produce a similar degree of bladder tissue destruction achievable with 200mg/kg ALA at a continuous light dose of 50J by decreasing the instilled ALA doses with 2 fixed light fractionations to a total dose of 50J.
7.3 MATERIALS AND METHODS

7.3.1 Chemicals

ALA powder (ALA.HCl, 98% purity, DUSA Pharmaceuticals Inc., New York, USA) was dissolved in normal saline and titrated to pH 5.5 with saturated sodium bicarbonate solution immediately prior to bladder instillation. The iron chelator, CP94, in the form of powder, was synthesised according to the published procedure (Hider et al, 1982) at the Department of Pharmacy, Kings College London, and was kindly donated by Dr John Porter (consultant haematologist at the University College Hospital, London). The purity of CP94 (95%) was confirmed by elemental analysis. The stability of the sample solution which had been dissolved in phosphate-buffered saline (PBS) at pH 7.4 and stored at 4°C for 1 week was monitored by high performance liquid chromatography (HPLC).

7.3.2 Animals and technique for drugs administration

ALA solutions of 10% (200mg/kg) and 1% (20mg/kg) were used for bladder instillation. The procedure for anaesthesia and intravesical instillation were the same as in the previous chapters.

In addition to ALA, CP94 at the dose of 200mg/kg body weight was injected intraperitoneally at the time of ALA instillation. Two rats receiving just 0.9% normal saline intravesically and two others having CP94 only, were killed at 5 hours to serve as controls. For each set of treatment values, 2-3 rats were studied. Bladder specimens were collected at 1, 2, 3, 4, 5, 7, 9 and 24 hours after drug administration by killing the rats with CO2 narcosis. All the residual ALA was washed out by rinsing the bladders twice with normal saline before removing them. Specimens were transferred to a liquid nitrogen container immediately after harvest. Frozen sections at 10 micrometer thickness were used for fluorescence imaging and quantification of the PpIX distribution.

7.3.3 Fluorescence microscopy with CCD camera

The presence of tissue protoporphyrin was confirmed by in vivo fluorescence spectroscopy using a Perkin-Elmer LS50B spectrofluorometer. Rat bladders instilled
with either normal saline or pH 5.5, 10% ALA for 3 hours were examined with a probe placed 3 mm above the urinary bladder which was exposed by a lower abdominal incision. The emission spectrum was recorded between 600 and 750 nm using an excitation wavelength of 410 nm. For quantification and microscopic imaging of PpIX fluorescence on the frozen sections of bladder, a technique combining phase contrast microscopy and a slow-scan charge coupled device (CCD) camera (Wright Instruments Ltd, model 1) was utilised. The details were discussed in Chapter 3.

7.3.4 Photodynamic therapy

7.3.4.1 Assessment of effects of iron chelator
A copper vapour pumped dye laser (Oxford Lasers Ltd.) emitting red light at 630 nm was used as the light source. A total of 36 rats was stratified equally into 4 treatment groups. Group A and B had ALA only. Group A had 1% ALA retained in the bladder for 2 hours and treated with light 2 hours later. Group B received 10% ALA instillation for 2 hours and waited for an additional 3 hours before commencing light illumination. In group C and D, in addition to the ALA, 200mg/kg CP94 was given intraperitoneally at the time of bladder sensitisation. For PDT treatment of bladder, the procedures were virtually the same as was described in Chapter 5. After PDT, assessment of PDT effects was achieved by comparing histological changes in bladder sections which were obtained serially by killing the rats 2, 3 and 7 days after treatment.

7.3.4.2 Assessment of light fractionation
Except for a slightly different ALA dosimetry and the fractionation of light, other procedures were essentially the same as described in the previous section. At a total light dose of 50J (100mW for 500 seconds), the illumination time was split in two fractions by imposing a 5 minutes break in the middle. Using Group B as the standard for further histological comparison, three additional groups with various ALA doses were studied. Group 1 (10 rats) had 5% ALA, Group 2 (8 rats) had 2.5% ALA, and Group 3 (6 rats) had 1% ALA. Without exception, the instilled ALA was retained in the bladder for at least 2 hours with a further wait of 3 hours before light illumination was initiated. During the dark interval, which was 5 minutes in this study, the bladder was not deflated and the Intralipid solution remained in situ until reinstitution of light. However, to avoid drying out of the bladder which had been exposed and placed externally, regular rinse of the bladder with 0.9% normal saline was carried out.
throughout the break period. After completion of treatment, the bladders were harvested at 2, 3 and 7 days after PDT and stained with H&E for examination.
7.4 RESULTS

7.4.1 Tissue fluorescence quantification

There was no detectable fluorescence in the control rats having normal saline. However, the fluorescence emission spectrum from the rats receiving intravesical ALA (pH 5.5, 10% ALA retention for 3 hours) was characteristic of protoporphyrin IX with peak emission at 635 and 710 nm (see Figure 5.2, Chapter 5) (Kongshang et al., 1989).

The microscopic fluorescence CCD pictures of bladder sections sensitised with intravesical ALA with CP94 are shown as Figure 7.2. The CCD picture without CP94 is demonstrated in Figure 5.3.

![Figure 7.2: CCD fluorescence microscopic picture of bladder 5 hours after ALA instillation and 200mg/kg CP94 intraperitoneal injection. (A) 10% ALA (200mg/kg), (B) 1% ALA (20mg/kg).](image-url)
Figure 7.2: (C) CCD fluorescence microscopic picture with (D) matching histology showing higher PpIX intensity in the urothelium. The fluorescence levels in the lamina propria and muscle layer are much less than the urothelium (X100, H&E stain).
The comparison of PpIX fluorescence in the layers of the bladder (urothelium, lamina propria and muscle) following intravesical instillation of 10% ALA at pH 5.5 with (Group D) and without (Group B) concomitant CP94 administration is plotted in Figure 7.3. The PpIX fluorescence in the urothelium was first detectable 1 hour after instillation. With or without CP94, the PpIX fluorescence intensity was similar in the first 4 hours after ALA instillation. However, a significant escalation of fluorescence signals was observed in the CP94 treated group in the time period between 5 and 7 hours, when urothelial fluorescence was twice the intensity of that seen in those without CP94 while the fluorescence in lamina propria and muscle remained low. By 24 hours, the PpIX signals from the two groups fell to near background levels.

Figure 7.3: Plot of bladder wall fluorescence intensity against time after instillation of pH 5.5, 10% ALA with and without CP94 (Data is shown as mean ± standard error).
With 1% ALA, the urothelial PpIX signals in the CP94 treated bladders (Group C) were no higher than those without CP94 (Group A) (Figure 7.4). The maximum PpIX intensity in the urothelium after 10% ALA was nearly three times higher than that seen after 1% ALA.

![Fluorescence intensity against time after instillation of pH 5.5, 1% ALA with and without CP94](image)

**Figure 7.4**: Plot of bladder wall fluorescence intensity against time after instillation of pH 5.5, 1% ALA with and without CP94 (Data is shown as mean±standard error).

The PpIX fluorescence ratios of the urothelium to muscularis propria after 1 & 10% ALA instillation are shown in Figure 7.5. With CP94, better urothelium selectivity was always possible especially in the period between 2-4 hours after ALA administration. At 10% ALA with CP94, the maximum ratio was 8 to 1 at 4-5 hours while it was 5.5 to 1 at 5 hours with ALA alone. PpIX intensity ratios between the urothelium and muscle layer with 1% ALA showed a maximum urothelial selectivity of 13 to 1 at 3 hours. At the peak of urothelium PpIX build-up (4 hours), the ratio remained as high as 12 to 1 (Figure 7.5).
Figure 7.5: Plot of fluorescence ratios between the urothelium and muscularis propria after instillation of pH 5.5, 1 and 10% ALA with and without CP94.

7.4.2 Photodynamic effects

7.4.2.1 CP94 in vivo effect

The early microscopic findings of the bladders in group A (1% ALA without CP94 treated at 4 hours), were patches of urothelial sloughing and diffuse swelling of the lamina propria with full thickness inflammatory cell infiltration (Figure 7.6). Muscle damage was not found. The lesions induced with 10% ALA without CP94 (Group B) were, on the contrary, more uniform than those seen with 1% when assessed 48 hours after PDT, and comprised generalised urothelial loss and areas of superficial ulceration which in rare cases, extended down to the muscle layer (See Figure 5.13, Chapter 5). However, the associated muscle damage was scanty and focal in distribution and never reached beyond the confines of superficial muscularis propria. Fibroblasts infiltration which could eventually lead to collagen deposition and scar formation began to appear in the lamina propria from the 3rd day after PDT. By the 7th day, regeneration of damaged urothelium was nearly complete although histological evidence of continuing tissue repair processes persisted. Healing of the
lamina propria was essentially complete by 7 days, at which time the microscopic signs of muscle layer damage were barely detectable (Figure 7.7).

**Figure 7.6:** Histological findings of bladder in Group A (1% ALA, no CP94) showing patchy urothelial sloughing, lamina propria swelling and inflammatory cell infiltration 48 hours after PDT (X40, H&E stain).

**Figure 7.7:** Histological changes of bladder in Group C (1% ALA plus CP94) showing full regeneration of urothelium with normal appearing muscularis propria 7 days after PDT. Only scanty fibroblasts are seen (X100, H&E stain).
The most prominent microscopic findings in the bladders receiving 1% ALA and CP94 (Group C) were extensive sloughing of the urothelium and a lesser degree of oedema and inflammatory cell infiltration in the lamina propria. The extent of tissue damage resulting from such a concentration, although less extensive, was similar to that seen after treating the bladder with 10% ALA without CP94. Despite a more diffuse urothelial and lamina propria destruction induced by 1% ALA in the presence of CP94, the lesions remained partially focal in distribution and varied in their degree of severity (Figure 7.8). Muscle damage was scanty and involved only the superficial portion of the muscularis propria. Fibrinoid necrosis of arterioles in the lamina propria was less prominent in the CP94 groups than in those with ALA only. With 10% ALA and CP94 (Group D), the urothelial damage was uniformly distributed throughout the bladder. As was observed in group C, full thickness infiltration of inflammatory cells and lamina propria swelling were also milder in group D than in group B (Figure 7.9). Unexpected muscle damage was occasionally seen if the light was not uniformly distributed (Figure 7.10). The histological findings of the bladders treated with different parameters are summarised in Table 7.1.

Figure 7.8: Microscopic findings of bladder in Group C (1% ALA plus CP94) showing focal urothelial damage with mild lamina propria oedema 2 days after PDT. Some urothelium remains intact (arrows) (X40,H&E stain).
Figure 7.9: (A) Microscopic findings of bladder in Group D (10% ALA plus CP94) 2 days after PDT showing diffuse urothelial destruction (X40, H&E stain).

Figure 7.9: (B) Close up view of (A) showing generalised urothelial ablation without lamina propria and muscularis propria damage. Inflammatory cell infiltration is not prominent (X100, H&E stain).
Figure 7.10: Histologic findings of degeneration of muscle layer in the bladder 7 days after PDT as a result of inhomogeneous light distribution. Damaged muscle bundle (large arrows), fibroblasts (small arrows) (X100, H&E stain).
Table 7.1: Histological findings of the bladder after PDT. Each group was treated with 50J

<table>
<thead>
<tr>
<th>Group</th>
<th>Urothelial Oedema</th>
<th>Superficial Sloughing</th>
<th>L.P.</th>
<th>M.P.</th>
<th>Ulceration</th>
<th>L.P.</th>
<th>M.P.</th>
<th>Ulceration</th>
<th>Muscle Damage</th>
<th>Fibroblasts in L.P. M.P. Serosa</th>
<th>M.P. Serosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (1% ALA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>patchy</td>
<td>++</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>B (10% ALA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>generalised</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>C (1% ALA &amp; CP94)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>diffuse</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>D (10% ALA &amp; CP94)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>generalised</td>
<td>+</td>
<td>--</td>
<td>++</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>--</td>
</tr>
</tbody>
</table>

--: negative, ±: minimal, +: mild, ++: moderate, +++: severe
L.P.: Lamina propria, M.P.: Muscularis propria
7.4.2.2 Light fractionation effect

In the study with light fractionation, 2 rats in Group 1 (5% ALA) died within 24 hours after PDT. Another 1 was very sick for 48 hours and was sacrificed accordingly. Post mortem inspection discovered extremely inflamed bladders which were densely covered with peritoneal adipose tissue and in association with profuse exudate in the peritoneum. There was no definite evidence of perforation of the bladder although they looked thin and flaccid. One animal in Group 2 (2.5% ALA) which appeared unwell in the early stage after treatment turned out to be alright at 48 hours. No rats were so generally unwell in Group 3 (1% ALA) after PDT. The gross appearance of bladder specimens harvested from rats having instillation of ALA with fractionation of light was basically similar to those without light fractionation (Section 5.4.3, Chapter 5).

Microscopically, a single break (25/25J) with a dark interval of 5 minutes at the ALA dose of 100mg/kg (Group 1) resulted in extensive full thickness destruction of the bladder at 48-72 hours. Almost all the cellular elements were destroyed by PDT, leaving behind the collagenous remnants for maintaining the bladder architecture (Figure 7.11). Muscle damage was so prominent that virtually no tissue selectivity could be found between the layers after PDT. At an ALA concentration of 1% (20 mg/kg) with light fractionation, the histological change in the bladder was so mild that hardly any pathology could be detected at 3 days (Figure 7.12). The microscopic findings which were most compatible to those without light fractionation were observed from Group 2 (50 mg/kg ALA). Urothelial sloughing was extensive together with a certain degree of muscle damage indicating partial thickness destruction of the bladder wall at this dosimetry (Figure 7.13). The microscopic findings of the three groups are summarised as Table 7.2.
**Figure 7.11:** Microscopic findings of the bladder 3 days after receiving light fractionation (25/25J) with dark interval of 5 minutes and bladder instillation of 100mg/kg ALA. The cellular elements are completely destroyed leaving behind only collagenous structure (X40, H&E stain).

**Figure 7.12:** Microscopic finding of bladder section 3 days after PDT with 1% ALA instillation and light fractionation. The urothelium is intact but stasis of small vessels in the lamina propria is seen (arrows) (X40, H&E stain).
Figure 7.13: Microscopic finding of bladder section 3 days after PDT with 2.5% ALA (50mg/kg) instillation and light fractionation showing diffuse urothelial sloughing with evidence of muscle damage (X40, H&E stain).

Table 7.2 : Histological findings of the bladder after PDT. Each group was treated with two fractions of light (25/25) with 5 minutes dark interval in the middle to a total light dose of 50J

<table>
<thead>
<tr>
<th>Group</th>
<th>Urothelial Sloughing</th>
<th>Oedema</th>
<th>Superficial Ulceration</th>
<th>Muscle Damage</th>
<th>Inflammatory cell Infiltration</th>
<th>Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L.P.</td>
<td>M.P.</td>
<td></td>
<td>L.P. M.P. Serosa</td>
<td>L.P. M.P. Serosa</td>
</tr>
<tr>
<td>B (10% ALA, without light fractionations)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>generalised ++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>I (5% ALA, 25+25J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>generalised ++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>7d</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>2 (2.5% ALA, 25+25J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>generalised ++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>3 (1% ALA, 25+25J)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3d</td>
<td>±</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

---: negative, ±: minimal, +: mild, ++: moderate, +++: severe
L.P.: Lamina propria, M.P.: Muscularis propria

211
7.5 DISCUSSION

7.5.1 Importance of tissue selectivity for PDT

Before more clinical PDT trials on the bladder are undertaken, further in vivo research on photosensitiser pharmacokinetics, light dosimetry and biological responses under rigorous experimental conditions are necessary, as the complications seen in previous clinical reports cannot be overlooked (Harty et al, 1989). To reduce urological side effects and undesirable cutaneous photosensitisation, common using the first generation porphyrin photosensitisers and which persisted as long as 4-6 weeks after treatment, attempts have been made to deliver the photosensitising agent by bladder instillation (Pope & Bown 1991; Chapter 5) or intratumour injection (Amano et al, 1988). As ALA is a small water soluble molecule, it was shown in my previous study that selective accumulation of PpIX in the urothelium was possible if the ALA was retained in the bladder for 5 hours (Chapter 5). The maximum PpIX intensity ratio between the urothelium and muscle layer, with 10% ALA, was 5.5 to 1 which was sufficient to result in diffuse destruction of the urothelium without apparent damage of the muscle layer when treated with an appropriate light dose (Chapter 5). It has been demonstrated that PDT following instillation of ALA not only resulted in better selective destruction of the urothelium but also induced less skin photosensitivity than administering the ALA via an oral route (Chapter 6). Further increasing the selectivity between the urothelium and the underlying structure is a good way to spare the detrusor muscle which is crucial for maintaining normal bladder function after PDT to the entire urothelium. The results from this study suggest that the addition of an iron chelator may be able to do this without causing unwanted systemic effects.

7.5.2 Mechanism of action of hydroxypyridinones (HPOs)

As ferric ion is essential for cellular homeostasis, all proliferating cells require iron for the functioning of the M2 subunit of ribonucleotide reductase which is responsible for the free-radical mediated reduction of ribonucleotides to deoxyribonucleotides, a key step in DNA synthesis (Thelander et al, 1983) and HPOs act particularly rapidly in this regard (Porter et al, 1993a). Interruption of iron metabolism with either DFO or HPOs may result in suppression of growth in various malignant cell lines (Becton & Robert 1989) and on bone marrow (Hoyes et al, 1992). Cells were also found
arrested in late G1 phase of cell cycle (Hoyes et al, 1993) and subsequently died by apoptosis (Porter et al, 1994). It seems possible that if an iron chelator is administered with ALA, in addition to its effect increasing PpIX levels, a further tumour cytotoxic effect might be anticipated if dysplastic cells are included in the PDT treatment field. A previous study by Bedwell et al (1992) indicated that the PpIX fluorescence intensity in a rat colon tumour was 6 times higher than in normal colonic mucosa 6 hours after systemic administration of ALA. Despite the need for further in vivo evidence, possibly at molecular level, on the cytotoxic effect of iron chelators with ALA, I foresee the possibility of having an even higher degree of selectivity, both in PpIX level and PDT effect between the tumour and urothelium than was achievable between the urothelium and muscularis propria, although such an ideal bladder tumour model growing transitional cell carcinoma in situ in the experimental animal currently is not available. In fact, in a recent in vitro study using a bladder cancer cell line (MGH-U1) and normal primary fibroblasts as models, my colleagues were able to obtain a factor of 5 in favour of cancer cells in PpIX build-up by giving ALA alone. Following administration of CP94 and ALA in the incubation medium, the PpIX level was doubled in the MGH-U1 cell line and the fluorescence intensity was 11-13 times higher than the fibroblasts which showed no additional elevation of PpIX level in the presence of the iron chelator (unpublished data).

7.5.3 CP94 and CP20 versus desferroxamine

In the HPO family, various substitutions at the R1 and R2 positions result in derivatives with different lipid solubility. This property has a profound bearing on iron mobilisation and toxicity with HPOs both in vivo (Porter et al, 1990) and in vitro (Porter et al, 1988). 1,2-dimethyl-3-hydroxypyridin-4-one (CP20) and the relatively lipophilic 1,2-diethyl-3-hydroxypyridin-4-one (CP94) have been studied in most detail clinically to date (Olivieri et al, 1990; Al Refaie et al, 1992). Pharmacokinetic data from patients showed that CP20 was absorbed rapidly and completely from the gastrointestinal tract, entered the blood stream and was then mostly excreted in the urine in the form of an iron complex or glucuronide (Kontoghiorghes et al, 1990). CP94, sharing a similar chemical structure with CP20, is also rapidly glucuronidated in humans but not in rats (Porter et al, 1993b). This compound accesses intracellular iron more rapidly than CP20 and is less inhibitory to murine haemopoeisis in vivo but not in vitro (Hoyes et al, 1993). As DFO has a high molecular weight, is relatively hydrophilic, and enters cells more slowly (Porter et al, 1993a), its ability to
induce tissue PpIX may not be as effective as that of CP94, although this has not yet been compared directly.

7.5.4 Optimal ALA concentration for instillation with CP94

In the present study I have demonstrated with fluorescence microscopy the feasibility of using CP94 to elevate the tissue level of PpIX. With CP94 and 10% ALA, the peak urothelial PpIX level was doubled at 6 hours compared with that using ALA alone. However the effect of CP94 with 1% ALA was marginal compared with that using 10% ALA. This differential effect observed using the higher ALA concentration may be attributable to saturation of the final conversion of PpIX to haem, with the rate being limited by the levels of available cellular iron and the amount of ALA substrate. It is noteworthy that the effect of CP94 was not evident until 3-4 hours after injection which again may be attributed to the saturation effect discussed above and possibly also to the excretion of CP94 in the urine, as some CP94 may be reabsorbed by the transitional cell lining of the bladder. With this concentration of intravesical ALA and intraperitoneal CP94, differential build-up of PpIX in the urothelium over the underlying layers was enhanced and the distribution of PpIX was much more uniform than had previously been reported with intravesical aluminium sulphonated phthalocyanine (AlSPc) (Pope & Bown 1991). It is probable that photosensitisers with different biochemical properties are taken up by a variety of mechanisms which will lead to variable tissue concentrations and distribution patterns with each route of administration.

7.5.5 Enhancement of PDT effects and selectivity with CP94

The most distinct histological changes with PDT following the combination of ALA and CP94 are a smaller degree of fibrinoid necrosis of the small arterioles and a seemingly milder degree of oedema in the lamina propria compared with the effects seen using ALA alone (Table 7.1). These differences may be attributed to the presence of the iron chelator (CP94), probably by decreasing lipid peroxidation secondary to iron induced hydroxyl radical damage or by the induction of cell cycle arrest and subsequent apoptosis, or of other unknown mechanisms. The presence of CP94 in the cell at the time of light illumination seems particularly useful for enhancing tissue destruction when the cellular PpIX is just above the threshold level, as is the case with 1% ALA in my study. At this ALA concentration, the urothelium which was not responsive to light illumination in the absence of iron chelator, was
ablated uniformly when CP94 was added. This effect seems less prominent when more PpIX has been transformed in the cell. For this reason, the addition of CP94 to the bladder which was instilled with ALA at a higher concentration (10%), did not enhance tissue destruction. The much reduced oedema and inflammatory cells infiltration of the lamina propria in Group D probably was caused by direct or indirect apoptotic effect of CP94 which has been shown to induce programmed cell death in thymocytes and tumour cells \textit{in vitro} (Porter et al., 1994). It is clear that apoptosis is one of the important mechanisms accounting for PDT mediated cell killing in some cell lines \textit{in vitro} (Oleinick et al, 1992; He et al, 1994) and \textit{in vivo} (Zaidi et al, 1993). However, this study did not look at the changes characteristic of apoptosis in the bladder. Further investigation is warranted to elucidate in more detail the role of CP94 on the tissue when acting together with photosensitiser for PDT.

7.5.6 The implication of PDT damage in normal urothelium

The bladder is an ideal organ for PDT as it is readily accessible and the transitional epithelium which is the site of origin of carcinoma-in-situ and superficial cancers can be treated as a whole. Although many urological oncologists are keen to apply PDT in treating papillary bladder tumours or carcinoma in situ, with current urological techniques, papillary tumours which can be visually identified at cystoscopy pose no particular therapeutic problem as they can be eliminated with laser coagulation or electrocautery. It is always my firm belief that only with complete removal of diseased urothelium can patients stand a chance for cure of bladder cancer, or at least have better chances for prevention of recurrence. It is with this rationale that I have focused on selective damage between normal bladder layers rather than on the difference between the urothelium and cancers. I also believe that any experimental tumour model which does not grow in the bladder (e.g. which grows in a physiologically inappropriate site like subcutaneously) and is not arising from, or histologically resemble, transitional cells is not appropriate for studies of PDT on urinary bladder. With the lack of an appropriate model in the experimental animals, I examined principally selectivity of destruction between urothelium and the muscle layer which is even more crucial if PDT with ALA is to be undertaken clinically.

7.5.7 The effect of light fractionation on the bladder

In accordance with other reports (van der Veen et al, 1994; Messmann et al, 1995) I have demonstrated an enhanced PDT tissue effect on the bladder if light fractionation
was employed. By varying ALA doses but with a fixed light fraction dosimetry, I showed that the bladder lesions sensitised with 50mg/kg ALA was roughly the same as those with 200mg/kg ALA but without light fractionation, an enhancing factor of 4. Although the exact mechanism(s) of action of light fractionation remains to be investigated, most studies suggested a reperfusion or reoxygenation theory as a possible explanation (Foster et al, 1991). The observation of much extensive muscle damage seen after light fractionation is disappointing despite the enhanced tissue destruction on the bladder as a whole. Unlike the common aims being pursued for the treatment of deeply infiltrated solid tumours which require a deeper and more extensive tissue necrosis to secure a prolonged effect, the success for photodynamic therapy of the bladder, depends more on the selective destruction of the diseased urothelium, whether exophytic of flat, than on the depth and extensiveness of PDT necrosis. Vessel constriction that occurs in the early phase of light illumination as was demonstrated by van der Veen et al (1994) may be protective rather than destructive to the muscle tissue which usually retains low levels of PpIX following bladder instillation of ALA. Reoxygenation of the lamina propria and muscle layer secondary to perfusion of blood from relaxation of constricted vessel during the dark interval may provoke a profound direct cytotoxic effect on those tissues upon subsequent illumination of light. The much higher incidence of full or partial thickness bladder wall damage after light fractionation as was demonstrated in this study, might imply that the total light dose used in this study was too high. If a light dose which is above the urothelial threshold but below the muscle's can be found, then light fractionation might be a convenient option over the standard PDT to treat bladder cancers in patients as the total illumination time can be much reduced. However, further study is needed to look at the selectivity between the urothelium and the underlying layers after PDT with light fractionation. The problem of whether reperfusion or reoxygenation will potentiate damage to the muscle and lamina propria remains to be evaluated.
I conclude that CP94 is effective in elevating urothelial PpIX levels after intravesical instillation of 10% ALA. However, at 1% ALA, the effect of CP94 is marginal with regard to PpIX levels, probably as a result of incomplete saturation of the process converting PpIX to haem in the cytoplasm or mitochondria of the urothelium. Although the PpIX level is not elevated with CP94 at 1% ALA, the enhanced selectivity between the urothelium and underlying layers, and the probable vascular sparing effect following CP94 administration makes PDT effects with this combination as good as those achieved with 10% ALA without CP94. The antiproliferative effect of an iron chelator on cancer cells might be a bonus if it is administered with ALA for PDT treatment of bladder cancer. The vascular endothelial protecting effect of CP94 in PDT, although largely speculative and awaiting further confirmation, is important to ensure a rapid regeneration of bladder tissue after treatment. Light dose fractionation has the potential to facilitate PDT effects in the bladder, but the mechanism of action and the usefulness of light fractionation remain to be determined. Clearly, light fractionation will lead to a shorter illumination time which is convenient in terms of clinical application when the total dose is 3000-5000J for a bladder volume of 150-200ml.
CHAPTER 8

INTERSTITIAL AND TRANSURETHRAL PHOTODYNAMIC THERAPY OF THE CANINE PROSTATE USING MESO-TETRA-(M-HYDROXYPHENYL)CHLORIN

| 8.1 | Background | 219 |
| 8.2 | Aims | 220 |
| 8.3 | Materials and Methods |
| 8.3.1 | Animals, anaesthesia and biopsy technique | 221 |
| 8.3.2 | Photosensitiser and pharmacokinetic study | 222 |
| 8.3.3 | Photodynamic therapy | 223 |
| 8.4 | Results |
| 8.4.1 | Pharmacokinetic study | 226 |
| 8.4.2 | General responses after PDT | 229 |
| 8.4.3 | Macroscopic findings | 229 |
| 8.4.4 | Microscopic findings | 237 |
| 8.5 | Discussion |
| 8.5.1 | Is PDT feasible for the prostate? | 242 |
| 8.5.2 | Tissue characteristics for PDT of the prostate | 242 |
| 8.5.3 | Urological complications of PDT on the prostate | 243 |
| 8.5.4 | Rectal injury after transurethral PDT | 244 |
| 8.5.5 | Prostate tissue selectivity on PDT | 244 |
| 8.5.6 | Optimum drug-light interval for mTHPC | 245 |
| 8.5.7 | BPH and PDT. Is there a hope? | 245 |
| 8.5.8 | Prostate cancer and PDT | 246 |
| 8.6 | Conclusions | 247 |
8.1 BACKGROUND

The available modalities for treating localised prostate cancer which offer the potential for cure or satisfactory long term disease control are radical surgery and radiotherapy, but both can be associated with considerable morbidity (Bagshaw et al., 1994; Murphy et al., 1994). The quality of life is just as important as the quantity (Madsen & Bruskewitz 1995) and recently, just observation has been proposed as an option for those with moderately to well differentiated localised tumours having a life expectancy of 10 years or less (Chodak et al., 1994). Prostate cancer affects about 250,000 men in the U.S.A. each year (Boring et al., 1994), there is a need for a new definitive treatment which has less morbidity than surgery and radiotherapy but remains as effective in controlling localised disease.

In a range of organs it has been shown that PDT destroys glandular tissue (normal or neoplastic) with little effect on connective tissue, and healing mainly by regeneration rather than scarring (Barr et al., 1987). This makes it an attractive option for treating localised prostate cancer as it has the potential to destroy malignant tissue in the prostate in situ with safe healing of necrosis in normal and neoplastic areas without destroying the connective tissue structure of the gland. Little work has yet been done on PDT on the prostate. Windahl et al. (1990) used the photosensitiser Photofrin successfully treated 2 patients with residual cancer after transurethral resection and reported significant reductions in the levels of prostate specific antigen (PSA) after PDT. However, Photofrin is a poorly defined mixture of porphyrins and causes troublesome skin photosensitivity for 2-3 months in patients, so various new photosensitising agents are being studied. There are several recent reports of studies with other photosensitisers looking at PDT effects on prostate cancer cells in vitro (Camps et al., 1985), in normal animal prostate in vivo (Pantelides et al., 1993; Shetty et al., 1994; Selman & Keck 1994), and the optical properties of human prostate ex vivo (Pantelides et al., 1990), all with encouraging results. In the present study I investigated PDT in the normal canine prostate using meso-tetra-(m-hydroxyphenyl) chlorin (mTHPC). This is a pure compound with an absorption peak at 650 nm allowing deeper tissue penetration than the red light at 630nm used with Photofrin. It is a potent photosensitiser and has been reported as having some tumour selectivity, although these experiments did not compare the tumour concentration of mTHPC with that in the normal tissue in which the tumour arose (Berenbaum et al., 1993).
8.2 AIMS

In the hope of providing prostate cancer patients with an alternative option which is less morbid than surgery and radiotherapy but remains as efficient in controlling localised disease, photodynamic therapy on the canine prostate was assessed as a preparatory step before extending it into clinical trials. Although the dog prostate is not an ideal model, it is the only reasonable animal available for such studies. In this chapter, I investigated the pharmacokinetics of mTHPC in the normal canine prostate and the short and long term effects of PDT after interstitial or transurethral light delivery. The extent of lesions in the prostate and the adjacent organs would be useful for planning light and drug dosimetry for clinical PDT. The elucidation of morbidity as well as treatment associated side effects from such in vivo studies would provide us with useful information toward prevention of complications in clinical trials. It is also the aim of this study to compare the treatment effects between the interstitial and transurethral routes of light delivery, as the optimum way of applying light to the prostate remains unclear.
8.3 MATERIALS AND METHODS

8.3.1 Animals, anaesthesia and biopsy technique

The only animal with a suitable size prostate for these studies was the dog. Sixteen mature ex-breeder beagles (3-7 years old) weighing between 12 and 21 kg were used and all were managed according to the guidelines of the Animals (Scientific Procedures) Act 1986 (Home Office, London, UK).

Multiple biopsies and therapeutic manipulations lasting for 15 minutes or more were undertaken under general anaesthesia with intubation. Pre-medication was with intramuscular fentanyl/fluanisone (Hypnorm) (Janssen, Oxford, UK) 0.1-0.2 ml/kg and subcutaneous prophylactic antibiotics (2.5% enrofloxacin) 1 ml/5kg. After intubation, inhalation anaesthesia was maintained with halothane 1-2% and nitrous oxide as a 50:50 or 60:40 mixture with oxygen on a Magills anaesthetic circuit. Stable anaesthesia was maintained throughout the procedures and recovery was uneventful in all experimental animals. Shorter procedures were done under heavy sedation with intramuscular Hypnorm (0.1-0.2 ml/kg) and oxygen through a mask.

Prostate biopsies were taken via the transperineal route under ultrasound guidance using a 7.5 MHz biplane transrectal probe (Aloka, San Jose, CA). The perianal and perineal area were cleaned and disinfected with chlorhexidine (DePuy, Leeds, UK), and the ultrasound probe then covered with a condom, lubricated and inserted into the rectum. With ultrasound guidance and a spring operated biopsy device, specimens were obtained with minimal discomfort to the animal (Figure 8.1). Liver biopsy was done under the guidance of a 3.5 MHz abdominal ultrasound probe and through a right subcostal approach obtaining 2-3 specimens at each time point. Postoperatively, animals were kept in subdued lighting to minimise the risk of cutaneous photosensitivity and received a 3-day course of analgesics with subcutaneous flunixin meglumine 1 mg/kg (Finadyne) (Schering-Plough, Suffolk, UK) and antibiotics for up to 5 days.
8.3.2 Photosensitiser and pharmacokinetic study

Meso-tetra-(m-hydroxyphenyl)chlorin (mTHPC) was kindly supplied by Scotia Pharmaceuticals Ltd (Guildford, UK), and dissolved in a solution composed of ethanol, polyethylene glycol and distilled water in the ratios of 2:3:5. This solution was freshly prepared immediately prior to injection and was given intravenously through the antecubital vein. After sensitisation, the beagles were kept in a room with dimmed light to prevent skin photosensitivity.

One dog was used for pharmacokinetic studies. Control biopsies of the liver and prostate were taken two weeks before intravenous sensitisation with 0.3mg/kg mTHPC for the pharmacokinetic study and biopsies taken after 1, 3, 5, 24, 48, 72 and 168 hours from each organ (2-3 at each time point). The specimens were placed on a sheet of paper and immersed in isopentane before being stored in liquid nitrogen.

mTHPC levels in the tissue were quantified by fluorescence microscopy on 10 micrometer thick sections using an inverted phase contrast microscope (Olympus IMT-2, Hamburg, Germany) attached to a high resolution (385 x 578 pixel) slow-scan charge coupled device (CCD) camera (model 1, Wright, Cambridge, UK) (Pottier et
al., 1986). The basic principles of fluorescence microscopy was discussed in Chapter 4.

### 8.3.3 Photodynamic therapy

The 14 dogs in the photodynamic therapy study were given 0.3mg/kg mTHPC intravenously prior to light illumination. The light source was a KTP/532 laser pumping a Dye Laser Model 630 (Laserscope, San Jose, CA) tuned to emit at 650nm. For both interstitial and transurethral PDT, a light dose of 100J (100 mW for 1000 sec) was delivered at each treatment site under general anaesthesia. A 0.6mm bare tip fibre was used for interstitial treatment and a 3cm long, 1.8 mm diameter diffuser fibre for transurethral treatment. The power was checked before and after each treatment. Figure 8.2 is the illustration of treatment.

![Figure 8.2: Illustration of PDT treatment of the prostate with transperineal approach.](image)

Two control animals were treated without prior administration of mTHPC to see if any thermal effects were produced with the laser power and exposure time used
(100mW for 1000sec). One was treated interstitially (one site only) and the other transurethrally. Both were killed 3 days after treatment for histological examination.

Reports on studies of mTHPC in other organs have suggested that the best ratio of mTHPC levels between tumour and normal are seen 72 hours after sensitisation (Ris et al., 1993) and so all but one animals were treated at this time, but as the absolute tissue level was highest at 24 hours, one dog was treated at this earlier time. For the interstitial study (8 animals, including the one treated at 24 hours), the fibres were inserted through a biopsy needle via the transperineal route under the guidance of transrectal ultrasound (Figure 8.3).

Figure 8.3: Transrectal ultrasonography of the prostate. (A) Longitudinal scan showing the position of the biopsy needle (long arrows) and the laser fibre (small arrow). (B) Radial scan showing the laser fibre as a white echogenic dot (arrows).

The target areas were the proximal prostate near the bladder neck, the mid prostate (near the urethra or against the capsule), and close to the apex. To minimise rectal injury, the laser fibre was positioned 10-12mm away from the rectal wall under transrectal ultrasound guidance. The number of treatment sites in each prostate was determined by the size of the gland as measured by ultrasound, and ranged from 1-4. For the transurethral study, five dogs were used. A sterile, transparent 10 Fr. nasogastric (NG) tube was inserted transurethrally to empty the bladder and the
cylindrical diffuser fibre advanced until its tip touched the blind end of the NG tube. The NG tube with the fibre in situ was then slowly withdrawn under ultrasound guidance until the tip reached the bladder neck and the full length of the diffuser lay in the prostatic urethra ready for light delivery (Figure 8.4). After treatment, all dogs were kept in subdued lighting and observed at least daily for any signs of discomfort or distress. Any animals having difficulty with voiding (spotting or retention) were catheterised once or twice daily until they could void adequately spontaneously. At times from 3 to 90 days after PDT, the animals were humanely killed with intravenous Expiral (Pentobarbitone, 120 mg/kg body weight) and the bladder and prostate removed en bloc. For macroscopic inspection, the specimens were sectioned serially from the bladder neck to the apical region at intervals of 5-7 mm. Selected areas were further processed and sectioned for histological examination (Haematoxylin and Eosin and Van Gieson stains).

Figure 8.4: (A) Transrectal ultrasonography with longitudinal probe showing N-G cannula (arrows) in the prostatic urethra, (B) 3-cm cylindrical diffuser fibre.
8.4 RESULTS

8.4.1 Pharmacokinetic study

The levels of mTHPC fluorescence in the liver and prostate seen at times from one hour to 7 days after sensitisation are shown in Figure 8.5. In the liver, a rapid surge of mTHPC was found 1 hour after sensitisation. The peak seen at one hour declined over the following 24 hours at which time the level was about half of the peak. A mild elevation of hepatic mTHPC was observed at 72 hours. The difference of mTHPC between 24 and 72 hours was not significant (student t-test, p>0.2). It was also noteworthy that at 7 days after sensitisation, mTHPC in the liver remained 5 times higher than that in controls. The ratios of mTHPC between liver and the prostate at each time point were in the range of 8-11 before 24 hours and 3-4.5 after 24 hours. In the prostate, the highest level was seen at 24 hours, significantly higher than that at 72 hours (student t-test, p< 0.01). A relatively high value was seen mainly in the capsule and stroma at the first time studied, 1 hour, which then declined before rising to the highest level seen throughout the glandular tissue proper, at 24 hours. By the third day, although the intensity was lower than at 24 hours, a substantial amount of mTHPC was still detected in the glandular structure whereas the stroma was relatively fluorescence free (Figure 8.6). By 7 days, the fluorescence level had fallen to about twice that seen before sensitisation.
Figure 8.5: (A) Plot of mTHPC concentration in the liver and prostate against time after intravenous injection. (B) Plot of mTHPC in the prostate. The difference of mTHPC between 24 and 72 hours is significant (p<0.005, student t test).
Figure 8.6: (A) CCD photograph showing microscopic details of mTHPC distribution in the prostate 3 days after injection. (B) Matching histology(X100, H&E stain) showing the highest concentrations of mTHPC are in the glandular areas (Gl) than in the stroma.
8.4.2 General responses after PDT

No evidence of skin phototoxicity was seen during the follow-up period in any animal, although all were kept in subdued light. Neither of the 2 animals having 2 sites treated interstitially had any problems, but 2 of the 5 having 4 sites treated had difficulty voiding and macroscopic haematuria with signs of general discomfort (poor appetite, slow gait and loss of body weight). One was killed at day 3 as scheduled, the other required daily catheterization for 5 days to relieve retention before resuming spontaneous urination. Urinary problems were more prominent in those treated transurethrally. Four out of 5 developed urinary retention 24-72 hours after treatment. One with partial voiding difficulty was killed on day 3 as scheduled. In spite of daily catheterization, one had to be put down at 5 days due to general malaise from persistent elevation of serum creatinine and urea nitrogen. No sign of urinary incontinence or passage of tissue debris through the urethra was observed in the 2 animals kept for more than 7 days. They voided spontaneously and smoothly after 5 days of catheterization.

8.4.3 Macroscopic findings

External examination of prostates treated with interstitial PDT at 2 sites showed only slight oedema but patchy subcapsular hyperaemia and marked oedema were seen in those treated at 4 sites. In 2 animals, the fatty and areolar tissue surrounding the prostate showed congestion and ecchymoses(Figure 8.7). On sectioning the gland, the lesion seen around each treatment site in the first couple of weeks after PDT was roughly spherical in shape and about 20mm in diameter (Figure 8.8). With multiple lesions, there was usually overlap and coalescence between lesions. Two fibres placed 2 cm apart produced a large confluent elliptical lesion (Figure 8.9). With 4 treatment sites, lesions up to 35x25x22 mm were seen covering more than 80% of the total prostate volume (Figure 8.10). The capsular structure overlying the prostate was intact in all cases. Lesions seen at 7 days were slightly smaller, but otherwise similar to comparable ones seen at 3 days (Figure 8.11). Four weeks after PDT, the prostate appeared macroscopically normal in size and texture, but on cutting, a scarred area was observed beneath the capsule, with or without periurethral congestion (Figure 8.12). Three months after PDT, the scarred area had shrunk further. There was no evidence of deformity or collapse of the prostate at any time after treatment(Figure 8.13).
Figure 8.7: Macroscopic appearance of the canine prostate showing hyperaemic changes on the capsule 3 days following PDT (4 treatment sites, 100J each).

Figure 8.8: Macroscopic appearance of the canine prostate 3 days after PDT showing extensive lesions confined to the prostate (2 treatment sites, 100J each).
Figure 8.9: Macroscopic lesion 3 days after PDT with 2 fibres 20mm apart (100 J each) measuring 20x40x22 mm in size.

Figure 8.10: Macroscopic lesions 3 days after PDT with 4 fibres. The lesions comprise up to 80% of the total volume of the prostate.
Figure 8.11: Macroscopic lesion 7 days after interstitial PDT with 2 fibres (one at left lobe, one near the urethra). The lesion shown here (20x22x20mm) is induced by one laser fibre.

Figure 8.12: Macroscopic lesion 28 days after PDT with a single fibre. A discoloured area (arrow) indicates PDT lesion that has healed without distorting the architecture of the whole gland.
Figure 8.13: Macroscopic lesions 3 months after interstitial PDT with 4 fibres. Scar is seen at different levels of the prostate. (A) Near the apex, (B) mid prostate, (C) posterior prostate, (D) reverse side of (C).
After transurethral PDT, the lesions were cylindrical, 30mm in length matching the length of the diffuser, and up to 20mm in diameter. These extended along the prostatic urethra from the bladder neck to the external sphincter (Figure 8.14). Two of the 3 dogs killed within 7 days of PDT showed an ulcer like hyperaemic erosion in the rectal mucosa immediately adjacent to the apex of the prostate (Figure 8.15). These lesions evolved very similarly to those produced interstitially, and by the 4th week there was little left to see (Figure 8.16). No lesions were seen in unsensitised control animals treated with light by either the interstitial or transurethral route. The dimensions of the lesions measured macroscopically are tabulated in Table 8.1.

Figure 8.14: Serial sections of a prostate 7 days after transurethral PDT. From left to right: apical-distal-mid-proximal-near bladder neck.
Figure 8.15: Gross specimen of the prostate and rectum showing a mucosal lesion (arrow) about 10x12mm in size at the rectal wall attached to the apex of the prostate.

Figure 8.16: Prostate 30 days after transurethral PDT showing the dilated urethra but with the rest of the gland appearing grossly normal.
Table 8.1: Demographic data and macroscopic changes of the prostate in the experimental animals

<table>
<thead>
<tr>
<th>Dog</th>
<th>Sensitisation Time (hours)</th>
<th>Route of PDT</th>
<th>Number of Treatment sites</th>
<th>Sacrifice Time (days)</th>
<th>Lesions size LxWxH (mm)</th>
<th>Complications and Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>--</td>
<td>IT</td>
<td>1</td>
<td>3</td>
<td>--</td>
<td>control</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>IT</td>
<td>4</td>
<td>3</td>
<td>42x22x20* 40x20x18*</td>
<td>retention for 1 day, no catheter</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>IT</td>
<td>2</td>
<td>3</td>
<td>20x40x22*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>IT</td>
<td>4</td>
<td>3</td>
<td>35x25x22* 22x21x17 22x22x20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>IT</td>
<td>2</td>
<td>7</td>
<td>20x22x20 18x18x18</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>IT</td>
<td>4</td>
<td>14</td>
<td>30x18x18* 28x14x18*</td>
<td>retention -daily catheter for 5 days</td>
</tr>
<tr>
<td>7</td>
<td>72</td>
<td>IT</td>
<td>1</td>
<td>28</td>
<td>12x14x11</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>IT</td>
<td>4</td>
<td>28</td>
<td>15x10x10* 12x10x9*</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>72</td>
<td>IT</td>
<td>4</td>
<td>90</td>
<td>8x8x8 7x8x8 6x6x8 5x6x5</td>
<td>vomiting for 4 days</td>
</tr>
<tr>
<td>10</td>
<td>--</td>
<td>TU</td>
<td>1</td>
<td>3</td>
<td>--</td>
<td>control</td>
</tr>
<tr>
<td>11</td>
<td>72</td>
<td>TU</td>
<td>1</td>
<td>3</td>
<td>30x15x20</td>
<td>partial retention, no catheter, rectal erosion (10x6mm)</td>
</tr>
<tr>
<td>12</td>
<td>72</td>
<td>TU</td>
<td>1</td>
<td>5</td>
<td>30x18x18</td>
<td>retention-daily catheter</td>
</tr>
<tr>
<td>13</td>
<td>72</td>
<td>TU</td>
<td>1</td>
<td>7</td>
<td>30x15x18</td>
<td>put down at 5 days (renal failure)</td>
</tr>
<tr>
<td>14</td>
<td>72</td>
<td>TU</td>
<td>1</td>
<td>14</td>
<td>30x14x16</td>
<td>retention-daily catheter for 2 days</td>
</tr>
<tr>
<td>15</td>
<td>72</td>
<td>TU</td>
<td>1</td>
<td>30</td>
<td>not visible</td>
<td>rectal scar (5x4mm)</td>
</tr>
</tbody>
</table>

*: Confluent lesion covering 2 treatment sites.
IT: Interstitial, TU: Transurethral
8.4.4 Microscopic findings

The extent of microscopic changes corresponded to those seen macroscopically. At 3 days there was extensive haemorrhagic necrosis in the glands with destruction of the ductal and acinar collagenous structure and haemosiderin deposition (Figure 8.17). In lesions involving the urethra, haemorrhagic necrosis as well as ulceration of the urethra was seen in the periurethral tissues with sloughing of the urothelium and inflammatory cell infiltration, particularly in the first week following PDT (Figure 8.18). These changes slowly evolved but up to 4 weeks after treatment there was little glandular regeneration and many areas of persistent atrophic glands interposed with haemorrhagic necrosis (Figure 8.19). Urethral healing, however, took place earlier, and by the third week, there was complete regeneration of the urethral epithelium with increased fibroblast activity in the peri-urethral tissue (Figure 8.20). No stricture formation was observed in the animal followed up to 30 days.

Figure 8.17: Microscopic finding of the prostate 3 days after PDT showing area of haemorrhagic necrosis and haemosiderin deposition (X150, H&E stain).

237
Figure 8.18: Microscopic finding of the prostatic urethrae 3 days after PDT showing extensive sloughing of the urothelium and destruction of the peri-urethral tissues (X40, H&E stain).

Figure 8.19: Microscopic finding of the prostate 28 days after PDT showing area of atrophic glands (arrows) and residual haemorrhagic necrosis (open arrows) (X60, H&E stain).
Figure 8.20: Microscopic finding of the prostatic urethrae 28 days after PDT showing (A) regeneration of urothelium with evidence of residual haemorrhagic necrosis in the glandular region (X40, H&E stain), (B) increased fibroblast activity with collagen deposition (red) in the peri-urethral area (X100, HVG stain).
Three months after treatment, although the glandular elements remained mainly atrophic with little fibroblast infiltration, fibrosis and dense collagen tissue deposition was seen in the periurethral area (Figure 8.21). With VanGieson’s stain, collagen fibrils in the ductal and acinar parts of the prostate were seen to be destroyed, but the supporting collagenous stroma which extended from the fibrous capsule to the interlobular septa of the glandular regions was well preserved (Figure 8.22). The erosion on the rectal mucosa showed intact lamina propria but the glandular elements had patchy interruption of the epithelium overlying the lesion. There was no fistulation between the 2 organs (Figure 8.23).

**Figure 8.21:** Microscopic finding of the prostatic urethra 90 days after PDT showing dense collagen deposits(X60, HVG).
Figure 8.22: Microscopic finding of the prostate 90 days after PDT showing atrophic glands, inflammatory cell infiltration and preservation of collagenous fibrils (X60, HVG).

Figure 8.23: Microscopic finding of the rectal mucosa 30 days after transurethral PDT showing regeneration of the mucosa and inflammatory cell infiltration (X60, H&E).
8.5 DISCUSSION

8.5.1 Is PDT feasible for the prostate?

This study looked at the effects of PDT using the sensitiser mTHPC on the normal canine prostate and has shown that it is possible to safely necrose most of the glandular tissue without changing the basic structure, shape or size of the organ and without unacceptable effects in adjacent tissues. If prostate cancer responds at least as well to PDT as normal prostate, this suggests that the technique has very considerable potential for treating cancer, wherever in the gland it may arise.

It is well documented that PDT necroses glandular elements in many of the hollow organs of the body with little effect on connective tissues like collagen, although additional collagen may be laid down during healing (Barr et al, 1987, Chapter 6). This has not previously been reported in solid organs like the prostate. The present study shows that the effects are very similar, although healing of the large volumes of necrosis seen in our experiments was much slower than healing in the mucosa of hollow organs, as would be expected.

8.5.2 Tissue characteristics for PDT of the prostate

For PDT to be of value clinically, it must be possible to treat all areas where the cancer may develop. This is particularly important next to the capsule. In this study animals with PDT lesions extending to the edge of the gland, some hyperaemia was seen over the capsule but there was no histological evidence of disruption or damage to the capsular architecture, presumably because it consists mainly of connective tissue. Further, the white coloured capsule is likely to reflect much of the light reaching it back into the main body of the gland, reducing both the amount of light absorbed in the capsule itself and the amount transmitted to other adjacent tissues. Other studies (Pantelides et al, 1990) have shown that light is extensively scattered within the prostate and these two factors make it feasible to treat essentially all the glandular tissue with a relatively small number of fibre sites. With mTHPC, the diameter of the zone of PDT necrosis around each fibre position is about 2 cm, so the requirement is that every point in the prostate should be no more than 1 cm from a fibre site.
8.5.3 Urological complications of PDT on the prostate

Early histological features of PDT lesions in glandular areas were haemorrhagic necrosis which healed with fibroblast infiltration but even after 90 days, there was still persistent glandular atrophy. This is functionally insignificant, but it is much more important to understand what happens to the urethra. Urethral damage was most severe in dogs treated transurethrally, but was also seen in some of those treated interstitially. It was characterised by sloughing of the urothelial epithelium with subepithelial oedema and haemorrhage. This was still seen after 14 days but the epithelium regenerated completely by 4 weeks. These changes would be expected to be associated with functional changes. No urinary incontinence was observed in any of these animals at any stage after PDT, but there were problems with urinary retention. Four animals treated transurethrally and two treated interstitially went into urinary retention 1-3 days after PDT. This was managed by once or twice daily catheterization, although one animal went into renal failure and had to be put down after 5 days. Of the other five, three were killed as scheduled at days 3 and 7, and had not resumed normal micturition by the time of their death, but the other two were able to void by a week and micturition was essentially normal by the time they were killed at 14 days. Inevitably, delivering light via a 3-cm diffusing fibre in the prostatic urethra included in the treatment field the external sphincter, located in the most distal portion of the gland. The most likely explanation for the retention is oedema in the treated urethra proximal to the sphincter, which would mean that any damage to the sphincter would not be apparent at this time. Another possibility is severe dysuria secondary to the extensive urethral necrosis caused by PDT, which inhibited them from voiding spontaneously. The study used a 3-cm cylindrical diffuser fibre for transurethral treatment which may not have been long enough to cover the full length of the prostate, which is usually about 4 cm. The lack of sphincter damage might be because very little light was delivered at that level. It is reassuring that incontinence was not seen in any of the animals at any stage after PDT, whether they had initial retention or not, so the drug and light dose used in this study cannot have been sufficient to cause clinically significant damage to the sphincter.

No animals were tested for sexual function after recovery from PDT to assess whether there had been any damage to the neurovascular bundle. There are no good studies yet reported of PDT on peripheral nerves, but it is unlikely that light can easily penetrate nerve sheaths even if they do take up significant amounts of photosensitiser.
8.5.4 Rectal injury after transurethral PDT

In the beagle, the rectum is close to the prostate, particularly at the apex in the region of the sphincter, and clearly some light passed through the capsule of the prostate to reach the rectum in 3 of the 5 animals treated transurethrally. This was seen as superficial ulceration during the first week, but had healed by 30 days, and no animal showed any sign of disturbance of bowel function at any time after treatment. Microscopically, there was interruption of the muscularis mucosa (a thin band of muscle located immediately beneath the mucosa), some fibroblast infiltration and a little scarring in the muscle of the rectum, but this would not be expected to cause any problems as long as it is only on one side of the rectum. The mucosa healed with regeneration.

8.5.5 Prostate tissue selectivity on PDT

This study only looked at PDT effects in normal prostate and the clinical challenge is to destroy areas of prostate cancer. There are no reports of experimental studies on prostate cancer growing in the prostate, although Windahl et al (1990) showed PDT destruction of residual cancer in two patients after transurethral resection. However, there are numerous reports of PDT effects on adenocarcinomas in other organs, all of which show that there is at least as much necrosis in the tumours as in the adjacent normal tissues in which the tumour arose (Barr et al, 1990). Thus it is reasonable to suppose that any part of the normal prostate that can be necrosed with PDT, would also be necrosed if it was replaced by cancer. Much has been written about selective necrosis of cancers with PDT. This might be difficult to achieve in practice, but in most organs, including the prostate, all that is required to achieve the desired clinical ends is that the cancer should be at least as susceptible as the normal tissue in which it arises (Bown 1990). Nevertheless, it is logical to choose the time interval between giving the photosensitising drug and delivering the light that gives the highest ratio of drug concentration between tumour and normal. Although the selectivity of photodamage between normal and cancer cells is likely to be marginal, there is another natural tissue selectivity between the cellular and the collagenous components of the prostate. I have demonstrated that the larger interlobular collagen fibrils remain relatively preserved after PDT, whereas the smaller ductal or intra-acinar fibrils were destroyed with the glandular tissues. The intact collagen in the stroma then serves as the frame structure for further tissue regeneration, keeping the prostate in shape and without causing severe fibrosis or tissue contracture. It is this character which is
specific to PDT and which makes it a potential option for treating the prostate, as the risk of a urethral stricture is low.

8.5.6 Optimum drug-light interval for mTHPC

The present study showed that the biodistribution of mTHPC in the prostate was a biphasic curve. The early peak was seen at the first time studied, 1 hour after injection, and was probably due to mTHPC in the blood. The second and highest peak occurred at 24 hours and was more due to photosensitiser in the glandular structure of the prostate. The differences between 24 and 72 hours were not great. Ris et al (1993) suggested that the best therapeutic ratio between tumour and normal tissue was seen at 3 days in mesotheliomas, and for this reason I chose 72 hours as the time interval for light illumination for most of my experiments. The one dog treated at 24 hours had a comparable size lesion to one treated similarly at 72 hours. However, no comment can be made in this report regarding urethral damage with a drug-light interval of 24 hours since there was no animal treated via transurethral route at this drug-light interval. After conventional dosage of mTHPC and a drug-light interval of 24 hours, a substantial damage to the urethra might be expected using the transurethral approach. Also, care must be taken when extrapolating data from one organ to another and from one species to another. The glandular to stromal ratio as well as the embryological development of the prostate in dogs and humans are different, which could influence the PDT effects, although this is unlikely (Laroque et al., 1995).

8.5.7 BPH and PDT. Is there a hope?

Recently, Selman and Keck (1994) used the photosensitiser tin(II) etiopurpurin dichloride (SnET2) with light delivered transurethrally for PDT on the normal canine prostate, and showed similar results to mines with good healing of the urethra. They concluded that its use for the treatment of benign prostatic hypertrophy (BPH) warranted further investigation. I have reservations about this conclusion as neither their experiments nor mine showed any objective evidence of reduction of prostate volume as required to reduce outflow tract resistance, which is the main aim of treatment for BPH. Moreover, the long term effects on the prostate, both functional and anatomical, remain to be assessed. Using conventional transurethral resection of the prostate (TUR-P) or contemporary laser coagulation and vaporisation techniques (Anson et al., 1994), BPH can be satisfactorily controlled with low morbidity in most
cases without the possible problems associated with PDT, particularly skin photosensitivity, although these other techniques can cause troublesome passage of debris per urethra for some time after treatment, which was not seen in my animals.

8.5.8 Prostate cancer and PDT

These experiments have shown that it is feasible to destroy large areas of the canine prostate without any unacceptable damage to immediately adjacent normal tissues, even when the PDT necrosis extends to the prostate capsule and includes the urethra. With careful positioning of the laser fibres, perhaps using both the interstitial and transurethral approaches, it should be possible to treat essentially the entire prostate safely. This has very considerable potential for treating primary or recurrent cancer (after radical surgery or radiotherapy) localised to the gland.

Recent reports show that elevated levels of PSA identify a group of patients at high risk of developing or already having prostate cancer (Parkes et al, 1995). This makes it likely that in the future, more early cancers localised to the gland will be diagnosed in asymptomatic patients. Current treatment of such patients with a life expectancy of more than 10 years with radical prostatectomy or radiotherapy is likely to be challenged by simpler options. Watchful waiting with delayed hormonal therapy which could achieve a 10 years disease specific survival as high as 87% in Grade 1 or 2 localised cancers has been proposed as a reasonable alternative (Chodak et al, 1994) although others have doubted the advisability of this (Menon et al, 1995).

Considering the low complication rate, the prolonged glandular atrophy with preservation of the connective tissue structure of the prostate and the prompt regeneration of urethral epithelium, PDT seems a promising option for the management of localised prostate cancer.
8.6 CONCLUSIONS

In this chapter, I studied the tissue biodistribution and photodynamic effects of meso-tetra-(m-hydroxyphenyl) chlorin (mTHPC), a potent second generation photosensitiser, on normal canine prostate in vivo. Using quantitative fluorescence microscopy, the highest concentration of mTHPC in the prostate was seen 24-72 hours after intravenous administration. For PDT, red light (650nm) was delivered to the prostate by laser fibres inserted via the transurethral or transperineal route under transrectal ultrasound guidance. PDT lesions up to 40 mm in diameter (using 4 fibre sites) were produced, characterised by swelling, inflammatory response and extensive glandular destruction. There was persistent glandular atrophy at 90 days, but no disruption of the main stroma and no change in the size or shape of the gland. Urethral damage sometimes caused temporary urinary retention, but this resolved by 7 days and no animal became incontinent. Occasional small lesions were seen in the rectum, but these healed without sequelae and there were no fistulae. As cancer and normal prostate are likely to respond similarly, PDT has considerable promise for treating cancer confined to the gland as large areas of glandular tissue can be necrosed with safe healing. As the structural integrity of the gland is maintained, PDT is unlikely to be of value in the management of benign prostatic hypertrophy.

Other than mTHPC which has been shown to hold great promises for clinical management of prostate cancer, I also tried to test other photosensitisers which are less potent but have the advantages of posing less risks of skin photosensitivity. A potent photosensitiser like mTHPC may be welcomed if the bulky prostate cancer is to be destroyed with other hypertrophic tissues. On the contrary, if the prostate cancer has been largely debulked by radical prostatectomy or has been shrinked by radiotherapy, a PDT treatment with potent photosensitiser brings concerns for overtreatment as the danger of damaging adjacent structure is enormous. In such cases, a less potent photosensitiser which is reliable and has less adverse effects might be more practical than mTHPC. In the next chapter, two less potent photosensitisers ALA and AlS2Pc will be tested on the canine prostate.
CHAPTER 9

INTERSTITIAL PHOTODYNAMIC THERAPY IN THE CANINE PROSTATE WITH DISULPHONATED ALUMINIUM PHTHALOCYANINE AND 5-AMINOLAEVULINIC ACID INDUCED PROTOPORPHYRIN IX

9.1 Background ................................................................. 249
9.2 Aims ......................................................................... 250
9.3 Materials and Methods
  9.3.1 Animals and anaesthesia .............................................. 251
  9.3.2 Photosensitisers .......................................................... 251
  9.3.3 Transrectal ultrasound guided prostate biopsy ............... 251
  9.3.4 Fluorescence microscopy ............................................. 252
  9.3.5 Laser and light delivery systems .................................. 252
  9.3.6 PDT treatment .......................................................... 254
9.4 Results
  9.4.1 Pharmacokinetic studies of the prostate ......................... 255
  9.4.2 Pharmacokinetic studies of liver .................................. 257
  9.4.3 Photodynamic effects
     9.4.3.1 Macroscopic findings ......................................... 258
     9.4.3.2 Histology .......................................................... 262
     9.4.3.3 Perioperative responses ...................................... 264
9.5 Discussion
  9.5.1 Light dosimetry for ALA based PDT on the prostate ......... 265
  9.5.2 PDT effect of ALA-PpIX on the prostate ....................... 265
  9.5.3 AlS2Pc based PDT on the prostate ............................... 266
  9.5.4 Tissue selectivity and PDT ....................................... 267
  9.5.5 Prostate PDT. Which photosensitiser? ......................... 268
9.6 Conclusions .............................................................. 269
9.1 BACKGROUND

Further to my study on mTHPC, this chapter looks at the effects of PDT in the same animal model with two further photosensitisers, 5-aminolaevulinic acid (ALA) induced protoporphyrin IX (PpIX) and aluminium disulphonated phthalocyanine (AlS2Pc) (Kennedy et al, 1990; Ben-Hur 1992). These photosensitisers have the major advantage of causing less skin photosensitivity, ALA because it is cleared from the body within 1-2 days and AlS2Pc because it only absorbs in the red and very little at other wavelengths in the solar spectrum (apart from some absorption in the ultraviolet) (Tralau et al, 1989).

ALA has been extensively studied experimentally (Kennedy et al, 1990; Bedwell et al, 1992, Loh et al, 1993a,b; Regula et al, 1994; Chapter 5, 6, 7). This agent has shown considerable potential for the management of superficial lesions in the gastrointestinal tract (Regula et al, 1995; Mlkvy et al, 1995), maxillofacial area (Hopper et al, 1994) and of course in the bladder, as in the earlier chapters of this thesis. However, its effectiveness for solid internal organs remains unclear. Johnson et al (1995) recently observed substantial depth of haemorrhagic necrosis in a normal canine prostate one week after PDT. They placed a 2 cm diffuser fibre transurethrally 8 hours after intravenous sensitisation of 100mg/kg ALA and treated the prostate with a high light dose (650 mW for 45 min, 1755J).

The phthalocyanines (PC) are synthetic azaporphyrin derivatives which chemically mimic naturally occurring porphyrins in many respects. With the incorporation of a diamagnetic aluminium ion in the structure and with various degrees of sulphonation, the aluminium sulphonated phthalocyanines (AlSPc) have some of the physical, chemical and biological characteristics of an ideal photosensitiser (Ben-Hur 1992). Detail discussions of phthalocyanines have been given in section 3.3 of Chapter 3. This agent has the advantages of lower risk of skin photosensitivity (Tralau et al, 1989), strong absorption of red light at 675-700 nm which is superior to that of Photofrin at 630 nm in terms of tissue penetration (50% deeper) and higher extinction coefficient (50 times higher) (Ben-Hur & Rosenthal 1986). Among the various aluminium sulphonated derivatives, the disulphonated one has been shown to be the best for PDT (Chatlani et al, 1991).
In this chapter, further to my experience on interstitial and transurethral PDT of the canine prostate with mTHPC, I examined the \textit{in vivo} efficacy of another 2 potential photosensitisers. Pharmacokinetic studies were performed before PDT treatment on 2 normal beagles which have been sensitised with ALA and AlS2Pc, respectively. After determining the optimum sensitisation time for treatment, PDT was accomplished by interstitial route at different light dosimetrys. The objective was to understand the biodistribution of these photosensitisers after drug administration and the nature and extent of PDT effects after interstitial light delivery.
9.3 MATERIALS AND METHODS

9.3.1 Animals and anaesthesia

Eleven mature beagles were used for this study (6-7 years old, 12-16 kg). The experimental design was the same as that described in Chapter 8. However, in this study, only interstitial PDT was performed.

9.3.2 Photosensitisers

5-aminolaevulinic acid in the form of powder (ALA.HCl 98%), was obtained from DUSA Pharmaceuticals Inc. (New York, USA), dissolved in normal saline and titrated with saturated sodium bicarbonate solution to pH 4 giving a final concentration of 10% (100mg/ml). AIS$_2$PC was supplied by the Department of Chemistry, Imperial College, London, and dissolved in 0.1M sodium hydroxide before being buffered to pH 7.4 with NaH$_2$PO$_4$. The photosensitising agents were given through a 0.2 micrometer filter into an antecubital vein. Four animals received 200mg/kg ALA, and 4 received 1mg/kg AIS$_2$PC (one for pharmacokinetics and 3 for PDT for each drug). An additional 2 animals received 100mg/kg ALA for PDT in an attempt to reproduce the results of Johnson et al (1995).

9.3.3 Transrectal ultrasound guided prostate biopsy

The technique for ultrasound scanning is the same as was described in Chapter 8. The biplaner ultrasound probe is shown in Figure 9.1.

Following photosensitiser injection, biopsies were taken from the prostate at 15, 30min, 1, 3, 5 and 24 hours for ALA, and 1, 3, 5, 24 and 48 hours and 7 days for AIS$_2$Pc. Biopsies in the first 5 hours were taken under the initial anaesthetic while those at subsequent times were obtained under separate heavy sedation with intramuscular Fentanyl/Fluanisone 0.1-0.2 ml/kg (Hypnorm) with a supplementary oxygen mask. Two or 3 biopsies were taken from each prostate at each time point. The time needed to take 3 biopsies was about 5-10 minutes. The specimens were placed on a sheet of paper and immersed in precooled isopentane (BDH Chemicals Ltd, Poole, UK) before being stored in liquid nitrogen for subsequent preparation of cryosections.
Figure 9.1: Transrectal ultrasound probe having two scanning plans for transverse and longitudinal imaging. A self-made puncture template is attached.

9.3.4 Fluorescence microscopy

An inverted phase contrast epifluorescence microscope (Olympus IMT-2) attached to an 8mW helium neon laser (632.8 nm) was used for examination of fluorescence from the biopsy specimens. The set up of this equipment has been discussed in great detail in chapter 3. After exposing tissue sections to the helium laser for 8 (AlS2Pc) and 25 (ALA) seconds respectively, the fluorescence data were recorded for further analysis. The quantification of fluorescence intensity at each time point was done by taking the average of results calculated from at least 6 different sections. After fluorescence imaging, the sections were fixed in formalin and stained with haematoxylin and eosin for histological study.

9.3.5 Laser and light delivery systems

Red light at the wavelength of 630 and 675 nm (for ALA and AlS2Pc, respectively) was supplied by a KTP (Potassium Titanyl Phosphate) laser pumping a Dye Laser Model 630 (Laserscope, San Jose, California) (Figure 9.2). For PDT, a biopsy needle was positioned transperineally under transrectal ultrasound guidance, and a 600μm
baretip fibre inserted through it until the tip was 8-10 mm beyond the bevel. The exposure time for AIS₂Pc sensitised beagles was 1,000 sec at 100mW, giving a total energy of 100J for each treatment. For ALA and AIS₂Pc, the drug and light doses used are shown in Table 9.1. The laser power was checked before and after each treatment.

Table 9.1: Treatment parameters and macroscopic results

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sensitiser(mg/kg)</th>
<th>Sensitisation time(h)</th>
<th>Power (mW)</th>
<th>Illumination light time(sec)</th>
<th>Light dose(J)</th>
<th>Animal killed(D)</th>
<th>Lesion size(mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>ALA -- AIS₂Pc --</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>3</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>ALA -- AIS₂Pc --</td>
<td>100</td>
<td>3600</td>
<td>360</td>
<td>3</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>100 ALA -- AIS₂Pc</td>
<td>8</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>3</td>
<td>2x2x2</td>
</tr>
<tr>
<td>19</td>
<td>100 ALA -- AIS₂Pc</td>
<td>8</td>
<td>300</td>
<td>3600</td>
<td>1080</td>
<td>3</td>
<td>9x9x9</td>
</tr>
<tr>
<td>20</td>
<td>200 ALA -- AIS₂Pc</td>
<td>3</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>3</td>
<td>2x1x1</td>
</tr>
<tr>
<td>21</td>
<td>200 ALA -- AIS₂Pc</td>
<td>3</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>3</td>
<td>Nil</td>
</tr>
<tr>
<td>22</td>
<td>200 ALA -- AIS₂Pc</td>
<td>3</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>3</td>
<td>2x1x1</td>
</tr>
<tr>
<td>23</td>
<td>-- 1 ALA -- AIS₂Pc</td>
<td>24</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>3</td>
<td>2x1x1</td>
</tr>
<tr>
<td>24</td>
<td>-- 1 ALA -- AIS₂Pc</td>
<td>24</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>7</td>
<td>12x10x10</td>
</tr>
<tr>
<td>25</td>
<td>-- 1 ALA -- AIS₂Pc</td>
<td>24</td>
<td>100</td>
<td>1000</td>
<td>100</td>
<td>7</td>
<td>9x9x9</td>
</tr>
</tbody>
</table>

h: hour; sec: second; J: joule; D: day

Figure 9.2: (A) A KTP laser, (B) pumped dye laser module 630. The wavelength of this dye laser is tunable in the range of 600-700 nm.
9.3.6 PDT treatment

Two animals were treated at the wavelength of 630 nm, and at a power of 100 or 300 mW to a total dose of 100-1080J without prior photosensitisation as controls. From the fluorescence study results, the time between sensitisation and light delivery chosen for ALA was 3 hours as this was the time at which the highest levels of PpIX were found, but in view of the findings of Johnson et al (1995), 2 further animals were treated with a time interval of 8 hours. For AlS2Pc, the time interval chosen was 24 hours as peak tissue levels were seen at this time. One or two sites were treated in each animal, one near the prostate capsule and the other near the urethra. Details are given in Table I. After treatment, the animals were kept in a dimly lighted chamber for careful further observation. They were killed with Expiral (pentobarbitone 120 mg/kg body weight) 3, 7 and 28 days after PDT and the bladder and prostate removed en bloc. For gross inspection, the specimens were sectioned serially from the bladder neck to the apical region at intervals of 3-5 mm according to the extent of the lesion. The maximum diameter of each zone of PDT induced necrosis was measured macroscopically on these blocks. Blocks of interest were then fixed and sent for histological assessment with H & E and Van Gieson's stains.
9.4 RESULTS

9.4.1 Pharmacokinetic studies of the prostate

In the control animal that received no photosensitiser, no PpIX or AIS\textsubscript{2}Pc fluorescence was detectable. In the animal given ALA, significant levels of PpIX were not seen until one hour after administration with a peak at 3 hours, returning to background levels by 24 hours (Figure 9.3). In the animal given AIS\textsubscript{2}Pc, the fluorescence intensity showed a steady increase in the first 5 hours and then maintained a plateau to 48 hours before starting to fall. AIS\textsubscript{2}Pc was still detectable at 7 days at about the same level as that found at 3 hours (Figure 9.4). Fluorescence microscopy 24 hours after AIS\textsubscript{2}Pc showed accumulation of the photosensitiser in both the stroma and glandular tissue (Figure 9.5).

\textbf{Figure 9.3:} Plot of protoporphyrin IX fluorescence in prostate against time after intravenous administration of ALA. Data shown is the mean from 10-12 blocks (50x50 pixels) from 3 biopsies at each time point. The single point in an animal given 100 mg/kg was from an experiment designed to reproduce the results of Johnson \textit{et al} (1995).
Figure 9.4: Plot of AlS\(_2\)Pc fluorescence in prostate against time after intravenous administration of 1mg/kg AlS\(_2\)Pc. Data shown is the mean from 10-12 blocks (50x50 pixels) from 3 biopsies at each time point.

Figure 9.5: Fluorescence microscopy showing accumulation of AlS\(_2\)Pc in the stroma and glands (Gl) 24 hours after sensitisation.
9.4.2 Pharmacokinetic studies of liver

The intensity of PpIX in the liver was constant in the first 30 minutes after ALA administration. However the PpIX level surged rapidly at 1 hour at which a level up to 4 times that at 30 minutes was observed before it elevated further to reach the peak at 5 hours. At 24 hours, the hepatic PpIX was about the same level as at 0 hour (Figure 9.). The concentration of AlS2Pc also showed a rapid elevation at one hour after sensitisation. However, the peak did not appear until 24 hours before it started to decline over the following days to reach a nadir at the 7th day (Figure 9.7).

![Graph showing ALA fluorescence in liver and prostate](image)

**Figure 9.6:** Plot of ALA fluorescence in the liver and prostate against time after intravenous administration of 200 mg/kg ALA. Data shown is the mean from 10-12 blocks (50x50 pixels) from 3 biopsies at each time point.
Figure 9.7: Plot of AIS₂Pc fluorescence in the liver and prostate against time after intravenous administration of 1mg/kg AIS₂Pc. Data shown is the mean from 10-12 blocks (50x50 pixels) from 3 biopsies at each time point.

9.4.3 Photodynamic Effects

9.4.3.1 Macroscopic findings

No lesion was seen in control animals receiving 100 or 360J at 100mW, but a large lesion (10x9x9 mm) was seen in the control animal receiving 1080J at 300mW (Figure 9.8). With ALA, only tiny haemorrhagic lesions 1-2 mm in diameter were found 3 days after PDT with the laser power at 100mW (360J) but a much larger lesion with central cavitation (9x9x9 mm) was created using 300 mW (1080J) 8 hours after ALA (Figure 9.9). There were no visible lesions at 7 and 28 days at a light dose of 100 J (Figure 9.10). In contrast, PDT with AIS₂Pc produced lesions up to 12mm in diameter with laser power and exposure times that had no effect in unsensitised animals. The 3 day lesion was a well circumscribed haemorrhagic necrotic area without cavitation, measuring 12x10x10 mm (Figure 9.11). The structures overlying the prostate were intact although the lesion did not quite reach the capsule. Lesions at 7 days were slightly smaller (10x9x9 and 9x9x8 mm) and less haemorrhagic than those seen at 3 days (Figure 9.12). Four weeks after PDT, wedge shaped fibrotic scars measuring 10x8x8 and 6x5x5 mm were formed beneath the prostate capsule (Figure 9.13), but no evidence of deformity of the gland as a whole was seen.
Figure 9.8: Control prostate at 300mW, 1080J but without ALA showing a lesion as large as 10 mm in diameter.

Figure 9.9: Prostate lesions 3 days after PDT with ALA (100mg/kg). Large arrow: 300mW, 1080J; Small arrow: 100mw, 360J.
Figure 9.10: Prostate section 7 days after PDT with ALA (100mg/kg). No visible lesion was found by serial sectioning the prostate at a thickness of 3mm.

Figure 9.11: Prostate lesion 3 days after PDT with AlS2Pc at a light dose of 100J (100mW for 1000sec) delivered interstitially through a single fibre.
**Figure 9.12:** Prostate lesion near urethra 7 days after PDT with AIS₂Pc at a light dose of 100J (100mW for 1000sec) delivered interstitially through a single fibre.

**Figure 9.13:** Subcapsular prostate lesion 28 days after PDT with AIS₂Pc at a light dose of 100J (100mW for 1000sec) delivered interstitially through a single fibre.
9.4.3.2 Histology

The control prostates treated at lower laser power (100mW) had no detectable lesions, macroscopically or microscopically. However, at the higher power of 300mW, peripheral coagulation necrosis and central cavitation were found around the fibre site 3 days after treatment, typical of a thermal effect (Figure 9.8). The tiny lesions seen after PDT with ALA at low laser power showed haemorrhagic necrosis, but the larger lesion seen after treatment at 300mW (Figure 9.9) had microscopic features similar to those of the control animal treated with 300mW, strongly suggesting that it was produced by a thermal and not a PDT effect. In contrast, the lesion seen 3 days after PDT with AlS2Pc showed extensive haemorrhagic necrosis of the glandular epithelium with relative sparing of interlobular collagenous fibrils. The demarcation between the necrosed zone and normal glandular areas was sharply defined (Figure 9.14).

Figure 9.14: Haemorrhagic necrosis in the prostate 3 days after PDT with AlS2Pc. There is a clear demarcation between the necrosed and undamaged areas (X40, H&E stain). The fibre was placed beneath the capsule and the lesion did not extend as far as the urethra.
By 7 days there was a marked infiltration of inflammatory cells. The glands were still atrophic at 28 days. There was damage to acinar collagen, but the collagen and other connective tissues in the interlobular stroma were well preserved throughout the follow up period (Figure 9.15). The lesion studied at 3 days did not include the urethra, but one of the lesions at 7 days showed suburethral oedema, congestion, fibroblast infiltration and early urethral re-epithelialisation without definite haemorrhage necrosis (Figure 9.16). By day 28, the epithelial lining of the prostatic urethra appeared normal in the treated area but the periurethral tissue showed evidence of inflammatory cell and fibroblast infiltration consistent with safe healing in this area.

Figure 9.15: Prostate 28 days after PDT with AlS2Pc showing atrophic glands with preservation of collagen fibrils in the stroma (X60, HVG stain).
Figure 9.16: Microscopic features of periurethral tissue response to PDT effect. The urethral epithelium has negligible destruction while the suburothelial tissue demonstrates oedema and blood congestion (X40, H&E stain).

9.4.3.3 Perioperative responses

No animal had any adverse effects associated with the laser procedures during this study. One animal developed a urinary tract infection with mild haematuria 5 days after prostate biopsy which was successfully treated with 2.5% Enrofloxacin (0.2 ml/kg) for 3 days, but no others had any macroscopic haematuria, difficulty in urination or sign of any general physical distress.
9.5 DISCUSSION

9.5.1 Light dosimetry for ALA based PDT on the prostate

The results of ALA mediated PDT in the canine prostate are disappointing. The only lesion more than 1-2mm in diameter was the one produced with the high laser power of 300mW, and an essentially identical lesion was seen in a control animal treated with the same laser power and treatment time but without ALA, strongly suggesting that the lesion in the sensitised animal was a thermal rather than a PDT effect. This was confirmed by the histological findings. Johnson et al (1995) recently reported haemorrhagic necrosis up to 10mm in depth in a normal canine prostate one week after PDT (8 hours after 100mg/kg ALA given intravenously) using a 2cm diffuser fibre transurethrally with a high light dose (650 mW for 45 min, 1755J). No lesion was seen in an unsensitised animal treated with the same light dose. These findings are inconsistent with the present results. They used a different light delivery system (diffuser transurethrally vs. bare fibre interstitially), with a higher laser power and longer sensitisation time (8 vs 3 hours), but the striking difference which cannot be explained by these factors is that they saw no lesion in their control animal whereas I did in the one treated at 300mW, even though I only used an energy of 1080J compared with their 1755J. Further experiments are required to resolve this discrepancy.

9.5.2 PDT effect of ALA-PpIX on the prostate

The depth of PDT effect may relate to the dose and route of administration of ALA as well as to the organ being treated. The maximum dose than can be tolerated clinically is 60mg/kg orally (equivalent to 30mg/kg intravenously) and with this it only appears possible to get necrosis up to 1-2mm in depth in gastrointestinal tumours (Regula et al, 1995). In contrast, lesions up to 8mm deep (comparable to the depth of effect reported by Johnson et al (1995) have been reported using 200mg/kg intravenously in papillomas in rabbits (Lofgren et al, 1995) and 400mg/kg orally (equivalent to 200mg/kg intravenously) in cancers transplanted into the hamster pancreas (Regula et al, 1994). Giving ALA intravesically to rats (200mg/kg), urothelial levels of PpIX are comparable to those seen in the prostate in the present work, and with appropriate light doses, can destroy the urothelium without damaging the underlying muscle (Chapter 5). However, the thickness of the rat bladder wall is less than 1mm, so the
absolute depth of effect is no greater than I found in the prostate. ALA looks promising for treating superficial lesions with PDT, but more questionable when a greater depth of effect is required as in the management of prostate cancer. From my results, PDT with ALA is not likely to be of value for treating the prostate unless ways are found to markedly increase the depth of necrosis produced. Just increasing the light dose seems unlikely to do this, although there could be some benefit from fractionating the light (Messmann et al, 1995).

9.5.3 AlS2Pc based PDT on the prostate

The situation with AlS2Pc is much more promising. The lesions produced in the prostate were well circumscribed and up to 12x10x10 mm in size. They healed without disruption of the basic connective tissue architecture of the organ as has been shown in other organs (Barr et al, 1987). This is of particular importance for treating prostate cancer since preservation of anatomical alignment between the bladder neck and external urethral sphincter is the best way to avoid the major complications that may arise from radical surgery. It is also likely to make it safe to treat large areas of the gland which is important as the disease is often multifocal and often occurs in the peripheral zone close to surrounding vital structures such as the neurovascular bundle, rectum and urethral sphincter. The prostate capsule seems unlikely to be significantly affected by PDT, but if PDT effects do extend to the rectum or major blood vessels, using AlS2Pc, it is well documented that the resultant lesions heal without any unacceptable effects on their structure or function (Barr et al, 1987; Grant et al, 1994). There are no good experimental studies on the effect of PDT using AlS2Pc (or indeed any other photosensitiser) on peripheral nerves, although fluorescence microscopy data from my colleague have shown that very little AlS2Pc is taken up in nerve bundles (unpublished data). It is one of the greatest attractions of PDT that it is possible to treat not only the target organ, but also adjacent normal tissues in the knowledge that if lesions are produced in these other tissues, they are likely to heal by regeneration without serious sequelae. Thus it should be feasible to treat the entire prostate and a margin of surrounding tissues safely. The one tissue that may be at risk is the sphincteric complex. Muscle heals better after PDT than after thermal injury, but there may be significant impairment of function (Pope & Bown 1991). No studies have yet looked at the effect of PDT on sphincter function, but it should normally be possible to treat the entire prostate without significant light doses reaching the sphincter region if the light can be properly applied interstitially or through a well designed transurethral balloon catheter. The results of my study suggest that although
the prostatic urethra may be damaged, it heals satisfactorily without stenosis at any
time after treatment, although to be sure of this it would be necessary to undertake
further experiments producing larger PDT lesions in the immediate vicinity of the
urethra. Another concern is urinary retention due to oedema which is likely if a large
volume of tissue is destroyed around the urethra. This may cause problems in
experimental animals, but is unlikely to pose a particular hazard in clinical practice as
it can be managed by temporary catheterization.

9.5.4 Tissue selectivity and PDT

PDT initially attracted so much interest because of selectivity of uptake of
photosensitisers between malignant tumours and the adjacent normal tissue in which
the tumour arose. This aspect, however, has been considerably over emphasised as it
is difficult to turn selectivity of uptake of a photosensitiser (typically in the range of
2-3:1) into selective tumour necrosis when both are exposed to the same light dose, as
will inevitably be the case when treating the region where tumour meets normal
tissue. Nevertheless, it is logical to choose the time interval between
photosensitisation and light delivery to be that at which the ratio of photosensitiser
between tumour and normal tissue is the greatest. There are no data on this for the
prostate, but in a rat colon cancer model, the best ratio (2:1) was found 48 hours after
giving AlSPc (a mixture of mono-, di-, tri- and tetrasulphonated derivatives),
although there was no great difference between 24 and 48 hours (Tralau et al, 1987a).
In the present experiments, the tissue level of AIS2Pc in the prostate was roughly
constant between 5 and 48 hours after photosensitisation, so it was decided to use the
middle of this range at 24 hours for the PDT study. Peak levels of AIS2Pc at these
times contrast with the results of Chatlani et al (1991) who reported a maximum level
of AIS2Pc in the mucosa of normal rat colon 1 hour after administration. Similarly,
Pope et al (1991) found highest levels of AlSPc (not AIS2Pc) in all layers of the
normal rat bladder one hour after photosensitisation, but by 24 hours there was more
in the mucosa than in the underlying muscle, although the absolute levels were lower
in both layers than at one hour. With different biological structures in various species,

despite the lack of a peak effect, further study is needed to look at the distribution of AIS2Pc in patients with prostate
cancer to define the relative concentration of photosensitiser in normal and malignant
tissues, but it is most unlikely that there will be less in the tumour than in the normal.
The present study did not look at skin photosensitivity, but one of the greatest
potential advantages of AIS2Pc over Photofrin is the likely lack of serious skin
photosensitivity (Tralau et al, 1989).
9.5.5 **Prostate PDT. Which photosensitiser?**

The selection of an appropriate photosensitiser for PDT is as important as selecting a suitable option for prostate cancer treatment. Photodynamic therapy of the prostate, although its future position for prostate cancer treatment remains to be defined, there is beyond question that PDT is far less morbid to the patient than a radical operation. Not infrequently we find patients who are too old or medically unfit to justify a major surgical procedure for curing a cancer, we then place the patient on the list for radiotherapy, either external beam or interstitial radioactive seeds implantation. The current project is a step to find a new weapon which is of reasonable effectiveness against prostate cancer, but not to challenge the value of radiotherapy. The two therapeutic options are complementary to each other. So is the choose of photosensitiser for prostate PDT. If the primary tumour is huge, it is imperative to remove as much malignant tissue as possible, then mTHPC is the ideal photosensitiser. However, for smaller lesions (as after TUR, or local recurrences radical prostatectomy) AlS2Pc might be more appropriate.
9.6 CONCLUSIONS

The biodistribution study of PpIX revealed that the peak prostate level was achieved 3 hours after ALA sensitisation. PpIX intensity in the liver, on the other hand, peaked at 5 hours, and resumed a nearly baseline level at 24 hours. Although prostatic AlSzPc reached its peak at 24 hours, the fluorescence levels were similar in intensity in the time between 5 and 48 hours. Hepatic AlSzPc despite having a peak at 24 hours showed a different pattern of pharmacokinetic action from that of the prostate. PDT with ALA does not look promising in the management of prostate disease as the largest lesion was only 1-2mm in size, but using AlSzPc, it is possible to necrose zones up to 12mm in diameter around each treatment site with safe healing. For interstitial PDT of the prostate, the laser power at the fibre tip should be kept at or below 100mW as thermal effects are likely to happen if the power is at 300mW. It would now appear justified to consider pilot clinical studies in carefully selected patients with small cancers localised to the prostate for whom there are no other treatment options available.
SECTION C: CONCLUSIONS AND FUTURE PROSPECTS

CHAPTER 10: Summary and Future Prospects of PDT of the Bladder and Prostate
CHAPTER 10

SUMMARY AND FUTURE PROSPECTS OF PDT OF THE BLADDER AND PROSTATE

10.1 Summary of Bladder PDT ................................. 272
10.2 Summary of Prostate PDT ............................... 274
10.3 Future Prospects for PDT in Urology ................. 276

10.3.1 Bladder cancer
   10.3.1.1 For prophylaxis of recurrence .................. 276
   10.3.1.2 For treatment of carcinoma in situ ............ 277
   10.3.1.3 For cancer detection ............................ 278

10.3.2 Prostate cancer ..................................... 278
   10.3.2.1 For primary localised disease ................. 279
   10.3.2.2 For cancer recurrence after irradiation .... 279
   10.3.2.3 For cancer recurrence after radical surgery .. 280

10.3.3 Other urological disorders .......................... 280

271
10.1 SUMMARY OF BLADDER PDT

In the first three chapters of this thesis the purpose was to investigate the biology of the urinary bladder after photodynamic therapy. I examined the possibility of intravesical instillation as a route of photosensitiser administration by delivering an endogenous substance, 5-aminolaevulinic acid (ALA) into the bladder, and studied the tissue biodistribution of its photoactive derivative, protoporphyrin IX (PpIX) and the photodynamic effects on the bladder and skin after light illumination at 630nm. The main conclusions from my experiments are:

(a) Without buffering to a less acidic condition, ALA solution is inappropriate for bladder instillation.

(b) Titration of ALA solution to pH 5.5 is suitable for bladder instillation without jeopardising its chemical stability.

(c) Selective retention of PpIX in the urothelium is possible if the ALA solution is retained in the bladder for at least 3 hours.

(d) The PpIX intensity ratio between the urothelium and muscle layer is in the range of 5-11:1, and the urothelium to lamina propria ratio is 7-9:1 which can be achieved if the concentration of instilled ALA is between 1 and 10%.

(e) In the range of 1-10% ALA, lower concentrations usually result in higher PpIX intensity ratios between the urothelium and the underlying layers.

(f) 10% intralipid is better than 1% as a light scattering agent in the rat urinary bladder.

(g) In this animal model where the bladder is small (volume: 0.3ml, surface area ≈ 2 cm²), a light dose of 50J seems adequate to achieve whole bladder illumination with generalised urothelium sloughing.

(h) 10% ALA is the optimum concentration in this study to induce homogeneous urothelial destruction with negligible muscle damage if the light dosimetry is well controlled.

(i) Regeneration of urothelium is evident 7 days after PDT, however, generalised and complete healing is achieved by 2 weeks.

(j) Liver and renal porphyrins are usually higher after oral intake in comparison to instillation.

(k) At the same ALA dose, oral intake leads to higher PpIX buildup in the urothelium, but the PpIX ratio between the urothelium and muscle layer is about the same as that with intravesical instillation.
(l) Urothelial PpIX is of a similar level at 3 hours after 100mg/kg oral ALA and at 5 hours after instillation of 200mg/kg ALA.

(m) PpIX intensity in the dermis and epidermis is generally 2-3 times higher after oral than after intravesical instillation for the same levels in the urothelium.

(n) As a result of higher PpIX levels, skin photosensitivity is more prominent with the oral route of administration under conditions where the urothelial effect is similar.

(o) Apart from the likelihood of direct cytotoxic effects with instillation of ALA, an indirect vascular mediated PDT effect is suspected if ALA is given orally (systemically).

(p) With concomitant administration of 10% ALA and the iron chelator CP94, the urothelial buildup of PpIX as well as its ratio to the muscularis propria is enhanced in Wistar rats.

(q) The addition of CP94 in the course of bladder PDT with ALA may stimulate unknown biochemical effects which are advantageous to cell killing. However, the mechanism for such effects remain undetermined.

(r) With 1% ALA and CP94, the degree of urothelial sloughing is approaching that achieved by 10% ALA alone.

(s) Light fractionation with a break of 5 minutes in the middle can induce a 4-fold increase in tissue damage.

(t) Light fractionation may be a convenient approach to reduce the total illumination time needed for whole bladder photodynamic therapy in patients.
10.2 SUMMARY OF PROSTATE PDT

In the second part of my thesis I looked at the possibility of producing PDT lesions in the canine prostate using 3 different photosensitisers; ALA, AlS2Pc, and mTHPC. These studies are essential prior to application of PDT as a modality for treating prostate cancer in men, whether primary or recurrent. Besides evaluating the nature of PDT induced prostate lesions, I also studied the effect of PDT on the adjacent structures surrounding the prostate. The conclusions are summarised below:

(a) The optimal sensitisation time for mTHPC in the normal prostate is 24-72 hours. However it may differ in cancer bearing tissue and this requires further investigation.

(b) PDT is feasible for producing haemorrhagic necrotic lesions which are confined to the prostate. The size of lesion varies with different drugs and light dosimetry.

(c) PDT lesions as large as 25x20x20 mm in size can be produced if mTHPC is given at a dose of 0.3mg/kg followed by interstitial light illumination at a dose of 100J (100mW for 1000sec) with a single bare fibre.

(d) The lesion produced by PDT has a sharp demarcation between the area necrosed and the surrounding viable tissues. No grey zone (coagulation necrosis) is seen in between the two.

(e) Treatment at 2 interstitial sites is well tolerated by the experimental animals. However, urinary retention lasting up to 1 week is a frequent finding if more areas are treated simultaneously, or if the light is delivered transurethrally.

(f) The urothelium of the prostatic urethra heals rapidly and is well regenerated by 4 weeks after PDT.

(g) The prostate glandular tissue remains atrophic when followed up at 90 days after PDT. The intra-acinar collagenous fibrils are gone whereas the interlobular collagen is well preserved which accounts for the maintenance of prostate architecture even after extensive glandular damage.

(h) Rectal injury was only seen in dogs treated with transurethral PDT, probably due to the close proximity of the rectal wall to the apex of the prostate.

(i) Despite rectal mucosal ulceration up to 10mm in diameter, the experimental animals did not show any evidence of rectal perforation or fistula formation in the long-term follow up, nor any abnormality of bowel function.

(j) With preservation of collagen fibres in the rectal wall after PDT, regeneration of rectal mucosa was complete by 4 weeks.
(k) Urinary incontinence was not observed in any of the 6 beagles treated with transurethral PDT.

(l) The optimal sensitisation time for AlS2Pc (1mg/kg) and ALA (200mg/kg) in the canine prostate is 5-48 hours and 3-5 hours, respectively.

(m) AlS2Pc induced lesions 3 days after PDT were up to 12x10x10mm in size around a single fibre and was similar to those seen using mTHPC.

(n) ALA induced lesions 3 days after PDT were no more than 2mm in diameter and seem to be of limited usefulness for prostate cancer treatment.

(o) mTHPC based PDT is more appropriate for debulking or treating larger prostate tumours whereas AlS2Pc is suitable for lesions of smaller size and located in the peripheral area of the prostate.
10.3 FUTURE PROSPECT OF PDT IN UROLOGY

PDT has been shown to hold promise for treating early cancers of the aerodigestive tract. Recently, the most studied photosensitiser, dihaematoporphyrin ester/ether (DHE) has officially been approved by the FDA for the prophylaxis of bladder cancer and for treatment of advanced oesophageal cancer. The Japanese government also granted its approval for the treatment of early gastric and lung cancers. From these developments, it seems that PDT is gaining public acceptance and has been officially recognised as a clinical rather than an experimental option for cancer treatment. This has turned PDT from a technique of interest to enthusiasts into a real weapon and has greatly promoted the likelihood of further clinical applications.

With the development of so called second generation photosensitisers, such as mTHPC, SnET2, NPe6, AlSPc, and ALA, which are mostly more potent than DHE (with the exception of ALA) in terms of their singlet oxygen extinction coefficient, and have a shorter duration of skin photosensitivity, clinicians are expected to face a dilemma of how to choose the most appropriate photosensitiser for their clinical uses. The great versatility of PDT for future oncological development lies in its close relations not only with the pharmacology of photosensitisers but also with laser light delivery, and to a lesser extent, with imaging and endoscopic advances. Simply by changing the route of administration, one may have ended up with a significantly different treatment effect as seen in chapter 6. Furthermore, by shifting light the wavelength from 630 to 635nm during ALA based PDT, an increase of 35% in the therapeutic effect has been reported. Fractionation of light illumination with a short break (dark period) causes a 4-fold increase in tissue damage. Other modifications of the therapeutic parameters, may also bring about new dimensions with an impact on outcome. Encouraged by the rapid advances in medical optics and molecular genetic technology, it is not over optimistic to foresee a bright future for PDT.

10.3.1 Bladder cancer

10.3.1.1 For prophylaxis of recurrence

With the approval of DHE for clinical prophylaxis of bladder cancer recurrence by both the Canadian Government, it is for sure that more experience and a wider understanding of bladder tissue biology to DHE based PDT will be accumulated in the foreseeable future. However, not too many urologists would like to pioneer this
clinical trial again, particularly with the nightmare that remains about muscle damage. Most in vivo studies have demonstrated that the preferential tissue retention of porphyrins (HpD or DHE) between tumour and the normal tissue in which they arose is usually in the range of 2-3 to 1. At such a narrow window, selective destruction of cancer is very difficult to achieve clinically. Currently, the knowledge as to what is the optimal drug and light dose to effect a prevention of papillary tumour recurrence remains unavailable, and there is little evidence that one can get a urothelial effect without muscle damage.

ALA has the advantages of being cleared quickly (24-48 hours) from the human body, and having a superficial tissue ablating effect, it is possible that ALA might be one of the drugs of choice for prevention of tumour recurrence. The objective is to eradicate the bladder urothelium (whether diseased or not) completely with PDT and to expect regeneration of normal urothelium from the prostatic urethra. It may be appropriate to start with patients who have had multiple low to mid grade bladder tumours, and have had at least one or two recurrences in the past 12 months. A prospective randomised study comparing the rate of recurrence between intravesical chemotherapy and ALA-PDT mediated prophylaxis is needed. If it proves to be effective and without major side effects, ALA-PDT could be extended to cover the whole spectrum for prevention of recurrence of papillary tumours.

To achieve this generalised urothelial ablating effect, I would suggest instilling weakly acidic ALA solution (pH 5.5) at a concentration of 1-10% for 3-5 hours followed by light illumination at a light dose of 10-20 J/cm². The key point to reduce side effects is to keep the bladder marginally inflated to a volume which is sufficient to unfold the epithelial lining (possibly 100-150ml) and to optimise light distribution by combining the use of a bulb tip laser diffuser, 1-5% intralipid, and ultrasonographic guided fibre positioning (both suprapubic and transrectal). A second or third treatment may be a reasonable approach to secure long-term prevention of recurrence.

10.3.1.2 For carcinoma in situ

Currently, one course of bladder instillation therapy with BCG is highly effective in treating carcinoma in situ of the bladder (section 1.3.3, Chapter 1). ALA-PDT, by removing all the urothelium, rather than only the dysplastic cells, might also be of value. If the dysplastic cells accumulate more PpIX than the surrounding normal
urothelium, it is a bonus as they are destined to be destroyed with other epithelia. The initial step to evaluate its efficacy can be accomplished by looking at its effects on those with resistant carcinoma in situ who failed BCG therapy. If it proves to be effective, a second stage prospective clinical trial aiming at comparing the therapeutic effectiveness of BCG therapy and ALA-PDT may be undertaken. For ALA-PDT to be a standard procedure, it should be at least as effective as BCG therapy, have fewer side effects, and be more convenient to the patient.

The procedure for carcinoma in situ treatment is virtually the same as that for prophylaxis of bladder cancer. However, in terms of the malignant potential of carcinoma in situ, it would be advisable to apply 2 sessions of PDT treatment (one month apart) as a standard protocol for comparison with BCG intravesical therapy which takes a 6 week course to finish.

10.3.1.3 For cancer detection

It is very likely that photodynamic diagnosis (PDD) will become a popular procedure for detection of bladder cancer or precancerous lesions (Section 4.1.6.3, Chapter 4). ALA based fluorescence diagnosis of bladder cancer has achieved a sensitivity as high as 97% and with a modest specificity of 70% (Kriegmair et al, 1996). Usual causes for false positive detection include inflammation, hyperplasia and trauma to the urothelium. Based on these encouraging results, particularly the very high sensitivity, it may be possible that in the future urologists will give up random biopsies (currently the standard procedure for bladder cancer diagnostic workup) and take biopsies only from areas where abnormal fluorescence is detected.

10.3.2 Prostate cancer

PDT of prostate cancer is a new approach which has been tried only in 2 cases (Windahl et al, 1990). However, with the accumulation of knowledge on light scattering and photosensitiser distribution in the prostate and tissue responses after PDT over the last few years (Section 4.2, Chapter 4, and Chapter 8,9), the interest in PDT for prostate cancer has started to emerge.

The discovery of more and more clinically silent prostate cancers by serum PSA measurement has changed the scenario of traditional treatment strategy. As a certain percentage of these patients may die with, rather than die of prostate cancer (lead time bias), treatment has posed a particular medicosocial problem. Aggressive treatment
with radical prostatectomy or definitive irradiation will result in increased medical expenses and negatively impact quality of life. In the absence of effective biological or molecular indicators to predict tumour progression potential, it is definitely necessary to find other options which are more friendly to those patients having localised prostate cancer at the time of diagnosis. PDT is potentially a modality to achieve this goal by necrosing glandular tissues (normal and malignant) up to the periphery of the prostate without causing catastrophic effects to the surrounding structures.

10.3.2.1 For primary localised disease
The aim of treatment is curative for T1 and T2 disease. Careful clinical trials will be required to optimise treatment, but a possible approach would be as follows: After sensitisation with a powerful photosensitiser (mTHPC, SnET2, or NPe6), the initial PDT treatment is undertaken by illuminating the prostate fossa (which is purposefully made or has been created by a previous TUR) with a diffuser fibre (with or without an intracavitary balloon). The purpose of this procedure is to achieve debulking of most of the glandular prostate which has been left behind after TUR. Perhaps a month or so later, a secondary interstitial treatment which would involve percutaneous insertion of a few laser fibres under the guidance of transrectal ultrasound to the peripheral zone suspicious of cancer. The aim would be to necrose those foci that lie immediately adjacent to the capsule. A milder photosensitiser such as AlSPc or benzoporphyrin derivative may be employed for this adjuvant therapy. It is theoretically possible to completely destroy the whole prostate gland with careful light dosimetry. Serum PSA and transrectal ultrasound guided biopsy could be used to assess the results with repeat PDT for persistent or recurrent disease. If PDT treatment can result in a long-term disease-specific survival rate comparable to that achieved by radical operation, the current therapeutic algorithm for prostate cancer could be changed significantly and the contribution to patient life and quality of life will be considerable.

10.3.2.2 For cancer recurrence after irradiation
Not infrequently we found patients whose PSA became elevating from a previous nadir level and biopsy proved to have recurrence after radical radiotherapy. For management of these patients, salvage prostatectomy or hormonal deprivation is the only resort. PDT is potentially helpful for these patients in terms of control of local disease, however, the impact on prolonging survival remains to be investigated.
Based on the results of this thesis, one patient in this category has been treated at the Middlesex Hospital. This clinical trial has been approved by the ethical committee of the UCL and is conducted by the collaboration of National Medical Laser Centre (Dr S-C Chang, Prof SG Bown), Department of Radiology (Prof WR Lees), Department of Oncology (Dr GM Duchesne), and The Institute of Urology (Prof AR Mundy). The technique involved sensitising the patient with 0.15mg/kg mTHPC 3 days prior to transperineal insertion of 2 laser fibres for light illumination. Good tumour necrosis was on contrast enhanced CT 4 days after treatment.

10.3.2.3 For cancer recurrence after radical surgery

The application of PDT for local tumour persistence or recurrence after radical prostatectomy is more or less similar to that after irradiation except that the former is technically more challenging. The change of architecture in the lower urinary tract and the effect of healing on the prostatic area after surgery has made followup imaging, particularly the interpretation of echotexture on the transrectal ultrasound more difficult. However, in the hands of an experienced ultrasonographer and with the aid of tissue biopsy, localisation of recurrent foci and PDT treatment is possible. The impact on survival with PDT treatment of local recurrence remains to be evaluated.

10.3.3 Other urological disorders

Since PDT is a technique developed mainly for the management of cancer, until recently its application for benign diseases has been rarely discussed. The principal side effect of PDT is prolonged skin photosensitisation which in some cases using HpD may be as long as 6-12 weeks. Rarely will any patient with a benign disease be willing to receive PDT for treatment and then endure an indoor life for 6-12 weeks, unless the therapeutic effect is overwhelming. No such condition is currently definable justifying the use of PDT for benign urological disorders, although if the period of skin sensitivity is only 1-2 days, as with ALA, the situation is quite different. Transurethral PDT treatment of BPH with SnET2 has been suggested by Selman & Keck (1994). However, it is my personal opinion that PDT is not at all suitable for that disease. Quite apart from the problems of photosensitivity, as PDT leaves the interlobular collagen unaffected, it is unlikely to achieve the main objective of treating BPH which is to reduce the bulk of the gland and so improve urinary flow. Considering a success rate as high as 85% can be achieved with TUR prostate with
negligible side effect, one has to justify carefully the usefulness of such an investigational option for benign diseases.

Interstitial cystitis, particularly those who have the most severe intractable pain, and are subject to cystectomy and augmentation cystoplasty as the final resort can be suitable candidates for ALA-PDT of the bladder before undertaking surgery. The symptomatology of interstitial cystitis is largely derived from the epithelial lining overlying the diseased submucosal fibrosis although the actual aetiology remains undetermined. Complete removal of the urothelium with PDT might provide the patient with a chance for symptom relief but is not likely to achieve a cure as the nature of disease is usually pancystitic.
APPENDIX

Figure. Fluorescence intensity of the urothelium and muscularis propria after instillation of ALA and ALA ester at 200mg/kg
Figure: Fluorescence intensity of the urothelium after ALA and ALA ester bladder instillation
Figure: Fluorescence intensity of the muscularis propria after ALA and ALA ester bladder instillation
Figure: Fluorescence intensity of the lamina propria after ALA and ALA ester bladder instillation
Figure: Fluorescence intensity of epidermis of back skin after ALA and ALA ester bladder instillation
Figure: Fluorescence intensity of dermis of back skin after ALA and ALA ester bladder instillation
Figure: Fluorescence intensity of the urothelium after BI ALA with CP94 delivered via different routes
Figure: Fluorescence intensity of the muscularis propria after BI ALA with CP94 delivered via different routes
Figure: Fluorescence intensity of the lamina propria after BI ALA with CP94 delivered via different routes
(A): CP94 IP at the time of BI ALA (200mg/kg)
(B): CP94 IP 2 hour prior to BI ALA (200mg/kg)

Figure: Fluorescence intensity of the urothelium and muscle after instillation of ALA with CP94 intraperitoneal injection
Figure: Fluorescence intensity of the urothelium and muscle after bladder instillation of ALA and oral CP94
REFERENCES


Al Refaie FN, Wonke B, Hoffbrand AV, Wickens DG, Nortey P, Kontoghiorghes GJ. Efficacy and possible adverse effects of the oral iron chelator 1,2-dimethyl-3-hydroxypyridin-4-one (L1) in thalassemia major. Blood 1992; 80: 593-599.


Catalona WJ, Smith DS, Ratliff TL, Basler JW. Detection of organ-confined prostate cancer is increased through PSA-based screening. JAMA 1993; 270: 948-954.


Epstein JI, Walsh PC, Carmichael M, Brendler CB. Pathologic and clinical findings to predict tumor extent of nonpalpable (stage T1c) prostate cancer. *JAMA* 1994; 271: 368-374.


Fitzpatrick JM, West AB, Butler MR, Lane V, O'Flynn JD. Superficial bladder tumors (stage pTa, grades 1 and 2): the importance of recurrence pattern following initial resection. *J Urol* 1986; 135: 920-922.


Jordan AM, Weingarten J, Murphy WM. Transitional cell neoplasms of the urinary bladder: can biologic potential be predicted from histologic grading? *Cancer* 1987; 60: 2766-2774.


Kontoghiorghes GJ, Bartlett AN, Hoffbrand AV, Goddard JG, Sheppard L, Barr J, Nortey P. Long-term trial with the oral iron chelator 1,2-dimethyl-3-hydroxypyridin-4-one(L1). *Br J Haematol* 1990; 76: 295-300.


Parsons JT, Million RR. The role of radiation therapy alone or as an adjunct to surgery in bladder carcinoma. Semin Oncol 1990; 17: 566-582.


Perez CA, Pilepich MV, Garcia D, Simpson JR, Zivnuska F, Hederman MA. Definitive radiation therapy in carcinoma of the prostate localized to the pelvis: experience at the Mallinckrodt Institute of Radiology. In: Gregoric FI, McCarthy A (eds), Consensus Development


Quilty PM, Duncan W. Treatment of superficial (T1) tumours of the bladder by radical radiotherapy. *Br J Urol* 1986; 58: 147-152.


Scardino PT. Is radiotherapy effective for locally advanced (stage C or T3) prostate cancer? *Prog Clin Biol Res* 1989b; 303: 223-239.


Sternberg JJ, Bracken RB, Handel PB, Johnson DE. Combination chemotherapy (CISCA) for advanced urinary tract carcinoma. *JAMA* 1977; 238: 2282-2287.


LIST OF PUBLICATIONS

Publications:


Presentations:


6. The effect of 1,2-diethyl-3-hydroxypyridin-4-one on tissue build up of protoporphyrin IX: A microscopic quantitative fluorescence study on rat urinary bladder. 5th Biennial Meeting of the International Photodynamic Association (IPA), Florida, September 1994.


10. Interstitial photodynamic therapy of canine prostate with meso-tetra-(m-hydroxyphenyl) choline and 5-aminolaevulinic acid: a preliminary study. European Biomedical Optics Week, BiOS Europe '95, Barcelona, September 1995.


12. Interstitial PDT on the canine prostate with 5-aminolaevulinic acid, meso-
tetra-(m-hydroxyphenyl)chlorin and sulfonated phthalocyanine. 6th Biennial Meeting of the International Photodynamic Association (IPA), Melbourne, March 1996.


15. Photoirradiation of rat urinary bladder: Comparison between oral and intravesical instillation 5-aminolaevulinic acid induced protoporphyrin IX tissue biodistribution and photodynamic effects. 91st Annual Meeting of the American Urological Association (AUA), Florida, May 1996.

Biodistribution of Protoporphyrin IX in Rat Urinary Bladder after Intravesical Instillation of 5-Aminolevulinic Acid
BIODISTRIBUTION OF PROTOPORPHYRIN IX IN RAT URINARY BLADDER AFTER INTRAVESICAL INSTILLATION OF 5-AMINOLEVULINIC ACID

SHI-CHUNG CHANG,* ALEXANDER J. MACROBERT AND S. G. BOWN

From the National Medical Laser Centre, University College London Medical School, England, and Tzu-Chi General Hospital, Hua-Lien, Taiwan

ABSTRACT

Purpose: Photodynamic therapy (PDT) has considerable potential for the treatment of superficial bladder neoplasia. Problems of detrusor muscle scarring and prolonged cutaneous photosensitivity with current photosensitizers may be reduced by using the new photosensitizer precursor, 5-aminolevulinic acid (ALA). We studied the fluorescence distribution of protoporphyrin IX (PpIX, the active derivative of ALA) in rat urinary bladder after intravesical administration of ALA as the first step to undertaking PDT using locally administered ALA.

Materials and Methods: Solutions of varying concentrations of ALA and pH were given intravesically to Wistar rats. The bladder was removed 1 to 24 hours later for measurement of the tissue levels of PpIX by fluorescence microscopy in the layers of the bladder wall.

Results: The stability of the ALA solution steadily decreased as the pH was increased from 2.1 to 7. The best value was pH 5.5, at which the stability of the solution and the urothelial tolerance were both acceptable. The maximum PpIX fluorescence intensity ratio between urothelium and the underlying muscle layer was 11 to 1. This was seen 5 hours after instillation of 1% ALA solution at pH 5.5. A 10% ALA solution gave higher levels of PpIX in the urothelium, but less selectivity between layers.

Conclusions: Under appropriate conditions, intravesical instillation of ALA can be used to sensitize the bladder urothelium with a ratio between urothelial and muscle concentrations of PpIX of up to 11:1.

Key Words: photosensitizing agents, phototherapy, protoporphyrins, aminolevulinic acid

Photodynamic therapy (PDT) is a technique currently under assessment for its possible role in the selective destruction of malignant tumors and premalignant lesions.1,2 Much work on PDT over the last 20 years has used the photosensitizer hematoporphyrin derivative (HpD),3 but this is far from satisfactory. An ideal photosensitizer does not yet exist. 5-aminolevulinic acid (ALA) is one of the most interesting new photosensitizing agents currently under assessment. Promising results have been reported for its use in treating basal cell carcinomas of the skin4 and for some small neoplasms of hollow viscera.5-7 In living cells, ALA is synthesized from glycine and succinyl coenzyme A and then metabolized through a series of intermediates to protoporphyrin IX (PpIX), the predominant porphyrin responsible for photosensitization, prior to its final conversion to heme.8 As the conversion of PpIX to photoinactive heme is the rate-limiting step between ALA and heme, bypassing the physiological feedback mechanism that controls the synthesis of ALA with excess exogenous ALA results in temporary accumulation of PpIX. As heme-containing enzymes are essential for energy production, cells with a higher metabolic turnover are likely to synthesize more PpIX when excess ALA is given. Mucosal cells are more active metabolically than muscle and have been shown to accumulate 7 to 10 times as much PpIX as the underlying muscle in the stomach, colon and bladder after oral or intravenous administration of ALA in rats.9,10

In the management of carcinoma in situ of the bladder, the challenge is to achieve generalized destruction of the transitional epithelium without damaging the underlying muscle layer. Although systemic administration of ALA has been shown to give selective sensitization of the urothelium,10 this is also likely to cause skin sensitization, even if this only lasts for 24 hours.5 In the present study we investigated the feasibility of local administration of ALA by the intravesical route. This was the first stage of a program to assess the feasibility of producing selective urothelial damage by PDT without administering the photosensitizer systemically.

MATERIALS AND METHODS

Preparation of ALA solutions. Purified ALA powder (ALA HCl 98%, DUSA Pharmaceuticals, New York, New York) was dissolved in normal saline and titrated with saturated sodium bicarbonate solution to a final pH of 2.1, 4.0, 5.5, or 7, at a concentration of 10% (100 mg./ml.). The solutions were stored in the dark at 37°C and in dim room light at 25°C for 72 hours for assessment of chemical stability. Stability was assessed by the severity of color changes during the observation period.

Intravesical instillation of ALA. Solutions with pH 4 and 5.5 at concentrations of ALA of 10%, 1% and 0.1% were selected for the instillation studies using female Wistar rats weighing 100 to 180 g. Under general anesthesia with Hypnorm (fentanyl and fluanisone, Jansen Pharmaceuticals, Ltd., Grove, United Kingdom), transurethral catheterization was carried out with an 18.5 gauge Teflon cannula after a small episiotomy (2 to 3 mm.) was made in the vestibule to expose the urethral meatus. The ALA solution was administered intravesically at a volume of 0.2 to 0.32 ml. (0.2 cc/100 g. body

Accepted for publication June 6, 1995.

* Requests for reprints: National Medical Laser Centre, The Rayne Institute, University College London Medical School, 5 University St., London WC1E 6JF, United Kingdom.

Dr. Chang is funded by the Compassion Relief of Tzu-Chi Foundation in Taiwan. Professor Bown is supported by the Imperial Cancer Research Fund, United Kingdom.
weight) and retained in the bladder for 2 to 2.5 hours. After that, the rats were allowed to wake up and void freely. Throughout this paper, the times from instillation given are the times from the initial infusion of ALA into the bladder.

With the exception of the animals killed 1 hour after ALA administration, all animals had ALA solution in the bladder for at least 2 hours. The exact time for the solution in the bladder varied and depended on when the animal voided after it was allowed to recover from the general anesthesia. For each ALA solution of different concentration and pH, 2 to 5 rats were tested. The control group consisted of 2 rats receiving no treatment and 2 others with bladder instillation of 0.3 ml. normal saline.

**Fluorescence microscopy: In vivo studies.** The fluorescence emission spectra of ALA induced porphyrins were measured in vivo in 5 rats: 2 controls without photosensitization, 1 examined 2 hours after 200 mg./kg. ALA given intravenously and 2 examined 3 hours after intravesical administration of a 10% solution of ALA at pH 5.5. In vivo fluorescence spectroscopy was carried out with a Perkin-Elmer LS50B spectrofluorimeter (Perkin-Elmer, Middlesex, United Kingdom) with a bifurcated fiber-optic bundle probe. At laparotomy, the probe was placed 3 mm. above the exposed bladder, which had been rinsed once and then filled with normal saline. The emission spectrum was recorded from 600 to 750 nm. using an excitation wavelength of 410 nm.

**Studies on tissue sections.** Sensitized rats were killed and the urinary bladders removed 1, 2, 3, 4, 5, 7, 9, and 24 hours after ALA instillation. Before the bladder was removed, it was rinsed transurethrally 3 times with normal saline and then filled with 0.3 ml. OCT embedding medium (Tissue-Tek, Miles Inc., Elkhart, Indiana) to keep the lumen distended. The samples were kept in liquid nitrogen prior to sectioning (10 µm.) for fluorescence imaging.

For fluorescence imaging and quantification of PpIX levels in the bladder sections, an inverted phase contrast microscope (Olympus IMT-2, Olympus, Hamburg, Germany) attached to a high resolution (385 × 578 pixels) slow-scan charge coupled device (CCD) camera (model 1, Wright Instruments Ltd., Cambridge, United Kingdom) was used. The set-up of this highly sensitive photometric system has been described previously. In brief, an 8 mW helium neon laser (632.8 nm.) was used to excite the tissue PpIX and the emitted fluorescence was detected between 665 and 710 nm. (maximum response at 690 nm.) by using a combination of bandpass and longpass filters. The fluorescence signal was processed by an IBM PC clone into a falsely color-coded image depicting the signal in counts per pixel. The image processing software permitted the fluorescence intensity in each tissue layer to be quantified digitally by averaging over specified areas. After fluorescence imaging, the sections were fixed in formalin and stained with hematoxylin and eosin for histological study. The use of a slow-scan CCD system with a fixed internal gain together with constant power laser excitation permitted highly reproducible signal calibration. Satisfactory correlation has been found previously between microspectrophotometric measurements obtained with this system and chemical extraction measurements in normal rat stomach and colon. In the present studies the excitation fluence was limited to 0.5 J/cm. and no effects attributable to photodegradation were observed under these conditions.

**RESULTS**

**Stability of ALA solution.** Color changes of the freshly prepared 10% ALA solutions at different pH and at body and room temperature were used as a simple measure of the stability of the solution. At both 37 and 25°C, the solution turned light yellow a few seconds after titration to pH 7 with saturated sodium bicarbonate solution (pH = 8.06) and became light brown at 5 hours, so no attempt was made to use the preparation at this pH for animal experiments. At pH 5.5, the solution was relatively stable in color for the first 5 hours although it became brown after 24 to 48 hours whether stored at body or room temperature. This was considered acceptable if the solution could be used within a few hours of preparation. At pH 4, the solution remained colorless over the first 24 hours, staining to light yellow through the following 2 days. Only the untitrated ALA solution with a pH of 2.1 exhibited no color change during the 72 hour observation period. The preparations stored at 37°C tended to discolor earlier than those at 25°C. The qualitative changes of color in the solutions are tabulated in the table.

**Fluorescence imaging: In vivo studies.** The fluorescence emission spectrum of the 2 rats having no ALA showed only background activity whereas the other rats, 1 with intravenous ALA and 2 with intravesical instillation of 10% ALA at pH 5.5, all exhibited emission spectra that were characteristic of protoporphyrin IX with maxima clearly observed at 635 and 710 nm.

**Studies on tissue sections.** The fluorescence microscopy image of a bladder after intravesical ALA together with the quantitative fluorescence profile across the wall is shown in figure 1. Fluorescence measurements for each specific tissue layer (urothelium, lamina propria and muscle) were averaged over more than 20 representative areas (minimum 10 × 10 pixels) taken from 2 to 3 rat bladders for each ALA solution pH and concentration, with correction for background autofluorescence. Following instillation of 10% ALA solution, total dose 200 mg./kg. body weight at pH 4, PpIX fluorescence in the urothelium was variable and unpredictable with a peak 4 to 7 hours after administration (fig. 2). This acidic preparation seemed uncomfortable for the animals as most of them moved around much less after recovery from anesthesia than those given ALA at a higher pH. With 10% ALA solution at pH 5.5, fluorescence signals were first detectable 1 hour after administration and the peak urothelial intensity was again reached between 4 and 7 hours. By 24 hours, the signal had fallen to near background levels (fig. 3). At a lower concentration (1%) at pH 5.5, the PpIX biotransformation curve was similar in configuration to that from 10% ALA, but the amplitude was lower and there was a small shift to the left (fig. 4). At all pH values, the fluorescence in the lamina propria and muscle layers was much less than in the urothelium. With the 10% ALA solution, a peak urothelium-to-muscle layer fluorescence ratio of 5.5 and urothelium-to-lamina propria ratio of 7.0 was

| The color changes of 10% ALA solution at body and room temperature at different pH |
|-----------|-----------|-----------|-----------|-----------|-----------|
| Conc. (mg/ml.) | 10% | 10% | 10% | 10% | 10% | 10% | 10% | 10% |
| pH | 2.1 | 4.0 | 5.5 | 7.0 | 2.1 | 4.0 | 5.5 | 7.0 |
| Temp. (C) | 37 | 37 | 37 | 37 | 25 | 25 | 25 | 25 |
| Grade of color | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Upon preparation:
- 10 min: 0 0 1 2 0 0 1 2
- 1 h: 0 0 1 2 0 0 1 2
- 2 h: 0 0 1 2 0 0 1 2
- 3 h: 0 0 2 3 0 0 2 3
- 4 h: 0 0 2 3 0 0 2 3
- 5 h: 0 0 2 3 0 0 2 3
- 6 h: 0 0 3 4 0 0 2 4
- 24 h: 0 1 4 5 0 1 3 5
- 48 h: 0 2 5 5 0 1 4 5
- 72 h: 0 2 5 5 0 1 4 5

Grading is indicated as follows:
- 0: colorless
- 1: very faint yellow
- 2: light yellow
- 3: golden yellow
- 4: light brown
- 5: amber
PHOTOSENSITIZATION WITH INTRAVESICAL ALA

Fig. 1. A, computer processed fluorescence microscopy image of bladder wall 5 hours after instillation of 10% ALA at pH 5.5. Color scale depicts signal in counts per pixel (white is high fluorescence, black low). B, quantification of tissue fluorescence intensity across line on (A).

Fig. 2. Plot of bladder wall fluorescence intensity against time after instillation of 10% ALA at pH 4. Each value is average of measurements from 20 to 30 areas in 2 or 3 rats.

Fig. 3. Plot of bladder wall fluorescence intensity against time after instillation of 10% ALA at pH 5.5. Each value is average of measurements from 20 to 30 areas in 2 or 3 rats.

Fig. 4. Plot of bladder wall fluorescence intensity against time after instillation of 1% ALA at pH 5.5. Each value is average of measurements from 20 to 30 areas in 2 or 3 rats.

DISCUSSION

The major attraction of PDT in the management of bladder disease is the possibility of ablating extended areas of abnormal urothelium without damage to the underlying muscle and doing so in such a way that the treated areas heal with regeneration of normal urothelium. Previous clinical reports of PDT for severe dysplasia and carcinoma in situ of the bladder have shown effective ablation of the abnormal areas, but also a high incidence of permanent damage to the bladder, sometimes severe enough to warrant total cystectomy in spite of the eradication of neoplastic disease. The purpose of the present study has been to assess whether it is possible to use intravesical ALA to produce selective urothelial accumulation of PpIX in the bladder and develop a window of effective doses as was achieved with intravenous aluminum sulphonated phthalocyanine (AlSPc). Skin photosensitivity is always a major concern with systemic administration of photosensitizers. Intravesical instillation is an attractive alternative as it should avoid systemic photosensitization and could give better urothelial selectivity. As the rat urinary bladder is small and difficult to dissect, assessment of PpIX content in different tissue layers by biochemical extraction would be technically difficult and inaccurate. However, satisfactory correlation (94%) has been found previously in normal rat stomach and colon between

achieved 5 hours after instillation. Although the absolute urothelial fluorescence at this time was not the maximum, it was the time showing the best selectivity between the urothelium and other layers. Figure 5 illustrates PpIX fluorescence against ALA concentration 5 hours after instillation when the pH was 5.5. As the concentration declined from 10 to 1 and to 0.1%, the change of fluorescence intensity was not linear. However, the urothelium remained the layer with the highest level and ratios as high as 11:1 (urothelium/muscle) and 9:1 (urothelium/lamina propria) could be obtained with the 1% solution.
microfluorometric measurements obtained with this system and chemical extraction measurements. Our study using quantitative fluorescence microscopy indicates that absorption of small molecules like ALA through the tight barrier of the transitional epithelium is possible and that a higher concentration of PpIX accumulates in the urothelium than in the underlying layers. Nevertheless, the rat bladder is very thin, so one would anticipate rapid diffusion across all layers. The wall of the human bladder is much thicker, so diffusion to the muscle layer may be less and could lead to even better selectivity. This hypothesis, however, awaits confirmation.

The distribution of PpIX through each layer of the bladder wall was much more uniform than had previously been reported with intravesical AlSPc. Possible reasons for the difference observed between ALA and AlSPc are the dose of photosensitizer, time of bladder retention (time of instillation plus postinstillation interval) and chemical properties of the specific drugs. In this study, the instilled dose of ALA was similar to that used in previous studies for systemic administration whereas in the previous AlSPc study, the dose used intravesically was much lower than that used systemically.

The time that the photosensitizers stay in the bladder is another factor that is likely to influence whether adequate and uniform urothelial buildup of PpIX is achieved. In this study, it was 2 hours whereas it was only 30 to 60 minutes in the AlSPc work. A third factor for the poorer absorption of AlSPc through the bladder wall might be the higher molecular weight of AlSPc (770 versus 168 for ALA). Although PpIX was virtually undetectable 24 hours after intravenous injection, the intravesical route is likely to be an effective means of reducing skin photosensitivity during this 24-hour period as the fluorescence signals from rat skin (epidermis and dermis) with intravesical administration are only 25 to 50% of those seen with oral administration of the same dose of ALA (unpublished data).

The urinary bladder is a specially designed reservoir for urinary storage and evacuation. Its inner surface is covered with urothelium, which constitutes an unique and integral barrier to urine. For any substance to be absorbed efficiently from the urothelium, it should have a low molecular weight (<200), be water soluble and yet be sufficiently lipophilic to bind with plasma membranes. The biochemical properties of ALA (low molecular weight, high water solubility and possibly high lipophilicity) fulfill these criteria. Despite the lack of direct evidence supporting the existence of a mechanism for transportation of small molecules across the urothelium, a concentration gradient from the mucosa to serosal surfaces has been demonstrated after instillation of amino acids. The finding of increased accumulation of PpIX in the urothelium after ALA instillation is good evidence of the permeability of the urothelium to ALA. After ALA penetrates the superficial umbrella cells, it may take some time to diffuse into deeper transitional cell layers and convert to PpIX. The relative speeds of absorption of ALA into the urothelium and conversion of ALA to PpIX once in the urothelium and other layers of the bladder wall are not known, but it would seem likely that the longer the ALA remains in the bladder, the more that will be absorbed, so the time of retention is an important factor influencing the PpIX levels in the layers of the bladder wall.

The pH of an agent delivered intravesically is also important. As urinary pH depends on the body acid-base balance, the urothelium is well adapted to substantial changes of pH although, under normal conditions, it rarely gets below 5.4. The buffered ALA solution has a pH of 2.1 which is unphysiological to the bladder and was not used in our study. An absorption spectroscopic study on the stability of aqueous ALA solution indicated that, upon titrating to neutral pH, dihydroxyprazine and pyrazine were formed as a result of condensation of ALA molecules, the former being the agent responsible for the yellow color change observed in our study. The authors concluded that the optimum ALA preparation for instillation is a solution of pH 5 and a concentration less than 5%. Our results showed higher levels of PpIX in vivo with pH 5.5 than with pH 4.0 solution. The fluorescence intensity of PpIX in the urothelium with pH 5.5 solution retained for 4 to 7 hours was 30% higher than that using ALA at pH 4 retained for the same time. It is probable that, at the lower pH, although the ALA is more stable chemically, some of the transitional epithelium might be damaged as a result of prolonged contact with the strong acid. The optimal pH for ALA instillation that achieves a balance between PpIX buildup and bioadaptability of the urothelium seems to lie in the range of 5 to 6, and it is with this rationale that we selected pH 5.5 for our study. The poor PpIX fluorescence detection after topical administration of ALA reported by Levecks et al. could be the result of instilling an ALA solution which was so acidic (pH = 2.1) that it reduced the ability of transitional cells to absorb ALA.

Following intravesical administration of 10% ALA solution, PpIX buildup in the bladder urothelium peaked at 4 to 7 hours, which is slightly later than after either intravenous (3 to 4 hours) or oral administration (4 to 6 hours). The absolute fluorescence levels in the urothelium are similar for the 3 different routes and comparable total administered doses. The selectivity of PpIX accumulation between the urothelium and muscle layer is also similar (5:1 with the intravenous route, 4:1 with the oral route and 5:1 with the intravesical route). The ratio of PpIX concentration between the urothelium and muscle layer is one of the best indicators for safe PDT treatment of the bladder, so the greater the ratio, the better the chance to selectively destroy the urothelium without harming the lamina propria and the detrusor muscle layer. The intravesical route has only shown a marginal benefit from this point of view, but it does avoid the transient liver damage that can occur after oral administration. Intravesical administration of ALA has rarely been tested and, as with other photosensitizers (AlSPc), the results from this route of administration seem conflicting. It is probable that different types of photosensitizer are taken up by different mechanisms, which will lead to different tissue concentrations and distribution patterns after different routes of administration. Even for closely related agents, the chemical structure, spatial configuration, number of side chains and molecular weight are all determinants of the final distribution. Our experiments suggest that intravesical instillation of ALA might be an attractive alternative to other routes because it leads to less skin photosensitization and retains considerable PpIX selectivity between the urothelium and other layers.

In this in vivo study of ALA as a photosensitizer precursor,
we conclude that intravesical instillation of ALA solution at an appropriate pH and concentration may be a clinically feasible route to achieve selective accumulation of PpIX in the urothelium with reduced systemic photosensitization.

REFERENCES

Photodynamic Therapy of Rat Urinary Bladder with Intravesical Instillation of 5-Aminolevulinic Acid: Light Diffusion and Histological Changes
PHOTODYNAMIC THERAPY ON RAT URINARY BLADDER WITH INTRAVESICAL INSTILLATION OF 5-AMINOLEVULINIC ACID: LIGHT DIFFUSION AND HISTOLOGICAL CHANGES

SHI-CHUNG CHANG,* ALEXANDER J. MACROBERT AND STEPHEN G. BOWN

From the National Medical Laser Centre, University College London Medical School, England, and Tzu-Chi General Hospital, Hua-Lien, Taiwan

ABSTRACT

Purpose: Photodynamic therapy (PDT) has the potential to treat extensive premalignant lesions and microinvasive tumors in the bladder, but its use has been hampered by the risk of detrusor muscle damage and prolonged skin photosensitivity. We have shown that the rat urothelium can be sensitized by selectively using a 10% solution of 5-aminolevulinic acid (ALA) at pH 5.5 administered intravesically. This paper evaluates the photodynamic effects on sensitized bladders.

Materials and Methods: The bladders of Wistar rats were instilled with ALA solutions of different concentrations at pH 5.5 and subsequently treated with laser light at 630 nm. Bladders were harvested 1 to 7 days after PDT for histological assessment.

Results: Under optimum conditions (10% intralipid diffusion medium, light dose 50 J) uniform urothelial necrosis was seen after 1 to 2 days; it healed in 7 days without damage to the underlying muscle layer although some increase in collagen was seen in the lamina propria. Over-treatment or poor light distribution resulted in muscle necrosis and scarring.

Conclusions: Selective urothelial necrosis is possible with PDT using intravesical ALA. There is now sufficient data for pilot clinical trials to start photodynamic therapy for management of superficial bladder cancer or carcinoma in situ.

Key Words: photosensitizing agents, phototherapy, protoporphyrins, aminolevulinic acid
Table 1. Treatment parameters for the PDT study groups. In all animals, ALA was instilled for 2 hours and treatment delivered 5 hours after initial instillation. The laser power was set at 100 mW.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Rat No.</th>
<th>Sensitizer</th>
<th>ALA Concentration</th>
<th>Laser Time (sec.)</th>
<th>Laser Energy (J)</th>
<th>Intralipid Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10%</td>
<td>1,000</td>
<td>100 J</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>10%</td>
<td>500</td>
<td>50 J</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>10%</td>
<td>500</td>
<td>50 J</td>
<td>10%</td>
<td>1%</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>1%</td>
<td>500</td>
<td>50 J</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0.1%</td>
<td>500</td>
<td>50 J</td>
<td>1%</td>
<td>1%</td>
</tr>
</tbody>
</table>

ALA, Intralipid: Concentration of ALA and intralipid.

through a urethral cannula with 0.3 ml of 10% or 1% intralipid solution to enhance isotropic light distribution. To optimize the laser fiber position, the fiber tip was pushed through the dome and placed 3 to 5 mm. from the top of the bladder, so the light distribution through the bladder looked uniform. The fiber was so thin that the bladder sealed around it without causing any visible leakage during and after treatment. The power from the fiber tip was calibrated to 100 mW before each treatment, which was for 500 or 1,000 seconds (50 or 100 J) per rat. After light exposure, the abdomen was closed and the animals allowed to recover consciousness. The rats were killed 1, 2, 3, or 7 days after laser treatment (at least 2 for each set of treatment variables) and the bladders removed and distended by instillation of 0.3 ml formalin.

RESULTS

Macroscopic findings. In the 6 control rats receiving ALA alone, laser illumination alone, or neither, the bladders were pinkish and easily dissectable from other surrounding tissues. Macroscopically, the bladder interior looked pale and smooth without evidence of ulceration.

The PDT treatment groups are listed in Table 1. Group 1 consisted of 10 rats sensitized with 10% ALA and treated with a light dose of 100 J (100 mW × 1,000 seconds) using 1% intralipid as the light scattering medium. This was the highest light dose used, and most rats were unwell after treatment. The power from the fiber tip was calibrated to 100 mW before each treatment, which was for 500 or 1,000 seconds (50 or 100 J) per rat. After light exposure, the abdomen was closed and the animals allowed to recover consciousness. The rats were killed 1, 2, 3, or 7 days after laser treatment (at least 2 for each set of treatment variables) and the bladders removed and distended by instillation of 0.3 ml formalin.

Two rats treated with 50 J were killed 3 months after PDT. Specimens were sectioned and stained with hematoxylin and eosin and Van Gieson stains for histological assessment.

Histological findings. On microscopic examination in control animals, the urothelium consisted of 3 to 6 tightly opposed layers of transitional epithelial cells. Deep to the urothelium, the lamina propria consisted of loose connective tissue with scanty cellular components and a few vascular structures extending to the muscularis propria. In the bladders which had been treated with laser light through a laparotomy but without prior ALA sensitization, mild inflam-
The most prominent findings in the bladder 1 to 3 days after PDT in group 1 (100 J) were diffuse swelling of the lamina propria and focal sloughing of tissue which, in some cases, extended from the urothelium through the muscularis propria to the serosa. The urothelial damage was patchy with areas of complete urothelial destruction and seemingly normal tissue in between (fig. 2). Acute inflammatory cell infiltration, mostly with mononucleated round cells, was seen throughout all tissue layers. Fibrinoid degeneration of small arterioles was evident by 3 days after PDT (fig. 3). Regions of full thickness necrosis and patches of superficial ulceration confined to the lamina propria were most striking 2 days after treatment. Extensive full-thickness bladder damage was seen in areas facing the laser probe, where the light dose per cm.$^2$ was greatest, indicating that the concentration of the intralipid used as light scatterer was too low to achieve isotropic light distribution. Treatment at the lower light dose of 50 J (group 2) produced similar focal lesions which were patchy in distribution with varying degrees of tissue destruction, but there was much less full thickness necrosis than in group 1 (fig. 4). With the higher concentration of intralipid used in group 3 (10%), the changes in the mucosa were much more uniform and essentially limited to the urothelium and lamina propria (fig. 5). The microscopic changes in groups 4 and 5 were even milder and ranged from small areas of urothelial sloughing to no damage at all. In all groups, edema of the lamina propria was the most constant histological finding in the first few days after PDT, although the severity varied markedly with the treatment parameters used. By the
seventh day after treatment, although the necrosed mucosa had healed with regeneration of healthy urothelium in all groups, there was persistent evidence of ongoing tissue repair processes such as increased mitotic figures and prominent hypnophymatomous nuclei in the basal cell layer. Healing in the lamina propria was also essentially complete by 7 days (fig. 6). In group 1, there was scarring in the muscularis propria at 7 days, but in the other groups, there was nothing more than very minimal changes in the muscle layer at this time in sections stained with hematoxylin and eosin or Van Gieson's stain. The principal finding of the bladders examined 3 months after PDT (group 2, 50J) was mild collagen fiber deposits with an increased fibroblast activity in the lamina propria compared with that of normal bladder. The collagen fiber formation in the muscularis propria was negligible (fig. 7). The principal histological findings are summarized in table 2.

**DISCUSSION**

Comparable to our previous results with AlSPc given intravenously, we have achieved selective urothelial necrosis with ALA given by the intravesical route and with a much shorter time interval between drug and light (5 instead of 24 hours). With inappropriate dosimetry, muscle damage is still inevitable. Nevertheless, histological data showed that the PDT effect was largely superficial and predominantly confined to the urothelium with a total light dose of 50J. We have not found a dose of ALA at which we could get completely uniform urothelial ablation but no muscle damage even with larger light doses, but from our results, it is possible that such a dose exists between 1% and 10% (20 mg/kg, and 200 mg/kg.). With any sensitizer given intravenously, the ratio of sensitiser concentration between urothelium and muscle is likely to be the same in rats and patients, but with the intravesical route, the need for drug to diffuse across a greater thickness of urothelium and lamina propria may give better selectivity in patients. Also, the red light used for PDT is attenuated rapidly as it goes through the bladder wall, so the relative intensity of light in muscle in the human bladder compared with that in the overlying urothelium is less than in rat bladder muscle compared with the overlying urothelium. This may further improve selectivity in clinical use. However, care must be taken with this aspect as clinical trials have shown that scarring in muscle is easily produced by using Photofrin as the photosensitizer with red light at the same wavelength as is used for ALA.6

Kriegmair et al. used a similar instillation technique and ALA dosimetry (pH 5.5, 20% ALA retained for 3.5 to 4 hours) and achieved selective damage to chemically induced bladder tumors. The normal urothelium was spared after laser treatment of rat bladder at various light doses (15 to 100 J/cm.2) although tumor destruction was more constant at higher light doses. It is possible that, during bladder illumination in their experiments, the laser beam was aimed chiefly at the tumor, and as a result, less light fell on the surrounding normal urothelium. Our experiments did not study bladder tumors, but showed selectivity between the layers of the normal bladder, particularly between urothelium and muscle, with treatment parameters comparable to those of Kriegmair et al. We believe that it is important to emphasize the need to destroy all the transitional cell lining as a crucial step in the management of bladder cancer. With current urological techniques, papillary tumors that can be visually identified under cystoscopy pose no particular therapeutic problem as they can be eliminated with laser coagulation or electrocautery. However, intractable problems remain with dysplastic or precancerous foci that can be distributed throughout the urothelium, are not easily detected macroscopically and may develop into invasive cancers. If the diseased urothelium can be eradicated completely with PDT and the bladder lining regenerates subsequently with normal urothelium and without causing underlying muscle damage, then PDT might be the most attractive modality for prevention of bladder cancer recurrence. It is with this rationale that we focused on selectivity of damage between normal bladder layers rather than on the difference between normal and neoplastic urothelium.

Previous PDT studies with HpD and Photofrin have shown that the cytotoxic effect of those photosensitizers was largely due to shut down of the tumor and normal tissue microvasculature. The site of ALA-associated cell killing has been shown to be the mitochondria; however, the possibility of damage to other cellular structures at a later time when most of the PpIX has accumulated in the stroma cannot be ruled out. The presence of arteriolar fibrinoid lesions raises the possibility that the photodynamic threshold (the product of tissue photosensitizer concentration and the light dose) for necrosis in the arteriolar smooth muscle is lower than for the

---

**Table 2. Histological changes of the bladder wall 1, 2-3, 7 and 90 days after PDT in different treatment groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Loss of urothelium</th>
<th>Edema</th>
<th>Necrosis of urothelium and L.P.</th>
<th>Muscle necrosis</th>
<th>Inflamm. cell infiltration</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L.P.</td>
<td>M.P.</td>
<td></td>
<td>L.P.</td>
<td>M.P.</td>
</tr>
<tr>
<td>1 (10% ALA, 100 J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>patchy</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>2-3d</td>
<td>patchy</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>7d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 (10% ALA, 50 J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>patchy</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>2-3d</td>
<td>patchy</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>7d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3 (10% ALA, 50 J, 10% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>diffuse</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-3d</td>
<td>near uniform</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>7d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 (1% ALA, 50 J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>rare</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-3d</td>
<td>occasional</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 (0.1% ALA, 50 J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>rare</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>2-3d</td>
<td>rare</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Treatment parameters: ALA concentration, light dose, concentration of intralipid
Changes within layers: -, none; ±, minimal; +, mild; ++, moderate; ++++, severe
L.P.: Lamina propria, M.P.: Muscularis propria
adjacent venules. It is also possible that the lesions are caused by disruption of the microcirculation, although this mechanism has not yet been clearly shown with ALA. The importance of vascular effects in the destruction of tissue, whether normal or neoplastic, remains to be established.

We also demonstrated that the better concentration of intralipid for light scattering in the rat bladder with a volume of 0.2 to 0.3 ml was 10%. At this concentration, uniform urothelial sloughing with edema of the lamina propria was observed. With the same treatment parameters but reduction of the intralipid to 1%, urothelial damage was patchy and fibrinoid change became more common in the arterioles of the lamina propria. This finding was in accord with the prediction by Baghdassarian and associates who tested the uniformity of light distribution at different intralipid concentrations in flasks of various volumes. In a sphere with a volume of 50 ml or less, light tended to focus on the site opposite to the tip of the laser fiber (180 degrees) and uniform distribution of light was not possible in the presence of 1% intralipid. Higher concentrations of scatterer lead to more uniform light distribution at the expense of greater energy loss. In the clinical situation, as the capacity of the human bladder is usually in the range of 300 to 450 ml, although it is not an ideal sphere, a 1% intralipid would be more likely to be the concentration of choice to obtain the maximum uniformity of light distribution with the minimum amount of light absorption in the scattering medium.

Before PDT is adopted for the treatment of multifocal carcinoma in situ or diffuse low grade papillary bladder tumors, it is worth emphasizing that, for any therapeutic modalities to be clinically applicable, the ability to achieve uniform urothelial necrosis is crucial, as complete removal of the urothelial lining is the only reliable proof of cure of this type of bladder malignancy. As we have demonstrated the possibility of achieving selective urothelial destruction with appropriate ALA concentrations and light dosimetry in the rat bladder, it may be feasible to extend PDT to treat patients with recurrent bladder cancer in whom intravesical chemotherapy or BCG instillation has failed. One advantage of ALA over other more potent photosensitizers is the feasibility of multiple treatments, as with chemotherapeutic agents, as skin photosensitivity only lasts 1 to 2 days. However, care must be taken in applying in vivo data from rodents to clinical trials as the bioavailability of ALA and PpIX and PDT treatment parameters may differ from species to species.

In this study, we have illustrated histologically that, with careful light dosimetry, a diffuse urothelial sloughing without damage to the underlying muscle layer, can be achieved with ALA. Treatment with ALA also has an advantage over AlSPc as urothelial regeneration is essentially complete by 2 weeks after photoactivation of endogenous porphyrins. Br. J. Cancer, 56: 815, 1987.

Acknowledgment. We are grateful to Dr. C. Parkinson, consultant histopathologist of the University College London Hospitals, for her help in setting up the scheme for histological assessment of tissue damage. We also thank Dr. G. Buonacorsi in the Department of Medical Physics for his constant laser technical assistance.

REFERENCES

Reprinted from

5th International Photodynamic Association Biennial Meeting

The Biodistribution and Photodynamic Effect of Protoporphyrin IX in Rat Urinary Bladder after Intravesical Instillation of 5-Aminolaevulinic Acid

21–24 September 1994
Amelia Island, Florida

©1995 by the Society of Photo-Optical Instrumentation Engineers
Box 10, Bellingham, Washington 98227 USA. Telephone 360/676-3290.
The biodistribution and photodynamic effect of protoporphyrin IX in rat urinary bladders after intravesical instillation of 5-aminolaevulinic acid

Shi-Qiung Chang, Alexander J. MacRobert, Stephen G. Bown

ABSTRACT

Photodynamic therapy (PDT) has considerable potential for the treatment of superficial bladder neoplasia. Complications such as scarring of detrusor muscle and prolonged cutaneous photosensitivity may be reduced by using the new photosensitiser precursor, 5-aminolaevulinic acid (ALA). After instillation of ALA, the concentration, pH and time of bladder retention of ALA solution were found to be the key factors to a satisfactory PpIX buildup in the mucosa. The optimum PpIX fluorescence intensity ratio between mucosa and muscle layer is 10 to 1 with a pH 5.5, 1% ALA solution retained for 5 hours. Higher concentration resulted in more mucosal PpIX formation, but less selectivity. Unbuffered ALA was unsuitable for bladder instillation. Two days after laser treatment with 25 J/cm² at 630 nm with optimal sensitisation, typical histological findings were urothelial sloughing and lamina propria edema without obvious muscle damage. After 7 days, recovery of the urothelium was almost complete and fibroblast infiltration was minimal. ALA induced PpIX after bladder instillation may be an appropriate photosensitizer for future management of superficial bladder cancer.

Keywords: photodynamic therapy (PDT), 5-aminolaevulinic acid (ALA), protoporphyrin IX (PpIX), tissue selectivity

1. INTRODUCTION

Photodynamic therapy (PDT) is a technique combining administration of a photosensitizing agent and subsequent illumination with light of appropriate wavelength to produce oxygen-dependent photochemical tissue destruction [1]. It was first used in urology for the diagnosis and treatment of small bladder cancers in 1976 [2]. Thereafter, in spite of some encouraging results reported using hematoporphyrin derivative (HpD) or its more purified ether/ester derivative (Photofrin) as photosensitizers in the treatment of dysplasia or superficial carcinomas of the bladder [3-5], PDT has been of limited clinical value because of prominent post-treatment side effects [5]. Another disadvantage of Photofrin is the skin photosensitisation that may last for several weeks after treatment. Nevertheless, Hpd and Photofrin have been widely studied and recently Photofrin has been approved for clinical use in Canada for prophylaxis of superficial papillary bladder cancer [7]. The advantages of minimal invasiveness, lack of cumulative toxicity, good mucosal healing and the possibility of repeat applications are extremely attractive.

Little work has been done to elucidate the mechanisms of PDT damage to the bladder muscle and to look for ways in which this might be avoided. In looking for the "window" of doses at which selective damage to the urothelium could be achieved was quite narrow. Pope & Bown [8] had some success on the rat bladder using the photosensitiser aluminium sulphonated phthalocyanine (AlSPc, which is a mixture of the mono-, di-, tri-, and tetrasulphonated derivatives). It was also necessary to wait 24 hours between giving the AlSPc and delivering the light. It is likely that similar results could be achieved using Photofrin, although despite its use in clinical trials over at least a decade, such experiments have not been reported. AlSPc does not have the same problems of prolonged skin photosensitivity to sunlight seen with Photofrin [9], but it must be given intravenously as experimental administration directly into the bladder has shown very patchy uptake [8].

One of the most interesting photosensitiser currently under assessment is 5-aminolaevulinic acid (ALA). Promising results have been reported for its use for PDT of basal cell carcinomas of the skin [10] and some small neoplasms of hollow viscera [11-13]. ALA is an intermediate synthesized from glycine and succinyl co-enzyme A in the biosynthetic pathway for the production of haem in mammalian cells. In this biochemical chain reaction, ALA is converted through a series of intermediates to protoporphyrin IX (PpIX), the predominant porphyrin species responsible for photosensitisation [14]. Since the conversion of PpIX to photoinactive haem is the rate limiting step in the chain of reactions between ALA
and haem, bypassing the physiological feedback mechanism with excessive exogenous ALA results in temporary accumulation of PpIX. As haem containing enzymes are essential for energy production, cells having greater metabolic turnover are likely to synthesize more PpIX in situ when excess ALA is given. Epithelial cells being more active metabolically than muscle, some degree of selective accumulation of PpIX would be expected in it. Preferential accumulation of PpIX in the mucosa layer of alimentary tract after injection and oral intake of ALA has been reported[11-13]. Intravesical instillation of photosensitisers seems an attractive route to avoid the problem of systemic photosensitivity. In this study we investigated the feasibility of using intravesical ALA for producing selective urothelial necrosis by PDT.

2. EXPERIMENT

2.1 Preparation of ALA solutions

Purified ALA powder (ALA.HCl 98%) was dissolved in normal saline and titrated with saturated sodium bicarbonate solution to a final pH of 2.1, 4.0, 5.5 or 7, at a concentration of 10%(100mg/ml). The solutions were then stored in the dark at 37 °C and in dim room light at 25 °C for 72 hours for qualitative assessment of chemical stability. At both 37 and 25 °C, the solution turned light yellow a few seconds after titration to pH 7 with saturated sodium bicarbonate solution (pH = 8.06) and became light brown at 5 hours. At pH 5.5, the solution was relatively stable in colour for the first 5 hours although it became brown after 24-48 hours whether stored at body or room temperature. This was considered acceptable if the solution could be used within a few hours of preparation. At pH 4, the solution remained colourless over the first 24 hours. Only the untitrated ALA solution exhibited no colour change during the 72 hour observation period. The preparations stored at 37°C tended to discolor earlier than those at 25°C.

2.2 Technique for intravesical instillation

ALA solutions of 10%, 1% and 0.1% concentration with pH 4 and 5.5 were selected for instillation into the bladder of female Wistar rats weighing between 100 and 180 grams. Catheterisation was performed with a 18.5 gauge Teflon cannula inserted transurethrally into the bladder after a small episiotomy(2-3 mm) in the vestibule to expose the urethral meatus. ALA solution was administered at a volume of 0.20 to 0.32 ml(0.3cc/100 gm body weight, 200mg/kg with 10% solution; 20mg/kg with 1% solution and 2mg/kg with 0.1% solution) and retained in bladder for at least 2 hours when the rats were still under the effect of general anaesthesia with Hypnorm (Fentanyl and fluanisone, Jansen Pharmaceuticals Ltd.). After that, the rats were allowed to wake up and void freely. For each ALA solution of different concentration and pH, at least two rats were tested. The control group consisted of 2 rats receiving nothing and 2 others with bladder instillation of 0.9% normal saline(0.24-0.3 ml).

2.3 Fluorescence microscopy with CCD camera

Bladder specimens were collected at 1, 2, 3, 4, 5, 7 and 24 hours after intravesical instillation of ALA solution. The bladder was rinsed 2 to 3 times with normal saline and then filled with 0.3ml OCT medium to keep the lumen distended before removing it. Frozen sections at 10mm thickness were used for fluorescence imaging.

In vivo fluorescence spectroscopy was carried out to confirm porphyrin synthesis using a Perkin-Elmer LS50B spectrofluorometer with a bifurcated fibre-optic bundle probe. The probe was placed 3 mm above the exposed bladder which had been rinsed once and then filled with normal saline. The emission spectrum was recorded from 600-750 nm using an excitation wavelength of 410nm. For quantification and microscopic imaging of PpIX fluorescence, an inverted phase contrast microscope(Olympus IMT-2) attached to a high resolution(385 x 578 pixels) slow-scan charge coupled device(CCD) camera(Wright Instruments Ltd, model 1) was utilized. In brief, an 8 mW helium neon laser (632.8 nm) was used to excite the tissue PpIX and the emitted fluorescence was detected between 665 and 710 nm (with the maximum response at 690 nm), using a combination of bandpass and longpass filters (Omega Optical Inc., Vermont). The fluorescence signal was processed by an IBM PC clone into a falsely colour-coded image depicting the signal in counts per pixel. The image processing software enabled fluorescence intensity in each tissue layer to be quantified digitally by averaging over specified areas. After fluorescence imaging, the sections were fixed in formalin and stained with haematoxylin and eosin for histological study. The use of a slow-scan CCD system with a fixed internal gain together with constant power laser excitation enables highly reproducible signal calibration and satisfactory correlation between...
microfluorometric measurements obtained with this system and chemical extraction measurements [16]. In the present studies, fluorescence emission spectra measured from 3 rats that had received ALA exhibited emission spectra which were characteristic of protoporphyrin IX with maxima clearly observed at 635 nm and 710 nm [17].

Fluorescence measurements for each specific tissue layer (urothelium, lamina propria and muscle) were the average count of more than 20 representative areas (minimum 10x10 pixels) with correction for background autofluorescence. Following intravesical instillation of 10% ALA solution (200 mg/kg BW, pH 4), PpIX fluorescence in the urothelium was variable and unpredictable. This pH 4 solution was more distressful to the rats than other less acidic preparations. With 10% ALA solution at pH 5.5, fluorescence signals began detectable 1 hour after instillation and the peak urothelial intensity was again reached between 4 and 7 hours. By 24 hours, the signal fell to near background level (Fig. 1). At all pH values, the fluorescence in the lamina propria and muscle layers was much less than in the urothelium. At pH 5.5, as the concentration declined from 10 to 1 and to 0.1%, the change of fluorescence intensity was not linear. The highest ratio of 11 to 1 between the urothelium and muscle could be obtained with the 1% solution.

2.4 Photodynamic therapy

The light source used was a copper vapour pumped dye laser (Oxford Lasers Ltd.) emitting red light at 630 nm and delivered through a 200μm silicon coated quartz fibre. Of the six rats in the control group, 4 received either intravesical ALA or laser illumination alone whilst another 2 had neither. In the experimental groups, laser treatment was applied 5 hours after ALA instillation as this was the time of maximum PpIX selectivity between urothelium and muscle. At the time of PDT treatment, a small laparotomy was made to expose the urinary bladder under general anaesthesia. The bladder was emptied of urine and filled through a urethral cannula with 0.3 ml of 10% or 1% intralipid solution to enhance isotropic light distribution. The tip of this alender laser fibre was pushed through the dome and placed 3-5 mm from the top of the bladder without causing any visible leakage during and after treatment. The laser power from the fibre tip was calibrated each time before treatment and used at 100 mW for an illumination time of 500 seconds. The rats (at least 2 for each time) were killed 1, 2, 3, 7 and 90 days after laser illumination and the bladders taken out for hematoxylin and eosin and Van Gieson for histological assessment.

2.4.1 Macroscopic findings

In the 6 control rats receiving ALA alone, laser illumination alone or neither, the bladders were pinkish and easily dissectable from other surrounding tissues. Macroscopically, the bladder interior looked pale and smooth without evidence of ulceration. Twenty-four hours after PDT, the bladders in group 1 and 2 (group 1: 10% ALA, 100mW x 500sec, 1% intralipid; group 2: 10% ALA, 100mW x 500sec, 10% intralipid) were grossly puffy and pale. On the second and third days, areas of petechial haemorrhage became prominent on the bladders which were covered by intraperitoneal fat. There were no PDT related deaths. The 20 rats in Group 3 and 4 treated at lower ALA concentrations had no identifiable abnormalities on gross inspection of the bladder at any time after treatment. Adhesion of peritoneal fat to the dome of bladder, oedema of the bladder wall and the presence of intraperitoneal fluid were found only in animals treated with 10% ALA.

2.4.2 Histological findings

Under microscopy, the urothelium of control rats was composed of 3-6 tightly opposed layers of transitional cells. Deep to the urothelium, the lamina propria consisted of loose connective tissue with scanty cellular components and a few
vascular structures, extending to the muscularis propria. In the bladders which had been treated with laser light through a laparotomy but without prior ALA sensitization, mild inflammatory cell infiltration in the serosa was a common finding 2-3 days after operation but had cleared by the 7th day. No abnormalities were seen in any other layers of the bladder wall.

The most prominent early findings in the bladder after PDT in group 1 were diffuse swelling of the lamina propria and focal sloughing of tissue which in some cases, extended from the urothelium through the muscularis propria to the serosa. The urothelial damage was patchy with areas of complete urothelial destruction and seemingly normal tissue in between. Acute inflammatory cell infiltration was seen throughout all tissue layers. Regions of full thickness necrosis and patches of superficial ulceration confined to the lamina propria were found 2 days after treatment. Extensive full thickness bladder damage was seen in areas facing the laser probe, where the light dose per cm² was greatest, indicating that the concentration of the intralipid used as light scatterer was too low. Increasing the intralipid concentration to 10% (group 2), the changes in the urothelium and lamina propria were much more uniform (Fig. 2). The microscopic changes in group 3 and 4 were milder and ranged from small areas of urothelial sloughing to no damage at all. In all groups, oedema of the lamina propria was the most constant early histological finding after PDT, although the severity varied markedly. By the 7th day, although the necrosed mucosa had healed with regeneration of urothelium in all groups, there was persistent evidence of ongoing tissue repair processes. Healing in lamina propria was also essentially complete by 7 days. There were no obvious changes in the muscle layer at this time in sections stained with H & E or Van Gieson's stain. The main histological findings are summarised in Table 1.

Table 1. Histopathological changes of the bladder wall 1, 2 and 7 days after PDT in different treatment groups

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (10% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>patchy</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2-3d</td>
<td>patchy</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2 (10% ALA, 50J, 10% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>diffuse</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2-3d</td>
<td>near uniform</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3 (1% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>rare</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2-3d</td>
<td>occasional</td>
<td>++</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>4 (0.1% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>rare</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2-3d</td>
<td>rare</td>
<td>++</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Fig. 2: PDT treatment effect of bladder with 10% pH 5.5 ALA and light dose(100mW, 500sec).

Table 1. Histopathological changes of the bladder wall 1, 2 and 7 days after PDT in different treatment groups

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (10% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>patchy</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2-3d</td>
<td>patchy</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2 (10% ALA, 50J, 10% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>diffuse</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2-3d</td>
<td>near uniform</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3 (1% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>rare</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2-3d</td>
<td>occasional</td>
<td>++</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>4 (0.1% ALA, 50J, 1% intralipid)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>rare</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2-3d</td>
<td>rare</td>
<td>++</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7d</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>±</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

*: negative, ±: negligible, +: mild; ++: moderate; +++: severe
L.P.: Lamina propria, M.P.: Muscularis propria
3. DISCUSSION

The major attraction of PDT in the management of bladder disease is the possibility of ablating extended areas of abnormal urothelium without damage to the underlying muscle and in such a way that the treated areas heal with regeneration of normal urothelium. Previous clinical reports of PDT for severe dysplasia and carcinoma in situ of the bladder have shown very effective ablation of the abnormal areas, but the price to pay has been a high incidence of permanent damage to bladder function (irritability and contraction), sometimes bad enough to warrant total cystectomy in spite of the eradication of the neoplastic urothelium and its replacement by essentially normal urothelium [6]. In this study we have achieved selective urothelial necrosis with a much shorter time interval between drug and light (5 hours), although with inappropriate dosimetry, muscle damage is still seen.

3.1 Measures to reduce systemic photosensitisation

Prolonged skin photosensitivity is the prime concern for systemic administration of photosensitisers. Although PpIX was nearly undetectable 24 hours after intravenous injection in our previous studies[11-13], we used the intravesical route as a means for reducing the possibility of skin photosensitivity during this 24 hour period. Our data firmly indicated that the PpIX signals of rat back skin (epidermis and dermis) are only 1/2 to 1/4 in intensity after intravesical route than with oral administration(unpublished data). For any solution to be instilled into the bladder, two factors, the biological adaptability of the bladder compartment and the chemical properties of the molecule in the solution deserve special attention. The inner surface of the urinary bladder is covered with urothelium which is an unique and effective barrier to urine. From the pharmacokinetic point of view, any substance to be absorbed efficiently from the urothelium should have a low molecular weight (<200), be water soluble and yet be sufficiently lipophilic for binding with plasma membranes [18]. ALA fulfills these criteria. Despite the lack of direct evidence supporting the existence of a mechanism for transportation of small molecules across the urothelium, a concentration gradient from the transitional cell lining to serosal surfaces has been demonstrated after instillation of amino acids[19]. The finding of increased accumulation of PpIX in the urothelium after retaining the ALA solution for several hours is good evidence to support the permeability of the urothelial barrier to ALA.

Intravesical instillation is an attractive option for administration of photosensitisers as it should avoid systemic photosensitisation and could give better urothelial selectivity. As the rat urinary bladder is so small and difficult to dissect, assessment of PpIX content in different tissue layers by biochemical extraction would be technically difficult and inaccurate [16]. Our study using quantitative fluorescence microscopy indicates that absorption of small molecules like ALA through the tight barrier of the transitional epithelium is possible and a higher concentration of PpIX has accumulated in the urothelium than in the underlying layers. The distribution of PpIX through each layer of the bladder wall was much more uniform than had previously been reported with intravesical ALSPc [8]. The possible reasons accounting for the difference between ALA and ALSPc are dose of photosensitizer, time of bladder retention and chemical properties of the specific drugs. In this study, the total dose of ALA for intravesical instillation is similar to that used for systemic administration which when compared with our previous study using ALSPc, is much higher. The time of retention of ALA in the bladder is another important factor to ensure adequate and uniform urothelial buildup of PpIX. A third factor for the poorer absorption of ALSPc through the bladder wall might be the difference in molecular weight which tends to be higher in ALSPc than in ALA. If the instilled ALA was absorbed rapidly by the urothelium and then redistributed via systemic circulation throughout the body, one might expect similar or even higher PpIX level in tissues receiving more blood supply which in bladder, is the muscle layer. However, after instillation of ALA, the PpIX intensity detected in the muscle layer was rather constant and lagged far behind that of urothelium that negatively supported the possibility of absorption and redistribution in the bladder.

3.2 Importance of time of retention of ALA

After penetrating the superficial layer of umbrella cells, ALA may take some time to diffuse into deeper layers of transitional epithelium and to convert it to PpIX. The time of retention thus is an important factor influencing the PpIX concentration in tissue layers. Since ALA is a precursor of PpIX, the absorbed ingredient in the cytoplasm has to diffuse into the mitochondria before starting the biochemical reactions for haem[10]. It also needs some time for the biosynthesis of PpIX in the mitochondria. It seemed clear from our study that the urothelium PpIX concentration built up rapidly in the first few hours after ALA instillation and reached a plateau between 4 and 7 hours before starting to decline. If the instilling ALA solution is of sufficient concentration, a retention of 2 hours in the bladder would be more than enough for the diffusion of ALA into the transitional cells. Using similar instillation techniques but a higher ALA concentration,
3.5 Importance of pH for bladder instillation

The pH of an intravesical agent is another variable influencing urothelial absorption. As urinary pH depends on the body acid-base balance, the urothelium must be able to adapt to substantial changes of pH although under normal conditions, it rarely gets below 5.4. The unbuffered ALA solution has a pH of 2.1 which is unphysiological to the bladder and was not used in our study. Our results showed higher levels of PpIX when ALA was instilled at pH 5.5 than at pH 4.0. With pH 5.5 and the solution retained in the bladder for 4-7 hours, the fluorescence intensity of PpIX in the urothelium was 30% higher than that using ALA at pH 4. A possible explanation is that at the lower pH some of the superficial transitional epithelium was damaged as a result of prolonged contact with the strong acid. The poor PpIX fluorescence detection followed by ALA topical administration reported by Leveckis et al [20] could be the result of instilling improper ALA solution which is so acidic (pH 2.1) as to deprive the vitality of transitional cells rendering themselves no chance to absorb ALA and to accumulate adequate PpIX for detection.

3.4 Tissue selectivity

Following intravesical instillation of 10% ALA solution, PpIX buildup in the bladder urothelium peaks at 4 to 7 hours which when compared with other routes is slightly longer than either injection(3-4 h) or oral(4-6 h)[13]. Although the absolute fluorescence levels in the urothelium are similar by the three different routes, what really matters is the ratio of PpIX intensity between the urothelium and muscle layer if a PDT treatment is planned to destroy the urothelial layer but not to damage the underlying structures. Generally, the greater the ratio, the better the chance to selectively destroy the urothelium without harming the lamina propria and the detrusor muscle layer. The PpIX selectivity between the urothelium and muscle layer is 5:1 with the intravenous route, 4:1 with the oral route[13] and 5.5:1 with the intravesical route. A ratio of 7 in PpIX buildup between the urothelium and lamina propria 5 hours after intravesical ALA is slightly better than the results attained by either the intravenous (4.8) or oral (3.8) route. Although the beneficial effect of tissue selectivity with intravesical instillation seems marginal compared with other methods of administration, avoiding the hepatic metabolism which certainly occurs after oral administration [13] and may occur after i/v is a clear bonus. It was disappointing that the selectivity between urothelium and muscle was no better than after systemic administration of ALA. Nevertheless, the rat bladder is very thin, so one would anticipate rapid diffusion across all layers, whereas the wall of the human bladder is much thicker, so diffusion to the muscle layer may be less which could lead to better selectivity. This hypothesis could be tested by looking at the PpIX distribution in the bladder of a larger animal like a pig. Although ALA has rarely been tested with intravesical route[17,20], like other photosensitisers(AISPC), the results from such route of administration seem conflicting[8,17,20,21]. It is probable that different types of photosensitiser are taken up by different mechanisms which will lead to different tissue concentrations and distribution patterns after different routes of administration. Even for closely related agents, the chemical structure, spatial configuration, number of side chains and molecular weight are all determinants of the final distribution. Our experiments suggest that intravesical instillation of ALA might be an alternative to other routes because it leads to less skin photosensitisation but retains considerable PpIX selectivity between the urothelium and other layers.

3.5 Optimal drug and light dosimetry

Histological studies showed that the PDT effect was superficial and predominantly confined to the mucosa with a total light dose of 50J. Higher doses lead to full thickness damage and a few deaths. This result is very similar to that found with AISPC when the dose of AISPC was above the minimal effective dose (0.5mg/kg). We have not found a dose of ALA similar to the 0.5 mg/kg of AISPC at which we could get uniform mucosal ablation but no muscle damage, even with larger light doses, but it is possible that such a dose exists between 1% and 10% (20mg/kg and 200mg/kg). In rats, 10% seems an appropriate ALA concentration for bladder instillation. Little effect was seen with 1% or 0.1%. With any sensitzer given intravenously, the ratio of sensitizer concentration between urothelium and muscle is likely to be the same in rats and patients, but with the intravesical route, the need for drug to diffuse across a greater thickness of urothelium and lamina propria may give better selectivity in patients. Also, the red light used for PDT only has a penetration depth of a few millimetres, so although plenty of light will reach muscle in the rat, relatively little will reach muscle in patients.
again improving the selectivity. However, care must be taken on this aspect as clinical trials have shown that scarring is easily produced in muscle [6].

3.6 Optimal concentration of light scatterer

We demonstrated that the better concentration of intralipid for light scattering in the rat bladder with a volume of 0.2-0.3 ml was 10%. At this concentration, uniform urothelial sloughing with oedema of the lamina propria and fibrinoid change of vessels were observed. With the same treatment parameters but reducing the intralipid to 1%, urothelial damage was patchy. This finding was in accord with the prediction by Baghdassarian and associates who tested the uniformity of light distribution at different intralipid concentrations in flasks of various volumes[22]. In a sphere with a volume of 50ml or less, light tended to focus on the site opposite to the tip of the laser fibre(180°) and uniform distribution of light was not possible in the presence of 1% intralipid. Higher concentrations of scatterer lead to more uniform light distribution but at the expense of more energy loss. In the clinical situation, although the bladder is not an ideal sphere, a 1% intralipid would be the concentration of choice to obtain the maximum amount of light dispersion with the minimum amount of light absorption.

4. CONCLUSION

Before adopting PDT for clinical use, especially for the treatment of multifocal carcinoma in situ or diffuse low grade papillary bladder tumours, it is worth emphasising that for any therapeutic modalities to be clinically applicable, the ability to achieve a uniform urothelial eradication is crucial as complete removal of the urothelial lining is the only reliable proof of cure of this type of bladder malignancy. Using ALA as a photosensitiser, a similar degree of urothelial destruction to that resulted from AISPC could be observed but with a seemingly lesser degree of muscle damage [8] because laser of shorter wavelength at 630 nm was employed for the present study. Another probable advantage of ALA for bladder PDT is the rapid urothelial regeneration that by the 7th day after treatment is microscopically normal. The underlying mechanism could be that only the urothelium rather than the microvasculature in lamina propria is damaged. Better preservation of the lamina propria is a proof for faster urothelial regeneration. This might be of clinical significance since early healing implies less full thickness damage and a shortened period of functional impairment. The negligible collagen deposition and fibrosis in the lamina propria and muscle layer of the bladder 90 days after PDT treatment indicates that ALA is a milder photosensitiser with great potential for clinical application.

5. REFERENCES


Reprinted from

5th International Photodynamic Association Biennial Meeting

Application of 1,2-Diethyl-3-hydroxypyridin-4-one to Enhance Tissue Selectivity for Photodynamic Therapy of the Bladder

21–24 September 1994
Amelia Island, Florida
Application of 1,2-diethyl-3-hydroxypyridin-4-one to enhance tissue selectivity for photodynamic therapy of the bladder

Shi-Chung Chang, Alexander J. MacRobert, John B. Porter and Stephen G. Bown

1National Medical Laser Centre and 2Department of Clinical Haematology, University College London Medical School, University of London, United Kingdom
3Department of Urology, Tzu-Chi General Hospital, Hua-Lien, Taiwan

ABSTRACT

5-aminolevulinic acid (ALA) induced protoporphyrin IX (PpIX) has been proved to be a useful photosensitizer for photodynamic therapy (PDT). In living cells, the conversion of PpIX to photoinactive haem is catalysed by ferrochelatase in the presence of tissue iron and inhibition of this final committed step results in increased accumulation of PpIX. The in vivo effect of a new iron chelator, 1,2-diethyl-3-hydroxypyridin-4-one (CP94) on the buildup of PpIX in different bladder layers was evaluated. In CP94 treated rats, 5-7 hours after intravesical instillation of ALA solution, the fluorescence intensity of PpIX in the urothelium was doubled whilst in the muscle layer it remained low at a similar level to those seen without the iron chelator. With CP94, further reduction of skin photosensitisation is possible as similar photodynamic effect on the bladder could be achieved at lower ALA concentration. The addition of CP94 seems an effective and convenient way to potentiate ALA induced PpIX tissue selectivity.

Keywords: 5-aminolevulinic acid(ALA), 3-hydroxypyridin-4-ones(HPOs), 1,2-diethyl-3-hydroxypyridin-4-one(CP94), bladder instillation, tissue selectivity, Desferrioxamine(DFO), photodynamic therapy(PDT),

1. INTRODUCTION

Despite the potential of having preferential retention of sensitizers in tumour cells followed by selective tissue destruction after light illumination, photodynamic therapy has remained of limited clinical value, although it received more attention than ever in the expertise searching for new methods for the treatment of various malignancies[1-5]. Previous PDT studies using haemtoporphyrin derivative (HpD) or its purified ester/ether compound (Photofrin) in bladder cancer treatment revealed that, with careful planning, promising results were achievable [6,7] but the complications such as scarring of detrusor muscle and reflux uropathy could not be ignored [8]. These results have prompted the search for more selective photosensitisers. One of the most interesting photosensitiser currently under assessment involved 5-aminolevulinic acid which is a naturally occurring intermediate in the biosynthesis pathway for haem in living cells. In the liver, ALA is synthesised from glycine and succinyl Co-A under the catalyse of ALA synthetase and then in a series of reactions is metabolised to protoporphyrin IX, a potent photosensitiser, before being converted to haem in matrix mitochondria[9]. Through the introduction of an excess of ALA, the regulatory pathway is overloaded with PpIX which may then be used as a photosensitiser for PDT [10,11]. The bladder is an ideal organ for PDT as it is readily accessible and the entire transitional epithelium which is the site where carcinoma-in-situ and most superficial cancers arise can be treated as a whole. To achieve satisfactory treatment results, selective destruction of cancerous tissue, would be extremely attractive. With current urological techniques, papillary tumours which can be visually identified under cystoscopy pose no particular therapeutic problem as they can be eliminated with laser coagulation or electrocautery. However, intractable problems remain with grossly undetectable dysplastic or pre-cancerous foci that distributed through out the urothelium which tend to recur after treatment. If the diseased as well as normal urothelium can be eradicated completely with PDT while the bladder lining regenerates later with normal urothelium and without causing underlying muscle damage, then PDT might be the most attractive modality for bladder cancer treatment. It is with this rationale that we have focused on selective damage between normal bladder layers rather than on the difference between the urothelium and cancers.

The conversion of PpIX to haem catalyzed by ferrochelatase in the presence of iron is the final committed step in haem biosynthesis. In order to enhance tissue selectivity of PpIX, some methods have been tried with various degree of success. Approaches using PpIX as a photosensitiser directly administered to cells have been unsuccessful because of its low water solubility prohibiting transmembrane absorption and therefore a poor tumor localiser[12]. Modifying the delivery vehicle results in better cellular absorption but at the expense of diminished selectivity and altered pharmacokinetic properties of the
original porphyrins [13,14]. Another possible alternative to elevate cellular PPIX concentration is to inhibit the enzyme, ferrochelatase which regulates the conversion of PPIX to haem in the presence of iron. Chelating ferrous(ferric) ions seems a simple alternative approach to retard the conversion of PPIX to haem. Basic in vivo work has shown that cellular porphyrins accumulated after administration of chemicals such as ethylene-diamine-tetra acetic acid (EDTA). However, clinical application of EDTA is limited due to its systemic toxicity. For a long time, the only clinically available iron chelator used to treat blood transfusion related iron overload has been desferioxamine(DFO) which is expensive, has toxic side effects, and has to be given subcutaneously with a portable pump[15,16]. With the pressing need for an orally effective inexpensive iron chelating drugs, 3-hydroxypyridin-4-ones(HPO compounds), a family of bidentate iron chelators was designed by Hider and Kontoghiorghes [17]. This agent can be easily synthesised from the natural plant product maltol in a one step reaction [18]. These chelators form strong complexes with iron in the ratio of 3 to 1 at physiological pH and remove iron effectively in mice and rabbits [17].

Previous work by Ortel and associates claimed that iron deprivation by addition of DFO accelerates the PDT effect, and makes it more efficient at lower ALA concentration[15]. In this study, we examined the effectiveness of CP94 in escalating tissue PPIX level by fluorescence microscopy and compared the PDT treatment results between those with and without CP94 under the same drug and light dosimetry.

2. EXPERIMENTS

In this study, physiologically acceptable pH 5.5 ALA solutions at 10% and 1% were selected for bladder instillation. The techniques for anaesthesia andatraumatic intravesical instillation were described in great details in previous session of this SPIE volume[19]. In addition to ALA, CP94 at the dose of 200mg/kg body weight was given intraperitoneally at the same time of instillation to female Wistar rats(100-180gm). Two rats received only CP94 but without ALA were killed at 5 hours which served as control. Bladder specimens(at least 2 rats) were collected at 1, 2, 3, 4, 5, 7, 9 and 24 hours after drugs administration. Before removal, the bladders were rinsed twice transurethrally with normal saline in order to remove all residual ALA. Frozen sections at 10μm thickness were used for fluorescence imaging and CCD camera quantification.

For photodynamic therapy of the bladder, the rats were treated with copper vapour pumped dye laser (Oxford Lasers Ltd.) emitting red light at 630 nm and delivered through a 200μm silicon coated quartz fibre 5 hours after ALA and CP94 administration. For each study group, 8-10 rats were used. During treatment, the bladder was filled with 0.2 ml 10% intralipid as light scatterer and received laser power of 100 mW over 500 second. After laser treatment, rats were killed 1, 2, 3 and 7 days for histological assessment of PDT effect.

3. RESULT AND DISCUSSION

Comparing the quantitative fluorescence intensity of the kidney and bladder tissues between those with and without CP94 administration, it seemed clear that CP94 had substantial effect on cellular buildup of PPIX, as the levels of tissue prophyrrins were much higher in the former than in the latter(Fig 1, 2). After instilling 10% ALA, the urothelial PPIX intensity peaked at 6-7 hours when its level was twice as much as observed without iron chelator. The fluorescence intensity of lamina propria and muscularis propria were negligible in comparison to that of urothelium, and a ratio as high as 10 to 1 could be achieved at 5 hours. At lower ALA concentration(1%), the maximum urothelial buildup of PPIX was similar in intensity whether having CP94 or not(Fig 3).
This differential effect observed at the higher ALA concentration may be attributable to saturation of the final conversion step with PpIX cellular concentrations exceeding levels of available iron. It therefore seems reasonable to propose a 1% solution as the lower limit of ALA concentration for intravesical instillation if an incremental increase of cellular PpIX is to be achieved by oral CP94 when administered at the same time as ALA. It is also noteworthy that the PpIX escalating effect of CP94 was not evident until 3-4 hours after injection which again may be attributed to the saturation effect discussed above and possibly the presence of CP94 being excreted in the urine. The effect of CP94 on PpIX levels was abolished 7 hours which is compatible to what was demonstrated by Kontoghiorghes et al who showed that most of the absorbed HPOs was excreted within 5-6 hours through the urinary tract[20].

The histological findings after PDT were typically diffuse swelling of the lamina propria and generalised urothelial sloughing(Fig 4). In rare cases, full thickness bladder damage was possible when light dosimetry was suboptimal(Fig 5). Forty-eight hours after PDT, areas of shallow ulceration down to the lamina propria were observed with mild fibrinoid change of the small arterioles. Destruction of the muscularis propria, although patchy in nature, was detectable in the 10% ALA group but negligible in 1%. Qualitatively, comparing histological changes of the bladder sections taken from CP94 rats with those having identical treatment parameters but without CP94, a stronger PDT response was apparent in the former group. However, a quantitative comparison was not possible, as even in the same bladder, some variation in response is inevitable, most likely the result of inhomogenous light distribution.
Iron is essential for cellular homeostasis as all proliferating cells require iron for the functioning of M2 subunit of ribonucleotide reductase[21]. This enzyme is responsible for the free radical mediated reduction of ribonucleotides to deoxyribonucleotides, a key step in DNA synthesis[22]. Previous studies have shown that interruption of iron mobilisation by either desferrioxamine or HPOs may result in antiproliferative effects on lymphocytes[23] and various malignant cell lines[24,25]. However, in our investigation, we tried with another way of approach and proved that CP94 is an effective agent to increase cellular PpIX level through a mechanism by retarding its conversion to haem. It is also well established that HPOs are highly specific and selective for combining tissue iron(III)[26], and CP94 that exhibits the best lipid solubility also demonstrates the most efficiency in iron chelation[27].

4. CONCLUSION

We conclude that CP94 is an effective agent in escalating urothelial PpIX levels after 10% intravesical ALA. At lower ALA concentration, such as 1%, the effect of CP94 is marginal possibly as a result of inadequate saturation of ALA in the cytoplasm or mitochondria of the urothelium. With concomitant administration of CP94, similar PDT effect on the bladder can be achieved at lower ALA concentration which induces less cutaneous photosensitisation. Although chronic administration of HPOs, especially with CP20(1,2-dimethyl-3-hydroxypyridin-4-one), has been proved to be harmless to human being, we remain conservative in reaching a conclusion from this more lipophilic agent until further clinical proof.

5. REFERENCES


Interstitial Photodynamic Therapy of the Canine Prostate with Meso-tetra-(m-hydroxyphenyl)Chlorin and 5-Aminolaevulinic Acid: A Preliminary Study

14-16 September 1995
Barcelona Spain
Interstitial Photodynamic Therapy of Canine Prostate with Meso-Tetra-(m-Hydroxyphenyl) Chlorin and 5-Aminolaevulinic Acid: A Preliminary Study

Shi-Chung Chang1,2, Gio Buonaccorsi2, Alexander J. MacRobert1, Stephen G. Bown1

1National Medical Laser Centre and 2Department of Medical Physics, University College London Medical School, London, UK and 3Department of Urology, Tzu-Chi General Hospital, Hua-Lien, Taiwan

ABSTRACT

Photodynamic therapy (PDT) is proved to have potential for managing various malignancies. We investigated tissue biodistribution and photodynamic effects on a canine model in vivo using second generation photosensitisers, meso-tetra(m-hydroxyphenyl)chlorin (mTHPC) and 5-aminolaevulinic acid (ALA) to evaluate the feasibility and possible future application of PDT on the prostate. Using fluorescence microscopy, the optimal sensitisation time of the prostate was between 24-72 hours with mTHPC and, 3 hours with ALA. After optimum time of sensitisation, prostates of mature beagle were treated with laser at various sites by placing fibre interstitially under the guidance of transrectal ultrasound. The light dose for each treatment site was 100J (100 mW for 1,000 seconds at the wavelength of 650 and 630nm, respectively). With mTHPC, single laser fibre was able to induce organ confined PDT lesion as large as 20x18x18 mm in size. However, the PDT lesion with ALA was negligible 3 days after treatment. Physical distress manifested as urinary retention, poor appetite and body weight loss, was more prominent with increasing number of treatment sites as a result of extensive prostatic swelling and urethral damages. However, these problems usually alleviated spontaneously 7 to 10 days after PDT. The characteristic histological changes were hemorrhagic necrosis and glandular destruction with preservation of interlobular collagen fibres. Urethral damage seen at the early stage healed by regeneration of urothelium in 4 weeks. We conclude that interstitial PDT with mTHPC is technically possible to produce extensive glandular necrosis in the normal prostate which heals safely and does not change the prostatic architecture. ALA, although seems promising for bladder tumours, is much less effective than mTHPC on the prostate. With mTHPC, it might have the potential for treating prostate cancers localised in the periphery of the gland.

Keywords: Photodynamic therapy, Prostate, Meso-tetra(m-hydroxyphenyl)chlorin (mTHPC), 5-aminolaevulinic acid (ALA), Photosensitisers, Hemorrhagic necrosis

1. INTRODUCTION

Prostate cancer is the most prevalent internal cancer in men [1] and the expected number of new cases in the U.S.A. is at least 200,000 in 1995 [2]. Since the current treatment options for localised cancer such as radiotherapy and radical prostatectomy are far too traumatic, the need for more effective treatments is becoming more urgent than ever. Photodynamic therapy (PDT) is a technique combining delivery of a photosensitiser and subsequent light illumination to produce photochemical tissue destruction [3]. Applying haematoporphyrin derivative (HpD) or the more purified ether derivative [Photofrin II) as photosensitisers, PDT has been shown to be rather promising in treating malignancies of the gastrointestinal tract [4], lung [5], oral and maxillofacial regions [6] and urinary bladder [7,8]. Despite encouraging experimental results in other organ systems and the potential for preferential destruction of malignant tumours with PDT [9], there are few relevant publications on the prostate. Camps et al [10], Pantelides et al [11,12], Chen et al [13] were among the pioneers performing basic PDT studies on the prostate. The only clinical report is from Windahl et al [14] who treated 2 patients using Photofrin mediated PDT to control residual prostate cancer after previous transurethral surgery. They reported significant reductions in the prostatic specific antigen (PSA) after PDT and in one patient who subsequently died of metastases, there was no evidence of residual cancer in the prostate itself. In the present study, we investigated PDT in the normal canine prostate using the new powerful photosensitiser meso-tetra-(m-hydroxyphenyl) chlorin (mTHPC), which has certain advantages over Photofrin II (15), and the endogenous photosensitiser precursor 5-aminolaevulinic acid (ALA), which has been extensively studied in various medical subspecialities [16-19]. mTHPC is a pure compound with an absorption peak at 650 nm allowing deeper tissue penetration than the red light at 630nm used for Photofrin and which has proven to be a potent photosensitiser with some tumour selectivity (20). ALA is an intermediate synthesized from glycine and succinyl coenzyme A in the biosynthetic pathway for the production of haem in mammalian cells. In this biochemical chain reaction, ALA is
converted through a series of intermediates to protoporphyrin IX (PpIX), the predominant porphyrin species responsible for photosensitisation [21]. Since the conversion of PpIX to photoinactive haem is the rate limiting step in the chain of reactions between ALA and haem, bypassing the physiological feedback mechanism with excessive exogenous ALA results in temporary accumulation of PpIX.

In this study, we investigated the pharmacokinetics of mTHPC and ALA in the prostate and the nature, extent and healing of PDT lesions produced with light delivered interstitially. As most normal glandular tissues heal remarkably well after PDT, usually by regeneration rather than by scarring, and with little effect on connective tissues like collagen [22]. If the glandular tissue of the prostate could be destroyed with interstitial photodynamic therapy while the architecture and continuity of the prostatic urethra was preserved, the clinical significance might be immense as this would offer the chance to eradicate cancer tissue in the periphery of the prostate without losing its anatomical alignment and functional integrity. There are unlikely to be any unacceptable effects in adjacent tissues such as the rectum, the urethral sphincter and the neurovascular bundle after having PDT.

2. EXPERIMENT

11 Animals and anaesthesia

Twelve mature ex-breeder beagles (3-7 years old) weighing between 12 and 21 kg were studied. They received pre-medication of Fentanyl/Fluanisone (Hypnorm) 0.1-0.2 ml/kg by intramuscular injection and prophylactic antibiotics (2.5% Enrofloxacin) 1 ml/5kg subcutaneously. After intubation they were maintained under inhalation anaesthesia using Halothane 1-2% and nitrous oxide as a 50:50 or 60:40 mixture with oxygen on a Magills anaesthetic circuit. Stable anaesthesia was maintained throughout the procedures and recovery was uneventful in all cases. Postoperatively, they all received a 3-day course of non-steroidal anti-inflammatory analgesic, Flunixin meglumine 1 mg/kg subcutaneously and were kept in subdued lighting during their subsequent period of observation.

12 Photosensitiser

mTHPC was supplied by Scotia Pharmaceuticals Ltd (Guildford, UK), and dissolved in a solution composed of ethanol, polyethylene glycol and distilled water in the ratios of 2:3:5. This was given intravenously at a dose of 0.3mg/kg through the antecubital vein. ALA was kindly donated by DUSA Pharmaceuticals Inc., (New York, USA), dissolved in normal saline and titrated with saturated sodium bicarbonate solution to a final pH of 5.5, at a concentration of 10% (100mg/ml). The agent was prepared immediately prior to intravenous injection and was given at a dose of 200 mg/kg.

2.3 Transrectal ultrasound guided biopsy

After sensitising the prostate with mTHPC, biopsies were taken from the prostate at 1, 3, 5, 24, 48, 72 and 168 hours (2-3 biopsies at each time point). For ALA, specimens were taken serially at 30 min, 1, 3, 5 and 24 hours after sensitisation. Biopsies in the first 5h were taken under the cover of the initial anaesthetic while the latter ones were done under heavy sedation with intramuscular Hypnorm (0.1-0.2 ml/kg) and an oxygen mask. After cleaning the perianal and perineal area with chlorhexidine, a biplanar transrectal ultrasound probe (Aloka, 7.5 MHz) was inserted into the rectum (Figure 1). Transperineal biopsy was then done under ultrasound guidance using an 18 G needle mounted on a spring operated device. The specimens were placed on a sheet of paper before being stored in liquid nitrogen.

Figure 1: Interstitial PDT via transperineal approach.
2.4 Fluorescence microscopy

An inverted phase contrast microscope (Olympus IMT-2) attached to a high resolution (385 x 578 pixel) slow-scan charge coupled device (CCD) camera (model 1, Wright Instruments Ltd, London) was utilized for quantification and microscopic imaging of tissue fluorescence from the prostate biopsies. The setup of this highly sensitive photometric system has been described previously [23] and is shown below (Figure 2). In brief, fluorescence was excited using an 1.8 mW helium neon laser operating at 543 nm, with the output directed through a liquid light-guide (via a 10 nm bandpass filter) on to a dichroic mirror. Fluorescence was detected in the range 630 to 680 nm, using a combination of bandpass and longpass filters (Omega Optical Inc., Vermont). The fluorescence signal was processed by an IBM PC clone into a falsely colour-coded image depicting the signal in counts per pixel. The image processing software enabled the fluorescence intensity in each tissue section to be quantified digitally by averaging over specified areas. After fluorescence imaging, the sections were fixed in formalin and stained with haematoxylin and eosin for histological study.

There was no detectable mTHPC fluorescence in the unsensitised control dog. The mTHPC fluorescence intensity in the prostate was evaluated up to 7 days. The highest levels of fluorescence were seen at the first time point, 1 hour after sensitisation. There was a biphasic distribution pattern with a second small peak at 24 hours. The ALA induced protoporphyrin IX fluorescence peaked at 3 hours and gradually returned back to baseline level 24 hours after sensitisation (Figure 3).

2.5 Photodynamic therapy

2.5.1 Light Dosimetry

A KTP/532 laser unit pumping a Dye Laser Model 630 (Laserscope, San Jose, CA) tuned to emit red light at the wavelength of 630 (ALA) and 650nm (mTHPC) and at the power of 100 mW was used in this study. After determining the appropriate treatment site in the prostate using transrectal ultrasound, a 600 micron bare tip fibre was advanced through the lumen of the biopsy needle until it was 8-10mm beyond the bevel. The power of the laser was checked before and after each treatment. The lasering time was 1,000s, making a light dose of 100 J for each treatment site.

2.5.2 Interstitial PDT treatment

One animal had laser illumination (100 J) only without photosensitisation. Three ALA sensitised beagles were treated 3 hours after drug administration with 1 treatment site in each. For the rest 6 animals, 72 hours after
sensitisation with mTHPC, through the transperineal route, 2 to 4 different sites were treated by inserting a laser fibre interstitially into the prostate under the guidance of transrectal ultrasound. The usual sites of treatment were one at proximal prostate near the bladder neck and capsule, one at apical region immediately adjacent to the urethra, 2, one in each lobe, at the middle portion of the prostate 8-12 mm lateral to the urethra. After treatment, the dogs were kept in subdued lighting and observed at least daily for any signs of general distress. The dogs were killed with intravenous Expiral (Pentobarbitone, 120 mg/kg body weight) 3, 7, 14, 28 and 60 days after PDT and the bladder and prostate removed en block. For gross inspection, the specimens were sectioned serially from the bladder neck to apical region at intervals of 5-7 mm. Selected areas were sectioned for histological examination with H & E and VanGieson's stains.

2.5.3 General responses after PDT treatment

The dogs were kept in a chamber with subdued light, and there was no overt evidence of phototoxicity observed throughout the follow-up period after PDT treatment. In the 6 animals having 1 or 2 treatment sites in prostate, there was no evidence of gross hematuria, difficulty in micturition or signs of physical distress, and their appetite remained roughly the same after PDT treatment as it had been before. However, with increasing treatment sites, micturition difficulty, mild hematuria and signs of distress (poor appetite, slow striding and loss of body weight) began to appear 24 hours after PDT. The PDT associated morbidity usually subsided spontaneously by the 7th post-treatment day.

2.5.4 Macroscopic findings

In the control dog receiving only light, no lesion was found macroscopically. In the ALA experimental group, neither gross swelling nor subcapsular hyperemia was discovered upon dissection. The prostatic cutting surface examined 3 days after treatment showed only negligible lesion (1 mm). There was no detectable ALA induced PDT lesion in the prostate at 7 and 28 days. For mTHPC sensitised group, early lesions were striking which comprised of focal ecchymosis on prostate capsule and boggy swelling. The intraperitoneal organs and the rectum immediately adjacent to the prostate were free from any gross lesions 3 days following PDT. On cutting, the lesion produced by 2 consecutive fibres was a large dumbbell shaped hemorrhagic necrotic area, centred around the two treatment points, measuring 40x25x25 mm which occupied the central portion of the prostate leaving a slender rim encircling the rest of the organ (Fig 4). With more treatment sites, elliptical lesions up to 40x35x35 mm were induced. The capsule overlying the prostate was intact although the lesion inside was extensive. The lesion seen at 7 days was slightly smaller, but otherwise macroscopically similar to that seen at 3 days. Four weeks after PDT, the prostate appeared normal grossly but on cutting, brownish scars measuring 8x6x6 mm in size was observed beneath the prostate capsule, with or without periurethral tissue congestion (Fig 5). There were no evidence of deformity or collapse of the prostate at any of the times after PDT studied in this project.

2.5.5 Microscopic findings

![Figure 4: PDT (mTHPC) lesion induced by 2 laser fibres.](image1)

![Figure 5: Gross change of the prostate 28 days after PDT.](image2)
ALA induced prostate lesion although barely detectable on gross inspection, was histologically characteristic of interstitial RBC extravasation and minimal haemorrhagic necrosis. At 7 days, focal inflammatory cells infiltration was salient. On the contrary, Early mTHPC induced lesions revealed extensive hemorrhagic necrosis and complete destruction of the glandular epithelium with hemosiderin deposition in the area corresponding to the lesion seen macroscopically (Fig 6). Urethral damage was extensive during the first 2 weeks, and was characterised by loss of urothelium and subepithelial hemorrhage and inflammatory cell infiltration. Despite complete healing of the urothelium of the prostatic urethra by 4 weeks after PDT, the glandular structure of the prostate was atrophic, and there was intense fibrosis in the periurethral area and some residual inflammatory cell infiltration. Generally, the slender collagen fibrils in the intraductal acinar were destroyed together with glandular tissue by PDT. Hoewever, for the supporting collagenous stroma which connecting the capsule and extending into the interlobular septa of the glandular proper, it remained well preserved after PDT treatment.

Figure 6: Microscopic change of PDT lesion(x40).

3. DISCUSSION

The number of cases in the United States died of prostate cancer was approximately 35,000 in 1994 [24]. Most, if not all would agree that organ confined cancer is potentially curable if the disease could be detected and treated earlier. Current treatment for localised cancer in patients having a life expectancy over 10 years with radical prostatectomy or radiotherapy is likely to be challenged by other options with less treatment associated morbidity in the foreseeable future. PDT is still largely experimental, but has the potential to destroy extensive prostate tissue while maintaining the structure of the organ after treatment, which is important for a satisfactory outcome. Encouraged by the preliminary clinical results from Windahl et al [14], we investigated PDT in the normal canine prostate using interstitial light illumination and the second generation photosensitisers, mTHPC and ALA. The biodistribution results in our study showed a biphasic configuration after mTHPC given intravenously. The early peak with the highest levels seen at the first time studied, 1 hour after injection, is probably due to mTHPC in the blood. The second, smaller peak at 24 hours is more likely due to photosensitiser in the glandular structure of the prostate. The differences between 24 and 72 hours are not great. Ris et al [25] suggested that the best therapeutic ratio between tumour and normal tissue was seen at 3 days in mesothelioma, we therefore chose 72 hours as the time interval for light illumination. However, care must be taken when applying data from beagles for further clinical trials as the glandular to stromal ratio as well as embryological development of prostate in the 2 species are different.

The results of ALA mediated PDT in the canine prostate are disappointing, as the lesion produced was only 1 mm in diameter, this could be due to low PpIX levels in the gland, or an inadequate light dose. At similar PpIX level in rat urinary bladder after intravesical instillation of ALA (as measured by fluorescence microscopy), we were able to destroy the urothelium without damaging the underlying muscle with appropriate light doses [17]. However, the thickness of the rat bladder wall is less than 1 mm, so the absolute depth of effect was no greater than we found in the prostate. Johnson et al [26] recently reported PDT induced haemorrhagic necrosis up to 10mm in diameter periurethrally using a higher light dose (650 mW for 45 min) to treat the canine prostate 8 hours after ALA 100mg/kg given intravenously. This finding contrasts with our recent clinical observation that it was only possible to get necrosis up to 1-2mm in depth in gastrointestinal tumors [27] although we did get larger necrosis in an animal tumor [28]. Differences in light dosimetry (100 vs 1755 J), laser power (100 vs 650 mW) and sensitization time(3 vs 8 hours) are possible explanations for the varied photodynamic findings in the prostate observed by the two groups. ALA looks very promising for treating superficial lesions with PDT, but more questionable when a greater depth of effect is required as in the management of prostate cancer. From our results, ALA is not very likely to be of value for PDT in
the prostate unless ways are found to markedly increase the depth of necrosis produced. Just increasing the light dose seems unlikely to do this, although there could be some benefit from fractionating the light [29].

The early histological findings were of haemorrhagic necrosis confined to the prostate tissue which healed with mild fibroblastic infiltration and glandular atrophy but without altering the basic structure of the prostate. The urethral damage characterised by urothelial sloughing and subepithelial oedema and haemorrhage was still present 14 days after PDT. By 28 days, regeneration of urothelium was complete, although there was evidence of subepithelial fibrosis. These findings of glandular atrophy with preservation of the prostate architecture and prompt regeneration of urethral epithelium without apparent functional disturbance are just what is required to treat localised prostate cancer. Recently, Selman and Keck [30] used the photosensitiser Tin(II) etiopurpurin dichloride (SnET2) with light delivered transurethrally for PDT on normal canine prostate, and showed similar results to ours with good healing of the urethra. They concluded that its use for the treatment of benign prostatic hypertrophy (BPH) warranted further investigation. We have reservations about this conclusion as neither their experiments nor ours showed any objective evidence of reduction of outflow tract resistance, which is the main aim of treatments for BPH. Moreover, the long term effects, both functional and anatomical, on the prostate tissue remain to be answered. Using traditional transurethral resection of the prostate (TUR-P) or contemporary laser coagulation and vapourisation technology, BPH can be satisfactorily resolved at minimal surgical risk and complications. There must also be concern over whether PDT might damage the urethral sphincter, bladder neck or the neurovascular bundle. These possibilities have not yet been adequately explored, but even if the risk is low, it would be a high price to pay for treating a benign disease. Further, there is the problem of skin photosensitisation which may necessitate avoiding bright lights for several weeks, depending on the photosensitiser used.

It is our belief that PDT is more suitable as a technique for treating localised prostate cancer than for the treatment of BPH. Recent reports show that elevated levels of PSA identify a group of patients at high risk of developing or already having prostate cancer [24]. This makes it likely that in the future, more early cancers localised to the gland will be diagnosed in asymptomatic patients. PDT could be an important new development in their management, particularly as the treatment seemed to cause so little in the way of general or urological distress to the animals.

4. CONCLUSIONS

Photodynamic therapy in the normal prostate with the photosensitiser mTHPC causes necrosis of the glandular tissue which heals safely without disturbance of the architecture of the gland. There is sloughing of the urothelium of the prostatic urethra, but this heals by regeneration without disturbing micturition at any time after treatment as long as the entire urethra is not fully involved. As malignant tissue in the prostate is likely to respond at least as well as the normal, we conclude that PDT with mTHPC is a promising option for the treatment of prostate cancer limited to the gland, although further investigation is needed to test its efficacy in the human prostate and to clarify the risk of unwanted effects on the adjacent normal structures. ALA is likely to benefit superficial lesions such as bladder carcinoma-in-situ or precancerous lesions of the alimentary tract, does not hold a same promise for prostate diseases as the depth of necrosis is limited.

5. ACKNOWLEDGMENT

We are grateful to Dr. D. Rickards, Consultant Radiologist at the Middlesex Hospital, London, for loan of the transrectal ultrasound scanner, and to LaserScope (California, USA) for loan of the KTP pumped dye laser system. Dr. Shi-Chung Chang is funded by the Compassion Relief of Tzu-Chi Foundation in Taiwan and Professor Stephen Bown by the Imperial Cancer Research Fund, London, UK.

6. REFERENCES


