Application of Photodynamic Therapy in Head and Neck Oncology

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by

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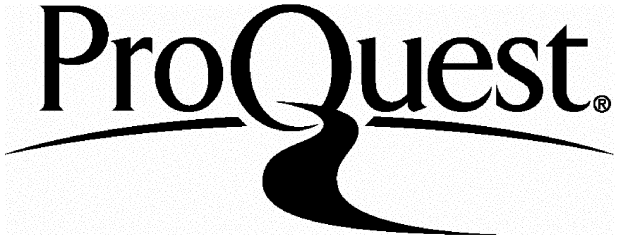

Abstract

Photodynamic Therapy in Head and Neck Oncology

Photodynamic therapy (PDT) involves the activation of a previously administered photosensitising agent by light resulting in the formation of cytotoxic oxygen species capable of tissue necrosis. The aim of this thesis was to evaluate the role of PDT in the treatment of tumours of the head and neck region both preclinically and clinically.

Preclinical studies were carried out on a rabbit model to determine the effect of PDT on normal bone using tumouricidal doses of drug and light. Minimal effects were seen on normal bone using three photosensitisers; Photofrin® , aminolaevulinic acid (ALA) and meta tetrahydroxyphenyl chlorin (mTHPC).

PDT is an attractive option for the management of nasopharyngeal carcinoma (NPC). Initial pharmacokinetic and PDT studies were carried out on normal rabbits using two photosensitisers, mTHPC and phthalocyanine (AlS2Pc) prior to embarking on clinical therapy. The concern with PDT in the nasopharynx is the close proximity of the brain. Both sensitisers were taken up in the nasopharyngeal mucosa, with no detectable levels in the brain. Using light doses that produced mucosal necrosis in the nasopharynx, no neurological deficit Nor histological damage was observed. Light transmission studies through the skull base confirmed that little light reached the brain from the nasopharynx.

The clinical role of PDT was evaluated in patients with oral cancer and dysplasia, using three photosensitisers; Photofrin®, ALA and mTHPC. Encouraging results were observed in patients with field cancerization following Photofrin® PDT, although the prolonged skin photosensitivity was a major disadvantage. ALA PDT was found to be very effective in superficial epithelial disease (dysplasia). For early invasive disease the best results were seen using mTHPC. Seventeen out of twenty-one T1 and T2 cancers showed complete local responses along with much lower light doses than required for other photosensitisers.

With an appropriate choice of photosensitiser, PDT is developing an important role in the management of premalignant and early invasive disease of the mouth.

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Dedication

To my parents
and family
for their love and support

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Statement of Originality

The experiments described in this thesis were performed by the author and has not previously been entered for a higher degree or award of this or any other University. The study on normal bone was planned following discussion with Professor S.G.Bown (Professor in Laser Medicine and Surgery). The pharmacokinetic studies in Chapter 6 were planned with the help of Dr A.J.MacRobert (Senior Lecturer), whilst advice was obtained from Professor Bown, Professor Speight (Professor in Oral Pathology) and Mr Hopper (Senior Lecturer and Consultant in Maxillofacial Surgery) concerning the PDT experiments in the nasopharynx. The light transmission study (Chapter 7) was my own idea, but planned with the help of Drs G. Buonaccorsi and P.Ripley. The clinical sections of this thesis (Chapter 8-10) were carried out under the guidance of Mr C. Hopper and Professor Bown.

Abbreviations

ALA	5-aminolaevulinic acid
AlS2Pc	Aluminium disulphonated phthalocyanine
CCD	Charge coupled device
CT	Computerised tomography
DHE	Dihaematoporphyrin ester/ether
H&E	Haematoxylin and eosin
HpD	Haematoporphyrin derivative
IV	Intravenous
Laser	Light amplification by the stimulated emission of radiation
MRI	Magnetic resonance image
mTHPC	meta tetra hydroxyphenyl chlorin
NPC	Nasopharyngeal carcinoma
$^1O_2$	Singlet oxygen
PDT	Photodynamic therapy
PII	Photofrin®
PpIX	Protoporphyrin IX
SCC	Squamous cell carcinoma
SnET2	Tin etiopurpurin dichloride
UDNT	Undifferentiated nasopharyngeal tumour

Chapter 1

ORAL SQUAMOUS CELL CARCINOMA AND NASO-PHARYNGEAL CARCINOMA

1.1 INTRODUCTION

Head and neck tumours encompass tumours affecting the following sites:

Lip, oral cavity (ICD-O 140, 141, 143-145)
Pharynx (ICD-O 141.0 , 145.3, 4, 146-148)
Larynx (ICD-O0161)
Maxillary sinus (ICD-O 160.2)
Salivary glands (ICD-O 142)
Thyroid gland (ICD-O 139)
(TNM Atlas, 4th edition, UICC (1987)

This thesis is restricted to carcinomas of the oral cavity and nasopharynx and investigates the role of photodynamic therapy with these neoplasms. Over 90% of all malignancies of the oral cavity are squamous cell carcinomas, whilst the most common form in the nasopharynx is the undifferentiated carcinoma.

ORAL CANCER

1.2 EPIDEMIOLOGY

1.2.1 Geographical Variations

The prevalence of oral cancer varies dramatically with geographical location (Parkin *et al.*, 1992). Oral cancer is the sixth most common form of cancer with an estimated 412,000 new cases world-wide (Parkin *et al.*, 1993). In England and Wales there are approximately 2400 newly diagnosed cases of oral cancer each year, with an overall incidence of 4.5 per 100,000 (Office of Population Censuses and Surveys, 1994). In most western countries oral cancer only accounts for 1-4% of all malignant disease (Johnson & Warnakulasuriya, 1993), however, in parts of India and South East Asia, these tumours are the most common form of cancer, sometimes causing over 40% of all cancers (CRC, 1993; Parkin *et al.*, 1992). High rates are also observed in four regions of France (Bas Rhine, Doubs, Calvados and Somme), whilst the lowest incidence rates have been reported in Japanese men and various population groups in China, Africa and America (Parkin *et al.*, 1993). There is evidence that the rate of oral cancer has been increasing over the last three decades in the United Kingdom (Johnson &

Warnakulasuriya, 1993), similarly increases in mortality have been observed in other parts of Europe including Denmark (Moller, 1989) as well as the United States (Davis & Saverson, 1987).

1.2.2 Age

The prevalence of oral cancer increases with age. In the West, 98% of cases are over 40 years of age (Kroll & Hoffman, 1976; Silverman, 1990) and the overall incidence rises from 4.5 per 100,000 to 100 cases per 100,000 in those aged over 75. In regions where there is a higher prevalence, the disease is more common in a younger group, often occurring before 35 (Davis & Saverson, 1987; Johnson, 1991). Of most concern are the large numbers of teenagers using oral snuff in America which has been linked to the development of oral cancer in the third and fourth decade (Davis & Saverson, 1987).

1.2.3 Mortality

Early detection of oral cancer is associated with better prognosis in terms of increased chance of survival and prolonged survival time (Silverman, 1988; Speight & Morgan, 1993). The overall age adjusted death rate for oral cancer (ICD9 140-149) is 2.77 per 100,000 in England and Wales, and 3.85 per 100,000 in Scotland. (Boyle *et al.*, 1990). The crude 5 year survival figure for intraoral cancer is approximately 30-40% (Johnson, 1991). In Scotland death rates from oral cancer have increased in younger age groups since 1960 (MacFarlane *et al.*, 1992). A similar trend can be seen in England where an increase in intraoral cancer has been observed, especially in females and in a younger age group of males (Hindle & Nally, 1991). Advances in treatment have been most marked in reconstructive techniques and though this has improved quality of life, cancer of the tongue and elsewhere in the mouth still has a disturbingly high mortality which has not improved for many decades (Aoki *et al.*, 1992; Miller *et al.*, 1992; Stell & McCormick, 1985) .

1.2.4 Sex distribution

In the UK oral squamous cell carcinoma (SCC) is almost twice as common in males compared to females with ratios 1.8:1 (Cancer Research Campaign 1993). This trend is generally reflected in most Western countries, however in regions of high prevalence, e.g. India, the converse is found which is thought to be related to tobacco and betel nut chewing (Boyle *et al.*, ; Johnson & Warnakulasuriya, 1993). A difference in smoking and alcohol intake is believed to account for the trend in the West (Moller, 1989).

1.2.5 Anatomical location

The most common site of oral squamous cell carcinoma is the tongue, followed by the floor of the mouth, the alveolar mucosa, buccal mucosa, the lower lip and the palate (Silverman 1988).

1.3 RISK FACTORS

1.3.1 Tobacco

It is known from many studies that oral SCC and other mucosal lesions such as leukoplakias are strongly associated with tobacco usage. Smoking has been associated with an increased risk of oral cancer (Sterling *et al.*, 1992). It has been reported that over 75% of cases of intraoral cancer can be accounted for by tobacco habits (IARC, 1986).

In Western Europe, the majority of tobacco related oral cancers result from active smoking (Johnson & Warnakulasuriya, 1993), whilst in high risk countries smokeless tobacco is more important. The risk from smoking is strongly dose related. People who smoke >40 cigarettes daily have been estimated to have a 6.9 times greater risk than non smokers (Keller and Terris 1965). There is ample evidence that tobacco in the non smoked form (i.e. chewed and snuff-dipping), most commonly consumed in a 'betel quid' or 'pan,' is the major risk factor associated with oral cancer.

1.3.2 Smokeless tobacco: Oral snuff

Many studies have shown a correlation between the use of smokeless tobacco and oral leukoplakia, a premalignant lesion (Grady *et al.*, 1990; Mehta *et al.*, 1981). In South East USA, an increased risk of mortality from oral cancer was observed in females, where the topical use of tobacco is common amongst textile workers (Mason *et al.*, 1977). A four-fold increase in the risk of oral and pharyngeal cancer and a fifty fold increase in cancer of the gingivae and buccal mucosa has been reported in a similar female group and in long term users of smokeless tobacco (Winn *et al.*, 1981). Conversely, smokeless tobacco (either as snuff or chewed) has also been reported not to increase the risk of oral cancer or cancer of the digestive organs (Sterling *et al.*, 1992).

1.3.3 Tobacco and betel quid chewing

A betel quid contains betel leaf with areca nut and slaked lime, and sometimes tobacco is added. A number of variations exist in different parts of India e.g. powdered tobacco and lime is used in Khaina. There is, significant evidence relating betel-quid chewing and oral cancer in the Indian population (IARC, 1985). Users of tobacco quid, especially if they also smoke, have a 10-12 times greater risk of developing cancer than those who neither smoke nor chew (Gupta *et al.*, 1982). The relative risk (rr) of developing oral cancer with tobacco chewing (rr=5.98) is greater than that of smoking (rr=2.82) and such habits are likely to be maintained in the Southern Asian immigrant populations to this country (Jayant *et al.*, 1977).

1.3.4 Alcohol

The association of alcohol and oral cancer is difficult to isolate as many who consume heavy quantities of alcohol also smoke. Nevertheless, the high intake of all forms of alcohol are associated with increased risks (IARC, 1988). The synergistic effect of both factors is well documented with figures of relative risk (rr)as high as 70 (Brugere *et al.*, 1986; McCoy & Wynder, 1979).

1.3.5 Other risk factors

A number of studies have suggested that various nutritional factors influence the likelihood of developing oral cancer. A low intake of foods containing vitamin A, β-carotene and vitamin C has been associated with an increased risk of oral cancer (McLaughlin *et al.*, 1988). By contrast, consumption of adequate quantities of raw green vegetables and fresh fruit are associated with a lower risk of oral/pharyngeal carcinoma (Steinmetz & Potter, 1991). There is also some evidence that dietary iron may play a role in maintaining epithelial thickness. Iron deficiency states may be associated with increased susceptibility to upper aerodigestive tract (UADT) cancer (Warnakulasuriya & Prabhu, 1992). High fat consumption has also been shown to be a risk factor for oral cancer, with an odds ratio of 1.3:2.1 (Marshall *et al.*, 1992).

The hypothesis that candida increases the risk of oral cancer, stems from findings of candidal hyphae in some dysplastic and neoplastic lesions (Pindborg, 1980). In addition to the higher frequency of malignant transformation in such lesions (Scully *et al.*, 1991). Other organisms which have been implicated in oral cancer include human papilloma virus (types 16 & 18) and Epstein-Barr virus. The question remains whether these organisms are coincident or causative. Physical factors such as mechanical and traumatic injury to the oral mucosa have also been suggested as causative agents, as well have mouth washes which contain alcohol. The risk of malignant transformation of precancerous lesions and conditions has been well studied and will be described later.

1.4 PATHOLOGY

1.4.1 Histopathological features

Many oral cancers arise in clinically normal mucosa but some will be preceded by a premalignant lesion. Oral SCC are most commonly classified histologically relative to the degree of differentiation or keratinisation according to the system originally devised by Broders in 1920, since when slight revisions have been made. Broders classified the

tumours into four groups based on the cellular differentiation and percentage of total cellular elements (Broders, 1941). However this system has been shown to be of poor prognostic value (Anneroth *et al.*, 1987; Bryne *et al.*, 1989) and reproducibility (Anneroth & Hanson, 1984). Subsequently, multifactorial grading systems, introduced by Jacobsson et al (1973), were reported to be of better prognostic value (Anneroth *et al.*, 1987). Numerous modifications of Jacobsson's system exist e.g. Anneroth et al (1987) which evaluates three tumour features in the less differentiated parts of the tumour and the histological relationship between the tumour cells and the surrounding stroma. Further modification was proposed by Bryne et al (1989), where only the most invasive areas of the tumour are graded.

Table 1.1 illustrates the factors used in the grading system.

Tumour cell features	**Score**
1. Degree of keratinisation	1-4
2. Nuclear polymorphism	1-4
3. Number of mitosis	1-4
Features of relationship between tumour cells and connective tissue	
4. Pattern of invasion	1-4
5. Leukocyte infiltration	1-4
Total malignancy score	(sum of scores features 1-5)

Adapted from(Bryne *et al.*, 1989)

1.4.2 Histopathological features of prognostic significance

Using the grading system shown in Table 1.1, correlation was observed between histological grading and the size and stage of the tumour and hence prognosis has been observed (Bryne *et al.*, 1989). Other histological features of prognostic value include the depth and pattern of invasion and DNA content (Bryne, 1991). It has been suggested that tumours which exceed 8mm in depth carry a worse prognosis (Shingaki *et al.*, 1988). The sites of the tumour must also be considered, as shallow tumours in one region may metastasise early due to superficial lymphatic drainage in that region.

The presence and extent (number of nodes involved) of metastatic disease in cervical lymph nodes, along with extracapsular spread is inversely related to survival (Woolgar *et al.*, 1995b). Perineural infiltration has also been shown to be adversely associated with prognosis.

In the UK, the death: registration ratio for oral SCC is approximately 0.5, but if lesions of the lip are excluded a ratio of 0.6 is recorded for other intraoral sites. This means that 60% of patients with intraoral cancer will die of their disease. The outcome depends on the site and size of the cancer. For small SCC, a retrospective study observed positive correlation between metastasis and Broders grade, pattern of invasion and invasive front total score. Whilst local recurrence was associated with Broders grade, keratinisation at the invasive front and pattern of invasion (Odell *et al.*, 1994). Using the TNM system (see below), the five year survival figures for T_1-T_2, T_3 and T_4 tumours of the buccal mucosa are 61-46%, 33% and 10% respectively (Gerbault & Pernot, 1985). Tumours in the retromolar trigonum range from 27-40% (Berktold, 1985) and the 5 year survival for carcinoma in the tonsillar region falls from 67% for T_1, N_{0-1} disease to 15% in the T_3, T_4, N_2, N_3 disease (Batani, 1986).

1.5 FIELD CANCERIZATION

One of the main problems associated with head and neck cancer is that even with the successful treatment of the initial primary cancer, a high percentage of patients develop additional malignancies. Slaughter et al (1953) reported a high incidence of second primary cancers in patients with head and neck squamous cell carcinomas and proposed the concept of "field cancerization" as the explanation. The was based on the histological observation of independent foci of in situ or invasive cancer surrounding the tumour. It was hypothesised that carcinogen-induced changes occur throughout the mucosa of the upper aerodigestive tract. Since then numerous authors have confirmed Slaughter's findings (Licciardello *et al.*, 1989; Lippman & Hong, 1989; Maisel & Vermeersch, 1981; McGuirt *et al.*, 1982).

Head and neck cancer is now believed to progress through a series of well defined clinical and histopathological stages (Licciardello *et al.*, 1989). There is evidence to suggest that the occurrence of multiple tumours arise from a single clone, following the observation of similar chromosomal changes in distinct specimens obtained from patients with multiple tumours (Bedi *et al.*, 1996). In a study on 87 head and neck lesions, adjacent areas of tissue with different histological appearance shared common genetic changes. The more histologically advanced the lesion, the greater degree of chromosomal change (loss of heterozygosity). Increasing genetic alterations were seen from benign hyperplasia to dysplasia to carcinomas *in situ* to invasive cancer (Califano *et al.*, 1996). This suggest that determination of the genetic status of the primary tumour and surrounding tissue may have important implications of the likelihood of recurrence and or progression.

The presentation of these further tumours is classified by their temporal sequence; new lesions may be simultaneous (present at time of diagnosis of primary tumour), synchronous (diagnosed within 6 months of initial cancer) or metachronous (those found later than 6 months of initial diagnosis). The magnitude and severity of this problem in patients with oral and oropharyngeal cancers has been demonstrated in several studies. The rate of development of second primary tumours was found to be in the region of 3-4% per year(Day & Blot, 1992; Licciardello *et al.*, 1989), with reported incidence ranging from 1-27% (Cohn & Peppard, 1980; Cooper *et al.*, 1989; Gluckman, 1979; Hordijk & de Jong, 1983; Jovanoic *et al.*, 1994; Maisel & Vermeersch, 1981; Tepperman & Fitzpatrick, 1981).

Patients with multiple primary cancers pose a particularly difficult therapeutic challenge. It has been reported that such patients appear to have a poorer prognosis compared to those with single tumours. Gluckman & Crissman (1993) reported the overall 5 year survival rate after diagnosis of second malignant neoplasm to be 22.3% compared with 35% in the control group. Cohn and Peppard(1980) observed that 75% of patients who developed second primary tumours of the UADT, died within 12 months of the second diagnosis (Cohn & Peppard, 1980) . Carr and Langdon (1989) found that the average survival of

patients with index oral cancers who developed second primary tumours was 7 months and two-thirds of this series died less than six months after diagnosis of the second tumour. The difficulties in the treatment of such lesions have been suggested as one of the potential reasons for the less favourable outcome, as radical therapy may have already been carried out on the primary lesion (Gluckman & Crissman, 1983). The morbidity associated with surgery and radiotherapy will be discussed later. However, there is a requirement for a modality which allows conservation of tissue and without cumulative toxicity, thus enabling repeated treatments if necessary.

1.6 CLINICAL ASPECTS OF ORAL CANCER

The signs and symptoms of oral cancer vary with the site and also the extent of disease. Typical clinical features include the presence of ulceration, induration, swelling (fungation and exophytic), erythema and in the more advanced cases fixation of the tissue.

The main problems associated with early tumours is that they are frequently asymptomatic and may therefore lead to a considerable delay in the diagnosis (Guggenherner 1989). In a study of approximately 1000 oral cancers, more than 65% were at least 2 cm at the time of admission for treatment (Krolls and Hossman 1976).

1.6.1 Diagnostic Methods

Although some encouraging results have been reported with the use of Toluidine Blue dye and the use of exfoliative cytology, these techniques can only be an adjunct and not a substitute for a biopsy (Mashberg & Barsa, 1984; Ogden *et al.*, 1994). Toluidine Blue dye primarily stain nucleic acids which are present in large quantities in malignant and premalignant tissue, unfortunately similar finding are seen with ulcerated and inflamed tissues.

1.6.2 Imaging Techniques

Computed tomography (CT) and MRI (magnetic resonance imaging) play a role in the determination of the extent of the primary tumour and the presence of associated lymphadenopathy. Knowledge of the extent of the cancer and what tissues are involved facilitate the decision of what therapeutic approach should be used. CT images are superior to MRI in their ability to directly detect and depict bone erosion and/or reorganisation (Silver 1983). However, when soft tissue differentiation is required, for example in the detection of the margins between tumour and muscle and fat, MRI may be the modality of choice. Unfortunately such techniques have difficulty in detecting thin lesions of a few millimetres depth and in such instances the use of techniques, such as high frequency ultrasound may provide a better indication of the depth of the superficial lesion (appendix).

1.6.2 Classification and Staging

The most commonly used staging system for head and neck cancer is the TNM System, which is based on the anatomical extent of the disease as determined by clinical examination. The most widely used system in Europe is the International Union Against Cancer (UICC, 1987) which is essentially the same as the American version (American Joint Committee on Cancer AJCC, 1988). The TNM Classification of oral cavity and oropharyngeal malignancies is based on the assessment of :

- tumour size (**T**)
- condition of the regional lymph nodes (**N**)
- absence or presence of distant metastases (**M**)

The 'T' category is assessed on the following criteria:

T_x - Primary tumour cannot be assessed
T_0 - No evidence of primary tumour
T_{is} - Carcinoma in situ
T_1 - Tumour 2 cm or less in greatest dimension
T_2 Tumour >2 cm but <4 cm
T_3 - Tumour >4 cm in greatest dimension
T_4 - Tumour invading adjacent structures,
e.g. through cortical bone or into deep (extrinsic) muscle of tongue

For the 'N' category:

N_X - Regional lymph nodes can not be assessed

N_0 - No regional lymph node metastases

N_1 - Metastasis to a single ipsilateral lymph node 3 cm or less

N_{2a} - Metastasis to a single ipsilateral lymph node >3 cm but <6 cm in greatest dimension

N_{2b} - Metastasis to a multiple ipsilateral lymph nodes but not >6 cm in greatest dimension

N_{2c} - Metastasis in bilateral or contralateral lymph nodes but not >6 cm in greatest dimension

N_3 - Metastasis to lymph nodes >6 cm

For the 'M' category

(metastasis to any lymph node other than regional nodes and into other organs):

M_x - Presence of distant metastases cannot be assessed

M_0 - No distant metastases

M_1 - Distant metastases

Table 1.2: TNM Staging

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IV	T4	N0/N1	M0
	Any T	N2/N3	M0
	Any T	Any N	M1

The stage of disease is a major determinant of survival. However, deficiencies exist with the UICC and AJCC TNM system, as only certain features are considered (Platz & Hudec, 1987). Following a retrospective study of 802 intraoral cancers, it was suggested that a combination of size and extent of infiltration of adjacent structures was required (Platz *et al.*, 1982). There is evidence that similar to melanomas the depth and pattern of invasion of oral tumours, correlate with the prognosis (Bryne *et al.*, 1989; Shingaki *et al.*, 1988). Other systems have been developed which include the histological and even

the site of disease e.g. the STNMP staging system takes account of pathology and site of the disease(Langdon *et al.*, 1977).

1.7 MANAGEMENT OF ORAL SCC

A number of therapeutic modalities are currently available for management of oral cancer. The most important of these include surgery, radiation therapy and to a much lesser degree, chemotherapy. Often, a combination of two or more of these modalities is appropriate. The treatment chosen depends on patient and tumour factors; the extent of the tumour, its location, the patient's physical and social state, along with the patient's and clinician's preference. Patients with head and neck cancer may present with additional medical problems associated with their social habits such as tobacco and alcohol intake. All of these factors can influence the quality of life and overall survival. These problems are estimated to account for 30% of deaths amongst patients with head and neck cancer (Jacobs 1990).

Routine work-up for any head and neck cancer patient usually includes either a CT or MRI scan along with complete examination of the head and neck region to detect the presence of a second primary as well as a bronchial and oesophageal endoscopy. It has been shown that the use of pan endoscopy combined with vital staining using toluidine blue may detect up to 19% of synchronous primaries of the UADT (Pradoura 1986).

1.7.1 Management of local disease

Surgery

Although the results of radiotherapy and surgery for small tumours (T1 & T2) are similarly effective, in larger tumours surgery is reported to be superior (De Vries, 1995). In many instances a combination of both surgery and radiotherapy will be indicated. The

choice of which modality is used depends on the likely morbidity resulting from that treatment.

Surgical management for oral and oropharyngeal cancers will frequently result in some morbidity in terms of function and or cosmesis. Excision of the tumour through the open mouth is really only possible for small accessible lesions, others will require some form of access surgery (e.g. splitting of the lip to enable a mandibulotomy or raising a cheek flap). The extent of the surgical margin depends on the site of the tumour, for tongue and floor of mouth lesions a 2 cm margin is often advised, whilst 1 cm is adequate for lip lesions (De Vries, 1995). Frozen sections are taken when there is doubt concerning the clearance.

Reconstruction of the residual defect is required with the exception of the smallest lesions, where primary closure will not result in severe tissue distortion. A wide variety of surgical techniques are now available for head and neck reconstruction including: skin grafts, local flaps, pedicle flaps and free flaps. The advent of microvascular surgery has opened up new horizons in reconstructive surgery.

Radiotherapy

The use of ionising radiation in the treatment of head and neck cancer is a well established modality. Small lesions within the head and neck have excellent cure rates with radiotherapy (RT) and this may be chosen over surgery. Surgical removal of small tumours may result in a considerable residual defect when conventional surgical margins are used. This can result in significant problems in terms of function (Fuller 1987) In situations where speech is of paramount importance to the patient socially or professionally, radiotherapy may be preferable. Advanced lesions present a therapeutic challenge and are frequently managed by combined surgery and radiotherapy. RT may be used in combination with surgery pre or post-operatively. The aim of preoperative RT is to reduce the bulk of the lesion, as the peripheral cells of the tumour are well oxygenated and likely to respond better to RT, whilst the central, more hypoxic cells, are

subsequently removed surgically. Concerns of possible adverse effects on wound healing have led surgeons to favour post operative RT.

RT may be administered by external beam radiation or by interstitial irradiation (brachytherapy). Brachytherapy is indicated for small (T1-T2) and well localised tumours, whereby a tumour dose of 60 Gy (6000 rad) is given in 4-7 days. In larger tumours (T2-T4) external beam irradiation is used allowing the treatment of tumour plus margin of tissue and the regional nodes. Doses of 60 Gy over 6 weeks is considered essential (Souhami & Tobias,).

Chemotherapy

Chemotherapy has been used in combination with surgery and/or radiotherapy for the treatment of advanced head and neck cancer. Enthusiasm for cytotoxic therapy is extremely varied because, although initial shrinkage of the tumour has been observed, studies have not indicated improved survival following neo-adjuvant chemotherapy (Stell & Rawson 1990). No improvements in outcome were observed in trials which use aggressive chemotherapy based on Cisplatin combinations. The use of intravenous chemotherapy also did not show significant differences in survival although two trials showed improved survival in patients with oral cancer (Arcangeli et al. 1993). An overview of 23 trials of adjuvant chemotherapy in the head and neck concluded, an insignificant overall improvement in cancer mortality (Stell & Rawson, 1990). An updated analysis by the same author also failed to show any benefit of chemotherapy (Stell, 1992). However, meta-analysis of results from 54 randomised trials, suggest that chemotherapy produces a small but significant improvement in survival (6.9%). This was more apparent with single agent chemotherapy given synchronous with radiotherapy (Munro, 1995). The problems with chemotherapy are the high rate of complications which frequently lead to contraindication of use except in well controlled clinical trials.

1.7.2 Management of Cervical Lymph Nodes

The incidence of cervical node metastasis depends on site and size of the primary tumour. Lymph node metastasis in the untreated neck has been observed to occur as a result of embolic spread (McKelvie, 1976), nevertheless *'en bloc'* resection of the primary tumour and regional lymph nodes is common practice. It is believed that a step wise spread occurs through the lymphatic chains, so if there is no disease of the higher node it is unlikely that the lower nodes will contain disease (Brown & Langdon, 1995). Woolgar et al. (1997) confirmed the observation of orderly progressive involvement of anatomical levels of nodal involvement, but "skipping " of levels II and III were seen in 10 % of the cases. Since the early description of the classical radical neck dissection, the procedure has remained virtually unchanged until in the last 2 decades when various modifications have been applied to preserved one of the three structures which are sacrificed, spinal accessory nerved, internal jugular vein and sternocleidomastoid (Crile, 1906; Martin, 1941; Robbins *et al.*, 1991).

1.8 COMPLICATIONS

1.8.1 Problems of Surgery

Neither surgery nor radiotherapy are without complications in the treatment of head and cancer. Surgical complications can arise as a result of anaesthesia in older patients who frequently have coexisting cardiorespiratory diseases from smoking. Functional and cosmetic changes result from tumour ablation and flap failure. The function of the mouth is so diverse that removal of any tumour is likely to cause functional deterioration, the extent depending on the nature and volume of tissue removed. Deglutition and speech intelligibility have been shown to be negatively correlated to the quantity of lingual tissue removed (Diz Dos *et al.*, 1994). Strong correlation has been observed between poor functional outcome and higher 'T' stage disease (Colangelo *et al.*, 1996).

1.8.2 Problems of Radiotherapy

There are a number of critical organs which must be considered when radiotherapy is used, these include the spinal cord, salivary glands, larynx, temporomandibular joint and mandible. The problems encountered with radiotherapy may be divided into acute and delayed.

Acute reactions

- *Mucositis (from 30 Gy):* caused by death of the rapidly dividing epithelial cells in the oral cavity. Signs include erythema, patchy fibrinous exudate and even ulceration with high doses. Changes appear about 12-14 days after the start of irradiation.
- *Xerostomia:* radiation causes atrophy of the salivary glands. Reduced salivary flow occurs after a few Gy. The effect is dependant on the total dose and volume of salivary gland with in the treatment field. The dry mouth can persist for years which is unpleasant for the patient, leads to taste dysfunction, and greater risk of tooth decay and candidal infection. Unfortunately no completely satisfactory treatment exists for xerostomia though sialogogues and parasympathomimetic agents may be of some use.
- *Taste loss:* most patients experience some disturbance or loss of taste.
- *Skin reactions:* skin erythema develops from 40 Gy with dry desquamation and even ulceration with high doses.

Late complications

- *Necrosis of soft tissue:* poorly vascularised soft tissue is more prone to development of necrosis and can lead to fistula formation following minor trauma injuries.
- *Osteoradionecrosis:* is believed to be caused by ischemia secondary to end arteritis obliterans. Predisposing factors include the dose delivered to the mandible and health of the overlying mucosa. Infection appears to be the result rather than cause of osteoradionecrosis. Therefore, tumours involving the mandible are best managed with surgery, as radiotherapy has been shown to be less effective as

well as being associated with the increased risk of osteoradionecrosis (Gerbault & Pomp, 1995).

1.8.3 Problems of Chemotherapy

The systemic side effects include of chemotherapy include:

- Nausea
- Vomiting
- Renal dysfunction
- Toxicity
- Bone marrow suppression

Such toxicity can be problematic and require careful considered particularly in patients with poor performance status (Tobias, 1994).

Head and neck cancer is thought to be one of the most traumatic types of tumour. Therefore quality of life issues are extremely important to consider along with the management. Factors of concern include physical functioning, occupational functioning, communication, eating and appearance and all factors are subject to changes with treatments.

1.9 PROGNOSIS

1.9.1 Prognostic Indicator

There are a number of indicators which have been reported to be associated with prognosis. Some of the histological prognostic indicators, such as histological grading of the advancing front of the oral SCC, have already been described. The extent of disease at the time of the initial diagnosis and therapy is important (Platz *et al.*, 1986). The presence or absence of regional metastases has been shown to have major influences not only on treatment but also outcome (Woolgar *et al.*, 1995a). The survival in patients with regional metastases is approximately half that of patients without clinical evidence of metastases (Spiro *et al.*, 1974). More specifically, extracapsular spread of the lymph node metastases

has been shown in number of studies to be the most important prognostic factor influencing survival (Johnson 1981). The survival for patients with metastatic disease confined to the lymph node was similar to those that had no evidence of metastasis. Death from recurrent regional disease was only seen in patients with macroscopic or microscopic extracapsular spread (1-6 years follow up) (Woolgar, 1997). Until the advanced stages, metastatic disease from oral cancer tends to remain above the level of the clavicle and general dissemination of tumour only occurs in much later stages. In the past most patients succumbed to local regional disease, but improvement in treatment has lead to increases in patients dying of distant metastases. In addition there are also increases in the number of patients presenting with second primary tumours in the head and neck and this has also contributed to the lack of improvement in survival over the past 30 years.

1.10 ORAL PRECANCER

Many oral cancers arise in clinically normal oral mucosa, but some will be preceded by a precancerous lesion (Speight *et al.*, 1996), In regions with higher incidence of oral cancer, clinically recognisable premalignant lesions are more common (Bouquot *et al.*, 1988; Gupta *et al.*, 1980).

1.10.1 Definition and guidelines (Axell *et al.*, 1996)

- A precancerous lesion is defined as a morphologically altered lesion in which cancer is more likely to occur than in its apparently normal counterpart e.g. oral leukoplakia.
- A precancerous condition is a generalised state associated with a significantly increased risk of oral cancer e.g. oral submucous fibrosis.

1.10.2 Pathology

The diagnosis of such lesions and conditions are made on the basis of both clinical and histological features. The degree of cytological atypia is one of the histological markers for the risk of malignant transformation. Potentially malignant lesions often present as red

lesions (erythroplakia or erythroplasia), white lesions (leukoplakia) or speckled lesions (speckled leukoplakia). Leukoplakia is the most common precancerous lesion of the head and neck mucosa (85%) (Bouquot & Whitaker, 1994). In the West it is typically found in 2-4% of adults, studies from various populations report figures of 0.4-11.7% (Banoczy, 1984). Erythroplakia is a less common precancerous lesion but is believed to have the highest risk of malignant transformation, possibly over 50%, and almost all erythroplakic lesions have been shown to contain dysplastic changes at biopsy, typically severe epithelial dysplasia or carcinoma situ (Bouquot *et al.*, 1988). Up to 10% also demonstrate micro-invasive SCC. The grade of epithelial dysplasia is dependant on the degree of cellular atypia and the level of change.

The rate of malignant transformation in oral epithelial dysplasia and leukoplakia with severe dysplasia have been quoted as 16 and 43% respectively (Axell, 1987; Lumerman *et al.*, 1995; Maerker & Burkhardt, 1978) There are a number of uncertainties associated with premalignancy. Firstly, there are no widely accepted criteria for defining the presence and degree of dysplasia. Secondly, it is not clear which lesions require treatment, nor how aggressively such lesions should be treated (Speight & Morgan, 1993).

Precancerous lesions and conditions of the oral mucosa are detailed overleaf.

1.10.3 Management of Oral precancer

There is considerable variation in the management of leukoplakias, the most common precancerous lesion. Depending on the aetiology, some clinicians adopt a "wait and see" approach and carry out treatment if malignant transformation occur. Cessation of smoking, surgical removal of the lesion, cryotherapy, topical cytotoxic agents and the use of vitamin A or β-carotene have all been used.

Precancerous lesions and conditions of the oral mucosa (clinical terms only)

Precancerous lesions:

Leukoplakia (a predominantly white lesion which can not be characterised as any other definable lesion; some will transform into cancers)

Proliferative verrucous leukoplakia

Smokeless tobacco keratosis

Candidal leukoplakia

Non-homogenous leukoplakia (erythroleukoplakia)

Erythroplakia (red lesion which can not be diagnosed as any other definable lesion; some will transform into cancers)

Precancerous conditions:

Oral submucous fibrosis

Actinic cheilitis

Lichen planus

Sideropenic dysphagia (Plummer-Vinson syndrome)

Discoid lupus erythematosus

Adapted from (Axell *et al.*, 1996; Speight *et al.*, 1996)

Surgical treatment

Frequently, removal of the lesion is carried out, however, such patients still remain at risk of relapse with further leukoplakia or even progression into cancer (Scully, 1995). In a study of 61 patients with premalignant lesions, following conventional surgical excision recurrence rates of 20% were observed and 3 of the 61 patients in the study developed carcinomas over an average follow up period of 3.9 years (Vedtofte *et al.*, 1987).

Chemoprevention

The increasing knowledge concerning the various stages of carcinogenesis and factors which may modulate the process has led to interest in methods of aborting or reversing the neoplastic process. Micronutrients such as vitamin A have been reported to be capable

of suppressing the process of carcinogenesis (Shklar *et al.*, 1980). Retinoids are the synthetic analogues of vitamin A and their use in leukoplakias have been evaluated over the past 30 years. Although results have been promising, unwanted side effects and recurrence of the lesion on cessation of the therapy slowed progress in this field (Hong *et al.*, 1986). The adverse reactions associated with retinoids include cheilitis, facial erythema, desquamation, conjunctivitis, photophobia, hypertriglyceridaemia and liver damage (Scully, 1995; Tanaka, 1989).

In view of the toxicity associated with the retinoids, there have been moves to investigate micronutrients such as β-carotene, vitamin E and C. A recent study using vitamin E in oral leukoplakia has shown promising results with beneficial responses observed in 46% of the 43 patients by 24 weeks with no serious side effects reported (Brenner *et al.*, 1993).

Thermal Lasers

Carbon dioxide lasers are widely used as a scalpel in the head and neck region. It has the advantage of sealing small blood vessels (0.5-1 mm diameter) and thus provide a relatively bloodless field (Carruth, 1990). The other lasers which are also used included include the argon ion laser (514 and 488nm), KTP laser (532nm) and Nd:YAG (Neodymium Yttrium Aluminium Garnet) laser (1.06μm).

Early studies report the advantage of minimal wound contracture and functional impairment following laser vaporisation or excision of premalignant lesions, however, recurrence rates of 22% were found (Frame *et al.*, 1984; Horch *et al.*, 1986). A recent study of 167 patient who underwent CO_2 laser resection of leukoplakias, over a 5 year period, also noted similar relapse rates in 19% of patients, with new lesions in 27 (16%), oral carcinomas developed in 5 patients (3%) and tumours in other organs in 6 patient (Cheisa *et al.*, 1993).

In conclusion, no one method fulfils the ideal criteria of eradication of disease with negligible adverse effects. The tendency for relapses following initial successful treatment of these premalignant lesions, indicate the need to determine an appropriate method to manage such lesions with minimal side effects and without cumulative toxicity.

NASOPHARYNGEAL CARCINOMA

1.11 EPIDEMIOLOGY

The prevalence of nasopharyngeal carcinoma (NPC) has a marked geographical and population distribution, with specific predilection for the southern provinces of China, Eskimos in Greenland and Alaska and the Philippines. The highest incidence is in South China with figures of 30-80/100,000 per year (Cvitkovic *et al.*, 1991). NPC is more common in males with a 2-4:1 male:female ratio.

Age

NPC affects a relatively younger population compared with other head and neck cancers. In endemic regions, at least 60% of the patients are under 50 years (Skinner *et al.*, 1991). In Hong Kong and Southern China, NPC is not infrequently found in those under 21 years. In the second decade it is the 4th most common malignancy in males and the 5th in females (Sham *et al.*, 1990).

Mortality

The overall survival rate for NPC is in the region of 35-40% (Molinari *et al.*, 1995), ranging from 20-70% in various series (Ho *et al.*, 1981; Huang *et al.*, 1985). The majority of failures following therapy will occur early, 91% of local failure will occur within 3 years and 94% of distant failures within 2 years (Vikram *et al.*, 1985; Vikram *et al.*, 1986).

1.12 RISK FACTORS OF NPC

The marked geographical and racial differences in NPC have stimulated much research in to the aetiology of this condition. Both genetic and environmental factors have been postulated. The serological and biological association of NPC with Epstein-Barr virus (EBV) was first established by (Old *et al.*, 1966). An aetiological relationship has been implicated, supported by the findings that all NPC patients are infected with EBV, in addition EBV DNA and antigens have been demonstrated in all biopsies (Pearson et al 1993). The characteristic EBV serological profile, in endemic regions, is now well established. There is elevation of IgA and IgG anti- viral capsid antigen (VCA) , early antigen (EA) and EBV-associated nuclear antigen. IgA anti-EA seems to be specific for early detection of NPC in high risk patients (Henderson 1988, Lynn 1984), IgG anti-VCA and IgG anti-EA levels may be related to tumoral levels. Follow up serology is not a good marker for cure because many patients without evidence of disease continue to have elevated serological levels after successful treatment (Hadar et al 1986, Henle et al 1977).

Many studies have looked into in the development of NPC and the possible role of carcinogens in salted fish have been implicated as an important factor amongst Chinese, Eskimos and North Africans. Chinese studies has shown a dose-related relationship between salt fish consumption and the incidence of undifferentiated nasopharyngeal tumour (UDNT) (Poirier et al 1987, Yu et al 1986) . Animal experiments in rats have shown that large amounts of nitrates, which are converted to carcinogenic nitrosamines in salted food, produce adenocarcinoma and undifferentiated carcinoma of the nasal and paranasal cavities in rats (Yu *et al.*, 1989).

1.13 PATHOLOGY

1.13.1 Histopathology

NPC is an epidermoid cell lineage carcinoma of the head and neck, with a variety of morphological degrees of differentiation, which has lead to controversy regarding its

histological classification. A number of histological classifications exist for NPC. The WHO (Shanmugaraytum K, 1978) recognises 3 types;

- Squamous cell carcinoma (SCC, keratinising)
- Non-keratinising carcinoma (poorly differentiated)
- Undifferentiated carcinoma (UDNT)

The most common histological type of NPC in endemic areas such as South East Asia is the undifferentiated squamous cell type, where it represents more than 90% of cases seen (Shanmugaratnam K, 1979). This histological type tend to occur at a younger age group. The primary tumour is of a smaller size, seems to affect patients of a younger age group and also displays a higher incidence of metastatic spread (Bedwinek et al 1980, Vikram et al 1986, Peraz & Brady 1987). However, they also exhibit greater radiosensitivity with better survival relative to stage of disease compared with the SCC type (Molinari *et al.*, 1995).

The squamous cell variety is the one most frequently seen outside endemic areas. In these cases the serological profile is less classical and the natural history of the disease is somewhat different to the UDNT variety.

1.13.2 Genetic Factors

The difference in incidence of NPC in people of different genetic origin has led to the suggestion that there may be a genetic factor involved. Epidemiological studies have highlighted the possibility of an inherited predisposition. The occurrence of multiple cases of NPC in first degree relatives has been well documented (Huang, 1991). Several genetic systems have been investigated and the HLA locus A and B antigen association in Chinese with UDNT is well established. HLA B17 and HLABw46 are associated with increased risk, whereas HLA A11 is associated with a decreased risk.

Unlike other head and neck cancers, NPC does not appear to be strongly associated with heavy smoking or alcohol abuse (Boyle *et al.*, 1990),

1.14 CLINICAL ASPECTS

1.14.1 Anatomy

The nasopharynx is a cuboidal space in direct continuity with the nasal cavity anteriorly, inferiorly the oral cavity, and laterally with the middle ear via the eustachian tubes, which open in the fossa of Rosenmüller (pharyngeal recess). The bony roof and posterior wall are formed by the basisphenoid, basiocciput and the arch of the atlas. The most common site of origin of NPC is the fossa of Rosenmüller and therefore local spread of disease can occur early owing to the proximity of the upper parapharyngeal space and the skull base.

Due to the anatomical position, tumours are initially symptomless. The classical presentation of NPC is a triad of a neck mass (90% of cases, or 50% bilateral), conductive hearing loss or serous otitis and nasal obstruction. When present, symptoms may be separated into those related to the neck mass, nasal, aural, neurological and others sites. The presence of a neck mass is the most common complaint prompting patients to seek medical advice (Molinari *et al.*, 1995). Proximity of the base of skull allows for direct intracranial extension and cranial nerve involvement occurs in 25% of cases (Molinari *et al.*, 1995).

1.14.2 Management of NPC

Due to difficult access, the frequency of clinical and subclinical retropharyngeal node involvement and the radiosensitivity of nasopharyngeal carcinomas (NPC), radiotherapy (RT) is the initial treatment of choice and is very successful in the control of disease particularly in the early stages.

Radiotherapy for NPC

The success of radiotherapy is dependant on the ability to deliver adequate doses to all affected areas whilst sparing the normal tissue. The nasopharynx is closely situated to many important structures, namely the eyes, ears, temporal lobes of the brain, cranial

nerves, spinal cord, brain stem and hypothalamus all of which have limited radiation tolerance, yet are often unavoidably included in the treatment field. In the early stages good local control of disease has been observed. However the response of locally or regionally advanced NPC to radiotherapy is poor. The median five year survival is less than 50%, even in patients with early disease, reducing to 30% for T_2 N_0 -N_1 disease (Souhami & Tobias,).

External beam irradiation

It has been observed that there is a positive relationship between tumour dose and tumour control (Bendwinek *et al.*, 1980; Moench & Philips, 1972; Yan *et al.*, 1990) . Most centres advocate radiation doses of 65Gy (Lee, 1991) which exceeds the tolerance dose for neurological tissues (Rottenburg *et al.*, 1977; Sheline *et al.*, 1980).

Brachytherapy

Brachytherapy, either by intracavity or interstitial techniques, has been used for residual disease following external irradiation. The rapid fall of radiation intensity at short distances allow delivery of high dose of radiation to tumour whilst minimising normal tissue effects.

Complications of radiotherapy

Treatment -related side effects are important especially in younger patients. The most prevalent long term complications are skin and/or subcutaneous fibrosis, osteoradionecrosis (John *et al.*, 1993; Li *et al.*, 1994), radiation myelitis, brain necrosis (Lee, 1991), temporomandibular joint ankylosis, hypopituitarism (Sham J 1994), and bone atrophy (Cvitkovic 1991). In addition, the complications described above in section 1.8.2 are also relevant following radiotherapy for NPC. A randomised trial of radiotherapy, which compared shielding of the pituitary and the anterior hypothalamus with standard radiotherapy covering the sphenoid sinuses was carried out in 152 patients. Shielding reduced neuroendocrine complications without jeopardising local control (Sham *et al.*, 1994), therefore some precautions can be taken to minimise side effects.

Nevertheless, complications can still be severe, with extensive osteoradionecrosis of the posterior maxilla, pterygoid plates and floor of the sphenoidal sinuses (Li *et al.*, 1994) possibly as a result of the large fraction dose and total dose of radiotherapy.

Neurological complications following radiotherapy constitute the major morbidity and account for the majority of treatment deaths (Lee, 1991). Cerebral necrosis post RT has been well documented. In a study of 102 patients, 31% of the patients presented with temporal lobe epilepsy (Lee *et al.*, 1988). Radiation damage to the optic nerve or chiasma resulting in blindness has been documented (Parsons *et al.*, 1983) as has hormonal dysfunction thought to be related to hypothalamic damage (Lam *et al.*, 1987). The most serious complication of radiotherapy is myelitis, which usually correlates with a high dose of radiation to the spinal cord (Hoppe RT et al 1978).

Chemotherapy

The role of chemotherapy in the initial treatment of NPC still needs to be determined. No improvement in survival was observed in a randomised study of 229 patients on the use of adjuvant chemotherapy (Rossi & al, 1988). Similarly, results following the use of induction chemotherapy concluded it to be of no benefit (Tannock *et al.*, 1987). Review of studies carried out with sequentially delivered neoadjuvant chemotherapy followed by radiotherapy or adjuvant therapy after radiotherapy have showed both primary and recurrent NPC to be sensitive to neoadjuvant chemotherapy, however the numbers in many of these studies were often small (Dimery & Hong, 1993). Tang et al (1990) found that the addition of chemotherapy did not significantly improve the survival for a group of 561 patients. With the controversial results concerning the impact of chemotherapy, complications related to this modality must not be forgotten.

1.15 RECURRENT NPC

Local failure after radiotherapy is not uncommon and the management of persistent and recurrent disease is a therapeutic challenge. The prognosis for recurrent NPC is grave as the surviving cells are likely to be more radioresistant and therefore require even higher

doses of radiation (Tsao, 1991). Reported incidence of local recurrence varies from 18% to 58% (Shan & Choy, 1990; Yasashita *et al.*, 1985) with medians at 34% (Bendwinek *et al.*, 1980; Schabinger *et al.*, 1985).

The risk of late irradiation complications is substantial and is often the major consideration that deters many from using adequate re-irradiation. A one year interval is recommended before further irradiation. However, the doses to the neurological tissues are still considered cumulative (Tsao, 1991). There is a wide variation in the incidence of major side effects such as neurological complications from 0% (Fu *et al.*, 1975) to more than 12% (Yan *et al.*, 1983). In a study of 891 patients with recurrent disease, re-irradiation was carried out in 706 patients with success in 32% of those re-irradiated. The authors reported the risks of re-irradiation as being substantial with a 24% incidence of complications. The most common sequelae are trismus (17%), deafness (5.9%) and neurological damage in 5% (Lee *et al.*, 1993). The reported five year survival for patients suitable for re-irradiation range from 15% (Chen *et al.*, 1984) to 41% (Fu et al 1975).

1.16 SUMMARY

A number of therapeutic options are available for oral cancer, precancer and nasopharyngeal carcinoma. However, these are often associated with some degree of compromise in terms of loss of tissue, function and with surgery cosmesis (surgery). Radiotherapy is associated with damage to normal adjacent structures (bone, salivary glands, mucosa, neurological tissue) along with cumulative toxicity and hence pose a problem for further disease resulting from field cancerization. The systemic toxicity associated with chemotherapy and chemopreventative agents may make them unacceptable to patients. These problems highlight the need for a modality which is tissue conserving, with minimal systemic toxicity and no cumulative toxicity. This thesis will evaluate the role of photodynamic therapy in the management of malignancy and premalignancy in the head and neck and whether the above criteria are fulfilled.

Chapter 2

AN INTRODUCTION TO PHOTODYNAMIC THERAPY

2.1 DEFINITION OF PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) involves the administration of a drug (a photosensitiser) which once in the target tissue can be activated by non-thermal light, usually from a laser. Neither the drug nor the light alone have any toxic effect but together in the presence of molecular oxygen, cytotoxic species are produced which cause cell death

2.2 HISTORY OF PDT

The therapeutic use of light was described by Niels Finsen in 1903, in the treatment of lupus vulgaris, with the use of an arc lamp as the light source (Finsen, 1903). Around the same time a German medical student, Oscar Raab, who was working under van Tappeiner, observed the lethal effect of light on paramecia in the presence of acridine and this is considered to be one of the earliest reports of a drug light interaction (Raab, 1900). Van Tappeiner continued the work and in conjunction with Jesionek carried out clinical treatment on patients with skin cancer, where a combination of artificial and natural light were used to activate topically applied eosin; some improvements were seen in most cases (Jesionek & Tappeiner, 1905). In 1904 van Tappeiner and Jodlbauer coined the phrase "photodynamische Wirkung" (photodynamic action) for such oxygen requiring photosensitised reactions (Tappeiner & Jesionek, 1904).

Interest in this field continued and in 1908, Hausmann reported the use of haematoporphyrin to kill protozoa paramecia before moving on to animal studies showing the differential photosensitivity effects of light on animals previously administered with haematoporphyrin (Hausmann, 1908; Hausmann, 1911). The corresponding clinical effects were observed, when Meyer-Betz injected himself with intravenous haematoporphyrin (200mg) followed by short exposure to sunlight. He experienced prickling and burning of the exposed areas followed by the development of erythema,

oedema and pain. This photosensitivity remained for a further 2 months (Meyer-Betz, 1913).

2.2.1 Tumour localisation and detection

Policard observed that certain tumours in man and animals showed fluorescence which he attributed to the accumulation of endogenous porphyrins resulting from the action of haemolytic bacteria (Policard, 1924). Auler and Banzer subsequently reported the affinity of exogenous haematoporphyrin for neoplastic tissues, which presented as fluorescence with the use of a woods lamp (Auler & Banzer, 1942); this was later confirmed by (Figge *et al.*, 1948), who postulated that "such substances could be utilised to improve the existing methods of tumour detection". After the observation by Schwartz that tumour localisation by crude haematoporphyrin was the result of the "impurities" and not of the pure substance (Schwartz *et al.*, 1955), Lipson refined the compound by acetylation with acetic-sulphuric acid followed by reduction, producing a mixture termed haematoporphyrin derivative (HpD) with improved tumour localisation (Lipson & Blades, 1960). Lipson and co-workers then observed preferential fluorescence/localisation in human tumours from various sites (Lipson *et al.*, 1961; Lipson *et al.*, 1964a; Lipson *et al.*, 1964b). However, Gregorie administered intravenous HpD in 226 patients and concluded that this technique was unreliable in the diagnosis of various malignant tumours (Gregorie *et al.*, 1968).

The tumour localising properties shown by the porphyrins have also been observed with other photosensitisers. There has been much speculation as to the mechanism of this feature. Explanations have been attributed to certain properties of the tumour tissue; leaky vasculature, poor lymphatic drainage, uptake of aggregated material by macrophages, low pH, higher concentrations of lipoprotein receptors and low ferrochelatase activity (Henderson & Dougherty, 1992; Moan, 1986b).

2.2.2 Early PDT

Lipson was the first to use photodynamic therapy with haematoporphyrin derivative (HpD) in the treatment of a patient with metastatic chest wall breast cancer (Lipson *et al.*, 1966). This was followed by the treatment of a patient with bladder cancer (Kelly & Snell, 1976). However, despite positive responses, neither group followed up with additional patients. Since these early days, thousands of patients have been treated with PDT and "nearly 10,000 publications relating to PDT have appeared, including almost all types of solid cancers" (Dougherty, 1993). The most widely investigated sensitiser, is Photofrin® (purified form of dihaematoporphyrin ether and ester, Quadra Logic Technology, Vancouver) and is the only sensitiser which has approval for clinical use in Canada, France, Japan, the Netherlands and USA for one non-malignant and five malignant conditions (Brown, 1996). Early clinical results from several centres reported encouraging outcomes following the use of Photofrin® (Dougherty, 1993; Pass, 1993) although results from the Phase III trial still need to be fully evaluated. Numerous second generation photosensitisers with improved properties such as shorter photosensitivity or which are more active and powerful, have been developed and are undergoing Phase I and II clinical evaluation at present. These include: aminolaevulinic acid, (ALA), meta tetrahydroxyphenyl chlorin (mTHPC), chlorin e6 aspartic acid (NPe6) and tin (II) etiopurpurin (SnET2). Even with these large numbers of photosensitisers currently available, the chemist still strives to produce the "ideal drug" for which the true test will ultimately be the clinical benefit.

2.3 GENERAL PRINCIPLES OF PHOTODYNAMIC THERAPY

In photodynamic therapy, the photosensitiser in the target tissue is excited by visible light of a wavelength matched to its absorption characteristics. In the presence of tissue oxygen the resultant effect is PDT induced necrosis.

2.3.1 Photosensitisers

One of the major tasks in tumour therapy is the development of tumour selective drugs and this is the rationale behind photosensitisers research. As with conventional cytotoxic drugs, the limiting factor will be the toxicity of the sensitiser. The hope is that selectivity in uptake/retention of the drug will translate into corresponding selective damage of the tumour along with preservation of normal tissue, minimum systemic toxicity, and negligible cutaneous photosensitivity. However, sensitiser selectivity is always relative rather than absolute and various methods have been tested in an attempt to improve on the selectivity following systemic administration. These include local sensitiser administration (e.g. topical application) and intraneoplastic administration, in addition to the use of radioprotective agents: monoclonal antibodies and bioreductive drugs (Bremner *et al.*, 1992; Oseroff *et al.*, 1987). Local administration will rely on adequate uptake of the sensitiser, whilst sensitiser distribution may not be uniform following intratumoral administration. Bioreductive drugs are metabolised under hypoxic conditions to produce cytotoxic species and therefore may take advantage of the hypoxic conditions, reached after vascular shut down following light activation. Such drugs have been shown to produce enhanced PDT effect, but as yet none have been tried clinically.

The properties of an ideal photosensitiser are summarised below (MacRobert, 1989)(Moan, 1990b):

- red and near infrared light absorption for high tissue penetration
- non-toxic, biochemically stable
- selective retention in target tissue relative to normal tissue
- little or no skin photosensitising potency
- an efficient generator of cytotoxic oxygen species (high triplet quantum yield)
- a good fluorophore- provides an easy method of detection
- defined chemical composition- simplify interpretation of dose: response relationship and consistency of the product
- water solubility for systemic administration

At present no sensitiser conforms completely to these characteristics. Careful manipulation of sensitiser and light irradiation regimens are required to obtain selectivity, such as low sensitiser doses combined with large light dose (photodegradable dyes) (Moan, 1986a). This section will contain descriptions of the properties of sensitisers used in this thesis.

2.3.1.1 Haematoporphyrin derivative (HpD) and Dihaematoporphyrin ester/ether (DHE, Photofrin®)

HpD

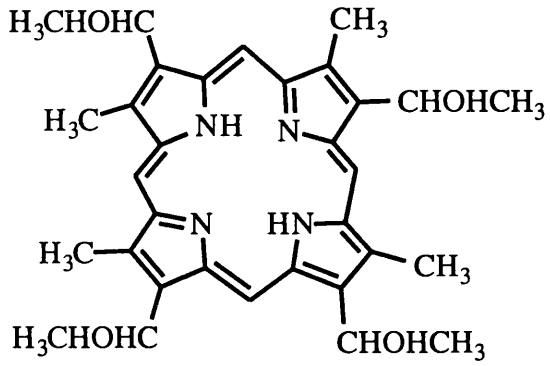


Figure 2.1 : Chemical structure of haematoporphyrin

Haematoporphyrin derivative (HpD) and the further purified version, Photofrin®, have been the most widely used sensitisers, and the latter is currently the only sensitiser which has approval for clinical use (Brown, 1996). It is made up of a complex mixture of oligomeric esters and ethers of haematoporphyrin. The active components of HpD have been identified using gel filtration and are usually referred to as dihaematoporphyrin esters and ether (DHE), although poly haematoporphyrin ester or ether may be more appropriate (Dougherty *et al.*, 1984). The commercial preparation of DHE is known as Photofrin® and is composed of a mixture of non-metallic oligomeric porphyrins predominantly linked by ether bonds, with between two to eight porphyrin units (Dougherty, 1987; Kessel & Thompson, 1987). However, Photofrin® remains a complex mixture with inherent variability (Fisher *et al.*, 1995). Although Photofrin® can be activated by light of various wavelengths e.g. 400nm (violet), ~500nm (green) or 625-

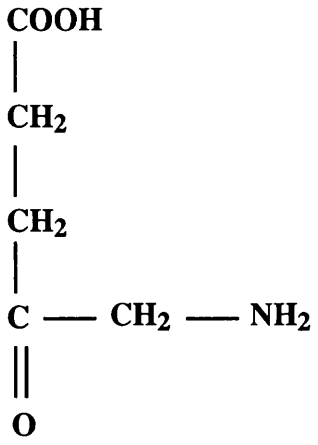
630nm (red), red light is used as it has the best penetration in biological tissues. However, this also corresponds to its weakest absorption peak (Star, 1990). In addition Photofrin® has the disadvantage of causing prolonged cutaneous photosensitivity, typically lasting 4-8 weeks although some patients report sensitivity persisting for several months (Dougherty & Marcus, 1992). Pharmacokinetic studies in mice utilising radiolabelling techniques have revealed the presence of approximately 1% of the injected dose at 24 hours, although a small quantity of DHE is still detectable at 75 days (Bellnier *et al.*, 1989a).

HpD and DHE exhibit some selectivity in tumour, with tumour:skin ratios in the region of 2:1 (Bellnier *et al.*, 1989b). The sensitiser also appears to persist in reticuloendothelial tissues (Gomer & Dougherty, 1979a). The exact mechanism of this retention is still unclear and has already been discussed (section 2.2.1). The optimal therapeutic ratio for Photofrin® has been reported between 24 and 96 hours after administration. The reason for the range of time may be batch to batch variability (Fisher *et al.*, 1995; Henderson & Dougherty, 1992). Photofrin® has been claimed to be taken up by virtually all solid tumour (Dougherty & Marcus, 1992) and has been used in the treatment of most types of solid tumours. However, it is not an ideal photosensitiser and hence the move to second generation sensitisers such as 5-aminolaevulinic acid and meta tetrahydroxyphenyl chlorin with improved properties.

2.3.1.2 δ-aminolaevulinic Acid

The use of the body's own mechanism to produce an endogenous photosensitiser was first proposed by Malik et al following the observation that protoporphyrin IX (PpIX) accumulated in solid tumours and leukaemic cells in the presence of abnormal porphyrin metabolism. (Malik & Lugaci, 1987) (Rubino & Rasetti, 1966; Smith, 1987). Kennedy was subsequently the first to introduce this technique clinically for topical use on cutaneous lesions (Kennedy & Pottier, 1992).

5-aminolaevulinic acid



Protoporphyrin IX

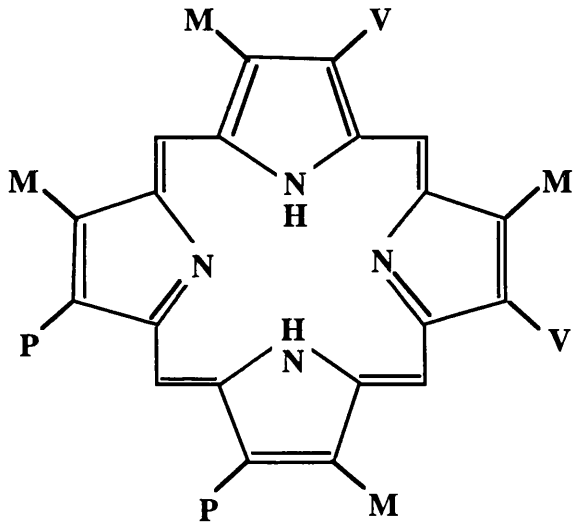


Figure 2.2: Chemical structure of ALA and PpIX

Aminolaevulinic acid (ALA) can induce the synthesis of protoporphyrin IX (PpIX) which is the immediate precursor of haem in the biosynthetic pathway. PpIX is believed to be the photoactive species in ALA PDT. Studies on haem biosynthesis in the liver demonstrated that the rate-limiting step of this pathway is the conversion of glycine and succinyl CoA to ALA, a reaction catalysed by the enzyme ALA synthetase (Marriot, 1968) (Figure 2.3). ALA synthetase is regulated by a feedback control mechanism determined by the concentration of free haem. Therefore exogenous administration of 5-ALA bypasses this control and leads to accumulation of haem intermediates, especially PpIX, since the second, rate limiting step along this pathway is the conversion of

protoporphyrin IX (PpIX) to haem. A number of studies suggest that ferrochelatase activity is low in neoplastic cells (van Hillegersberg *et al.*, 1992). In-vitro studies on erythroleukaemic cells incubated with ALA demonstrated the presence of uro and coproporphyrin along with PpIX (Malik & Djaldetti, 1979). Loh et al, in an in-vivo study on tumour and normal rat colon demonstrated that PpIX was the predominant porphyrin species present following systemic administration of ALA (Loh *et al.*, 1993b). Protoporphyrin IX exhibits fluorescence and PDT potential which has been shown (in-vitro) to be directly related to the PpIX concentration (Kennedy & Pottier). ALA induced PpIX is unique in that a pro-sensitiser (ALA) is administered with the subsequent production of an endogenous sensitiser (PpIX).

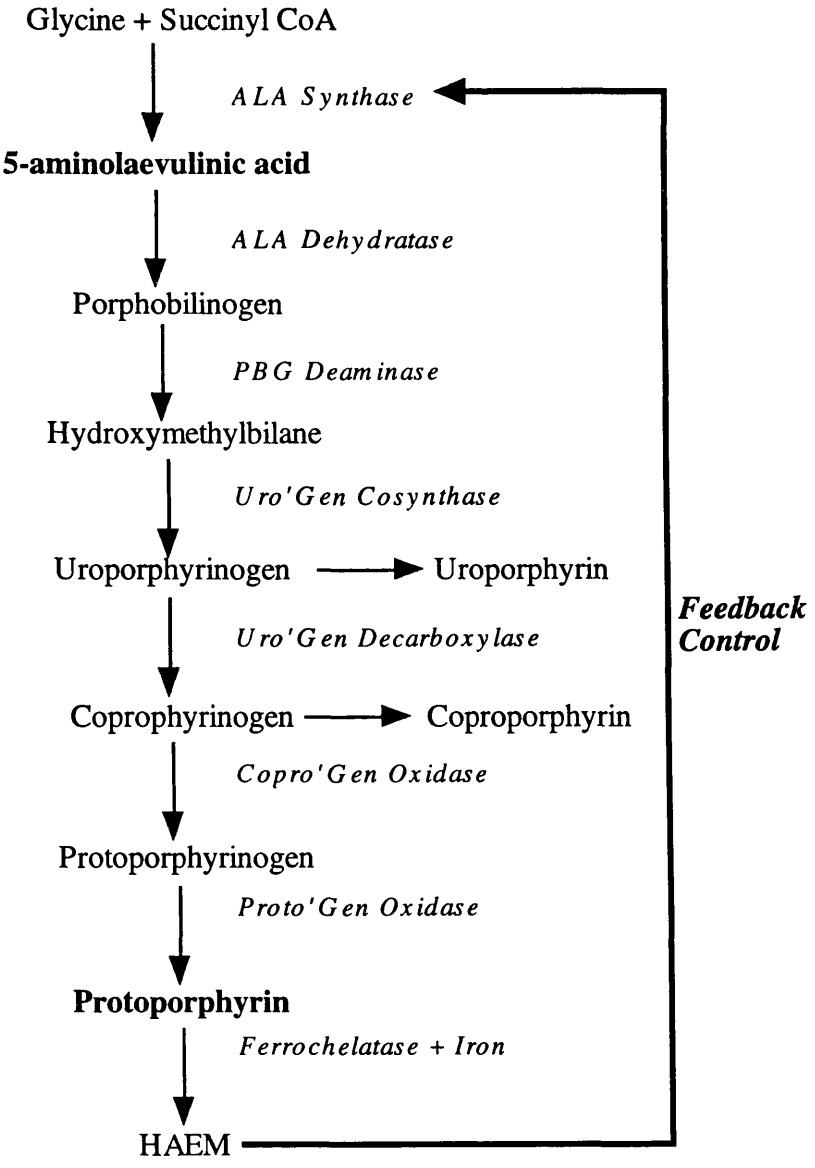


Figure 2.3: Haem biosynthetic pathway

Most nucleated cells are capable of haem synthesis and PpIX formation. However, there is a marked difference in the level of photosensitisation of various tissue structures following administration of exogenous ALA to mice. Strong fluorescence was seen in the skin, oral mucosa and mucosa of the larynx, vaginal and anal cavities (surface lining tissues), salivary glands , bile ducts and gall bladder whilst negligible fluorescence was observed in skeletal or cardiac muscle (Divaris *et al.*, 1990; Kleemann *et al.*, 1996). Some degree of selectivity has been suggested following ALA sensitisation, with tumour to normal mucosa ratios of 6:1 observed by fluorescence in the rat colon, ratios of 30:1 for normal mucosa to submucosa and 60:1 for normal mucosa to muscle, indicating that there is tumour selectivity and also selectivity within certain tissue layers (Bedwell *et al.*, 1992).

The mechanism of PDT damage with ALA has not been investigated as thoroughly as Photofrin®. However as PpIX is formed within the mitochondria, it has been proposed that this may be the initial site of PDT damage (Iinuma *et al.*, 1994; Kennedy & Pottier, 1992). The lack of fluorescence observed in vascular tissue led to the speculation that the ALA induced photodamage was by direct cell kill and not secondary to vascular damage as observed with other sensitisers (section 2.5.3) (Bedwell *et al.*, 1992; Divaris *et al.*, 1990; Peng *et al.*, 1992). Tumours excised immediately following light exposure and plated, demonstrated a significant reduction in clonogenic survival (77% reduction) suggesting that direct cell kill is produced. However, very few animals survived 24 hours following the same treatment regimen so it was more difficult to assess vascular damage with the same protocol (Henderson *et al.*, 1995). Conversely, damage to the endothelium and basal lamina has been seen under light and electron microscopy (Peng *et al.*, 1992) and reduction in blood flow has also been observed, although complete shut down was not found (Henderson *et al.*, 1995; Roberts *et al.*, 1994).

Loh et al observed maximal porphyrin fluorescence in the mucosal layers of the rat colon and stomach, whilst the rise to peak levels were slower in the muscularis layers following 200mg/kg (IV) ALA (Loh *et al.*, 1993b). In a subsequent study the same group observed

PpIX to preferentially accumulate in the mucosal layer following both IV and oral ALA administration. The peak concentration of PpIX was reached at 4 hours with an ALA dose of 200mg/kg (IV) and 400mg/kg (oral); however lower ALA doses corresponded to an earlier peak (Loh *et al.*, 1993a). This confirmed the earlier findings of Bedwell et al who observed peak fluorescence in the normal rat colon at 4 hours, followed by a rapid drop off by 6 hours and subsequent return to background values by 24 hours (Bedwell *et al.*, 1992). This suggests that the therapeutic window with ALA is relatively narrow (i.e. hours). Phototoxic necrosis of the lining mucosa has also been shown in the rat colon and rabbit larynx (Bedwell *et al.*, 1992; Kleemann *et al.*, 1996).

Clinical ALA PDT has been received with much enthusiasm, principally as a result of the cutaneous phototoxicity lasting only 24 hours, a major advantage over other currently available sensitisers. Since Kennedy's early patients, it has been shown that ALA PDT has significant promise in dermatological conditions following topical applications of ALA PDT. A short-term complete response rate of 90% has been reported for a group of 80 superficial basal cell carcinomas, with 89% complete responses observed in patients with Bowen's disease (Cairnduff *et al.*, 1994; Kennedy & Pottier, 1992). Unfortunately, topical application of photosensitisers is not really feasible in most other organs and hence the move to a systemic mode of administration. Grant et al (1993) reported the first use of systemic ALA-induced phototoxicity in four patients with advanced squamous cell carcinoma. Since then there have been further reports of the use of systemic ALA in gastrointestinal tract, where superficial mucosal necrosis has been observed (Mlkvy *et al.*, 1995; Regula *et al.*, 1995).

Various methods have been shown in animal models to enhance the PDT effect produced following ALA-induced PpIX; these include the use of various fractionated light regimens (dark periods interspersed amongst irradiation periods) (Hua *et al.*, 1995; Messmann *et al.*, 1995; van der Veen *et al.*, 1994), the use of iron chelators to slow the conversion of PpIX to haem (Berg *et al.*, 1996; Chang *et al.*, 1994), in addition to other factors such as

method of drug administration, fluence rate and wavelength of the incident light (630 or 635 nm). There is a need to assess the efficacy of these changes clinically.

2.3.1.3 meta tetrahydroxyphenyl chlorin (mTHPC)

m-THPC

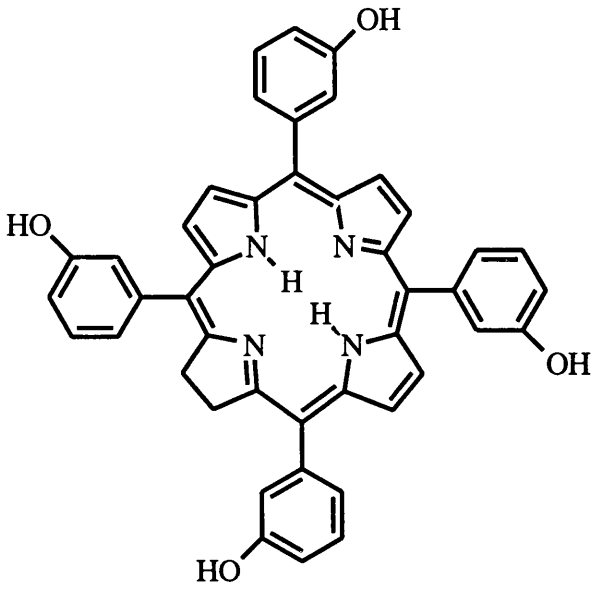


Figure 2.4 : Chemical structure of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin

Chlorins are produced following the di-imide reduction of porphyrins, followed by dehydrogenation with σ-chloranil (Bonnett *et al.*, 1989) and is available at a purity >99%. The other advantage of these structural changes from porphyrins is the stronger absorption at a longer wavelength in the red region of the spectrum, resulting in a maximum absorption at ~650nm and corresponding improved light penetration in tissue (Bonnett, 1995). It has been shown that both m-THPC and p-THPC have greater efficacy and selectivity as a sensitiser compared with Photofrin® and that the meta-isomer is better than the para-isomer. In a mice model, cutaneous photosensitivity as measured by skin oedema, was found to return to almost normal two weeks after mTHPC administration, whilst it was still appreciable after 4 weeks following Photofrin® sensitisation (Berenbaum *et al.*, 1993).

Lofgren et al carried out a study on a rabbit papilloma model and showed higher concentration of mTHPC in the tumour relative to the normal tissue at all times and that the optimal selectivity between tumour to normal tissue occurred at four days. In addition, analysis of the sensitiser levels in plasma by fluorimetric analysis revealed a double exponential decay, with half-lives of 26 hours and 185 hours. However, the authors noted some individual variation in the plasma levels with higher sensitiser levels correlating to better tumour response (Lofgren *et al.*, 1994). Using the same method, this group observed the half-life of mTHPC (0.0375-0.3mg/kg), in a group of 14 patients, to be 44.5 hours with a near total disappearance of the sensitiser in 8-10 days (Ronn *et al.*, 1995). Whelpton et al also demonstrated a biexponential decay of plasma mTHPC levels in a murine model, but their half-life values were earlier at 0.6 and 13.9 hours respectively (Whelpton *et al.*, 1995).

In-vitro studies using fluorescence spectroscopy revealed that maximal uptake of mTHPC occurred at 24 hours and localised diffusely in the cytoplasm, with minimal fluorescence observed in the nuclear region which the author speculated may indicate low genotoxicity and risk of mutation (Ma *et al.*, 1994).

The first clinical use of mTHPC was in a group of 4 patients with mesotheliomas. It was observed that 0.3mg/kg mTHPC followed by activation with 10J/cm^2 light dose at 48 hours was capable of producing 10mm depth of necrosis. Tumour:normal tissue ratios of up to 14:1 were observed in two of the patients following chemical extraction assays by high performance liquid chromatography (HPLC) (Ris *et al.*, 1991). Since these early patients, this photosensitiser has been used in the head and neck region by Savary et al in Lausanne and Dilkes et al in London and will be addressed later in Chapter 3 (Dilkes *et al.*, 1995; Savary *et al.*, 1993).

2.3.1.4 Phthalocyanines

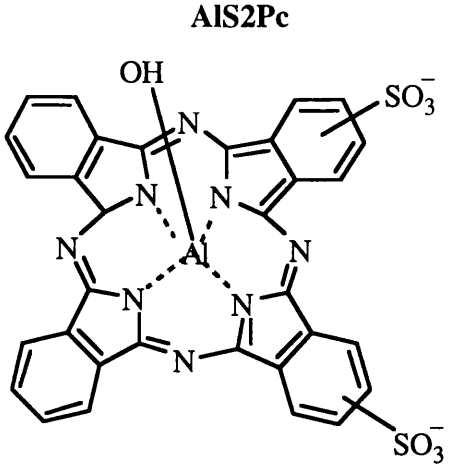


Figure 2.5 : Chemical structure of aluminium disulphonated phthalocyanine

Phthalocyanines (Pc) are synthetic porphyrins sometimes termed aza-porphyrins. In contrast to haematoporphyrin the phthalocyanine pyrrol rings are extended by condensation with an extra benzene ring and bridged by aza-nitrogens rather than methine groups (Figure 2.5). These structural changes result in strong absorption bands in the red region of the visible spectrum 670-675nm (Edwards & Gouterman, 1970). The phthalocyanines can chelate with many metal ions and it is the nature of this ion that affects the photochemical properties of the Pc. A diamagnetic metal such as aluminium and zinc extends the triplet lifetime of the sensitiser and hence enhances the phototoxicity, whilst metal-free phthalocyanine molecules are less effective (Evensen & Moan, 1987; Sonoda *et al.*, 1987).

The Pc has a tendency to aggregate in aqueous solution which is its photochemically inactive form (Darwent *et al.*, 1982) . Disaggregation can be attained by the addition of organic solvents, detergents or serum proteins (Ben-Hur *et al.*, 1987b). The introduction of sulphonate groups to substitute the hydrogen ion on the benzene ring will produce a water soluble compound and therefore reduce aggregation. In aluminium sulphonated phthalocyanine (AlSPc), the aluminium ion also acts as an extraplanar ligand which also

inhibits aggregation. The work for this thesis used AlS2Pc, prepared by Dept. of Chemistry, Imperial College, London.

Most studies have been carried out with the sulphonate phthalocyanines, which are the most efficient phthalocyanine photosensitisers (Rosenthal, 1991). An inverse relation has been observed between phototoxicity and the degree of sulphonation, with the exception of the monosulphates of Al and Zn e.g. where AlPcS2 > AlPcS1 >AlPcS3 >AlPcS4 (Rosenthal, 1991). Similarly, there also appears to be an inverse correlation between the number of sulphonate substitutes and increased lipophilicity, and cellular uptake (Berg *et al.*, 1989) .

For over 30 years it has been known that phthalocyanines preferentially accumulate in tumours (Wrenn *et al.*, 1951); however interest has been roused because of recent publication of its use as possible sensitiser for PDT. Preferential sensitiser distribution in the region of 2-3:1 was observed for tumours outside the CNS. Peak sensitiser concentrations were seen 24-48 hours after intravenous injection of AlSPc, whilst peaks levels in normal tissue were seen at 1-3 hours (Tralau *et al.*, 1990) In the larynx, AlS2Pc was seen to accumulate in the submucosa and muscle at the 1 hour time interval, whist at 24 hours the sensitiser predominantly located in the mucosa, indicating the importance of PDT at the optimal time interval to avoid unacceptable normal tissue damage (Kleemann *et al.*, 1996). These studies suggest that there may be a wide window for tissue destruction.

There are suggestions that skin photosensitivity may be less of a problem with the phthalocyanines than with Photofrin® . Comparisons of the absorption spectra indicate that Photofrin® has maximal absorption around 400nm which constitutes a large proportion of sunlight, therefore, Photofrin® would absorb more sunlight. This has been supported by a controlled study on mice using Photofrin® and AlSPc as photosensitising agents (Tralau *et al.*, 1989)

2.4 LASER AND LIGHT DELIVERY SYSTEMS

Delivery of light allowing optimal irradiation of the whole tumour is essential to the success of photodynamic therapy. The distribution of light must not only match the geometric but also the optical characteristics of the target tissue, in order to optimise tumour kill and minimise damage to the normal surrounding tissue (Wilson & Patterson, 1986). The light source used for PDT must be capable of providing the appropriate wavelength of light matched to the sensitiser to be used, of an adequate intensity to allow treatment to be carried out in a reasonable length of time and be coupled to an appropriate light delivery system (which can be selected for different geometries).

2.4.1 History of Lasers

Laser is an acronym for Light Amplification by the Stimulated Emission of Radiation. The theory of laser was first postulated by Einstein (1917). In 1955, the predecessor to the laser the "maser" (the microwave amplifier) was constructed (Gordan *et al.*, 1955). Five years later, Theodore Maiman demonstrated lasing action in a ruby ($Cr:Al_2O_3$) crystal on May 16, 1960 (Maiman, 1960) . Some 18 months after Maiman's discovery the first laser patient was treated for a retinal tumour (Koester *et al.*, 1962). The potential for laser therapy in the head and neck was recognised very early (Sataloff, 1967). The discovery of various gas and solid state lasers followed quickly in succession. The carbon dioxide (CO_2) laser was reported only four years later (Patel, 1964) and is still widely used in clinical practise as is the neodymium-YAG (solid state laser) (Geusic *et al.*, 1964) and diode laser (Ripley, 1996).

2.4.2 Principles of Laser Action

A laser consist of an active medium placed between two mirrors which form the laser cavity. One of the mirrors is fully reflective while the other is partially reflective and forms the output for the laser beam. The lasing medium is excited (pumped) to invert the population ratio of excited to non-excited atoms/molecules. The excited atoms/molecules relax to the ground state by release of a photon (quantum of radiant energy). When a

photon of appropriate energy strikes another excited atom/molecule in the chamber, it stimulates emission of an identical photon (coherent); the excited molecule then decays back to the ground state. The reflections of these released photons from the mirrors into the lasing medium will cause a cascade effect, i.e. amplification , resulting in the rapid build up of photons within the laser cavity This coherent, monochromatic light is released from the cavity through the partially reflective mirror. The laser medium determines the range of wavelengths and the mirrors determine the specific wavelength of the laser light. In summary the are four basic characteristics of light produced by a laser are:

- *Monochromatic*
- *Coherent*
- *Highly directional*
- *Capable of producing high energies*

The monochromatic nature of laser light is important for PDT as a specific wavelength of light is required for sensitser activation. All of the light produced shares the same optical properties this improves efficiency, and the high directionality enables the light to be channelled down an optical fibre. The highly directional nature also allows the beam to be focused into a very small spot, resulting in a high concentration of energy.

2.4.3 Light Tissue Interactions

When a photon of light hits a tissue surface, four interactions occur and are summarised as follows:

1. Specular reflection at the tissue surface- due to the difference in optical properties of air and tissue.
2. Transmission through the tissue (no interaction).
3. Scattering within the tissue- by small particles such as mitochondria in cells and at cell interfaces.
4. Absorption by chromophores within the tissue can lead to the production of heat. By the appropriate adjustment of laser wavelength, certain chromophores can be targeted for clinical use e.g. intracellular water is highly absorbing for CO_2 radiation and therefore can be used for cutting or vaporisation and is the mechanism

seen with thermal surgical lasers (Carruth, 1984). Alternatively, the wavelength can be used to target photosensitisers and cause a photosensitised change to the cell (photodynamic therapy)

2.4.4 Light penetration into tissue

The depth of light penetration in tissue is a very important feature in photodynamic therapy, as the action of light on the photosensitiser is crucial to the destruction of tumours. The depth of light penetration through different tissues is a function of the optical properties of that tissue specifically absorption and scattering coefficients and the scattering angular distribution, these factors are related to the tissue chromophores in addition to the wavelength of the laser light itself.

The opacity of a tissue is defined as the attenuation coefficient whilst its inverse is the penetration depth of light, which is the depth when the incident light intensity drops to approximately 37% of the initial value (1/e). Penetration depths into tissue typically range from 1-2mm to nearly 5mm at a wavelength of 630nm used for sensitiser activation with Photofrin® and PpIX (Dougherty & Marcus, 1992), while the penetration depth is almost doubled at 700-850nm [Wilson, 1989]. This improved penetration depth at longer wavelengths has prompted the development of sensitisers which absorb in this region. Examples of such sensitisers include, benzoporphyrin derivative which absorbs at 690nm and theoretically would have between 30-50% increase depth of penetration compared with Photofrin®; the chlorins (652-660nm) have a somewhat lower increase and the napththalocyanines which absorb strongly at 760nm (Dougherty & Marcus, 1992). However, absorption of light by the photosensitiser itself can limit tissue penetration and is particularly pronounced with high concentrations of sensitisers which act as very strongly absorbing chromophores at the treatment wavelength, (Wilson *et al.*, 1986).

2.4.5 Light Sources

Various types of light sources are available for photodynamic therapy as lasers are not essential. Historically sunlight has been used (Jesionek & Tappeiner, 1903), whilst wavelength filtered lamps have been used in the past with a recent revival of interest due to low cost production, running cost and size (Dougherty *et al.*, 1975; Morton *et al.*, 1995).

The most commonly used lasers for PDT are tuneable dye lasers which are wavelength conversion devices. The dye laser is "pumped" by another laser and converts the energy from the pump laser by the use of fluorescent dyes such as rhodamine. This results in a tuneable output over a range of 30-50 nm (620-650 nm), and such devices have been used extensively in PDT related research. The advantage of dyes laser is their tunability over parts of the visible spectrum. Copper, gold vapour and argon ion lasers are frequently used as the "pump laser" due to strong absorption by Rhodamine dyes at the green wavelength. The dye can be changed to provide different range of wavelength.

The copper and gold vapour lasers are bulky and too expensive for most hospitals to purchase specifically for PDT. However, many surgical units already have a potassium titanyl phosphate (KTP) Nd:YAG laser which can also be used to pump a dye module. This creates a slightly more portable system which is becoming an important and attractive light system for PDT. The ultimate "black box" laser for clinical use must be the diode lasers which make use of semiconductor technology to emit light with high power output and are currently available at 652 and 675nm for use with ALA, mTHPC and phthalocyanines. These compact size laser are not only portable but significantly cheaper and more user friendly than the other laser systems making them particularly attractive for PDT use in non specialised units who do not have easy access to technical support. The disadvantage of the diode lasers is their lack of tunability which means that extra systems would need to be acquired if using more than one sensitiser at different wavelengths.

There has also been renewed interest in non-laser source particularly for dermatological applications. A lamp incorporating a 300W xenon short arc plasma discharge with a band width filter of 30nm has been developed (Whitehurst *et al.*, 1993; Whitehurst *et al.*, 1995). Such light devices may further complicate light dosimetry, as a percentage of the incident light may not be of an appropriate wavelength to activate the sensitisers especially with sensitisers that have very narrow absorption bands. Consequently more energy may be required than with laser light to achieve the same PDT effect, the presence of infrared light will further complicate matters.

2.4.6 Light delivery system

The light generated by a laser can be coupled to an optical fibre which allows delivery to the target tissue either by surface irradiation for easily accessible sites, or modified for more complex geometries. Surface irradiation for the oral cavity can be carried out simply with the use of a polished bare optical fibre. However, the beam profile of such optical fibres provide a peak intensity centrally with significant drop off towards the edge of the treatment field (bell shape). The better alternative is to use a microlens coupled to the optical fibre (Doiron, 1985). A good microlens is designed to give a homogenous spot of light with a top hat profile (Figure 2.6). Therefore the entire irradiated field should receive the same light dose. However, the coupling of the microlens to the optical fibre can lead to energy loss occurring in the coupling systems, which is up to 25% in most instances (Milam, 1994), and may make treatment times inconveniently long.

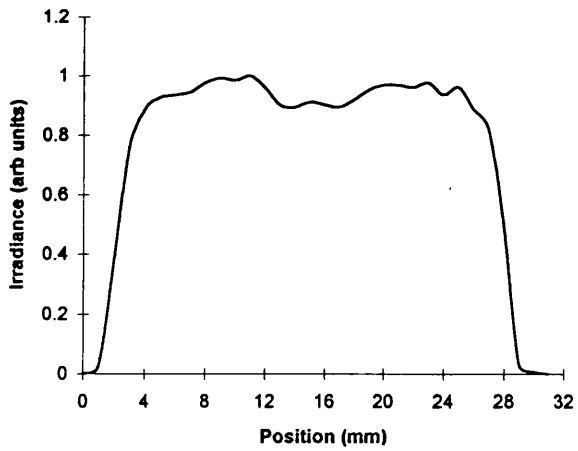


Figure 2.6 Irradiance profile of a typical microlens
(from personal communication Dr. M.L. de Jode)

For the treatment of many other organs, direct surface irradiation will not suffice. Nevertheless, in these situations various strategies have been developed to modify the spatial light distribution to match the target tissue configuration by modification of the optical fibre (Carruth & McKenzie, 1986). Modification of the output fibre tip to enable interstitial, intralumenal or intracavitary illumination (Doiron, 1985), the use of multiple fibres placed interstitially (McPhee *et al.*, 1984), or with various light-diffusing medium and scattering devices have been investigated (Baghdassarian *et al.*, 1985; Jocham *et al.*, 1984; Marijnissen *et al.*, 1989) .

2.4.7 Light Dosimetry

The power of a light source is expressed in Watts (Joules/second).

The energy delivered is:

Power (Watts) x irradiation time (seconds) = Energy (Joules)

As PDT is mostly carried out on a flat surface knowledge of the power per unit area or irradiance (W/cm^2) is important. It is believed that hyperthermia may occur if the surface irradiance exceeds $200mW/cm^2$ (Svaasand, 1985). However, PDT response may be enhanced by hyperthermia, (Waldow *et al.*, 1985). To truly assess the efficacy of PDT alone, the irradiance ideally should be kept below $200mW/cm^2$. Unfortunately this may lead to unacceptably long treatment times when the laser output is low. The "light dose" applied to the target area for superficial PDT is calculated from multiplication of the irradiance of the incident beam by the treatment time (J/cm^2) (Star, 1990).

$$\text{Dose } (J/cm^2) = \frac{\text{Power (W) x Time (s)}}{\text{Area } (cm^2)}$$

2.5 MECHANISM OF ACTION

2.5.1 Photochemistry

Upon absorption of light, the sensitiser molecule is transformed to an excited higher energy singlet state which is unstable and short lived (nanoseconds); from this excited state it may decay to a more stable energy state either by heat loss, fluorescence emission or undergo electron spin conversion (intersystem crossing) to a metastable stable triplet state. The reaction resulting in fluorescence decay, is the basis for the detection of tissue fluorescence of a photosensitiser for pharmacokinetic studies, whilst the conversion to the triplet state is the photoactive state responsible for the photochemical generation of cytotoxic species. Triplet state sensitiser can undergo either Type I or Type II photochemical reactions. Type II reaction (singlet oxygen production via energy transfer) is generally agreed to predominate over Type I reactions (hydroxyl and superoxide radical formation by electron or hydrogen atom transfer) (Bonnett, 1995; Fisher *et al.*, 1995). Intermolecular energy transfer from the sensitiser in the triplet state to molecular oxygen results in singlet oxygen production (Foote 1968). These processes are summarised in Figure 2.7. Singlet oxygen is an electronically excited state of oxygen because of the anti-parallel valence electron spin, and hence is extremely reactive and is able to cause damage to essential cellular components e.g. amino acids, unsaturated lipids etc. (see later) (Davies, 1994). Once energy transfer has occurred from the sensitiser to oxygen, the sensitiser reverts to the ground state where it may be further excited by absorbing more light.

2.5.2 Role of Oxygen

The evidence for the requirement of oxygen for PDT cytotoxicity has been widely investigated. Many groups have shown that in anoxic conditions, PDT effects are suppressed (Gomer & Razum, 1984; Lee See *et al.*, 1984; Moan & Sommer, 1985). Gomer and Razum reported the complete resistance of hypoxic tissue to PDT, similarly Bown et al showed that liver damage resulting from PDT was inhibited during occlusion of blood supply to the liver (Bown *et al.*, 1986; Gomer & Razum, 1984). One reason for

possible failures in tumour PDT is either tumour hypoxia or the rapid depletion of oxygen during treatment.

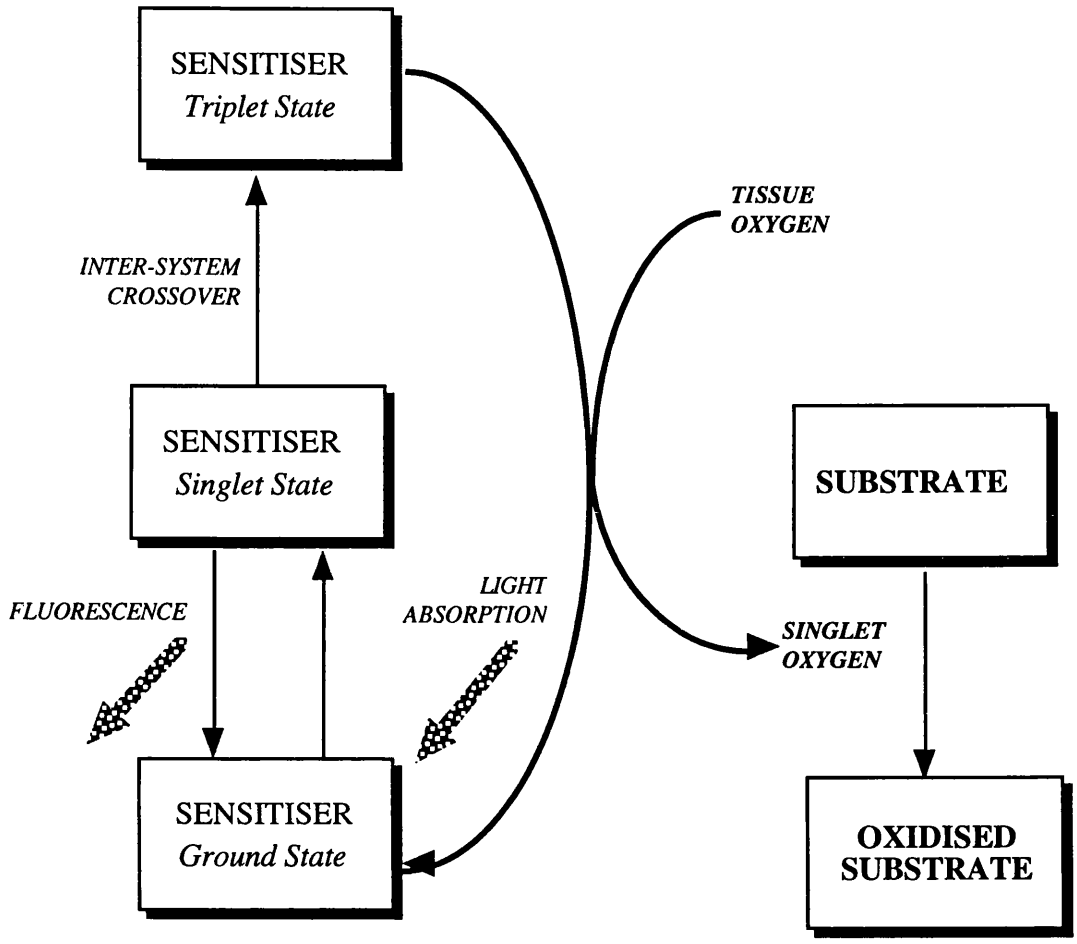


Figure 2.7: Type II Photochemical Reaction

There is much indirect evidence to suggest that singlet oxygen is the principal damaging species in photodynamic therapy. However at present its direct measurement is extremely difficult (Weishapt *et al.*, 1976). It has been estimated that the diffusion distance of singlet oxygen ($^1O_2$) is in the region of 0.1μm and therefore cell damage mediated by $^1O_2$ is likely to be close to the site of generation. As a result of the wide and varied distribution of sensitisers, virtually all cellular components are likely to be affected (Moan, 1990). It has also been reported that photochemical reactions may lead to radical chain auto-oxidation and further oxidative reactions, thus often making it difficult to identify the initial site of effect (Foote, 1984).

2.5.3 Photosensitiser Targeting

2.5.3.1 Membrane Damage

Cell membranes are believed to be among the primary sites of photodynamically induced cellular damage. Lipid peroxidation, as well as protein cross-linking of the membranes have been observed in mammalian cells (Girotti, 1976; Girotti, 1990; Moan, 1986b). Following PDT, damage to porphyrin sensitised cells are characterised by swelling of the cells with 'bleb' formation on the outer cell membrane(Moan *et al.*, 1982). The membrane damage can then lead to inactivation of membrane transport systems, depolarisation of plasma membrane and increased permeability (Kessel, 1986b). PDT-induced cellular damage has been reported to be enhanced by the use of membrane active drugs, such as potassium proton ionophore nigericin in cells pre-treated with unsulphonated aluminium phthalocyanine (Varnes *et al.*, 1990).

2.5.3.2 Mitochondrial Damage

Preferential accumulation of certain cationic sensitisers in the mitochondria has been observed and may account for the damage induced by these drugs (Gomer *et al.*, 1988; Salet & Moreno, 1990). The results of mitochondrial damage include inhibition of oxidative phosphorylation, electron transport enzymes and reduction in cellular adenosine triphosphate levels. The inactivation of mitochondrial enzymes such as cytochrome C oxidase and succinate dehydrogenase have been frequently considered a key event in PDT cell damage and has been demonstrated by biochemical assay. Following Photofrin® mediated PDT immediate mitochondrial changes have been observed with progressive swelling and structural disruption (Hilf *et al.*, 1984; Kessel, 1986a; Kessel, 1986b).

2.5.3.3 DNA Damage

Despite the almost non-detectable accumulation of the more lipophilic porphyrins and phthalocyanine sensitisers in the nucleus, DNA damage has been documented (Gomer, 1980; Ramakrishnan *et al.*, 1989). DNA synthesis in mammalian cells has been shown to be very sensitive to PDT and even low doses of HpD and light which have no effect on

cell survival may reduce DNA synthesis to zero, although recovery can occur (Moan *et al.*, 1983). Despite the findings of DNA strand breaks and chromosomal aberrations, it is believed that such lesions are not necessarily responsible for cellular death (Gomer, 1980; Moan *et al.*, 1980) and HpD-PDT is generally regarded as non-mutagenic (Evensen & Moan, 1982; Fiel *et al.*, 1981; Pass, 1993). In support of this, DNA-DNA cross-links have never been found (Dubbleman *et al.*, 1983) and the DNA and chromosome damages seen with PDT are significantly less than observed following radiation (Moan *et al.*, 1980). The mutagenic potential of phthalocyanines as tested by resistance to 6-thioguanine and oubain, was believed to be small even though single strand breaks and DNA-DNA cross-links have been reported (Ben-Hur *et al.*, 1987a).

2.5.3.4 Apoptosis

Apoptosis is a form of programmed cell death, which is gene directed and commonly occurs in normal healthy tissue as well as in many pathological settings (Alison & Sarraf, 1992). Apoptotic cells have the following characteristic features: chromatin condensation; reduction in cell volume, with frequent splits into apoptotic figures, dilatation of endoplasmic reticulum, loss of specialised surface and finally elimination by phagocytosis (Wylie *et al.*, 1980). Since the first demonstration that PDT can induce cell death by apoptosis, there has been a surge of interest in this topic (Agarwal *et al.*, 1991). PDT-induced apoptosis and physiologically-induced apoptosis probably operate through a different mechanisms (Olienick *et al.*, 1993). It has been proposed that PDT induced membrane changes cause activation of phospholipase C and phospholipase A2 along with the release of intracellular Ca^{++} and the subsequent activation of an endonuclease which cleaves nuclear DNA in to a series of fragments (Olienick *et al.*, 1993). Apoptosis has been observed with a number of sensitisers and in numerous cell types including oral mucosal keratinocytes (Amir Ketabchi personal communication). However, it has also been shown that PDT does not lead to apoptosis in all cell types (Agarwal *et al.*, 1991; Olienick *et al.*, 1993).

It is probable that different sensitisers may initiate cell death at different intracellular sites via different mechanisms which may be related to the specific patterns of intracellular localisation of each drug. Whatever the primary lethal effects of photosensitisation, the end result is the rapid decline of cell integrity due to the membrane damage and ultimate cell death.

2.5.3.5 Vascular Damage

PDT effects on blood vessels have been known for sometime and were described as early as 1911 by Hausmann. Numerous studies support the view that damage to the microvasculature and the resulting indirect effects are important. Vascular changes following photodynamic therapy (Photofrin®) have been well described, within seconds of irradiation, platelet aggregation can be seen, followed by transient vasoconstriction, vasodilatation and eventual complete blood stasis. The major determinant of vascular photosensitivity is the circulating levels of sensitiser and with most photosensitisers vascular occlusion can be induced soon after drug administration (Henderson & Dougherty, 1992). A number of studies have indicated that both normal and neoplastic microvasculature undergo microscopic arterial vasoconstriction and thrombus formation, as well as endothelial cell necrosis following PDT induced tumour destruction (Star *et al.*, 1986). Henderson et al showed in an *in vivo/in vitro* study that cells from tumours explanted immediately after PDT were found to be viable whilst tumours left in-situ after PDT under went necrosis. The authors therefore speculated that vascular damage was essential for tumour cure (Henderson *et al.*, 1985). It has also been suggested that damage to the normal microvasculature adjacent to the tumour may be important as tumour regrowth has otherwise been observed (Star *et al.*, 1986). PDT treated tumours (Photofrin® and aluminium tetrasulphonated phthalocyanine) exhibit significantly reduced tumour blood flow within five to ten minutes following light activation, thus suggesting that vascular damage is an important feature (Selman *et al.*, 1986; Wieman *et al.*, 1988).

The consequences of severe microvascular damage and the rapid shift of cells to hypoxia is of concern. As fully hypoxic cells are completely protected from further PDT, it is possible that a hypoxic subpopulation may survive and allow the tumour to regrow. A recent vascular perfusion study using Photofrin® has shown that the area of tumour hypoxia increased from 4% to over 50% of the total tumour area following light activation (van Geel *et al.*, 1996).

It would therefore appear that there is much evidence to support the role of vascular damage as the major pathway of PDT damage with many sensitisers. However, direct cell cytotoxicity may be more important with the endogenous sensitiser PpIX and *in vitro* studies support the role of direct cell-kill. It is probable that tumour destruction probably occurs by a combination of direct and indirect cell kill and the importance of each will vary depending on the sensitiser used and the drug-light interval.

This chapter has described the mechanism of photodynamic therapy and the properties of the photosensitisers that will be investigated further in this thesis. The applications of photodynamic therapy in the head and neck will be further discussed in the next chapter.

Chapter 3

LITERATURE REVIEW OF PHOTODYNAMIC THERAPY FOR HEAD AND NECK CANCER

3.1 INTRODUCTION

Although the first report of a patient receiving photodynamic therapy was in 1966 it was not until the early 1970's that Dougherty and co-workers reported the results of PDT on an appreciably larger group of patients with a variety of diseases (Dougherty *et al.*, 1978; Lipson *et al.*, 1966). Soon after, photodynamic therapy caught the attention of the head and neck surgeons and one of the earliest reports of PDT use in head and neck cancer appeared in 1983 by Dahlman et al. Since then there has been abundant reports of PDT research literature in the head and neck. The predominant sites for clinical PDT has been for diseases affecting the skin, aerodigestive and the gastrointestinal tract. These sites are inherently accessible directly or with the use of various scopes. Despite the enormous amount of clinical research within the upper aerodigestive tract much is anecdotal and preclinical research is relatively sparse. A review of the clinical and preclinical literature will be presented in the following sections.

3.2 CLINICAL TRIALS

Dahlman et al reported the use of PDT in 37 patients, twenty of whom presented with "ENT squamous cell carcinomas" with a 65% favourable response (complete response or partial response). This study did not provide clear definition for the outcome of the disease, however later studies in this region followed similar criteria, where:

- Complete Response (CR): no evidence of disease clinically or pathologically
- Partial Response (PR): reduction in size of the lesion by at least 50%
- No/Non Response (NR): reduction by less than 50% of the maximal diameter of the lesion

Even in this early study the problem encountered with inadequate light delivery during PDT was noted. Implantation of the optical fibre into the tumour mass was carried out in

two of the patients with bulky tumours, however despite this, no obvious success was observed. The following year (Wile *et al.*, 1984) reported a series of 39 patients with complete response observed in 25% and partial response seen in 37%, moreover the follow up period for this group was also of a similarly short period as Dahlman (4 weeks). Carruth and Mckenzie were the first to report PDT in a group of 10 patients with either cutaneous metastasis or superficial tumour in the UK. They observed complete response in two of the three patients with head and neck tumours (Carruth, 1985).

Schuller et al reported the results of PDT using haematoporphyrin derivative (HpD) in a group of 24 patients with recurrent or metastatic disease in the head and neck. This group also used interstitial techniques for the thicker tumours, whilst using surface irradiation for the superficial areas. In three patients, adjunctive intraoperative PDT was used following resection of the tumour and negative frozen sections at the margins. Like many of the early reports, this was a feasibility study and disease progression was observed in 15 patients within 6 weeks (Schuller *et al.*, 1985). Complications were reported in 5 patients, the most serious being exsanguination of a patient 24 hours following PDT for a tumour involving the carotid artery.

The move away from the use of bare fibre as the light delivery source to microlens and diffuser tips (cylindrical or spherical) was seen in the study by Keller et al, where Photofrin® with 25-60 J/cm^2 of light was used. Following the treatment of 11 head and neck tumour patients, it was concluded that diffuse superficial lesion responded well to PDT, whilst only limited palliation was observed in the advances cases (Keller *et al.*, 1985). The disappointing overall response seen with the advanced cancers were also observed by a number of other investigators, with the suggestion that in these patients the skin photosensitivity may in fact worsen the quality of life (Calzavara *et al.*, 1989; Gluckman, 1991a). For this group of patients, improvements must be found in terms of a more powerful photosensitisers with improved depth of damage, or the use of a photosensitisers with much shorter cutaneous photosensitivity, so allowing repeated treatments without the morbidity of prolonged skin sensitivity. In summary, the

disappointing results of PDT with the advanced bulky tumours are generally believed to be the result of inadequate light delivery to the whole tumour which is another area that research needs to be targeted (Dahlman *et al.*, 1983).

3.2.1 Superficial Disease

More favourable findings in patients with early superficial and diffuse disease have been well documented (Gluckman, 1991a; Schweitzer, 1990). Gluckman treated 13 patients with early oral and oropharyngeal lesions, following HpD or DHE (dihaematoporphyrin ether/ester) sensitisation with activation using red light from an argon ion pumped dye or gold vapour laser with light doses of 50-100J/cm^2. He observed an initial 85% complete response with 4 recurrences within 1 year. However, in the 8 patients with condemned mucosal disease (field change disease) complete response was seen in all except one patient with follow up between 6 -53 months (Gluckman, 1991a).

Grant *et al* reported the results in a similar group of 11 patients and observed similar success at the six to eight week stage. Ten patients in this study had a complete response, whilst the remaining patient had areas of residual leukoplakia (Grant *et al.*, 1993b). There is a need to establish longer follow ups on such patients, as such conditions are notoriously difficult to manage by conventional means.

The following tables (3.1 and 3.2) summarises the results in 12 studies reported before the start of the work for this thesis. Table 3.1 summarises the results for the head and neck and Table 3.2 illustrates the number of cases treated with oral disease. The latter reports the main area of interest for this work.

These tables illustrate the variation in drug dose, drug-light interval, light dose and light delivery systems. The aim is to establish specific treatment parameters for use with a particular photosensitiser, and one method to evaluate this would be to establish what effects are achieved when a group of patients with the same disease under go the same treatment. The ultimate aim in clinical PDT would be to establish a protocol whereby a

specific drug and light dose combination is used dependant on the depth of tissue necrosis required. The dream of matching the light/drug dose to tumour depth and possibly even the tumour type would eliminate many of the clinicians' uncertainties concerning PDT.

3.2.2 Depth of PDT damage with Photofrin®

There is surprisingly sparse literature on the comparison of the depth of histological damage relative to the treatment dose in patients. There have also been much speculation as to the selectivity of clinical PDT, yet little data is available on this subject. The pathological changes 24 hours following PDT using HpD was examined in 10 patients, however no correlation was made between the depth of necrosis achieved and the drug and light dose used (Zhao *et al.*, 1987). In a similar study, 11 patients received Photofrin® (2mg/kg) followed by irradiation at 48 hours using 50J/cm^2 light energy. However, in this study the treated tumour was often resected at a later time interval, within 7 days in most cases, therefore allowing adequate time for maximal tumour necrosis to occur. The authors observed the absolute depth of necrosis to range from 1.1-4.1mm (mean 2.1$\pm$0.9 s.d.) and no selectivity in PDT damage was seen both clinically and histologically (Grant *et al.*, 1997). The variable depth of damage is of concern, but corresponds to full thickness mucosal necrosis. However, most worrying is the evidence of viable tumour seen in two patients below the depth of necrosis.

3.2.3 Second Generation Photosensitisers

The move towards clinical treatment with more recently developed drugs possessing improved properties commenced with 5-aminolaevulinic acid (ALA). The basic properties of this agent have already been discussed in Chapter 3. ALA is a pro-drug which is converted in the body to the photoactive substance protoporphyrin IX (PpIX). The biggest advantage of using ALA induced PpIX in PDT is the extremely short cutaneous photosensitivity, which typically last 24 hours. Grant *et al* were the first to report tumour necrosis following photodynamic therapy after an oral dose of ALA. As with most Phase I studies, this was a feasibility study and 4 patients were sensitised with either 30 or

Table 3.1: Summary of photodynamic therapy in the treatment of head and neck cancer (excluding cutaneous and lung lesions)

Author	Pt No. (*)	PS dose (mg/kg)	D-L interval (hours)	Light Dose J/cm^2	Response (%)	Length of follow up
(Dahlman *et al.*, 1983)	12	HpD (2-5)	72-120	5-20	5CR (42) 7PR (58)	30 days
(Wile *et al.*, 1984)	21	-	17-91	-	6CR (29) 12PR (57)	-
(Carruth, 1985)	3	HpD	-	-	2CR 1PR	-
(Keller *et al.*, 1985)	11	HpD (2-3) DHE (1.5-2)	48-72	25-60	4CR (36) 6PR (55)	up to 17 months
(Schuller *et al.*, 1985)	24	HpD (3-5)	72	not specified	NA	NA
(Gluckman & Weissler, 1986)	16	HpD(3) or DHE (2)	-	-	9CR (56) 4PR (25)	3-19 months
(Zhao *et al.*, 1987)	94	HpD (2.5-5)	48-72	200-1440	62CR (66) 25PR (27)	1-4 years
(Calzavara *et al.*, 1989)	5	Hp (5) HpD (2.5)	-	120-150	5NR advance	-
(Feyh *et al.*, 1990)	8	HpD ? dose	48	100	7CR	≤14 months
(Schweitzer, 1990)	12	Photofrin® (2)	48-72	50-150	5CR 6PR 1NR	1-48 months
(Wenig *et al.*, 1990)	26	Photofrin®	24	125 (lens) 75J/cm- interstitial	20CR (77)	6-51 months
(Grant *et al.*, 1993b)	11	Photofrin® (2)	48	50-100	10CR (91)	6-8 weeks

Note:

D-L -drug-light

Pt No.-patient numbers

NA- not applicable

HpD- haematoporphyrin derivative

DHE- dihaematoporphyrin ether/ester

Photofrin®-commercial form of DHE

Table 3.2: Summary of photodynamic therapy in the treatment of oral/oropharyngeal cancer

Author	Patient number(*)
(Wile *et al.*, 1984)	21
(Dahlman *et al.*, 1983)	12
(Carruth, 1985)	3
(Keller *et al.*, 1985)	3
(Schuller *et al.*, 1985)	7
Gluckman, 1986	10
Zhao *et al.*, 1987)	66
(Calzavara *et al.*, 1989)	5
(Feyh *et al.*, 1990)	8
(Schweitzer, 1990)	2
(Wenig *et al.*, 1990)	19
(Grant *et al.*, 1993b)	11

60mg/kg ALA and multiple biopsies were taken to assess concentrations of PpIX by fluorescence microscopy study. The correlation between fluorescence and absolute PpIX levels, as determined by chemical extraction methods (HPLC) has already been discussed in Chapter 3. Three of these patients went on to receive a second dose of ALA with laser irradiation of the tumour between 4-6 hours post ALA administration, which corresponds to the peak PpIX fluorescence (and hence sensitiser concentration). All irradiated sites exhibited ulceration over 24 hours (Grant *et al.*, 1993a).

The search for a more powerful photosensitiser and the exciting clinical results following its use on patients with mesotheliomas, lead to the evaluation of meta tetrahydroxyphenyl chlorin (mTHPC) in the management of head and neck tumours (Ris *et al.*, 1991). Savary et al were the first to report the use of mTHPC-PDT on 13 patients with early SCC in the upper aerodigestive tract, oesophagus and tracheobronchial tree. This group suggested that 0.1mg/kg mTHPC is not sufficient to induce necrosis with light doses up to 90J/cm^2, whereas good results were observed with 0.15mg/kg (Savary *et al.*, 1993).

3.2.4 Light Dosimetry / delivery specific to the oral cavity

The literature illustrates the wide variation in light doses used for treatment of head and neck tumours with ranges from 17-1620J/cm^2. The light dose chosen is often based on empirical data following the testing on normal tissue. There is also a wide variation in the power density used (12- 800mW/cm^2) (Zhao *et al.*, 1987)(Zhoa et al 1990)(Edge & Carruth, 1988), it has been suggested that power densities above 200mW/cm^2 may result in thermal damage (Svaasand, 1985). Although thermal effects may enhance the PDT necrosis produced, undesirable features such as scarring may occur (Barr *et al.*, 1987a).

The geometry of the upper aerodigestive tract makes uniform light delivery particularly difficult. Even with the use of a conventional microlens it can be difficult to deliver light perpendicular to the lesion. Consequently, light delivery systems have been constructed at Lausanne where the cylindrical diffusers are positioned on a Savary-Gillard dilatation bougie, which can be inserted directly into tumours e.g. posterior tongue lesions (Monnier *et al.*, 1990). There is also interest in developing a light delivery device within a highly reflective cone which may be held over a particular lesion so that the whole of the lesion may be equally irradiated and loss of energy from reflection will be reduced significantly. Such light delivery systems are already available for cutaneous treatments (Diomed LED light source). The other aspects of light dosimetry and delivery have already been discussed in the last chapter.

3.2.5 Fluorescence detection

Photodetection of cancers by light induced fluorescence has been exploited since the very early studies and provides a convenient and non invasive method of analysing the tissue status (Policard, 1924). This relies on the detection of either autofluorescence, resulting from endogenous chromophores such as NADH, collagen, flavins, haem or tryptophan containing proteins and endogenous porphyrins or fluorescence following administration of exogenous substances e.g. Photofrin®. The use of exogenous agents depends on the preferential accumulation of the drug in tumour. These methods may enable detection of disease distant from clinically visible limits or aid the determination of tumour margins

and aid pharmacokinetic studies. There are two methods of utilising fluorescence detection in-vivo and ex-vivo, the latter method will be described in Chapter 6.

In-vivo fluorescence detection

Determination of tissue fluorescence depends on the excitation of the exogenous photosensitiser or endogenous chromophores in the tumour by the application of an appropriate wavelength. This is then followed by the detection of the fluorescence produced (emitted). When photosensitisers are used, it is necessary to deduct the fluorescence signal reading from that caused by the tissues own chromophores and thus it is possible to determine areas which exhibit higher signal. Theoretically this corresponds to the tumour as most sensitisers are preferentially taken up by neoplastic tissue as discussed in Chapter 2. In addition, with the changes in levels of photosensitisers, it would be possible to monitor its pharmacokinetic profile by repeated measurements of fluorescence with minimal inconvenience and discomfort to the patient. Numerous in-vivo fibre optic fluorescence detection systems have been developed that included endoscopes connected to devices such as charge coupled device cameras or photomultipliers and promising results have been observed clinically (Braichotte *et al.*, 1995a; Braichotte *et al.*, 1995b; Profio *et al.*, 1983).

3.2.6 PDT in the nasopharynx

Much of the work so far has concentrated on the tumours within the oral or oropharyngeal regions. Accordingly, there is interest in the use of this modality in the treatment of nasopharyngeal carcinomas (NPC). A disease where the primary option is radiotherapy with it attending morbidities and cumulative toxicity. The surgical management of recurrent disease in this area is particularly difficult, therefore a modality such as PDT is extremely attractive. In an early study, two patients with NPC received either HpD or DHE followed by light activation with 200J/cm^2. Complete responses were observed in both cases, however one patient died with liver metastasis, though still clear of local disease at 1 year, whilst the other developed recurrence at 14 months (Buchanan *et al.*, 1989). A series of 57 cases of NPC were reported following PDT with HpD

(5mg/kg). Complete response rates of 44% (disappeared of disease for 1 month) were seen with identical numbers exhibiting "marked response" (over 50% reduction in tumour size) (Sun, 1990). A later report by the same author on 137 patients describes complete response in 55% of the patients. Both red (630nm) and green (488 and 514.5) activating light was used with better responses in the group irradiated with green light. These studies show encouraging results, however, PDT in this region is accompanied not only by light dosimetry difficulties but also the concern of possible damage to normal adjacent structures such as the brain, especially in cases with advance or recurrent disease. PDT is certainly an attractive option, but requires further evaluation prior to commencing a controlled clinical trial.

3.3 PRECLINICAL STUDIES

Surprisingly few studies had been carried out to assess the PDT effect on normal tissue in the vicinity of the treatment area, prior to a number of the early clinical trials. Meyer et al (1990), evaluated the PDT effect on normal tissue in a rabbit model following sensitisation with di-sulphonated aluminium phthalocyanine (AlS_2Pc). It was found that bone was resistant to PDT damage, whilst muscle and salivary glands damage was related to the administered light dose. Mucosal and salivary gland damage regenerated following the PDT injury, but muscle healed with evidence of scarring (Meyer *et al.*, 1991). However, phthalocyanines have have yet to be used clinically as photosensitising agents, so ideally the same study should be carried out on photosensitisers that are in clinical use.

The effect of PDT damage on the normal rabbit tongue following sensitisation with HpD (10mg/kg) has been evaluated. Activation was carried with $100J/cm^2$ of light at 625nm, the authors observed mucosa and muscle damage up to 8mm deep with subsequent healing by a mixture of regeneration and scarring (Jefferis *et al.*, 1991). The PDT damage on skeletal muscle using 6 photosensitisers (HpD, Photofrin®, meta-, para-, and ortho-isomers of tetra (hydroxyphenyl)porphyrins (THPP) were evaluated in the hind leg of mice. Histological examination was carried out 48 hours following laser irradiation and

the most severe damage was seen with para-THPP. Similar long term results were seen by Jefferies (1991), showing muscle healing with regeneration and scarring, along with full return of muscle function (Chevretton *et al.*, 1992). The same group evaluated the cost-benefit of Photofrin®, the meso-tetra hydroxyphenyl porphyrins (mTHPP) and chlorins (mTHPC) in a mice model with implanted plasma cell tumour and concluded that mTHPC was the most selective for tumour compared with normal tissue whilst Photofrin® was the least, in addition to producing greater necrosis (Berenbaum *et al.*, 1993).

3.4 CONCLUSION

Starting with the early reports, which should be considered feasibility studies, there has been a move to treating disease with a curative intent by selecting earlier and more superficial disease. However, there is the need to compare PDT with conventional therapy along with long term follow up and survival data. The majority of clinical studies carried out have used either haematoporphyrin derivative (HpD) or di-haematoporphyrin ether/ester (DHE/Photofrin®) but, as previously discussed (Chapter 2), these are not ideal photosensitisers and hence the move towards the newer agents with improved properties. The different effects observed with different photosensitisers suggest that there is a need to fully evaluate each sensitiser and not simply extrapolate the data already available with other sensitisers. Therefore each photosensitiser needs to be fully investigated in appropriate preclinical models prior to clinical evaluation.

Chapter 4

CURRENT PROBLEMS WITH CLINICAL HEAD AND NECK PDT AND THESIS AIMS

4.1 PDT ON NORMAL TISSUE

Conventional therapies for head and neck cancer are associated with various problems including disfigurement and functional changes. Radiotherapy, though tissue conserving, is often associated with damage to various adjacent structures. Complications include xerostomia, mucositis and even osteoradionecrosis. The suitability of PDT as a modality in the treatment of head and neck cancer depends not only on its ability to eradicate tumour, but also the absence of potential side effects. Studies will therefore be carried out to evaluate the effect of PDT on normal rabbit bone and mucosa using photosensitisers that are undergoing clinical evaluation.

4.2 PDT FOR NASOPHARYNGEAL CARCINOMA (NPC)

As a result of difficult surgical access to the nasopharyngeal region, the first line treatment for NPC is radiotherapy. Response and survival following radiotherapy is high in the early stages (Qin *et al.*, 1988). Nevertheless, complications may arise from damage to the normal adjacent structures e.g. brain. Re-irradiation of local recurrence is associated with considerably higher morbidity. A modality without cumulative toxicity and which allows control of the treated field, such as PDT, would be beneficial. However, prior to formal clinical trials, it is necessary to determine the pharmacokinetic profile of various photosensitisers within the nasopharyngeal (NP) and surrounding tissues in order to define the optimal treatment parameters. This will be followed by the evaluation of the extent of PDT damage to the NP mucosa and adjacent structures. Of most concern is the possible PDT damage to neurological tissues and this will be examined by a study into the quantity of light transmitted through the base of skull in a rabbit model and any PDT effects this might produce.

4.3 CLINICAL PHOTOFRIN® PDT

Although numerous clinical studies have been carried out using PDT in the head and neck region, a large number of questions and problems remain. The predominant sensitiser used to date is Photofrin®, or its precursor HpD. The problems associated with this agent have already been discussed (Chapter 2, section 2.3.1.2). The most serious clinical limitation stems from the prolonged cutaneous photosensitivity. Variations in the chemical composition potentially introduce further problems but despite this, encouraging results have been observed (see Chapter 3). When this research was started, it was the only suitable photosensitiser available and so further studies were undertaken using it. The reported treatment parameters, especially the energy density used, varied enormously (see Chapter 3). Therefore, there was the need to evaluate the response following treatment with standardised parameters following sensitisation with Photofrin®. PDT has been claimed to be particularly suitable for patients with "field cancerization" so the long term outcome of such individuals was studied.

4.4 CLINICAL ALA INDUCED PPIX PDT

The interest in reducing cutaneous photosensitivity has led to the use of the endogenous photosensitiser, ALA induced protoporphyrin IX. At the start of this thesis, there was one publication on the use of systemic ALA in 4 patients (Grant *et al.*, 1993a). In three of the four patients with advanced oral cancer, it was shown that superficial tumour necrosis could be produced. The main advantage of this sensitiser is the short period of cutaneous photosensitivity, however, the time of irradiation and light dosimetry still needed to be optimised. Two different irradiation protocols were evaluated to attempt to improve the clinical effects observed and to establish the most appropriate way to use this agent in clinical practice.

4.5 CLINICAL mTHPC PDT

The problem of inadequate depth of PDT effect has always been of concern and has led to the search for more powerful sensitisers. The second generation photosensitiser, meta tetrahydroxyphenyl chlorin (mTHPC) has shown encouraging preclinical and clinical results, both in terms of the greater depth of effect produced and in the lower light doses required. Light dosimetry studies were performed, using previously reported sensitiser doses, to assess the clinical efficacy of this sensitiser. The difference in response between patients with isolated tumours and those with 'field cancerization' will be determined.

4.6 SUMMARY OF THESIS AIMS

Laboratory studies

a) To determine the effect of tumouricidal doses of PDT on normal bone.

b) To determine the pharmacokinetics of mTHPC and AlS2Pc in nasopharyngeal tissue and brain.

c) To determine the local and intracranial effects of PDT in the nasopharynx.

d) To determine the quantity of light transmission through the skull base.

Clinical studies

a) To determine the efficacy of PDT using Photofrin® in oral cancer.

b) To assess the role of ALA PDT for premaligant and early malignant lesions of the mouth.

c) To study the applications of mTHPC PDT for invasive lesions of the mouth.

Chapter 5

PDT ON NORMAL BONE IN A RABBIT MODEL

5.1 SUMMARY

There have been limited numbers of studies looking at the effect of PDT on normal tissue. Meyer et al (1991) reported a study of PDT applied to normal rabbit bone and mucosa with aluminium disulphonated phthalocyanine at a doses of 5mg/kg, and irradiated with red light at 675nm. They observed that bone was totally resistant to PDT. However no study has looked at the effect on bone with the clinically relevant photosensitisers, Photofrin®, ALA and mTHPC (Dilkes *et al.*, 1995; Fan *et al.*, 1996; Grant *et al.*, 1993a; Levy, 1994).

This study investigates the effect of photodynamic therapy (PDT) on normal bone, using photosensitisers with irradiation regimens capable of causing tumour and normal tissue necrosis.

Rabbits have been used as the jaw bones are of an adequate size to enable easy removal of their incisor teeth producing a bone defect which simulates tumour infiltration of bone. The model also enables comparison of results with previously published data, though modifications have been introduced such as - producing a more uniform illumination of the bone defect. The second part of the study, on buccal mucosa, was carried out to verify that the treatment parameters used in the bone experiments were capable of causing tissue necrosis.

5.2 INTRODUCTION

Numerous authors have used PDT as a modality for cancer of the oral cavity. PDT may be used as a primary modality or as an adjunctive intra-operative therapy, since the surgical management of tumours in this region may result in significant morbidity in terms of function and cosmesis. Radiotherapy, although tissue sparing, is associated with other complications including osteoradionecrosis. The suitability of PDT to eradicate oral

tumours as a primary modality or as an adjunct to surgery depends on the effects to normal tissue especially bone. All the three sensitisers used in this study are currently undergoing clinical investigation.

5.3 MATERIALS AND METHODS

5.3.1 Animal Model

Fifty-eight female New Zealand White rabbits (2-3kg, approximately 3 months old) were used. The experiments were divided into two sections. The initial experiments were carried out on the bone of the tooth socket (48 rabbits) and later experiments on the buccal mucosae (10 rabbits) to verify that the irradiation regimens used were capable of producing tumour necrosis. All animals used in this thesis were housed in temperature controlled quarters in cages with open mesh floors. Throughout the experiments, animal care and operating techniques were in accordance with the Animal (Scientific Procedures) Act 1986.

5.3.2 Photosensitisers

The three photosensitising agents used were; dihaematoporphyrin ether/ester (Photofrin®, QLT Phototherapeutics, Vancouver, Canada), 5-aminolaevulinic acid (ALA, DUSA Pharmaceutical Plc, USA) and meta tetrahydroxyphenyl chlorin (mTHPC, Scotia Pharmaceuticals Plc, Guildford). Photofrin® was reconstituted using 5% dextrose, to make a 2.5mg/ml solution for intravenous injection (IV) at a dose of 3mg/kg. ALA was obtained as the hydrochloride in 98% pure crystalline form and was dissolved in phosphate buffered saline, giving a 100mg/ml solution ready for administration via oral gavage at the dose of 400mg/kg. The mTHPC crystals were dissolved in a mixture of polyethylene glycol, water and ethanol to produce a 4mg/ml concentration and this drug was administered at a dose of 0.3mg/kg body weight (IV).

The ALA dose was delivered in a fractionated form with the total dose (400mg/kg) divided into three equal fractions with an one hour interval between each administration. This is consistent with our current clinical protocol and that of other authors (Fan *et al.*, 1996; Regula *et al.*, 1995). The fractionated ALA dose was chosen on the basis of established pharmacological research which has shown that this method produces a higher blood concentration (Regula *et al.*, 1995). This concept has also been demonstrated recently in a rat mammary carcinoma model where increased levels of fluorescence and better maintained levels of porphyrin were observed when two doses of 300mg/kg (total 600mg/kg) were given as opposed to a single dose of 300mg/kg ALA (Hua *et al.*, 1995) .

PART I

5.3.3 PDT to Normal Bone

In the first experiment 48 rabbits were divided into six groups as described in Table 5.1. The four treatment groups consisted of two Photofrin® groups, an ALA and a mTHPC group. Two "light only" control groups were also included.

Table 5.1: Treatment protocol for drug and light administration to rabbit tooth socket

	Photofrin® 1 Hour	**Photofrin® 48 Hours**	**ALA**	**mTHPC**	**Light alone**	**Light alone**
Dose/ route	3mg/kg (IV)	3mg/kg (IV)	400mg/kg (Oral)	0.3mg/kg (IV)	NA	NA
Wavelength	630nm	630nm	630nm	650nm	630nm	650nm
Drug/Laser interval (hrs)	1	48	2.5 & 4	72	NA	NA
Light Dose	100J	100J	2x100J	100J	100J	100J
No of rabbits	9	9	9	9	6	6
Time to Sacrifice (days)	3x3 3x10 3x21	3x3 3x10 3x21	3x3 3x10 3x21	3x3 3x10 3x21	2x3 2x10 2x21	2x3 2x10 2x21

The rabbit model used in these experiments has been previously described (Meyer *et al.*, 1991). However certain refinements have been incorporated. Firstly a cylindrical diffusing fibre was used instead of a bare cut fibre, to enable more uniform illumination of the whole of the tooth socket.

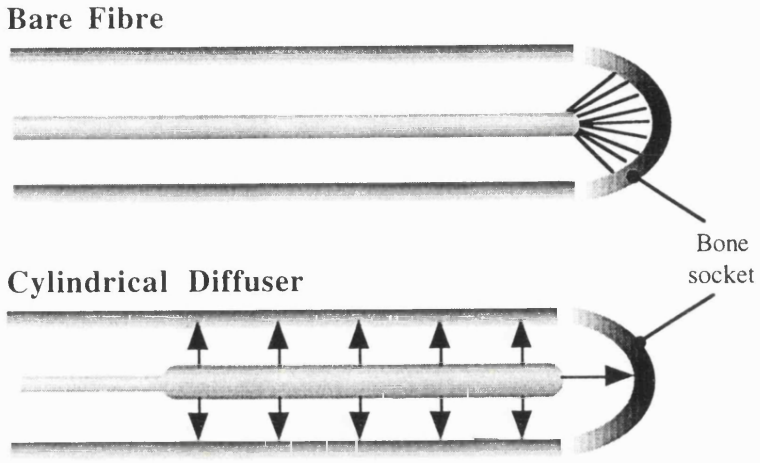


Figure 5 : Difference in distribution of light in tooth socket with bare fibre and cylindrical diffuser.

In addition, in the study by Meyer et al (1991) the photosensitising agent used was disulphonated aluminium phthalocyanine-which was irradiated at a wavelength of 675nm. In our study three different photosensitisers were used; Photofrin®, ALA and mTHPC, which were illuminated at wavelengths of 630nm, 630nm and 650nm respectively.

To simulate tumour infiltrating bone, all rabbits in the first part of the study had removal of the mandibular (lower) incisor. The ipsilateral maxillary incisor was also extracted in the majority of rabbits, to act as a control for the effect of the photosensitiser and trauma of the extraction procedure. Therefore, each rabbit produced two samples of tissue for analysis (except Photofrin® controls and extraction controls). Ideally, the drug controls should have been carried out on the contralateral mandibular incisor, however this would have led to possible feeding problems.

There were 9 rabbits in each treatment group as summarised in Table 5.1. Table 5.2 shows all the different experimental groups and Figure 5.0 the distribution of the samples.

Table 5.2: PDT and Control group Labels

Key		**No of Specimens**
PDT PII 1H:	Photofrin®, irradiation to mandibular socket at 1 hour post administration of photosensitising agent	9
PDT PII 48H:	Photofrin®, irradiation to mandibular socket at 48 hour post administration of photosensitising agent	9
PDT ALA:	Irradiation to mandibular socket after sensitisation with ALA (2.5 & 4 hours)	9
PDT mTHPC:	Irradiation to mandibular socket after sensitisation with mTHPC (72 hours)	9
Control PII:	Photofrin®, extraction of maxillary incisor, no irradiation.	9
Control ALA:	ALA, extraction of maxillary incisor, no irradiation.	9
Control mTHPC:	mTHPC, extraction of maxillary incisor, no irradiation.	9
Extraction alone:	No sensitiser, extraction of maxillary incisor, no irradiation.	6
Control 630nm:	No sensitiser, extraction of mandibular incisor, irradiation with 630nm	6
Control 650nm:	No sensitiser, extraction of mandibular incisor, irradiation with 650nm	6

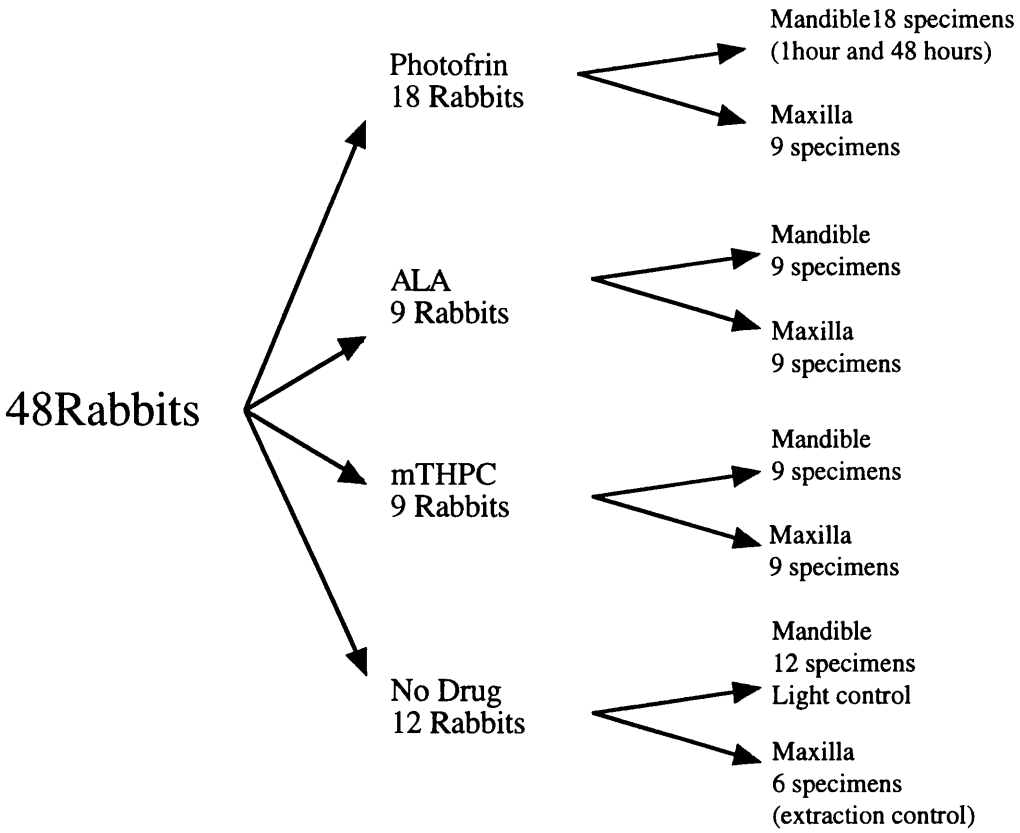


Figure 5.0: Distribution of specimens

Anaesthesia

Oral delivery of ALA required that the rabbits be sedated prior to drug administration. For removal of the tooth and subsequent laser irradiation the animals were anaesthetised using 2mg/kg diazepam (administered subcutaneously and maintained via the intravenous route) and 0.3ml/kg hypnorm (fentanyl and fluansone, Jansen Pharmaceuticals Ltd) administered intramuscularly.

5.3.4 Laser

The light source was a pulsed (12kHz) copper vapour pumped dye laser (Cu25/DL1O, Oxford Lasers Ltd, Abingdon) and the output was focused into a 0.4mm core diameter optical fibre with a 1cm cylindrically diffusing tip (PDT Systems, Santa Barbara, CA). This fibre was passed into the base of the tooth socket thus allowing near uniform irradiation of the whole of the defect (Figure 5.1). The power output was set to supply 100mW at the fibre tip, with an exposure time of 1000s to achieve a total dose of 100J. This dose was previously used in the study by Meyer et al (1991) and has been shown in

numerous other studies to be capable of causing tissue necrosis (Kleeman et al, 1991; Berenbaum et al, 1993).

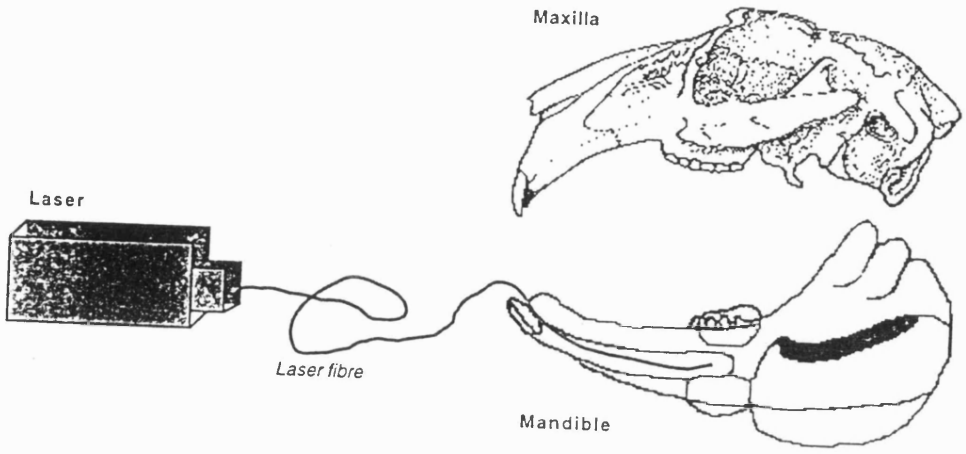


Figure 5.1: Experimental set-up for PDT to the rabbit mandible

5.3.5 Irradiation regimen

Light therapy was carried out at 1 and 48 hours post sensitisation with Photofrin®, and 72 hours post sensitisation with mTHPC. A fractionated irradiation regimen was used with the sensitiser ALA, with a 75 minutes break or dark period halfway through illumination, (100J at T=2.5 hours and 100J at T=4 hours after administration of the first dose of ALA). The rationale for this is described below:

Drug-Light Interval with mTHPC and Photofrin®

Laser light was applied 72 hours post sensitisation with mTHPC, as described by Ris (Ris *et al.*, 1991). The drug light intervals chosen with Photofrin® were 1 and 48 hours post sensitisation. Forty-eight hours has been a standard interval used in many clinical treatments (Grant *et al.*, 1993b; Levy, 1994). The one hour time interval was chosen because a major determining factor for vascular photosensitivity appears to depend upon the level of circulating sensitiser and with lipophilic compounds such as Photofrin®, the concentration of sensitiser reduces with time (Henderson & Dougherty, 1992). Numerous studies have shown that the predominant mechanism of tumour kill with Photofrin® is the direct result of vascular damage (Henderson & Dougherty, 1992) and

PDT carried out with an earlier drug-light interval may therefore produce an enhanced effect.

Drug-Light Interval with ALA

In clinical studies carried out with systemic ALA, only superficial necrosis of tumour has been observed (Grant *et al.*, 1993a; Regula *et al.*, 1995). Modifications to the irradiation regimen have shown promise in improving the PDT effect, hence the fractionated irradiation protocol used with ALA in this study. Reduction in the fluence rate of irradiation has been widely investigated and shown to be of benefit. For instance, in a rat model using Photofrin®, reducing the power density from 200 to 50 mW/cm^2 resulted in a significant delay in tumour growth, when the same total fluence was used (Gibson *et al.*, 1990a). Foster et al found that the tumour doubling time in the same rat model was significantly increased by using a 30s "on-off" irradiation regimen, (Foster *et al.*, 1991).

A study on the fluorescence kinetics with ALA in a rat skinfold observation chamber showed an immediate decrease in fluorescence following irradiation with 100J/cm^2 of light, but after 90 minutes new fluorescence was observed. The animals which received the dual fractions of light at 100J/cm^2 (total dose of 200J/cm^2), with a recovery period of 75 minutes between irradiations, experienced increased damage compared with those that received only one light dose (van der Veen *et al.*, 1994). Enhanced PDT efficacy with ALA via the incorporation of breaks during irradiation has also been observed in a normal rat colon model. In this work a single interruption of 150 seconds during irradiation with 25J resulted in a five fold increase in the necrotic area, from 13 to 94mm^2 (Messmann *et al.*, 1995). The dual irradiation regimen used with ALA in this study has also used in our clinical protocol in attempt to increase tissue necrosis with this sensitiser (Chapter 9).

5.3.6 Histology for the Bone Specimens

The maxillae and mandibles of the rabbits which had tooth extraction with or without PDT, were decalcified using trichloroacetic acid. Following this, longitudinal sections were taken through the tooth socket and the tissue sections were processed in paraffin. Haematoxylin and eosin (H&E) stained sections were examined for damage or necrosis to the normal bone in addition to the various stages of bone healing. The following occurs as stages of healing: haematoma, granulation tissue, inflammatory response, osteoid and woven bone formation. In this study, in each specimen the end point of healing was qualitatively assessed by measured by the amount of woven bone formation. This was scored on a 0-3 scale as :

'0' = absence or minimal presence of woven bone

'1' = presence of woven bone along the socket margin

'2' = socket half filled with new bone

'3' = socket was at least 3/4 filled with bone

5.4 RESULTS

5.4.1 Control Groups

The results from the control studies are shown in Figure 5.2. All the data shown represents the modal value to the most common set of observations from the three rabbits in each sub-group (data in appendix). No differences were seen between the amount of woven bone in any of the groups . In each of the drug only groups there was woven bone formation at the edge of the socket by 3 days, which then filled the socket by 10 days.

In the extraction alone group the level of woven bone formation present at days 3 and 21 was the same as that observed in the corresponding drug controls. In the laser light control group using light at 630nm, woven bone formation was identical to the three drug

groups but, at 650nm, was slightly slower - with a score of 3 observed only in the 21 day specimen.

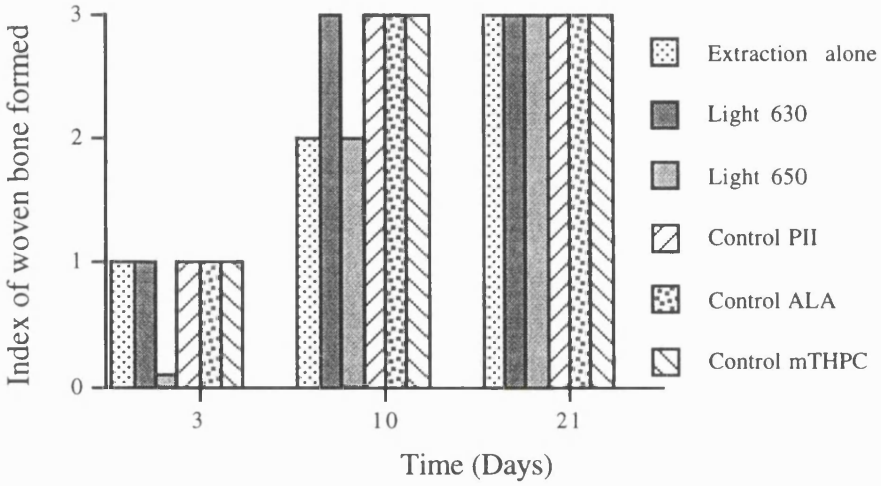


Figure 5.2: Woven bone formation in photosensitiser and light control tooth sockets

5.4.2 Bone healing in the PDT groups

Figures 5.3- 5.5 compare woven bone formation in the mandibular tooth sockets which had been irradiated following sensitisation to the maxillary sockets of the same rabbits which had extraction of the maxillary tooth but no irradiation. Using Photofrin® (Figure 5.3), woven bone formation was more rapid in the drug only control groups, but by 21 days a woven bone score of 3 was observed in Photofrin® (PII) PDT specimens from both groups. In the ALA rabbits (Figure 5.4) more woven bone was present in the PDT group at 3 days, but by 10 days this was reversed and woven bone formation of score 3 was again present in both groups by 21 days. With mTHPC (Figure 5.5) more woven bone was present in the control group at three days but, by 10 days, the level of bone healing was identical in both drug alone and drug plus irradiation groups. When healing of all four PDT groups were compared, those that had been sensitised with mTHPC had more rapid complete bone healing than those who received Photofrin® or ALA. The most important and encouraging feature noticed in these sections was the absence of any necrotic bone or non viable osteoblast in any of the experiments undertaken.

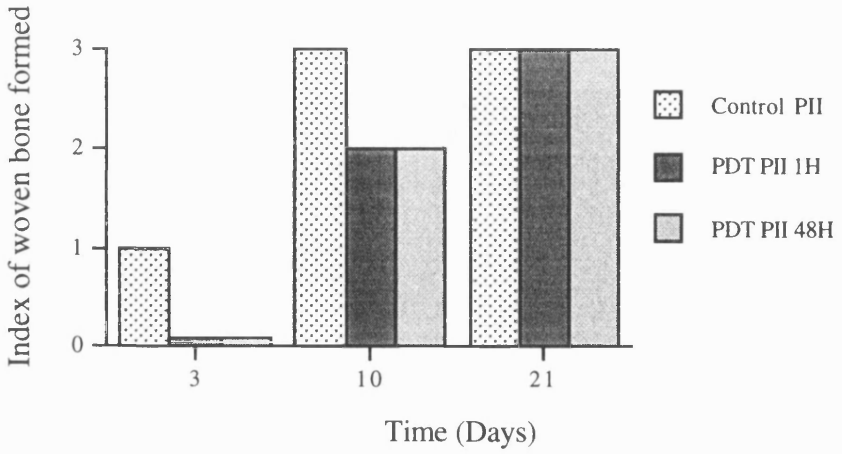


Figure 5.3: Woven bone formation with Photofrin as photosensitiser, with and without light irradiation

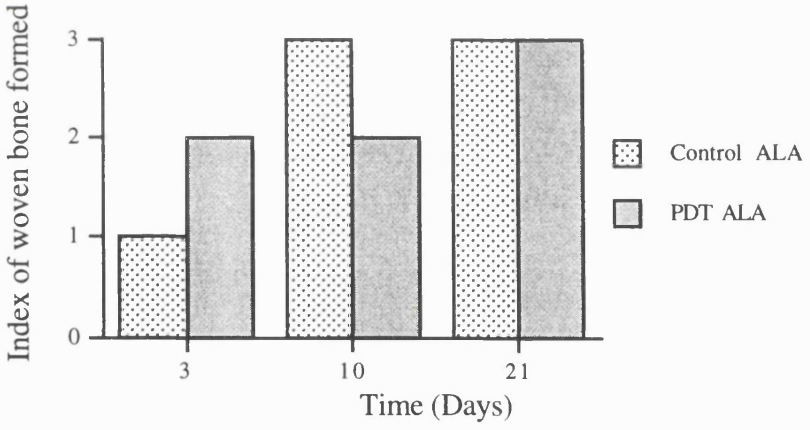


Figure 5. 4: Woven bone formation with ALA as photosensitiser, with and without light irradiation

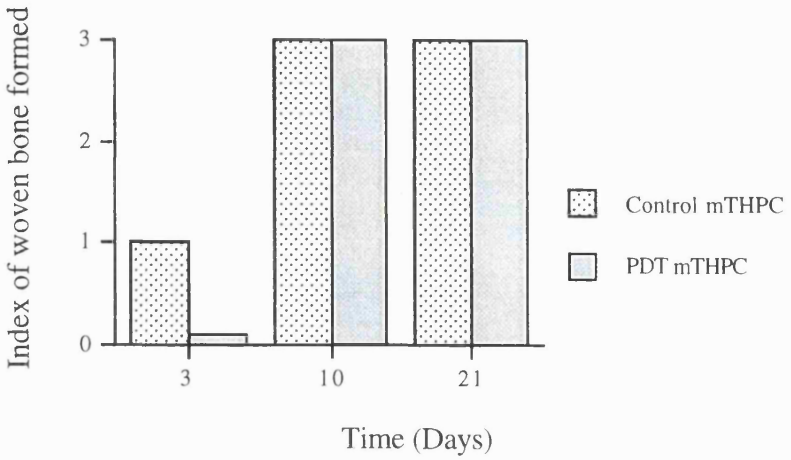


Figure 5.5 : Woven bone formation with mTHPC as photosensitiser, with and without light irradiation

On histological examination, osteoid formation was present as an early feature by three days in most specimens (Figure 5.6B). Likewise granulation tissue was most abundant at 3 days (Figure 5.6A). Although some myositis was present, especially in the mTHPC group, no muscle scarring was evident and no damage was seen in the adjacent salivary tissue in any group.

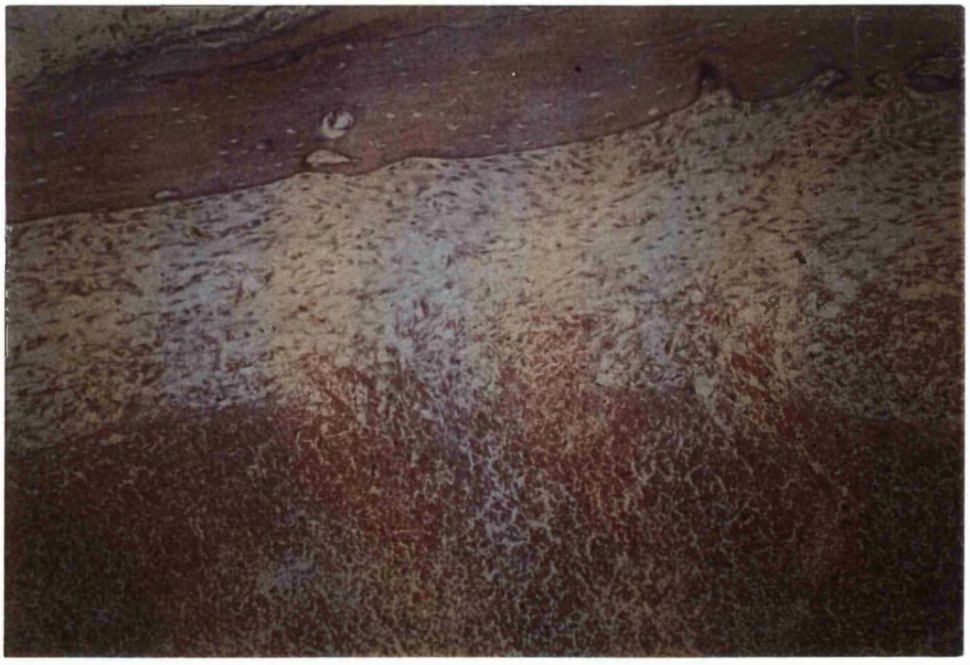


Figure 5.6: (A) Early stage healing showing presence of haematoma with replacement by granulation tissue.

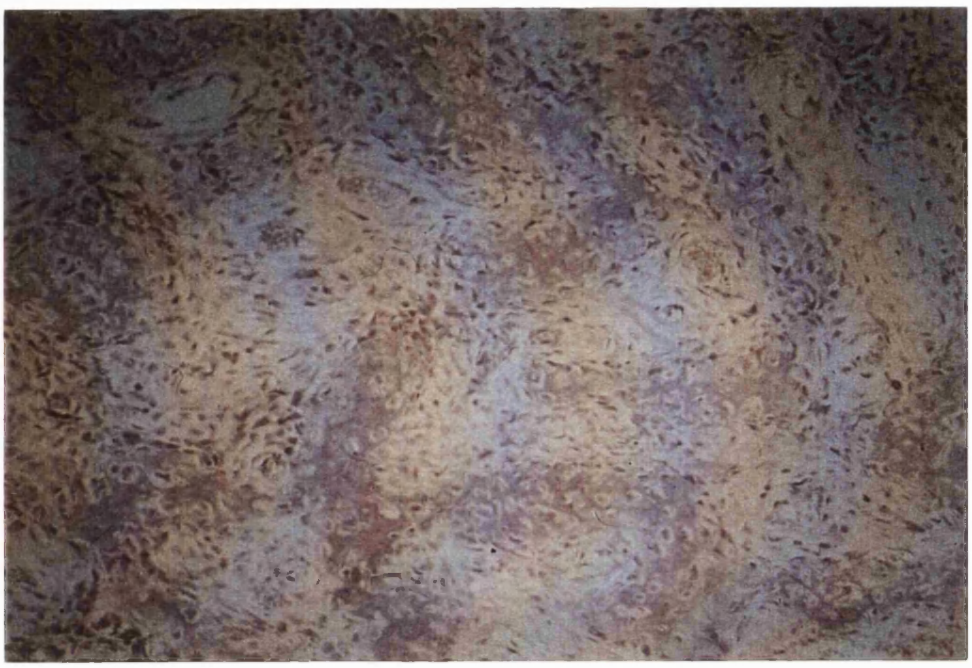


Figure 5.6: (B) healing with evidence of osteoid and woven bone.

PART II

5.5 PDT to Buccal Mucosa

The second series of the experiments were carried out on both the left and right buccal mucosae of ten rabbits (20 specimens), to confirm that treatment parameters used in Part I were capable of causing tissue necrosis. Following sensitisation with the drug doses described earlier, Photofrin 1 hour (n=2), Photofrin 48 hour (n=2), ALA (n=2), mTHPC 125J/cm^2 (n=2) and mTHPC 31J/cm^2 (n=2)) the rabbits were anaesthetised and treated with red light at either 630nm (Photofrin® and ALA) or 650nm (mTHPC). The same drug light-interval described earlier were used. To enable uniform surface illumination, thus eliminating the possibility of uneven treatment due to "hot spots" and avoiding the thermal effects which could arise from the contact of the bare fibre with the mucosa, a microlens was used. The laser beam was focused into a 0.4mm core diameter microlens fibre (QLT Phototherapeutics, New York, USA) allowing delivery of a uniform, 5 mm

diameter circular spot of light to the buccal mucosa with a power density of 125mW/cm^2. Rabbits sensitised with Photofrin® received an energy density of 125J/cm^2; those sensitised with ALA, 2x125J/cm^2 and those with mTHPC either 125J/cm^2 or 31J/cm^2. The higher energy densities (125 or 2x125J/cm^2) used were determined to approximately matched those delivered to the bone defect, calculated from the surface area of the removed tooth as measured with callipers (for results see appendix). The lower fluence was initially used with mTHPC to avoid extensive damage observed in clinical cases irradiated with 20J/cm^2, but these experiments were later repeated with the higher energy density to facilitate comparison. Three days following PDT the animals were sacrificed and the ulcers and a margin of normal surrounding buccal mucosa were excised.

5.5.1 Histology after PDT to Buccal Mucosa

Following PDT to the buccal mucosa, the ulcers and a margin of normal surrounding buccal mucosa were excised and immediately fixed in neutral buffered formalin and processed to paraffin blocks. Five micrometer sections were cut and stained with H&E for comparative light microscopy studies. Due to variable amounts of sloughing between the different specimens, the depths of necrosis were gauged by the different tissue levels affected and not by an absolute value. The depth of necrosis was defined as the deepest point at which there was necrosis of any tissue layer.

5.5.2 Results following PDT in buccal mucosa

Necrosis was present in all specimens (Table 5.3). The depths of necrosis in all Photofrin® and mTHPC specimens were comparable, whilst the depth of damage in those sensitised with ALA were much more superficial. In all the ALA mucosal sections necrosis of the epithelium with the presence of exophytic slough was observed, whilst the underlying lamina propria was intact. In two of the sections there was evidence of minimal necrosis to the superficial muscle and salivary gland necrosis was seen in one section.

The low energy density (31J/cm^2) was used initially with the mTHPC rabbits after our early clinical experience showed dramatic effects on soft tissue at a very low light dose. Subsequently, the experiments were repeated with the higher dose of 125J/cm^2: a slightly greater depth of damage was observed in the limited number of sections studied.

Table 5.3: Results of PDT to buccal mucosa

	Photofrin® 1H 125J/cm^2	**Photofrin® 48H125J/cm^2**	**ALA 125J/cm^2**	**mTHPC 31J/cm^2**	**mTHPC 125J/cm^2**
Depth of necrosis (layer)	muscle and subcutaneous fat	muscle and subcutaneous fat	epithelium, with minimal necrosis of the superficial muscle	muscle	muscle
Extent of muscle necrosis	severe	severe	mild	severe	severe
Thrombosis	yes	yes	no	yes	yes
Salivary gland necrosis	yes	yes	yes (minimal)	yes	yes

In the Photofrin® sections, there were no obvious differences in the histological effect achieved between the two irradiation times. Similar histological features were seen in the Photofrin® and mTHPC samples. The necrosis extended well into the muscle layer and as far as the subcutaneous fat in the Photofrin® sections. The muscle damage was severe, with necrosis of the salivary tissue. Extensive vascular damage with fibrinoid degeneration and thrombus formation was also observed. Subtle differences were seen in the nature of the inflammatory response. A dense inflammatory response, often with prominent eosinophila was noted, was characteristic of ALA PDT. With mTHPC the inflammatory response was minimal with scattered eosinophils and extensive oedema. The response in the Photofrin® samples was somewhere between that observed with ALA and mTHPC. An interesting feature seen in two of the mTHPC sections was the presence of intact epithelium, while there was necrosis of the underlying connective tissue (Figure 5.7).

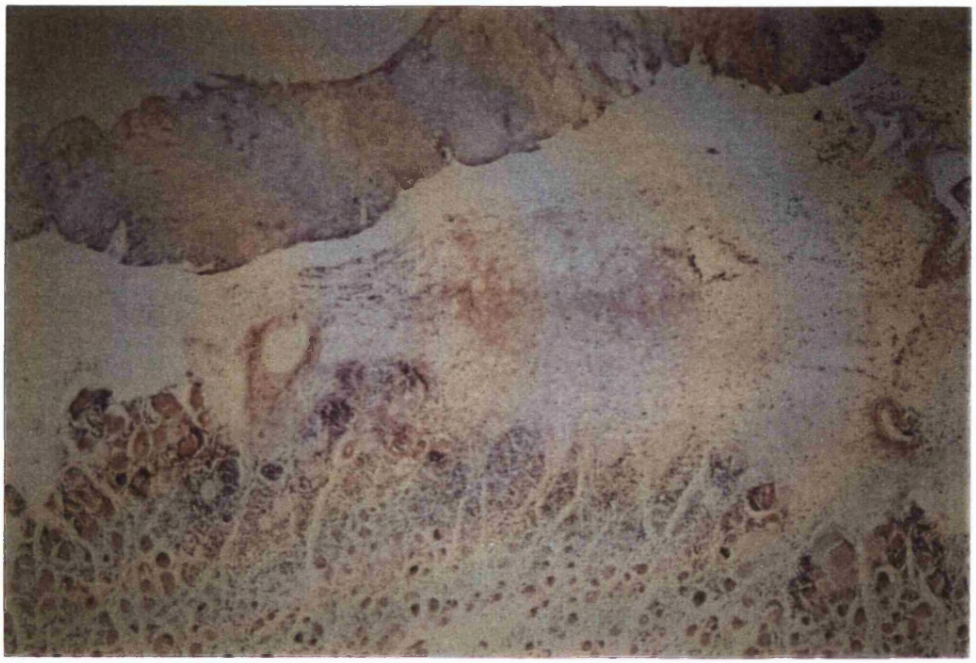


Figure 5.7: H&E section showing necrosis of connective tissue with intact epithelium after mTHPC PDT to rabbit buccal mucosa (day 3).

5.6 DISCUSSION

These experiments created a defect in normal bone (by tooth extraction) and showed that treatment of the tooth socket with PDT did not cause any bone necrosis and only caused a minor delay in healing of the defect. Previous work with the sensitisers investigated in this study have shown that parameters capable of causing normal tissue necrosis are also associated with tumour necrosis (Berenbaum *et al.*, 1993; Fingar & Henderson, 1987; Barr *et al.*, 1990). This study shows that using PDT treatment conditions which were

capable of creating necrosis to normal mucosa and, therefore, also to tumour, produced negligible effect on normal bone. This is in sharp contrast to thermal treatment and radiotherapy (Marks *et al.*, 1983; Stein *et al.*, 1990). In this model, photodynamic therapy retards bone healing in the early stages, but does not affect the long term outcome. At the concentrations used, none of the photosensitising agents on their own showed any adverse effects on the overall rate of bone healing. Similarly the light alone at 630nm had no apparent effect. The rabbits which had received light only at 650nm exhibited bony healing at a comparable rate to the untreated group. Laser light activation of each photosensitising agent is necessary for toxicity and all rabbits which had PDT to the buccal mucosae developed ulceration and necrosis at the treatment site when the same drug and light doses as applied to the bone were used.

5.6.1 PDT to Normal Bone

The photodynamic therapy caused an initial retardation in the rate of bone healing in all groups. This delay was transient and all groups showed the same level of bone healing by 21 days. Healing of the bony defect caused by tooth extraction follows the same stages as any bone healing: filling of the intervening space with a haematoma; the ingrowth of fibroblast and granulation tissue; followed by osteoblast laying down osteoid which becomes calcified into woven bone. The administration of the photosensitising agents did not impair healing sequence. Conversely more rapid infilling of bone was observed in the drug control groups compared with the extraction alone group. The model used eliminates the problems of individual variability. However, I have made the assumption that the rate of healing in the maxilla is identical to the mandible. Unfortunately, there is no published data comparing the difference in rate of healing or bone densities between the maxilla and mandible in rabbits. but the rate of bone infilling of extraction sockets was observed to be more rapid in the maxilla relative to mandible in dogs (Tanaka, 1989). The effect seen in the treated sockets therefore may not be completely due to a PDT effect alone, but a combination of the PDT and slower healing observed in the mandible.

Importantly, our results showed that PDT using these photosensitising agents did not cause mandibular bone necrosis. Neither non viable osteoblast nor sequestration of bone were seen in any specimen. There was, some initial delay in the rate of new bone formation with mTHPC and with both Photofrin® groups, at 3 days and with ALA the delay in woven bone formation appeared to extend to 10 days. However by 21 days no significant differences were seen in any of the specimens. Therefore this study demonstrates that PDT using these agents initially retards bone formation, a result which is different to that of Meyer (1991) who did not observe any delay in ossification with the use of phthalocyanines.

5.6.2 PDT Effect on Other Tissues

An interesting feature seen in specimens of all groups was the presence of a very dense calcified material which, when stained with Schmorl's picrothionin, demonstrated the presence of dentinal tubules. This was believed to be due to the continued tooth formation potential of any pulpal tissue remaining at the base of the tooth socket in the rabbits.

In contrast to Meyer *et al*, although some myositis was present in the PDT bone specimens, especially in the PDT mTHPC group, no muscle necrosis or scarring was evident (Meyer *et al.*, 1991). Similarly, no damage was seen in the salivary tissue in any of the bone PDT groups. These observations may be explained by the more uniform illumination of the bone defect provided by the cylindrical diffuser fibre, as compared to the use of a bare fibre where the light will be concentrated in the apex of the socket (Figure 5.8). Bone is also highly scattering and this would further reduce any light reaching the adjacent muscle. The irradiance at a particular site with the bare fibre is likely to be significantly higher than with a diffuser. Therefore, even with the protective optical properties of bone, sufficient light may transmit to the muscle and reach the threshold for PDT damage to occur.

5.6.3 PDT Damage to Normal Buccal Mucosa

The depth of necrosis produced in buccal mucosa with ALA (400mg/kg) was in agreement with our clinical findings where consistent epithelial necrosis was found (Fan *et al.*, 1996). The extension of the necrosis into muscle, although only to the superficial fibres, is very encouraging in view of the superficial effects created in clinical cases (Fan *et al.*, 1996; Regula *et al.*, 1995). Lofgren *et al* were able to achieve 8mm of necrosis in rabbit papilloma model following intravenous administration of ALA (Lofgren *et al.*, 1995) and Hua et al (Hua *et al.*, 1995) observed extensive PDT damage resulting in 90% mortality in a rat model, when the ALA dose was increased from 600mg/kg or the fluence was increased to 270J/cm2. It may be that depths of necrosis would be enhanced if patients could tolerate either higher drug doses, an intravenous preparation or modification in irradiation regimens.

The hope that Photofrin® PDT with an hour drug-light interval would produce enhanced PDT effect, on the supposition that photosensitiser would be predominantly localised in the vasculature, was not seen in normal tissue. This is in contrast to the findings in a tumour model where an early drug-light interval after Photofrin® administration was shown to enhance PDT damage (Olivo, 1990). In an animal model Barr et al observed maximum sensitiser concentration 1 hour post sensitisation with phthalocyanine; this time interval also correspond to maximum PDT damage (Barr *et al.*, 1987b).

The depths of damage produced with Photofrin® and mTHPC were not significantly different, in contrast to clinical observations (Grant *et al.*, 1992; Ris *et al.*, 1991). This may suggest that mTHPC has a better therapeutic ratio between normal and tumour tissue, hence producing less damage to normal tissue. This was shown by Berenbaum (Berenbaum *et al.*, 1993). However larger numbers of animals would need to be studied. The purpose of this part of the study was solely to verify that the treatment parameters used in the bone study were capable of causing tissue necrosis, and this is the reason why only small number of animals were used in this experiment.

5.6.4 Clinical Implications

Before adopting PDT as a modality for tumours of the oral cavity, adjacent to or invading bone, the ability to completely destroy tumour whilst leaving the normal bone intact is essential to ensure cure with minimal morbidity. The results indicate that PDT using these agents will initially delay bone ossification. The clinical significance of this may be that healing will need to be monitored more carefully during this early period after treatment. The bone defect created in this model was only in continuity with the oral environment via a very small opening. Conventional surgical techniques recommend removing a wide margin (2cm) of normal tissue during resection of oral tumours (Westbury, 1981). This would mean that a potentially large surface area of bone could be exposed to the oral environment following PDT treatment used as an adjunct to surgery. Although PDT alone does not appear to significantly influence bone healing, it might be that the adverse oral environment (saliva, bacteria and food) could have a detrimental effect on the healing of large areas of exposed bone.

In the edentulous mandible, it has been shown that the preferential pathway of tumour invasion is through the occlusal aspect of healed sockets in extracted teeth (McGregor & MacDonald, 1988a; McGregor & MacDonald, 1988b). In the western world, oral cancer predominantly affects those over 50 years of age, with a consequent increased likelihood of patients being edentulous. In these situations PDT may have a role to play as the primary treatment modality, as bone seem so resistant. Moreover patients with these tumours are frequently older and medically compromised which makes subsequent rehabilitation following radical surgery more difficult.

5.7 CONCLUSION

The aim of this study was to determine whether PDT is a safe modality in the treatment of tumours closely related to bone.

- Photodynamic therapy was found to be none toxic to normal bone in this model. Retardation of bone healing was observed in the early stages of repair, but there were no detrimental long term effects.

- The buccal mucosa experiments showed that the drug and light doses used were sufficient to cause PDT damage to the soft tissue.

- Factors such as the oral environment may influence healing, especially in situations where there are large areas of exposed bone following surgical resection. This needs to be investigated in future work.

Chapter 6

PHARMACOKINETIC AND PDT STUDIES OF NASOPHARYNGEAL TISSUES

There has been no published preclinical data concerning treatment parameters for PDT to the nasopharynx. Part I of this chapter looks at the change in distribution and concentration of two sensitisers with time in rat nasopharyngeal mucosa and brain. Part II of the study was designed to assess PDT induced damage in the rabbit nasopharynx and brain using the same sensitisers. Rats were used in the first part of the study to comply with guidelines of the Animal (Scientific Procedures) Act 1986, that the smallest animal possible is used. Rabbits were used in part II of the study as this was the smallest species that allowed passage of an optical fibre into the nasopharynx.

6.1 INTRODUCTION

Dissatisfaction with current therapy for nasopharyngeal carcinoma has led to increasing interest in alternative modalities such as photodynamic therapy. Radiotherapy remains the primary treatment option for this disease, and local control is a crucial factor in determining survival. However, management of this condition is particularly difficult because of the tumour's characteristic predilection for extensive infiltration and its invasiveness (Sham *et al.*, 1994) along with the proximity of the primary site to critical structures such as the eyes, ears, temporal lobe of the brain, cranial nerves, spinal cord, brain stem and hypothalamus (Tsao, 1991).

Successful radiotherapy requires the delivery of an adequate radiation dose to the affected area to provide effective treatment of the disease. Nevertheless it is essential that the delivery is precise to avoid excessive damage to the adjacent normal tissues. The risks of complication following radiotherapy are higher for patients with either residual disease or recurrent disease subsequent to previous radiotherapy (Lee, 1991; Lee *et al,* 1993). The early complications after radiotherapy for head and neck tumour have been discussed in chapter 1. Nearly one third of patients are affected by late sequelae (Haghbin *et al.*, 1985; Lee *et al.*, 1992), mostly soft tissue damage, including persistent otitis (Huang & Chu, 1981; Marks *et al.*, 1982), xerostomia and marked fibrosis of the neck tissues. Neurological complications, however, constitute the major cause of morbidity and

neurological damage was observed in 10% of the patients in one study (Lee *et al.*, 1992). For these reasons there is increasing interest in PDT as an alternative modality since, with careful manipulation of the various parameters, it offers the potential of tumour necrosis with no unacceptable damage to the normal tissue.

Previous clinical work with PDT using HpD for nasopharyngeal carcinoma has shown promising results. In a study of 57 cases complete response was achieved in 43.9% following irradiation of the tumour with light at 630 nm with a total dose of 2000J (Sun, 1990). No apparent attempts were made to determine the size of the irradiation site. In another study on a group of 20 patients complete remission was found in eight, with a further 10 patients showing greater than 60% reduction in tumour volume. The PDT treatment was carried out with 2.5 mg/kg HpD and a dual irradiation protocol at 48 and 72 hours post sensitisation. Unfortunately, no details on the irradiation wavelength, fluence, fluence rate nor tumour dimensions were given (Zhao *et al.*, 1996). A recent study described the dramatic response seen using HpD (5mg/kg, 200J/cm^2) as sensitiser in 12 patients who had recurrent NPC after conventional radiotherapy. At 12 months follow up three out of eight patients deemed "curable" were disease free after a single treatment and three of the remaining four patients achieved useful palliation (Tong *et al.*, 1996) .

PART I: PHARMACOKINETIC STUDIES

6.2 AIM

The aim of the following pharmacokinetic studies was to determine the temporal and spatial distributions of two photosensitisers within the tissues of the nasopharynx and the brain in rats and rabbits. Both of the sensitisers under evaluation fluoresce following excitation with an appropriate wavelength of light. The fluorescence intensity has previously been related to the photosensitiser concentration in tissue measured by chemical extraction (Chatlani *et al.*, 1991; Loh *et al.*, 1993b). Good correlation of these two methods was shown with the photosensitisers aluminium phthalocyanine and 5-

aminolaevulinic acid. The fluorescence detected has also been compared to the photosensitising potential for aluminium disulphonated phthalocyanine (AlS2Pc), and greater PDT damage was observed when irradiation was carried out at times correlating to maximum fluorescence (Chatlani *et al.*, 1991).

The kinetic profiles and biodistributions of meta tetrahydroxyphenyl chlorin (mTHPC) and AlS2Pc were initially determined in the rat nasopharynx and brain. A limited repeat study in rabbits was used in phototherapy studies presented later in this chapter.

6.3 MATERIALS AND METHODS

6.3.1 Animals

Stage 1 of the studies was carried out on 57 adult rats (250-300 grams) and 5 adult female New Zealand White rabbits (2-3kg body weight). All animals were housed and cared for as described in chapter 5.

6.3.2 Photosensitisers

mTHPC was supplied by Scotia Pharmaceuticals, Guildford in the crystal form with an accompanying solvent. The crystals are dissolved in the solvent mix consisting of 20% ethanol, 30% polyethylene glycol and 50% water. The aluminium disulphonated phthalocyanine was obtained from the Chemistry Department at Imperial College London. It was initially dissolved in 0.1M NaOH and subsequently buffered to a pH of 7.4 prior to systemic administration.

In the rats, each photosensitising agent was administered intravenously (IV) under inhalational anaesthesia using a combination of halothane and oxygen. For the rabbits, each sensitiser was injected without sedation, via the marginal ear vein.

Sensitisation with mTHPC

Thirty-six rats received IV mTHPC at either 0.3mg/kg (n=15) or 1mg/kg.(n=21). Sacrifice of the rats was carried out using rising concentrations of carbon dioxide and subsequent cervical dislocation at serial times ranging from 0.5-192 hours. There were 3 rats per time point. To ensure that the rat pharmacokinetic study could be translated to rabbits, two rabbits were sensitised with 0.3mg/kg and 1mg/kg mTHPC with subsequent sacrifice at 96 hours by pentobarbitone overdose (Expirial ®, Sanofi Animal Health Ltd, Watford), thereby enabling the determination of the presence of mTHPC. No published data exist as to how the different methods of sacrifice between rats and rabbits will influence fluorescence studies. Standard practice of sacrifice was carried out and was identical within each species.

Sensitisation with AlS2Pc

Six groups of rats (3 in each group) were administered 1mg/kg AlS2Pc with subsequent sacrifice at intervals between 30 minutes and 72 hours. Again, to ensure translation of rat data to the rabbit, two rabbits were given 1mg/kg AlS2Pc and sacrificed at 1 and 24 hours post sensitisation .

Controls

Control samples were taken from three unsensitised rats and one unsensitised rabbit for analysis with both mTHPC and AlS2Pc.

6.3.3 Collection of specimens

In all the animals, the nasopharyngeal (NP) tissue and brain closest to the skull base were exposed via a sagittal section through the head. The NP mucosa and brain were immediately harvested, laid flat, divided into two portions and frozen by submerging in a bath of isopentane (pre-chilled in liquid nitrogen). In the five rabbits (2 mTHPC, 2 AlS2Pc and 1 control), additional samples of the dura mater were dissected and collected.

6.3.4 Fluorescence Microscopy

The frozen sections were kept in liquid nitrogen until sectioned. Three 10μm sections were prepared using a Cryocut E microtome (Reichert-Jung). These were stored in a freezer at -20^{0}C. The slides were thawed immediately before fluorescence microscopy (Chan et al 1989). The set up for the fluorescence microscopy studies is shown in diagram in appendix to Chapter 6. The excitation source was an 8 mW helium-neon laser (632.8 nm). The phthalocyanine fluorescence emissions was detected between 665 and 710nm. The fluorescence signal was detected by a highly sensitive, cryogenically cooled, CCD (charge-coupled device) camera (Wright instruments, Model 1) fitted to the microscope. With mTHPC, the fluorescence excitation was carried out at 543nm and emission detected in the range 630-680nm.

Image processing and control operations were carried out using an IBM personal computer which generated false colour coded images depicting the fluorescence intensity as counts per pixel in arbitrary units. A minimum of 3 images were taken from each of the 3 sections of 3 rats sacrificed at each time point. The autofluorescence of the tissue was measured from the control sections of unsensitised animals. The slides were subsequently fixed in formalin and stained with haematoxylin and eosin, thus allowing conventional light microscopy to be carried out for comparison. Mean fluorescence values were calculated at each time point to obtain a profile of the change in concentration of each photosensitiser with time.

Determination of mTHPC in tissue by HPLC

The second portion of the mTHPC specimens were sent to Dr CK Lim, MRC Laboratory (Leicester), for determination of mTHPC concentration by high performance liquid chromatography (HPLC). Details of the HPLC techniques are given in the appendix to Chapter 6.

6.4 RESULTS

6.4.1 Fluorescence distribution of mTHPC in rat samples

Following IV administration of 0.3mg/kg and 1mg/kg mTHPC the photosensitiser fluorescence intensities (expressed in arbitrary units) in different tissue compartments are summarised Figures 6.1 and 6.2. The relative fluorescence intensities in both the respiratory epithelium and underlying connective tissue follow similar profiles (Figure 6.3). The times of peak fluorescence with 0.3mg/kg appear to be 24 hours in connective tissue and 96 hours in respiratory epithelium (Figure 6.1). Fluorescence of both layers is decreased at 72 hours relative to 24 and 96 hour specimens. With the higher drug dose (1mg/kg), the fluorescence peak in the connective tissue and respiratory epithelium coincide at 24 hours (Figure 6.4). Not surprisingly, the magnitude of the peak intensity in the NP tissues was found to be dose dependent with over 2.5 fold greater intensity in the respiratory epithelium of the 1mg/kg mTHPC rats relative to the lower drug dose (values of fluorescence: 91.46 with 1mg/kg mTHPC and 36.01 with 0.3mg/kg). After the peak at 24 hours, the fluorescence in the mucosal layers gradually declined, but even at 192 hours, with 1mg/kg, the count was still higher than autofluorescence. With both drug doses, the fluorescence intensities at 96 hours were almost identical values (Figure 6.5).

The fluorescence intensities in normal connective tissue and respiratory epithelium compared to those in brain was maximal at 24 and 96 hours respectively with 0.3mg/kg mTHPC. With 1mg/kg mTHPC, the maximal differential was at 24 hours. At all time points and with both drug doses, the fluorescence intensity in the brain tissues did not increase significantly beyond that of the control level.

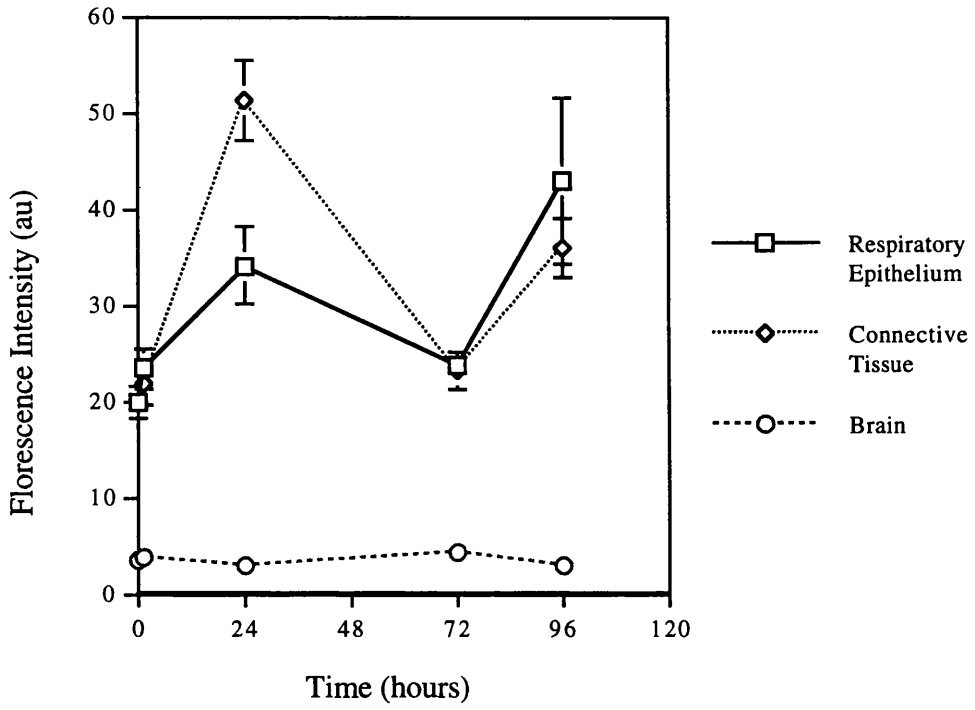


Figure 6.1: Comparision of fluorescence intensity in the nasopharyngeal tissues and brain following sensitisation with 0.3mg/kg mTHPC. Each value is the mean from 15-30 areas in 3 rats ± SEM.

Time 0 is unsensitised animals (autofluorescence)

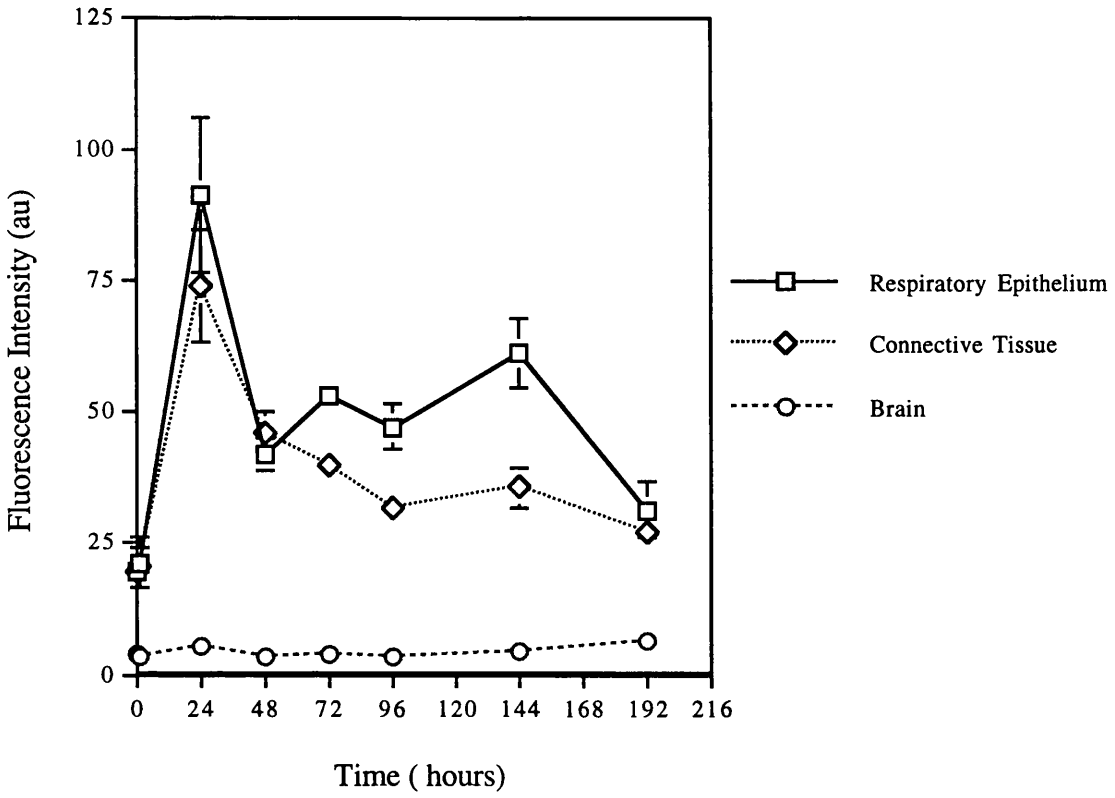


Figure 6.2: Comparision of fluorescence intensity in the nasopharyngeal tissues and brain following sensitisation with 1mg/kg mTHPC. Each value is the mean from 15-30 areas in 3 rats ±SEM.

Time 0 is unsensitised animals (autofluorescence)

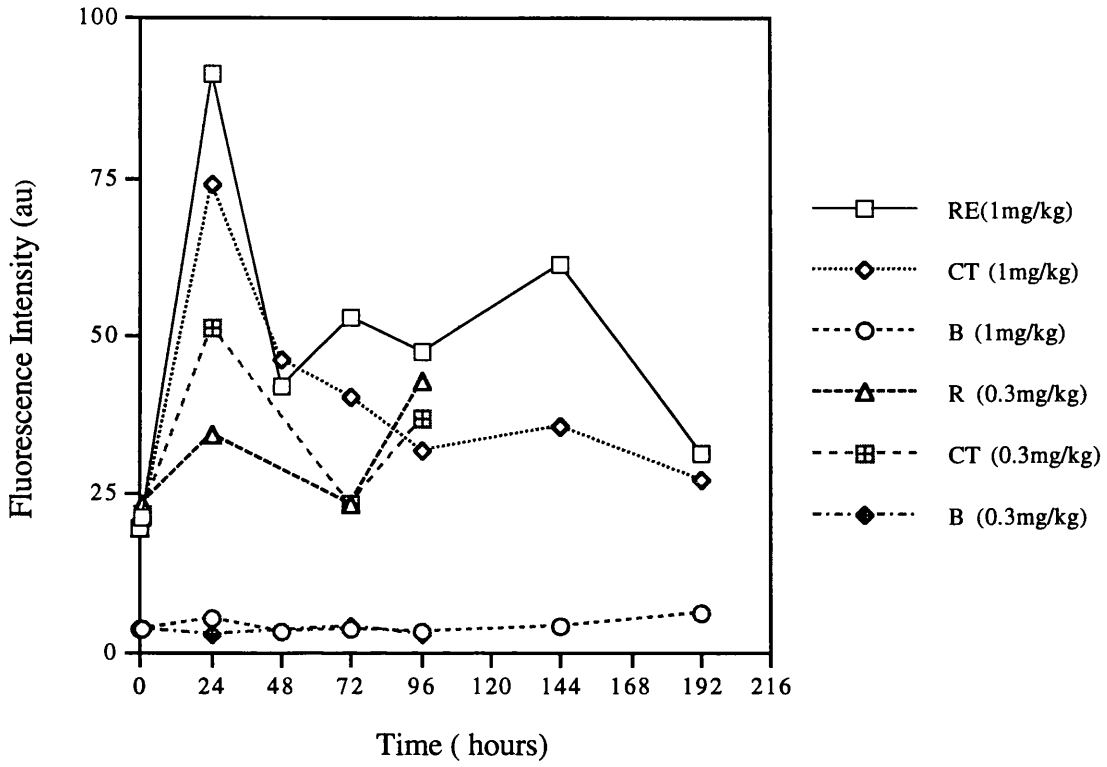
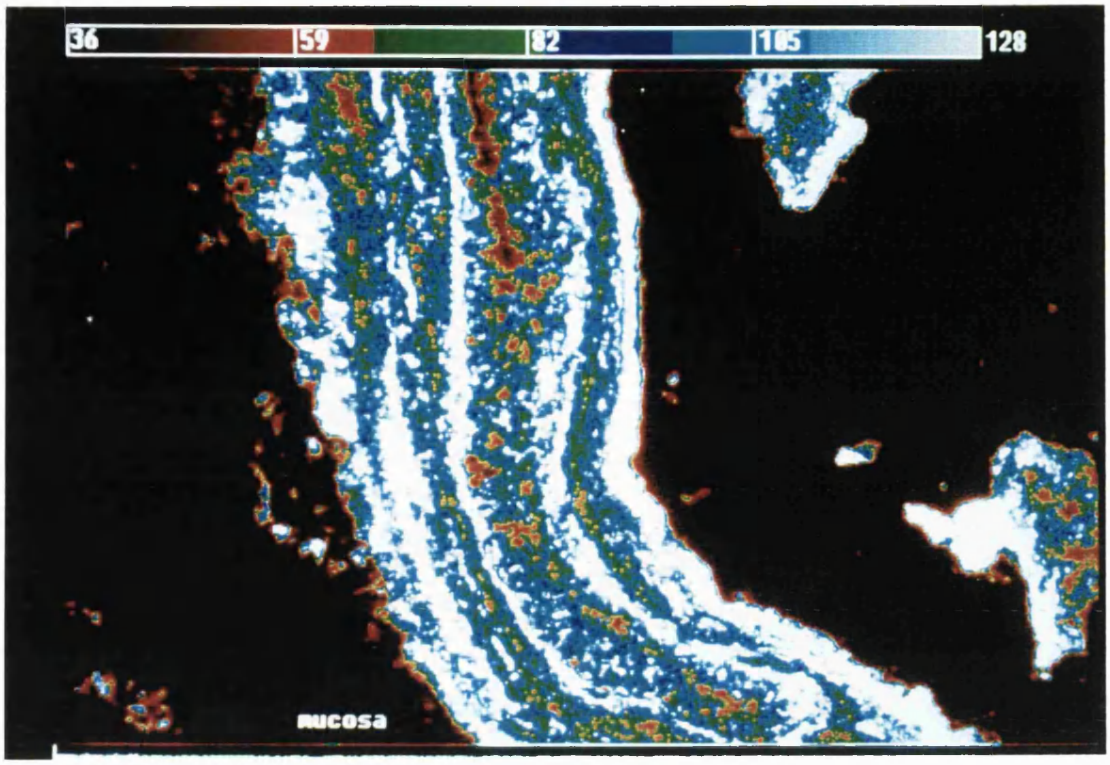


Figure 6.3: Cummulative data, for comparision of fluorescence intensity in the nasopharyngeal tissues and brain following sensitisation with 0.3mg/kg and 1mg/kg mTHPC. Each value is the mean from 15-30 areas in 3 rats.

Time 0 is unsensitised animals (autofluorescence)

RE: Respiratory Epithelium; CT: Connective Tissue; B: Brain;



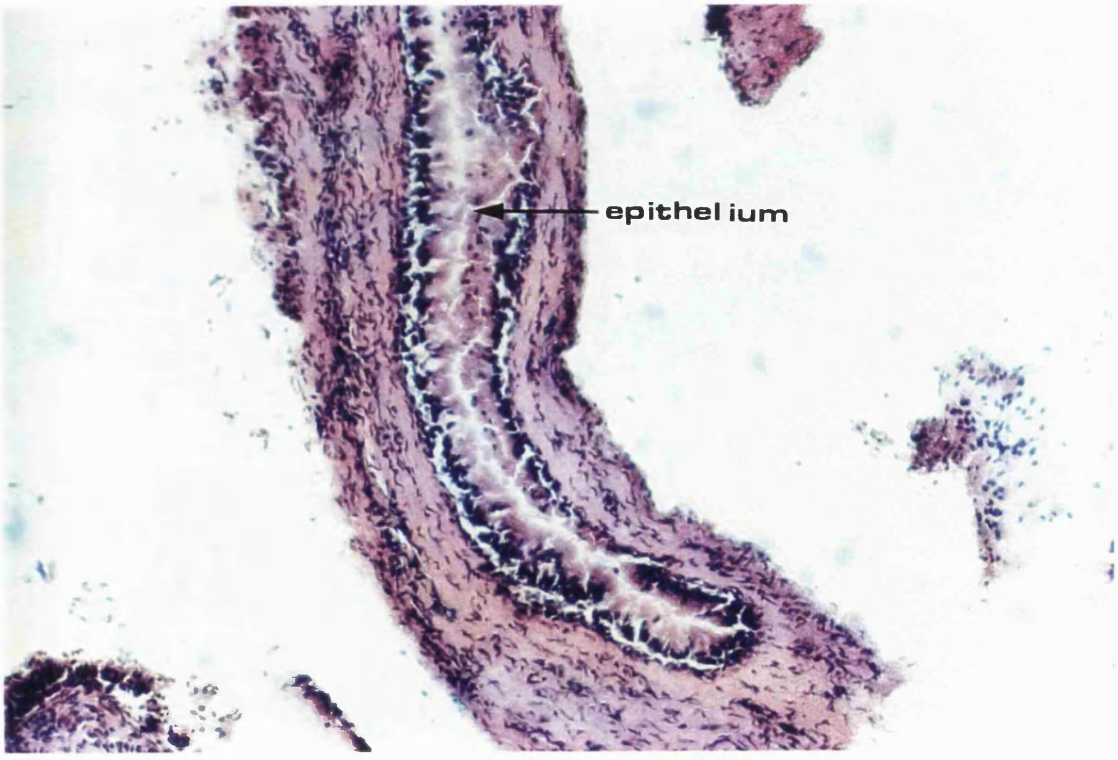


Figure 6.4: Computer processed fluorescence miscroscopy image of the NP tissues 24 hours after sensitisation with 1mg/kg mTHPC. The colour scale depicts the signal in counts per pixel (white is high, black is low fluorescence) and corresponding H&E.

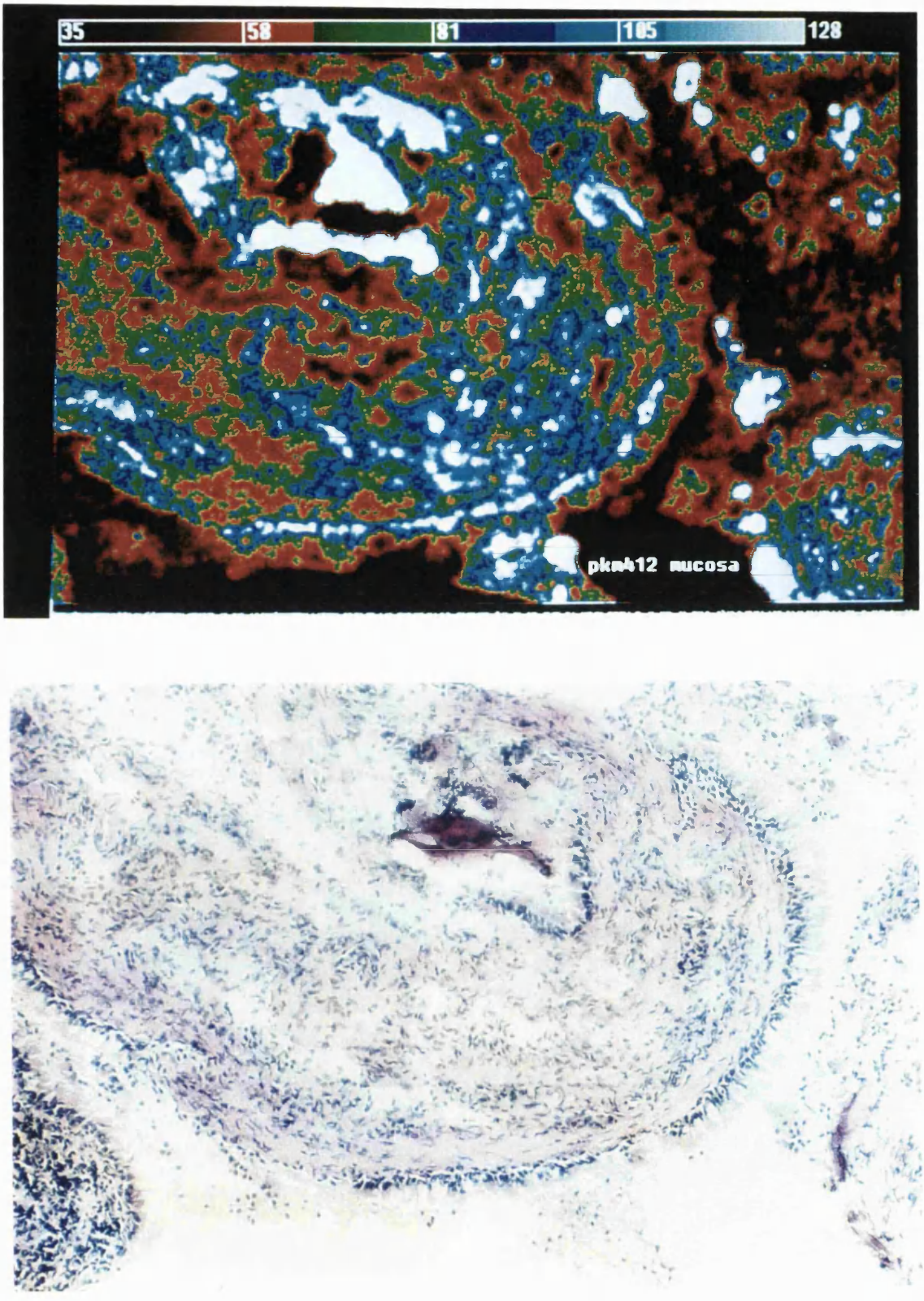


Figure 6.5: Fluorescence micrograph and corresponding H&E of NP mucosa, 96 hours following sensitisation with 1mg/kg mTHPC.

mTHPC Concentration by HPLC

Irrespective of the drug dose (0.3 or 1mg/kg mTHPC), or the time interval to sacrifice, there was no detectable quantity of mTHPC in any of the 36 brain specimens. Surprisingly only seven mucosal samples had detectable quantities of mTHPC and all except for one were day 1 or day 2 samples. Drug levels may be below the sensitivity of the assay due to the small size of the tissue.

6.4.2 Fluorescence distribution of AlS2Pc with the rat specimens

Intravenous injection of AlS2Pc resulted in the fluorescence intensities summarised in Figure 6.6. The relative fluorescence intensity was higher at 0.5 and 24 hours in the connective tissue. The fluorescence in the respiratory epithelium was lower than the corresponding connective tissue reading at all but 4 and 24 hours. As with mTHPC, fluorescence intensities from the brain tissues were low, regardless of the time interval and did not significantly increase above baseline levels. The optimal difference between the brain and connective tissue and respiratory epithelium occurred at 24 hours, when the fluorescence was highest in these tissues.

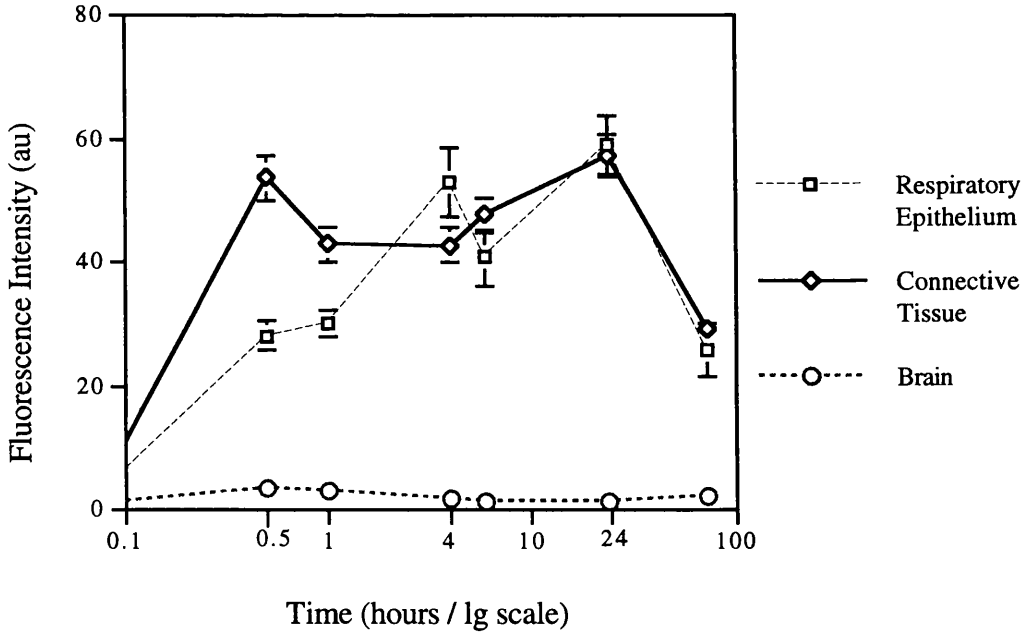


Figure 6.6: Comparision of fluorescence intensity in the nasopharyngeal tissues and brain following sensitisation with 1mg/kg AlS2Pc. Each value is the mean from 20-30 areas in 3 rats ±SEM.

Time 0 is unsensitised animals (autofluorescence)

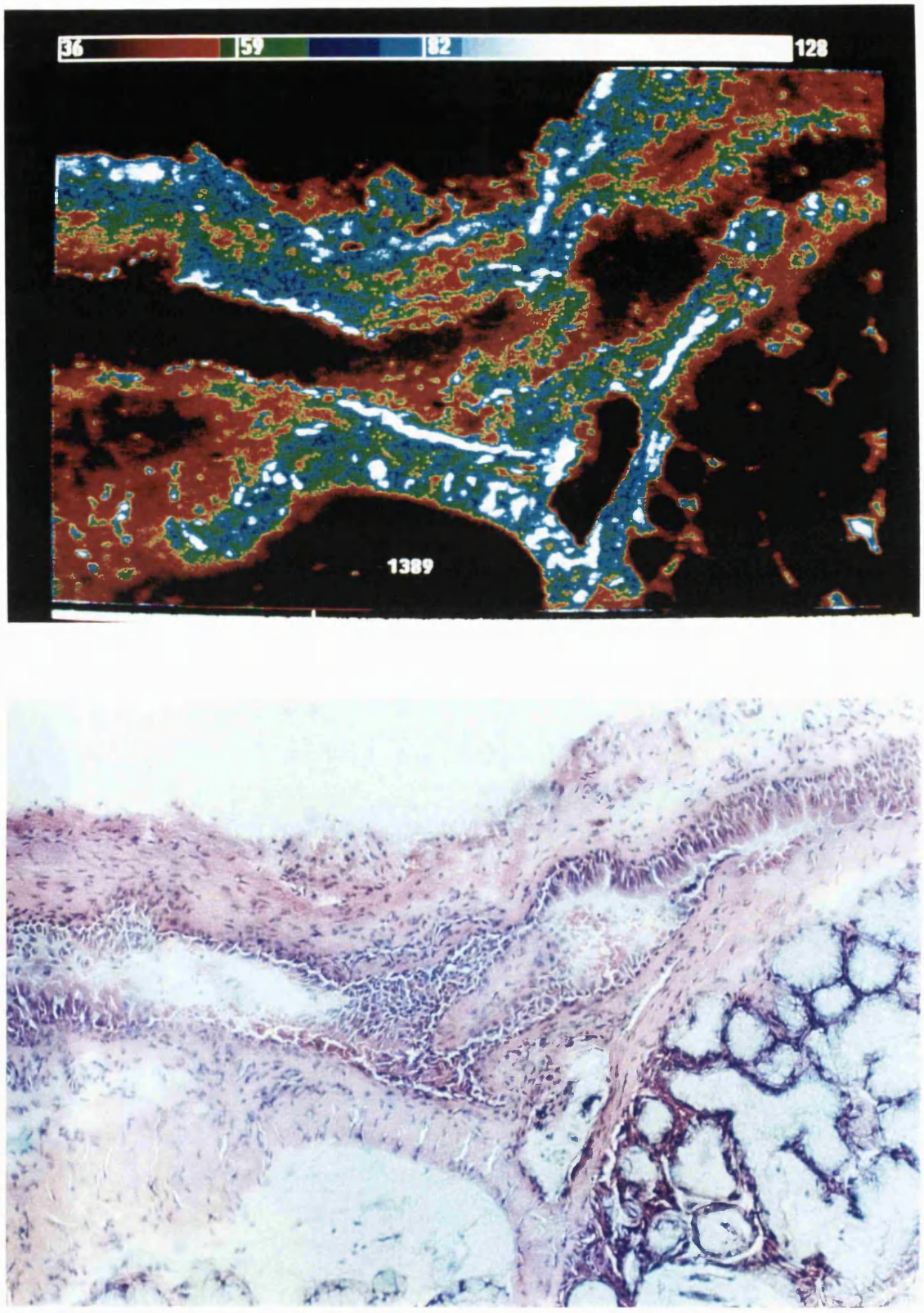


Figure 6.7: Fluorescence micrograph and corresponding H&E of rat NP mucosa 1 hour after sensitisation with 1mg/kg AlS2Pc

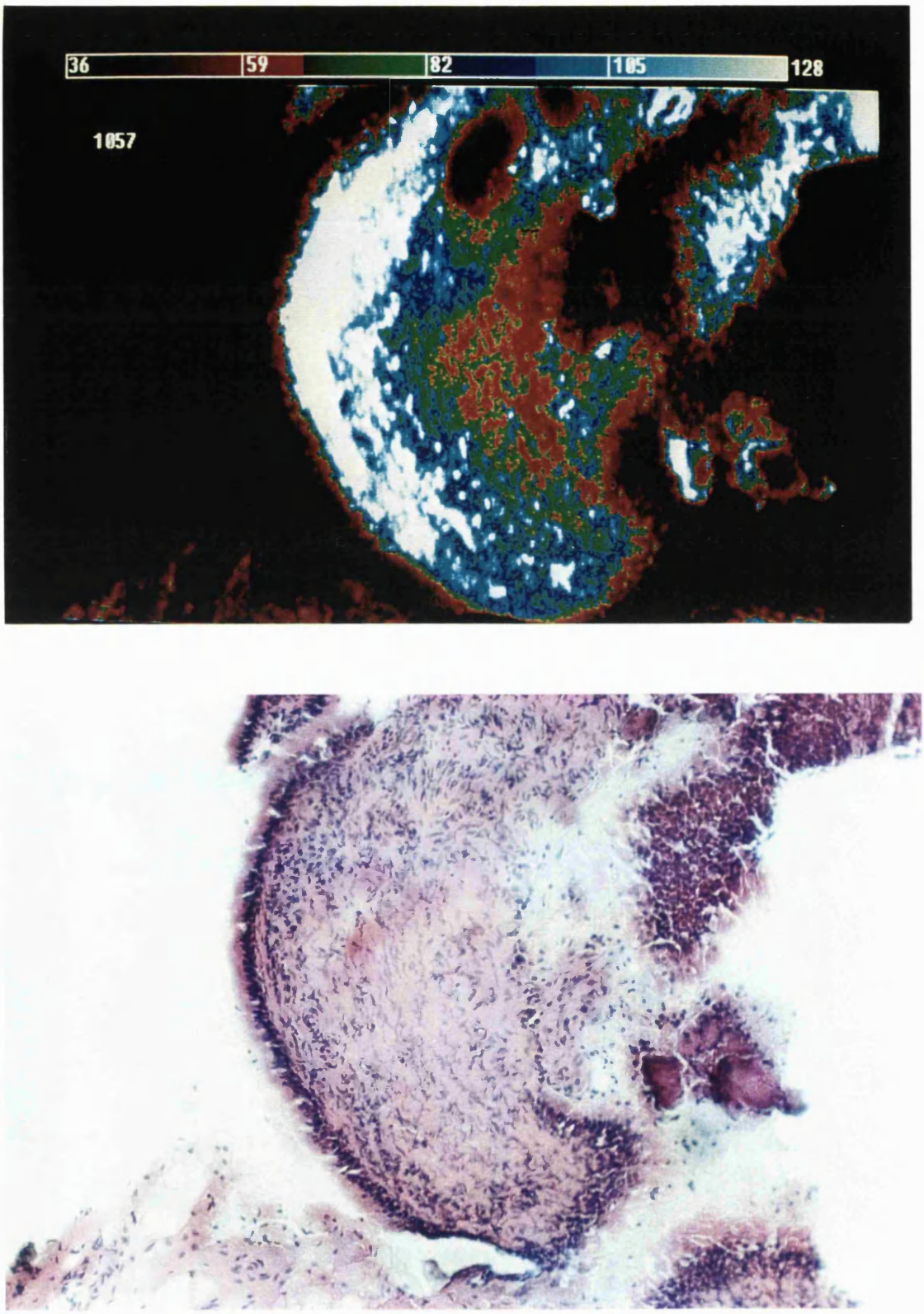


Figure 6.8: Fluorescence micrograph and corresponding H&E of rat NP mucosa 24 hour after sensitisation with 1mg/kg AlS2Pc.

Fluorescence distribution with the rabbit specimens

The relative photosensitiser fluorescence intensities in the nasopharyngeal and neurological tissues in the rabbit are shown in Figures 6.9 and 6.10. Sacrifice took place at 96 hours after sensitiser administration. The fluorescence of the normal mucosa was dose dependent: higher fluorescence was seen in the mucosa at 1mg/kg mTHPC, compared to 0.3mg/kg which is therefore in agreement with the rat study. Fluorescence was seen in the brain tissues at both drug doses, but this was barely above background levels. The fluorescence in the dura mater was surprisingly high in both the control and 1mg/kg mTHPC specimens, although the values observed with the 0.3mg/kg samples appeared to be below that of the control.

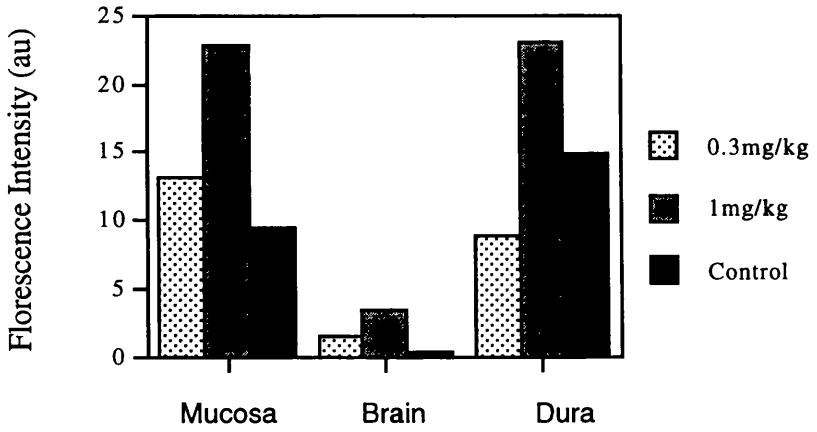


Figure 6.9: Comparision of fluorescence intensity in the rabbit nasopharyngeal mucosa and brain following sensitisation with 0.3mg/kg and 1mg/kg mTHPC. Each value is the mean from 20-30 areas in 2 rabbits.

The fluorescence intensity in the rabbit mucosa was similar in both the 1 and 24 hour samples following AlS2Pc administration, and was over six times higher than the control (Figure 6.10). Fluorescence observed in any of the brain samples was negligible. The fluorescence in the dura was higher in the 1 hour than the 24 hour samples, with ratios of 5:1 and 3:1 relative to control dura mater respectively.

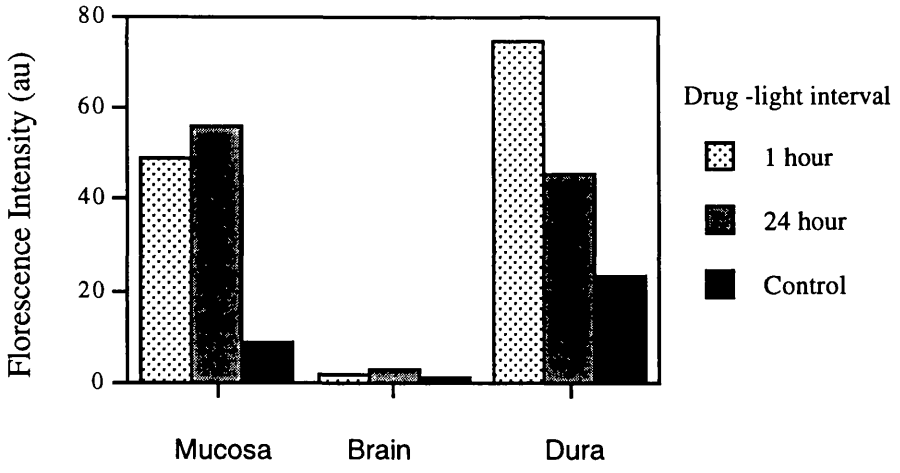


Figure 6.10: Comparision of fluorescence intensity in the rabbit nasopharyngeal mucosa and brain following sensitisation with 1mg/kg AlS2Pc. Each value is the mean from 20-30 areas in 2 rabbits.

Analysis of Data

Analysis of the fluorescence images produced from the rat pharmacokinetic studies were carried out using analysis of variance (ANOVA, Statview) to ascertain if there were changes in fluorescence with time. Subsequently, unpaired t-test were then applied to the specific time points. The data obtained from the mean fluorescence intensity of each image is presented in the appendix to Chapter 6.

6.5 DISCUSSION

The two sensitisers investigated in this study both distributed to the NP tissues. At the drug concentrations used and the times evaluated, both mTHPC and AlS2Pc exhibited strong accumulation in the NP mucosa with no detectable concentration found in brain tissues.

6.5.1 Fluorescence in rat specimens with mTHPC

The experiments with the higher mTHPC dose (1mg/kg) were initially used following previous fluorescence work by Van der Veen et al (van der Veen *et al.*, 1994), who observed negligible fluorescence in a rat skin-fold model following administration of 0.3mg/kg mTHPC compared to that seen with 1mg/kg. As 0.3mg/kg is a more likely therapeutic dose, the experiments were repeated and the fluorescence observed to be reduced relative to that obtained with the 1mg/kg samples. At all time points investigated the fluorescence in the brain tissue did not change significantly relative to the control. This is in agreement with previous studies carried out with mTHPC using both fluorescence intensities and chemical extraction analysis (Morlet *et al.*, 1995; Peng *et al.*, 1995). It would therefore appear that the normal brain is protected by the intact blood brain barrier, with only very little photosensitiser entering from the systemic circulation.

The two different concentrations of mTHPC follow a similar kinetic pattern of distribution in the respiratory epithelium and underlying connective tissue. The concentration of dye in these tissues peaked at 24 hours and remained higher than the control samples even at 192 hours for the 1mg/kg samples. Peng et al observed maximum concentrations of mTHPC between 24-48 hours in the normal tissues (including skin and muscle) of mice bearing mammary carcinoma, using chemical extraction assays (Peng *et al.*, 1995)., and using confocal laser scanning fluorescence microscopy, mTHPC fluorescence was seen to originate from the cytoplasm of the neoplastic cells. The same group also observed decline in individual tumour cells fluorescence by 96 hours, but even at 144 hours post injection of mTHPC fluorescence was still present in the tumour tissue.

mTHPC Fluorescence in Connective Tissue

The fluorescence in the connective tissue at 96 hours were similar with both drug doses which may suggest that the rate of mTHPC clearance is related to the concentration of dye i.e. faster clearance with the higher drug dose (1mg/kg) and slower clearance with the lower dose. No significant difference was seen between the fluorescence detected at 96,

144 and 192 hours following 1mg/kg mTHPC (Students t-test for unpaired data, t= -0.736 for 96 and 144 hours, t=1.15 for 96 and 192 hours, t=1.69 for 144 and 192 hours with P>0.05), difference was seen between 72 and 96 hours (P< 0.05). Similarly, a difference in fluorescence was detected between 72 and 96 hours with 0.3mg/kg mTHPC (Students t-test for unpaired data, t= 2.2 and P> 0.05) .

mTHPC Fluorescence in Respiratory Epithelium

No difference was seen in the respiratory epithelium fluorescence between 96 hours and 72, 144 and 192 hours, following sensitisation with 1mg/kg mTHPC (Students t-test for unpaired data, t= -1.49 for 96 and 144 hours, t=1.98 for 96 and 192 hours, t=3.09 for 144 and 192 hours with P≥0.05). With the lower mTHPC dose (0.3mg/kg) a difference was detected between 72 and 96 hours (Students t-test for unpaired data, t= -2.26 with P<0.05).

6.5.2 Fluorescence in rabbit specimens with mTHPC

The fluorescence intensity in the rabbit specimens also exhibited a dose dependant relationship, with higher fluorescence in the 1mg/kg mucosal samples relative to 0.3mg/kg mucosa. The fluorescence in the control samples was low in the mucosa and undetectable in the brain tissue. Low readings were observed in the brain samples from both mTHPC doses. The dura surprisingly showed fluorescence comparable to that observed in the mucosa, although at 0.3mg/kg the fluorescence in the dura was lower than in the control sample. The thinness of the dura made preparation of the frozen sections more difficult,; therefore some sections may be thicker and may have led to higher fluorescence signal as a result of artefact.

Chemical Extraction Assays

The undetectable levels of mTHPC in the brain tissues is in agreement with a previous reported study (Morlet *et al.*, 1995). This also correlates with the negligible fluorescence signal from the CCD (charged couple device) studies. Surprisingly, however is the undetectable mTHPC levels in the mucosal samples. One of the reasons for this may be related to the small quantity of NP tissue available for analysis (less than 20mg). Morlet et al (1995) were also able to observed fluorescence at mTHPC doses of 0.8mg/kg. Ideally an animal model with a larger nasopharynx is required. However economic restraints and Home Office guidelines make it difficult to study large animals.

6.5.3 Fluorescence with AlS2Pc

The fluorescence in the NP connective tissue reaches an initial peak at 30 minutes followed by a subsequent plateau, with the maximum fluorescence in the connective tissue reached at 24 hours Figure 6.6 ($P<0.05$ at all time intervals except 30 minutes). Overall the fluorescence in the connective tissue was higher than in the epithelium except at 4 and 24 hours post sensitisation. The maximum concentration of AlS2Pc in the NP tissues was reached between 30 minutes and 24 hours post sensitisation. The difference in fluorescence between 30 minutes and 1 hour was not significant for both epithelium (RE $P>0.05$) and connective tissue (CT $P>0.05$). Peng et al (1995) observed maximum concentration in normal and tumour tissue between 2-24 hours post administration of AlS2Pc, following chemical extraction assays. Chatlani et al (1991) observed maximum fluorescence in a rat colon model with AlS2Pc at 1 hour. However this was the earliest time interval studied. Barr et al (1987) demonstrated peak levels of phthalocyanine 1 hour post sensitisation by a chemical extraction, also in a normal colon model, but AlSPc was used instead of AlS2Pc. Peak phthalocyanine levels (AlSPc not AlS2Pc) were seen in various tumours at 24-48 hours, with the peak in normal tissue again seen between 1-3 hours (Tralau *et al.*, 1987). In Tralau 's study, peak levels of sensitiser in the normal brain was observed immediately following administration of the dye, with rapid a decline until it was no longer detectable by 10 weeks. The earliest sample taken in this present study was 30 minutes, which did not have a significantly higher concentration of AlS2Pc

compared to samples taken at later times. However, the Tralau study was in normal brain containing a tumour, so the early peak may be a feature of a breach in blood brain barrier.

6.5.4 Fluorescence in rabbit specimens with AlS2Pc

The results from these sections indicate the presence of AlS2Pc in both the mucosa and dura mater. The fluorescence signal obtained from the 24 hour specimens were slightly higher than the 1 hour samples. The brain samples exhibited negligible fluorescence. However these results were from a limited number of samples.

6.5.5 Photosensitiser selectivity

Unselective PDT effects may cause long term damage to certain structures A number of studies have shown excellent healing following PDT with various sensitisers, including phthalocyanines (Barr *et al.*, 1987b; Grant *et al.*, 1994). However, the normal brain has been shown to be highly sensitive to PDT (Lilge *et al.*, 1996) and any damage occurring as a result of PDT, in an organ without any capacity to repair, would clearly be highly unacceptable. With 12.5mg/kg Photofrin®, the threshold of damage to normal brain was indicated to be as low as 1.47J/cm^2. However this model used a much higher drug does than is generally used clinically. A subsequent study with AlSPc, also in a normal rat model, demonstrated that with a sensitiser dose of 2.5 mg/kg and irradiation at 24 hours post injection, brain damage should not occur below 7.85J/.cm^2 (Leach *et al.*, 1993). Both of these studies emphasise the sensitivity of normal brain to PDT and indicate the necessity for further investigations and the reason for carrying out the PDT studies in the rabbit nasopharynx.

This study showed that the peak fluorescence of mTHPC in epithelium and connective tissue of the NP region was at 24 hours. However, in clinical PDT the most important quantity is high level of sensitiser concentration in the tumour. Ris et al found that 72 hours post sensitisation produced the best therapeutic window for mesotheliomas (Ris *et al.*, 1993). As there appears to be no statistically significant difference between the fluorescence intensities following mTHPC sensitisation between 96 and 192 hours, 96

hours was chosen to be the drug-light time interval for the phototherapy studies with mTHPC presented later in this chapter. This time point also matches our current clinical protocol (Chapter 10).

The peak level of AlS2Pc in the connective tissue was observed at 24 hours. Between 30 minutes and 1 hour the AlS2Pc levels had already reached a plateau. From these results the following PDT studies to evaluate the effect on photodynamic therapy on the normal NP tissues were carried out at 1 and 24 hours post sensitisation.

PART II: PHOTODYNAMIC THERAPY TO THE NORMAL NASOPHARYNX

6.6 AIM

This second part of the study was to determine the effects of photodynamic therapy on the normal NP tissues using the same photosensitisers as in the pharmacokinetic studies described previously. The main concern with PDT in the nasopharynx is the difficulties of light dosimetry within such a confined space. The aim is to evaluate the damage caused to the rabbit nasopharynx and to find whether any unacceptable damage is caused within the intracranial tissues.

6.7 MATERIALS AND METHODS

6.7.1 Animals

The animal model used was adult female New Zealand White rabbits (2-3kg body weight). This is one of the smallest animals with a suitable size nasopharynx to enable the passage of a 400μm optical fibre.

6.7.2 Photosensitisers

Both sensitisers mTHPC (meta tetrahydroxyphenyl chlorin) and AlS2Pc (phthalocyanine) were freshly prepared prior to administration. Fifty-one rabbits received either intravenous mTHPC at the dose of 0.3mg/kg (n=15) or 1mg/kg AlS2Pc (n=36). Each sensitiser was injected without sedation via the marginal ear vein.

6.7.3 Drug light interval

The drug-light interval used for mTHPC was 96 hours. One and 24 hour was used as the time interval with aluminium disulphonated phthalocyanine (AlS2Pc). The rationale for this has already been explained (6.5.5).

Controls

Four unsensitised rabbits were used as light control samples whilst the rabbits in the pharmacokinetic experiments were also used as the drug only controls (two sensitised with mTHPC and two sensitised with AlS2Pc).

6.7.4 Photodynamic therapy

Prior to the PDT studies correct positioning of the optical fibre into the nasopharynx was practised in post mortem rabbits. The average size and shape of the NP cavity was also determined in post mortem samples by the use of a silicone base impression material (Extrude™, Kerr, Romulus, USA). Such materials are initially adequately fluid to enable them to be syringed via a narrow plastic tube into the cavity. The average size of the rabbit nasopharynx was determined in 3 post mortem specimens.

6.7.4.1 Light Source

The light source used was a copper vapour pumped dye laser (Oxford Lasers), with a 1 cm cylindrical diffuser fibre as the light delivery system (PDT Systems, Santa Barbara, CA). The laser was tuned to 652±2 nm (for mTHPC) or 675±2 nm (for AlS2Pc). A cylindrical diffuser was chosen to match the anatomy of the rabbit nasopharynx, though

the fibre of choice in patients would depend on the lesion and, in clinical practice, a spherical diffuser may be used.

One of the concerns with photodynamic therapy of the nasopharynx is the difficulty with accurate determination of true fluence rate at the mucosal surface relative to the fluence rate due to the primary (non scattered) light beam. It has been shown in both an optical phantom model and in the dog bladder that the former may be 5-6 times larger than the primary light beam due to the strong scattering of light by tissue (Star *et al.*, 1987). Similar results were observed in the human bladder where the magnitude of augmentation was 4.8± 1.2 SD (Marijnissen *et al.*, 1993). This phenomenon is known as the integrating sphere effect. As the nasopharynx is also a confined space a similar problem might be expected.

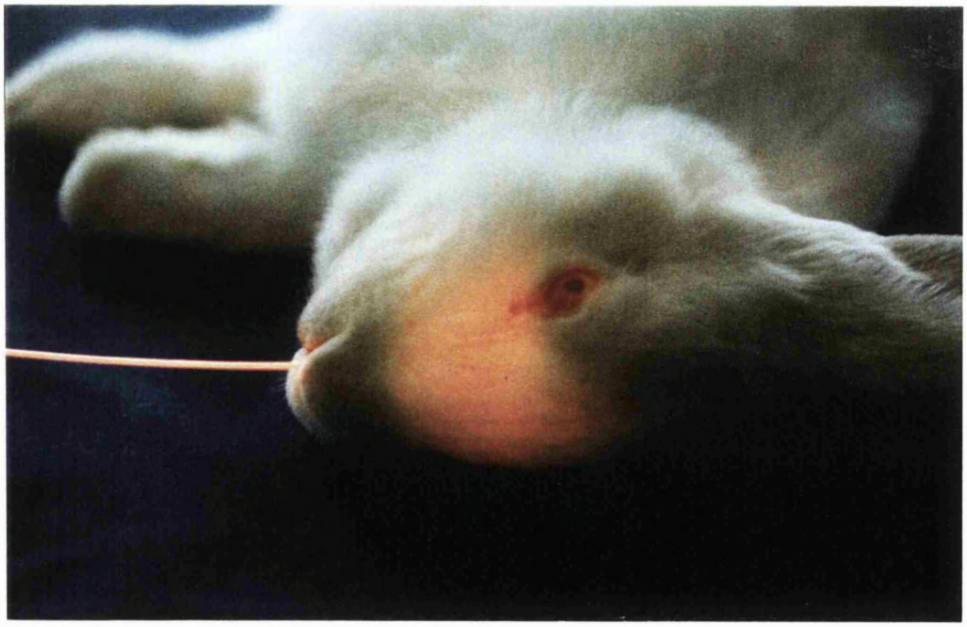


Figure 6.11: PDT of an experimental animal. The cylindrical fibre has been inserted into the nasopharynx.

6.7.4.2 PDT with mTHPC

Initially, two rabbits were sensitised with 0.3mg/kg mTHPC followed by laser activation using 50J/cm^2. This light dose was chosen as significant necrosis had been observed clinically with light doses as low as 5J/cm^2, hence the reluctance to administer 100J/cm^2 as in the bone study (Chapter 5). As both rabbits survived with no complications, the energy density given to the remaining 13 rabbits was 80J/cm^2. The rabbits were subsequently sacrificed at 3, 10 and 21 days after laser treatment by pentobarbitone overdose.

6.7.4.3 PDT with AlS2Pc

An initial 9 rabbits were irradiated with 100J/cm^2 following sensitisation with 1mg/kg AlS2Pc at either 1 hour (n=6) or 24 hours (n=3). This dose was chosen as it has been previously used in the PDT studies of the rabbit larynx (Kleeman *et al* , 1996). This was the highest light dose used and 6 rabbits died peri-operatively, due to local complications. Following this the energy density applied to the NP tissues was reduced in the next five rabbits to 25/cm^2. Due to the uneventful recovery following this dose (25/cm^2), the remaining 22 rabbits were irradiated with 50/cm^2 and killed at the same interval following PDT as above.

6.7.4.4 Harvesting of tissue

As the tissue of interest is not limited to the NP tissue but also the adjacent structures such as the base of skull and brain, it was important to maintain all these structures within the same anatomical relation yet allowing adequate fixation and preparation of the specimens. After sacrifice the rabbit heads were bisected to enable appropriate fixation with 5% neutral buffered formalin; subsequently, the samples were decalcified with trichloroacetic acid. The sections were then stained with haematoxylin and eosin for histological examination.

6.8 RESULTS

The 8 rabbits which received sensitiser alone (mTHPC or AlS2Pc), or just laser illumination, did not show any evidence of mucosal necrosis of the NP tissues or brain necrosis, either macroscopically or under histological examination. All the histology slides were evaluated in collaboration with an oral pathologist (Dr AW Barrett) and a selection of the slides were further examined by a neuropathologist (Dr T Ravesez) for evidence of necrosis in the brain. Haematoxylin and eosin (H&E) stained sections were examined for damage or necrosis to the normal nasopharyngeal mucosa as well as adjacent structures, including the salivary glands, muscles and brain.

Table 6.1 illustrate the histological findings observed at 3 days after PDT. The large numbers of peri-operative deaths in rabbits sensitised with AlS2Pc following irradiation with 100J/cm^2, were of most concern. Three of those in group 3 (AlS2Pc, 1 hour drug-light interval, 100J/cm^2) died along with all three rabbits in the 24 hour group (group 2). Soon after treatment the rabbits were observed to experience nasal obstruction, and subsequent respiratory distress and death was observed between 1 and 4 hours of irradiation. Subsequent post mortem examination revealed oedema and swelling locally at the treatment site. Only 3 rabbits irradiated with this light doses at the 1 hour interval survived, and they were subsequently sacrificed at 3, 10 and 21 days post PDT.

6.8.1 Response at 3 days with AlS2Pc

The only surviving rabbit from group 3, presented with extensive, full thickness mucosal necrosis and slough formation. Severe inflammatory response was seen in both the salivary glands and muscle. The response with group 6 (AlS2Pc, 24 hour, 25J/cm^2), and group 7 (AlS2Pc, 1 hour, 25J/cm^{2}25J/cm^2) were less severe with partial thickness mucosal necrosis seen. The same was found in group 4 animals (AlS2Pc, 24 hour, 50J/cm^2). The rabbits in group 5 (AlS2Pc, 1 hour, 50J/cm^2) had deeper necrosis (full thickness) and a more significant response compared with those treated at 24 hours with the same irradiance (Figure 6.12 A & B). Overall the response seen at day 3 with

phthalocyanine was greater with a 1 hour interval between sensitisation and irradiation than with a 24 hour interval.

Table 6.1 : Histological Findings at 3 days after PDT

Group / Drug	*Drug-light interval*	*No*	*Peri operative death*	*Light J/cm^2*	*Ulceration*	*Inflam*	*Slough*	*Salivary gland damage*	*Muscle damage*	*Brain*
1) mTHPC	96 H	6	0	80	N	++	N	N	N	N
2) AlS2Pc	24 H	3	3	100	-	-	-	-	-	-
3) AlS2Pc	1 H	4	3	100	FT	+++	+++	+++	+++	N
4) AlS2Pc	24 H	4	0	50	PT	++	+	+	+	N
5) AlS2Pc	1 H	4	0	50	FT	+++	+++	+++	++	N
6) AlS2Pc	24 H	3	0	25	PT	++	+	++	+	N
7) AlS2Pc	1 H	2	0	25	PT	+++	+++	+	N	N

PT/FT: Partial thickness/Full thickness mucosal ulceration

+++: severe, ++: moderate, +: mild, N: negligible/ no damage

Inflam: extent of inflammatory response

All result indicate the modal response .

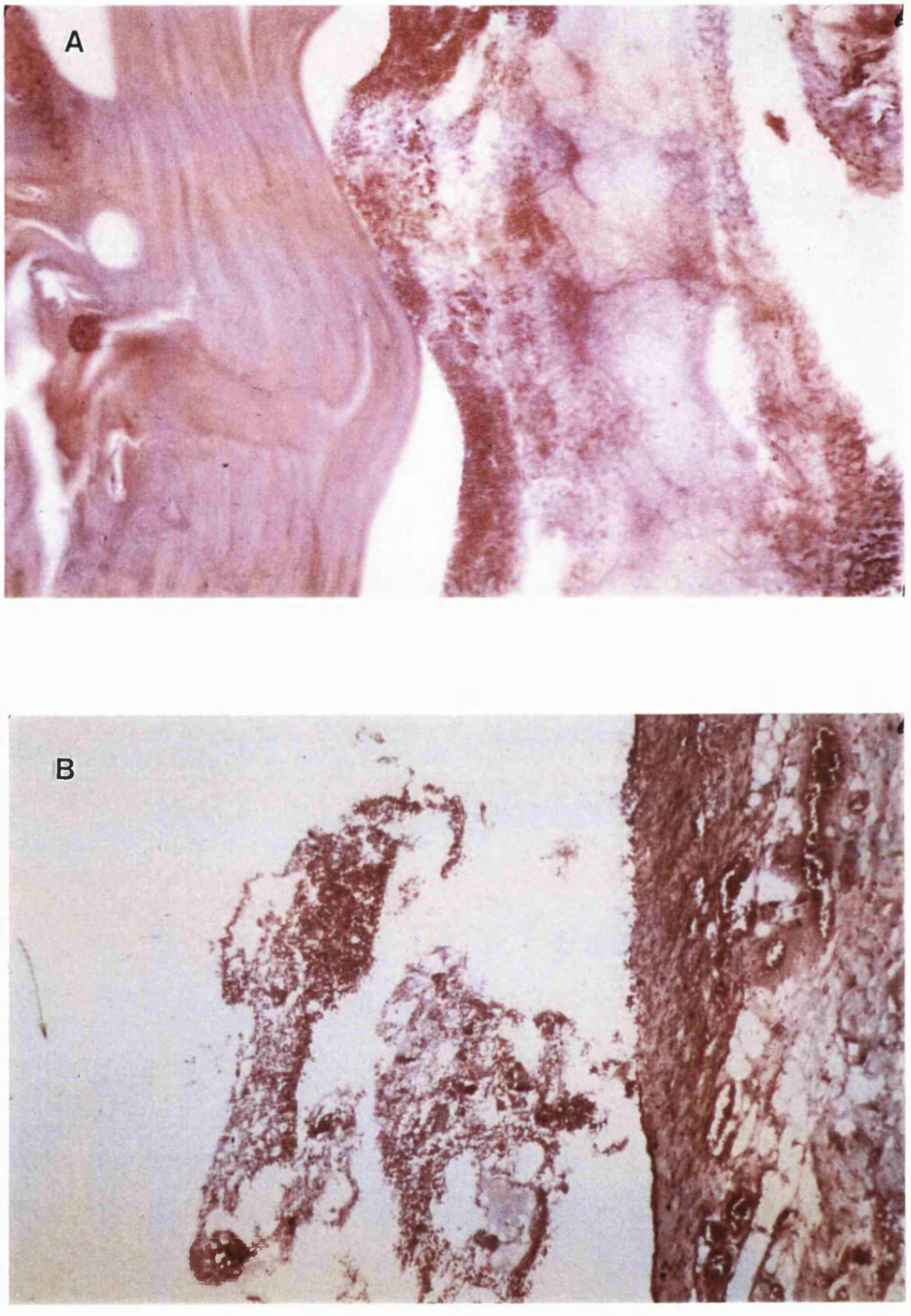


Figure 6.12: (A) Full thickness necrosis down to bone 3 days after PDT with AlS2Pc (1hour interval).(B)Superficial necrosis 3 days after PDT with AlS2Pc (24hour interval).

6.8.2 Response with mTHPC

The rabbits sensitised with mTHPC (0.3mg/kg, 96 hours) showed an unpredictable effect. Two of the 6 specimens exhibited full thickness mucosal necrosis, but the remaining four did not show frank necrosis (Figure 6.13 A & B). Not surprisingly, the 10 and 21 day specimens did not reveal any evidence of necrosis (Table 6.2).

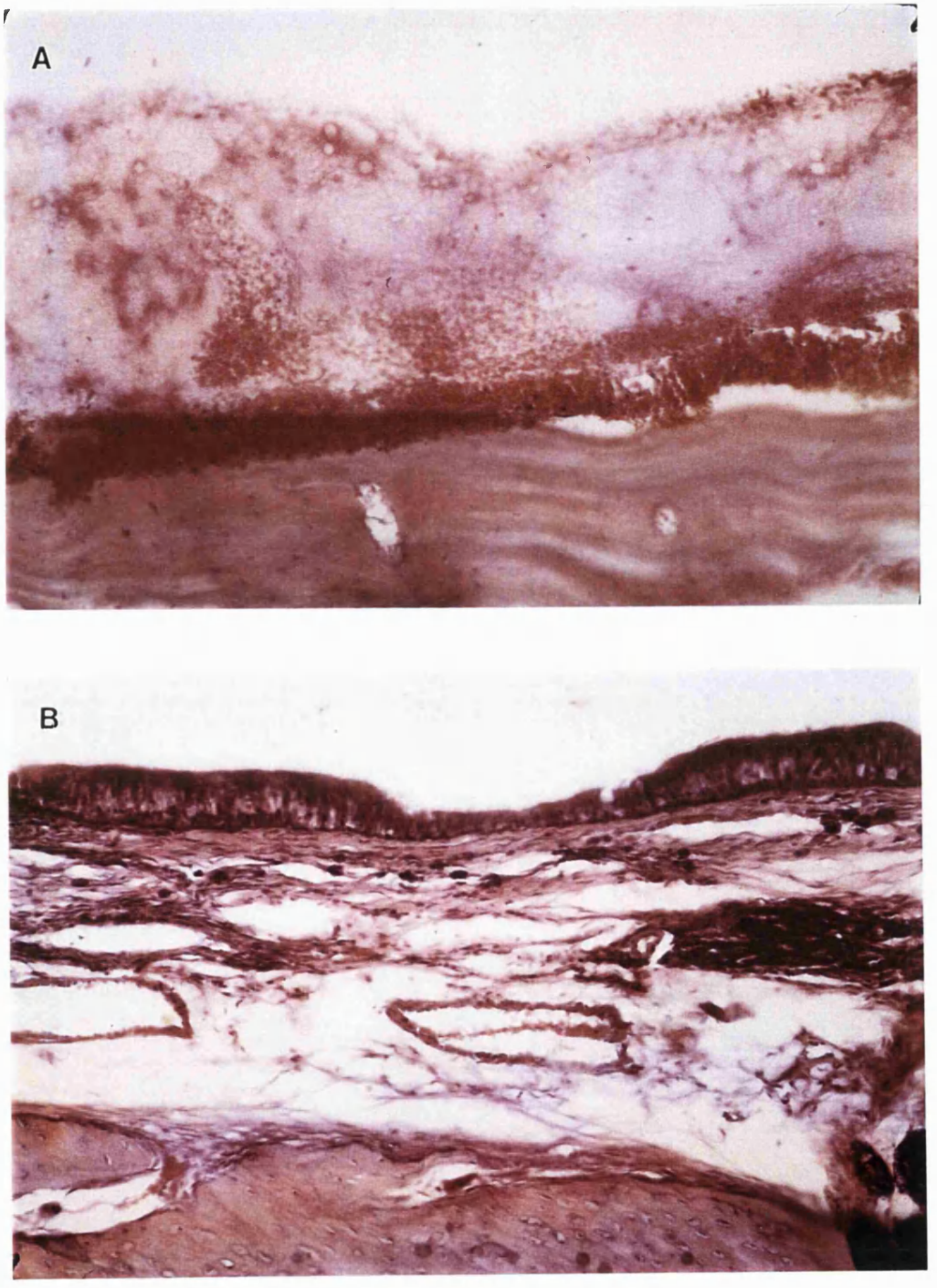


Figure 6.13: (A) Full thickness necrosis and
(B) no necrosis 3 days after PDT with mTHPC.

Table 6.2 summaries the modal observations of the response obtained in mucosa and brain following PDT with AlS2Pc. With one hour drug-light interval (50J/cm^2), full thickness mucosal necrosis was observed at day 3 and day 10 but had healed by 21 days. Inflammatory response was seen in the salivary glands and the muscle at 3 and 10 days, but no residual damage was evident at 21 days in muscle - although some fibrosis was seen in the salivary glands. With a 24 hour interval (50J/cm^2) partial thickness mucosal necrosis was produced at day 3, and maximal effects were seen at 10 days with full thickness necrosis. The mucosal inflammatory response was less than seen with the 1 hour interval. Despite the apparent lesser damage seen initially in the mucosa, more residual fibrosis was observed in salivary tissue (Figure 6.14) and muscle. The rabbits irradiated with 100J/cm^2 at 1 hour post sensitisation with AlS2Pc had the most severe damage with full thickness necrosis observed from day 3 through to day 21. Severe inflammatory response was seen in both salivary gland and muscle which resulted in significant fibrosis in the salivary tissue and moderate damage to muscle fibres at day 21.

No neurological deficit was observed in any of the rabbits. Figure 6.15 illustrates the relation of the nasopharynx to the brain. There was no obvious visual loss at any stage and, other than occasional mild transient nasal obstruction, there was no other evidence of respiratory obstruction except in the AlS2Pc rabbits irradiated with the highest light dose (100J/cm^2). General behaviour and ambulation was affected to a degree consistent with recent anaesthesia and treatment. Swallowing and appetite were affected, initially in those with severe NP damage, but these improved when the animals were placed on a soft diet.

continued...

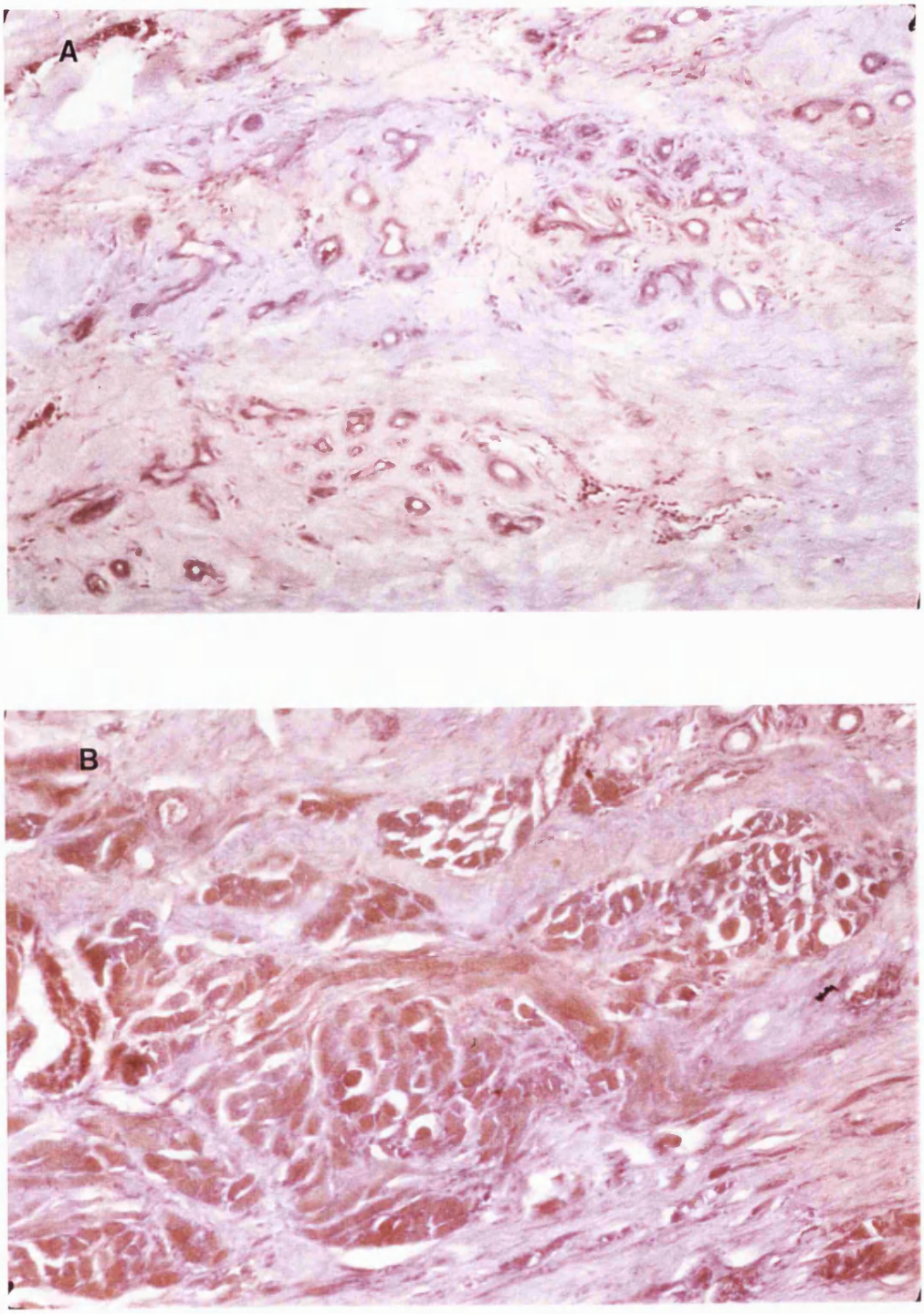


Figure 6.14: (A) Salivary gland fibrosis

(B) Muscle fibrosis 21 day after PDT using AlS2Pc (50J/cm^2) H&E x10

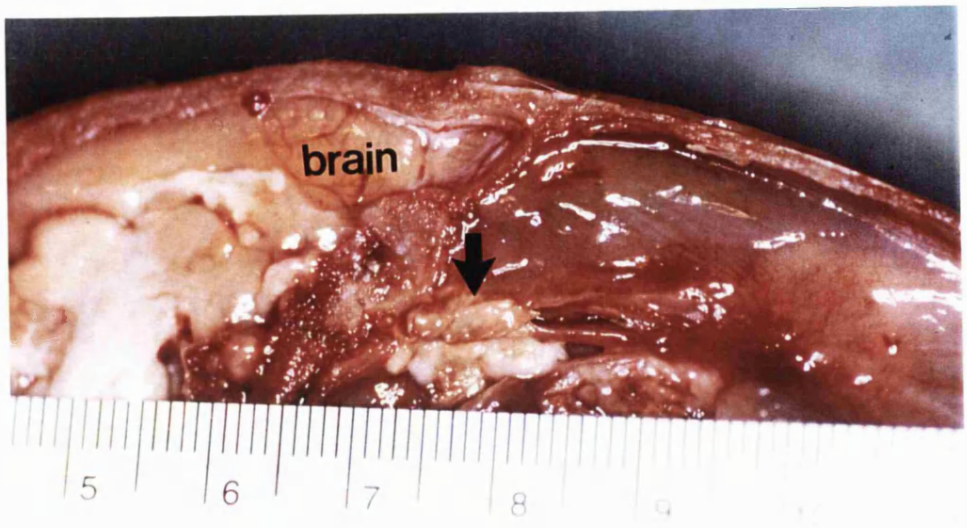


Figure 6.15: Necrosis in the nasopharynx (arrow) 3 days after PDT with AlS2Pc (1 hour 50J/cm^2).

6.9 DISCUSSION

The results from this study, illustrate that even when significant necrosis is produced within the nasopharynx, neither brain necrosis nor neurological signs result. The initial mortality associated with the AlS2Pc sensitised rabbits irradiated with the highest fluence (100J/cm^2) highlight possible local complications associated with over-dosage of light. However, none of the rabbits were given steroids to minimise swelling and oedema pre or post PDT which might have reduced the initial mortality observed. More significant damage was observed in the rabbits with the 1 hour interval relative to those in the 24 hour group. Similar results have been reported in numerous studies. Kleemann et al (1996) observed deeper necrosis of the rabbit larynx when PDT was carried out at 1 hour relative to 24 hours after sensitisation with 1mg/kg AlS2Pc. A more effective PDT effect was observed in a transplanted mouse mammary tumour model, when irradiation was carried out at 2 hours compared with 24 and 72 hours post AlS2Pc injection (Peng & Moan, 1995). Maximum PDT damage has also been observed in the normal colon of rats following light activation at 1 hour compared with those treated at 48 or 168 hours (Chatlani *et al.*, 1991). The more extensive response seen at the early time interval is likely to be due to vascular localisation of the photosensitiser. In the above study by

Chatlani et al (1991) maximal fluorescence was observed at one hour with greatest fluorescence in the submucosal blood vessels.

Table 6.2: Histological Changes of the nasopharyngeal and neurological tissues 3, 10 and 21 days following PDT.

Drug	Day of Sacrifice	Ulceration	Inflam response	Slough	Salivary gland damage	Muscle damage	Brain necrosis
1. (mTHPC 0.3mg/kg, 96 hour drug-light interval, 80J/cm^2)							
mTHPC	3	N	++	N	N	N	N
mTHPC	10	N	+	N	N	N	N
mTHPC	21	N	N	N	N	N	N
2. (1mg/kg AlS2Pc, 1 hour drug-light interval, 50J/cm^2)							
AlS2Pc	3	FT	+++	+++	+++	++	N
AlS2Pc	10	FT	+++	++	++	++	N
AlS2Pc	21	N	+/++	N	+	N	N
3. (1mg/kg AlS2Pc, 24 hour drug-light interval, 50J/cm^2)							
AlS2Pc	3	PT	++	+	+	+	N
AlS2Pc	10	FT	++	++	++	N	N
AlS2Pc	21	N	N	N	+++	++	N
4. (1mg/kg AlS2Pc, 1 hour drug-light interval, 100J/cm^2)							
AlS2Pc	3	FT	+++	+++	+++	+++	N
AlS2Pc	10	FT	+++	+++	+++	++	N
AlS2Pc	21	FT	+++	++	+++	++	N

No bone necrosis seen in any of the specimens.
PT/FT: Partial thickness/Full thickness mucosal ulceration
Histological changes seen: +++: severe, ++: moderate, +: mild, N: negligible/ no damage

The lack of response seen in the rabbits sensitised with mTHPC was surprising as fluorescence had been detected in the NP mucosa (Figure 6.9). However, the inconsistent PDT effects at day 3 suggest that a 96 hour interval with 0.3mg/kg mTHPC

and fluence of 80J/cm^2 is near the threshold dose. Lack of necrosis has also been observed following PDT to the normal lung in rats with a 96 hour interval (0.3mg/kg mTHPC), whereas necrosis was observed on subsequent repetition of the studies with a 72 hour interval (D. Fielding-personal communication). A difference in fluorescence intensity of the connective tissue was observed between a 72 and 96 hour interval in the rat studies with both 0.3mg/kg and 1mg/kg mTHPC (Student's unpaired t-test; t=2.85 and t= 2.19 respectively with $p<0.05$) (Figures 6.1-6.3). A rabbit papilloma model study, with 0.3mg/kg mTHPC, showed optimal difference in sensitiser level of normal skin relative to papilloma between days 4-8 (Lofgren *et al.*, 1994). It is likely that sensitiser levels in the normal NP tissues were too low for a PDT effect to occur with 80J/cm^2. This illustrates the differences in drug metabolism that exist between human and animal models, as extensive NP tumour necrosis has been observed clinically with fluence of 5J/cm^2, 4 days after administration of 0.15mg/kg mTHPC (author's unpublished data in patients).

6.10 SUMMARY

This study demonstrates the ability of both AlS2Pc and mTHPC to distribute to the NP tissues, with negligible accumulation in the normal brain. Nasopharyngeal tissue necrosis has been produced with AlS2Pc with both drug light intervals (1 and 24 hour) and with more prominent damage seen at the earlier time interval. Reassuringly, no neurological damage was seen in any of the rabbits. The results with mTHPC suggest that a threshold dose exist for normal rabbit mucosa and illustrate the difficulties of interchanging human and animal data. Repetition of the mTHPC studies with a 72 hour drug light interval needs to be carried out.

Chapter 7

LIGHT TRANSMISSION THROUGH THE SKULL BASE DURING PDT

7.1 SUMMARY

There has been little published data on photodynamic therapy for the management of nasopharyngeal carcinomas. This preliminary study addresses this deficiency and is divided into three sections; the first part of this study investigates the amount of light transmitted through the base of skull during laser irradiation within the nasopharynx. The second part of the study correlates the amount of light transmitted through a known thickness of bone and adjacent intact dura mater with that transmitted through bone alone. The protection afforded by the intact dura mater will also be assessed. This part of the experiment was carried out on the temporal bone due to the complexity of the skull base. The third part to the study was a Monte Carlo simulation for light propagation in tissue, carried out using computer technology. Rabbit heads were used in part 1 and 2, maintaining the same species as in the previous Chapter.

7.2 INTRODUCTION

To date the mainstay of treatment of nasopharyngeal carcinoma has been radiotherapy. However the dose that can be delivered is limited by tissue tolerance. Although the optimal dose still needs to be determined most centres advocate the use of 65 Gy (1850 ret) which exceeds the accepted tolerance limit for neural damage (1700 ret) (Rottenburg *et al.*, 1977; Sheline *et al.*, 1980). Complications are therefore substantial. Cerebral necrosis has been recorded by many authors with 1% of neurological complications observed in one study of 105 patients (Chatani *et al.*, 1986). Encephalomyelopathy with a median incidence of 1.7 % has been reported (Marks *et al.*, 1982) and studies have shown that the risk scales with increasing dose to the tumour. The balance therefore lies between the dilemma of inducing possibly fatal complications versus leaving residual or recurrent tumour. Peripheral nerve damage has also been reported, as has blindness following radiation damage to the optic nerve or chiasma.

A modality capable of tumour eradication without such non-selective effects would therefore be extremely attractive, both as a primary modality and in the management of recurrent or persistent disease. PDT has the advantage of having no known cumulative toxicity and can therefore be repeated if necessary. Some early clinical studies have been carried out, (reviewed in Chapter 3). However, larger clinical trials with standardised conditions are required. To ease the uncertainties associated with possible complications during PDT of the nasopharynx it is important to ascertain the effect on the normal surrounding tissue. The normal brain has been shown to be extremely sensitive to PDT damage and animal studies have shown that there may be a threshold, below which brain damage is not observed (Chen *et al.*, 1996; Dereski *et al.*, 1991; Leach *et al.*, 1993). Knowledge of the quantity of light transmitted through the skull base during irradiation of the nasopharyngeal region is therefore of paramount importance. Moreover in many clinical scenarios the bone at the base of the skull may have been eroded by the carcinoma. Therefore, it would be desirable for the clinician to also have prior knowledge of the amount of light transmitted through a known thickness of bone. This information is crucial for the prediction of any non-selective PDT effect and, more significantly, may provide an insight into the possible complications that may occur as a direct consequence of therapy.

Photodynamic therapy of the nasopharynx may also be complicated due to the geometry of the region, which resembles an enclosed cavity. Previous investigations into the light fluence during PDT in enclosed organs such as the bladder have demonstrated an increase in the true light irradiance due to the "integrating sphere effect" (Staveren *et al.*, 1995). The integrating sphere effect occurs when light which is redistributed from the tissue bulk due to reflection/scattering is trapped within the enclosure. This light entrapment process increases the relative irradiance by returning the light lost from the surface as a result of reflection/scattering during irradiation into the cavity, where it can re-irradiate another part of the cavity surface. This physical process might be expected to occur in the nasopharynx (and is discussed in Chapter 6). As yet there is no published work on this topic.

7.3 MATERIALS AND METHODS

In this chapter two separate experiments were performed. These were to determine the amount of light transmitted through the base of skull and the temporal bones. In the next section the experimental equipment common to both sets of studies will be described in detail, followed by the specific procedure used in each study.

7.3.1 Animal Model

The New Zealand White rabbit (2-2.5kg) used as the model for these experiments was the same as that used in the PDT experiments on the nasopharyngeal tissues (Chapter 6). All the samples were post mortem specimens from rabbits which had been heparinised prior to sacrifice to avoid local collections of post mortem intravascular thrombus formation which might have influenced light transmission. Five rabbits were used in the first part of the study to evaluate the percentage of light transmitted through the skull base. Two rabbits were used in the second part to determine the quantity of light transmitted through the left and right temporal bones (4 specimens). A craniotomy was carried out using a hand saw to provide access to the cranial cavity in all cases. In the experiments all tissues were maintained in the anatomical positions to reduce the error in variation of response by the isotropic detector.

7.3.2 Laser

The light source used was a copper vapour pumped dye laser (CU10 with DL1OK Oxford Instruments, Abingdon, UK) which lased at a selected wavelength of 652nm to match one of the photosensitisers used in the previous experiments (mTHPC). The light delivery system used for the base of skull studies was a 1cm long cylindrical diffuser fibre (PDT systems Inc. Santa Barbara, CA). This was chosen to match the geometry of the rabbit nasopharynx and to provide approximately uniform illumination over the entire cavity. Unlike the human, the nasopharynx in the rabbit is cylindrical rather than cuboid. This was confirmed by impressions of the region. The output of the diffuser was

measured in an integrating sphere power meter, adjusted to 200mW before commencing each experiment and checked at the completion of each series of measurements.

For the base of skull studies, the correct positioning of the treatment fibre within the nasopharynx was monitored using an image intensifier. The image of the radioluscent fibre was enhanced by closely adapted copper foil proximal to the diffusing tip. The copper marker permitted visualisation of the location of the fibre tip and thus helped to ensure the correct positioning of the cylinder diffuser fibre (Figure 7.1 and 7.2 correct and incorrect positions). Prior to these experiments, the mean distance to the nasopharynx from the right nostril was found to be 5cm (range 4.8-5.3), which also corresponded to the mid-pupillary position. This was determined in three post mortem rabbits following bisection of the head in the sagittal plane.

In both experiments the light irradiance was kept at approximately the same setting of 250mW/cm^2 to the nasopharyngeal mucosa and temporal bone, thus allowing a valid comparison of light transmission through both structures. In order to preserve the light irradiance in both sets of experiments different fibre delivery systems were used. A microlens fibre was used to deliver the light to the temporal bone after removal of the overlying skin. With this fibre a 5mm diameter spot was irradiated at a power setting of 50mW, giving the previously stated irradiance of 250mW/cm^2. In the nasopharynx a higher power of 200mW was applied through the diffusing tip. The surface area of the nasopharynx was estimated by taking impressions from three post-mortem subjects. The mean surface area was found to be 4cm^2±0.3 (appendix to Chapter 6). Hence a power of 200mW produced a direct irradiance of 50mW/cm^2. Previous studies in enclosed organs such as the bladder revealed the true fluence at the surface to be an average 5 times higher (Marijnissen *et al.*, 1993) with the increase in irradiance being attributed to the "integrating sphere effect". Such studies have yet to be performed in the nasopharynx. However, it was estimated that the true irradiance inside the nasopharynx may be in the region of 250mW/cm^2 (5 x 50mW/cm^2) due to the integrating effect of the cavity.

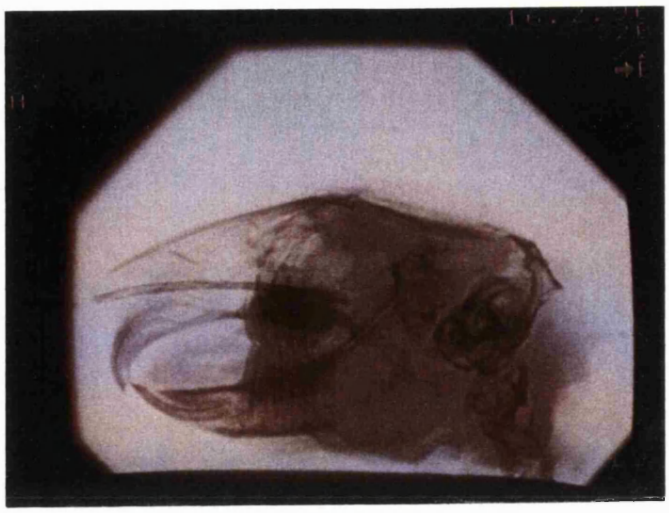


Figure 7.1: Correct positioning of the cylinder diffuser fibre in the nasopharynx as visualised using an image intensifier.

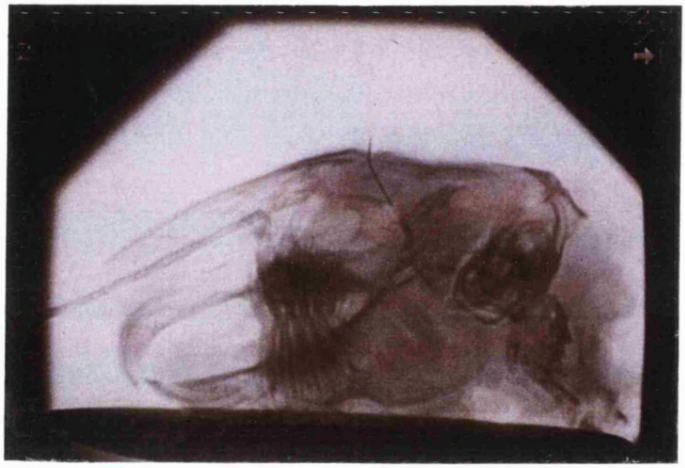


Figure 7.2: Incorrect positioning of the cylinder diffuser fibre in the nasopharynx as visualised using an image intensifier.

7.3.3 Light Detection System

An isotropic detector fibre (Medical Light Technologies, Edinburgh, UK) was used in both studies. The detector consisted of an optical fibre with a light scattering bulb of 0.5mm diameter at the distal end of the fibre (Figure 7.3). The biological effect of PDT is largely dependent on the amount of light energy available for absorption by the photosensitiser. At the wavelengths of light used for clinical PDT, the scattering coefficient in biological tissues is relatively large compared to the absorption coefficient and as a consequence, scattering is very efficient. As a result the photons are scattered in

all directions. In order to measure the fluence rate (W/m^2), a light detector which accepts light equally from all directions (i.e. isotropically) was chosen. The detector will give different readings depending on whether it is surrounded to air or tissue.

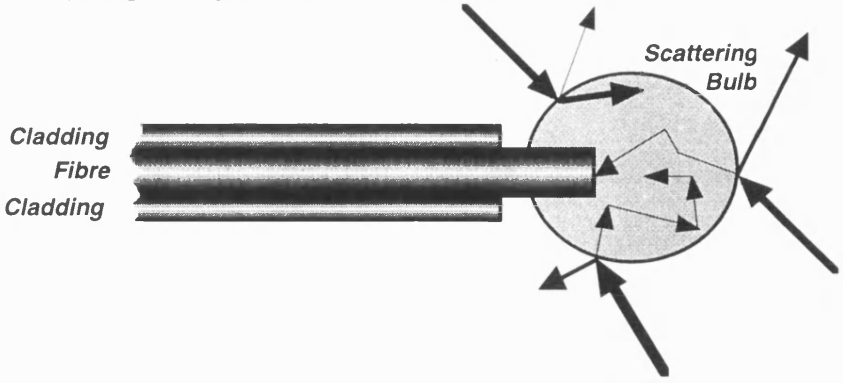
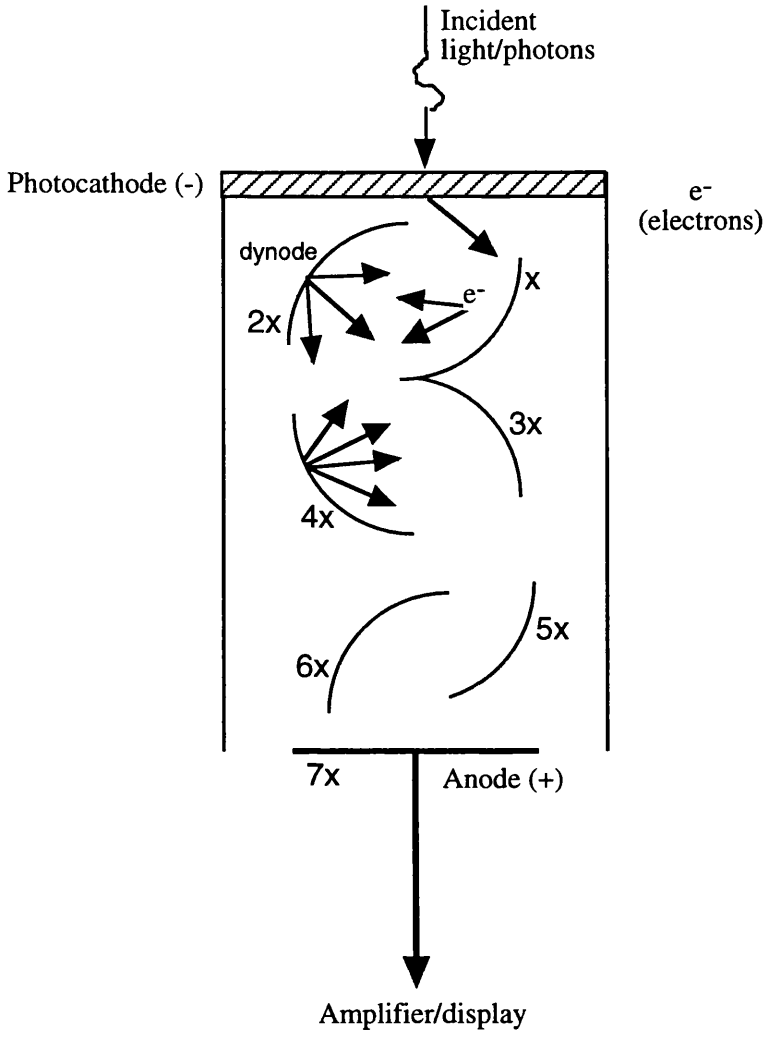


Figure 7.3: Isotropic detector, adapted from Marijnissen & Star, showing the action of the scattering bulb, directing light from all direction into the fibre.

The proximal end of the detector fibre was connected to a photomultiplier (PMT, Oriel, Stratford, Connecticut). The energy fluence rate (W/m^2) at a specific point within the tissue is defined as the total energy of photons incident per second on a small sphere centred at that point divided by the cross-sectional area of the sphere (Patterson *et al*, 1991). From this value and with knowledge of the absorption coefficient of the chromophores in the tissue, it is possible to estimate the energy absorbed per second and per unit volume.

A photomultiplier tube (PMT) is one of the most sensitive instruments devised to measure radiation in the visible regions of the spectrum. It has high current amplification and low noise (fluctuation). Photons of light entering the PMT are incident upon the primary photocathode. The photons liberate electrons from the photocathode via the photoelectric effect. These electrons are then accelerated to other cathodes (dynodes) where more electrons are liberated by collision (Figure 7.4). This multiplication process produces an electric current which gives an accurate measure of the amount of incident light.

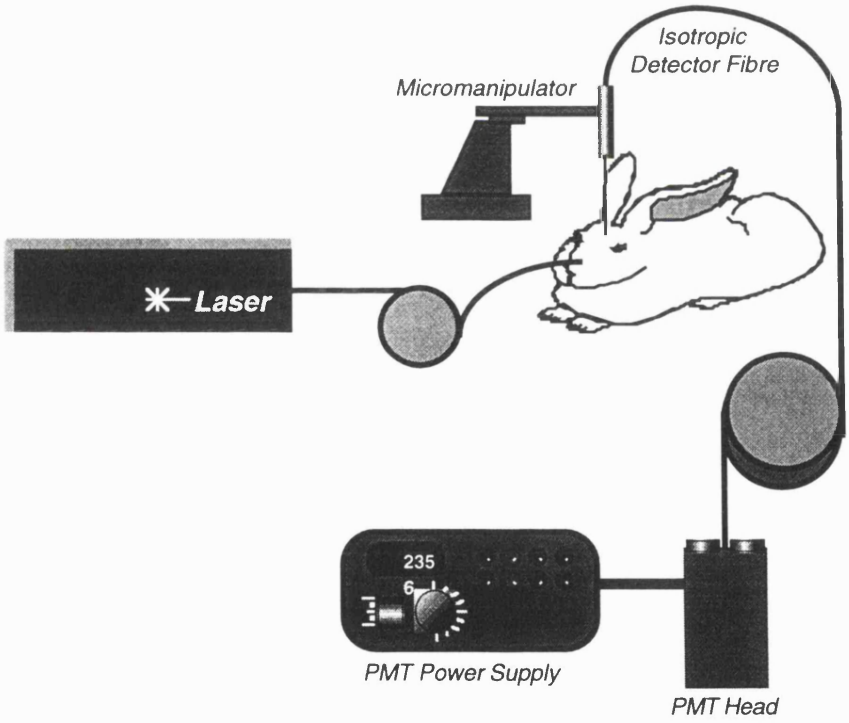
Figure 7.4 Schematic diagram of photomultiplier



7.3.4 Quantification of light transmission through skull base

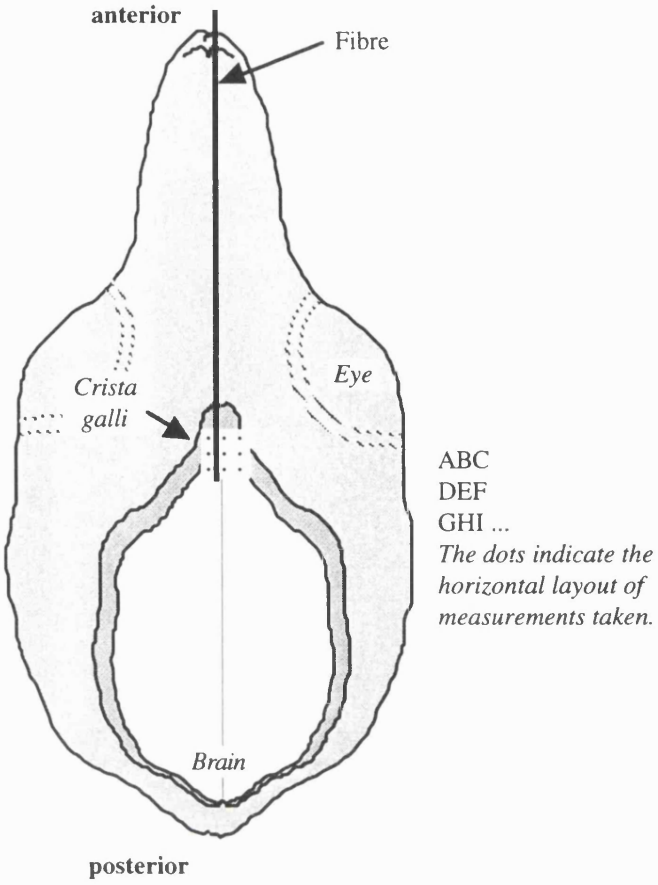
The experimental set-up used to measure the quantity of light in the rabbit brain is illustrated in Figure 7.5. An isotropic fibre mounted upon a collected the light transmitted through the base of skull. The micromanipulator enabled accurate positioning of the isotropic detector in 3 dimension. A horizontal grid was constructed on a clear acetate sheet with openings at regular 2.5mm intervals to allow the passage of the isotropic detector fibre. The vertical reference point for all the measurements was the surface of the brain and the horizontal reference point was the longitudinal fissure of the brain. The clear acetate sheet was placed so that it was centred on the crista galli of the ethmoid bone.

Measurement of the relative light intensity was afforded by coupling the isotropic fibre to the photomultiplier tube. Readings were taken starting with the isotropic detector fibre on the surface of the brain, lateral to the crista galli, and advancing the fibre towards the skull base by 0.5 mm increments. Three rows of readings were taken; mid-line and 2.5mm laterally into each hemisphere (Figure 7.6). This set up enabled the vertical and horizontal position of the isotropic detector fibre to be recorded along with each reading from the PMT. The readings were then repeated as the fibre was withdrawn. The distance between each horizontal point was 2.5mm apart. Between 9 and 21 horizontal reference points were evaluated in each of the 5 specimens. Fewer readings were taken in later specimens due to the low intensities recorded.



Figures 7.5: Experimental set-up for studies of light transmission through the base of skull

Figure 7.6 Schematic diagram of the superior aspect of the rabbit brain following craniotomy



7.3.5 Light Transmission through the Temporal Bone

The complexity and variability of the skull base made multiple measurement of bone thickness very difficult. The squamous temporal bone provided an easily accessible and measurable alternative. Following a craniotomy as previously described (7.3.1), the brain and overlying pia and arachnoid mater were carefully dissected to allow the positioning of the isotropic detector fibre in the subdural space lateral to the craniotomy site (Figure 7.7). A 5mm circle was marked out on the temporal bone after removal of the overlying tissue and the microlens was subsequently positioned to irradiate this area. The isotropic detector was positioned, and maintained by the micromanipulator, behind the irradiated bone. PMT readings were taken as the detector was moved across the calvarial bone behind the irradiated area. A 5mm disc of bone was then carefully removed, using a high speed dental drill, whilst protecting the meninges. The same readings were then repeated. The temporal bone was studied on both the left and right sides of each rabbit head.

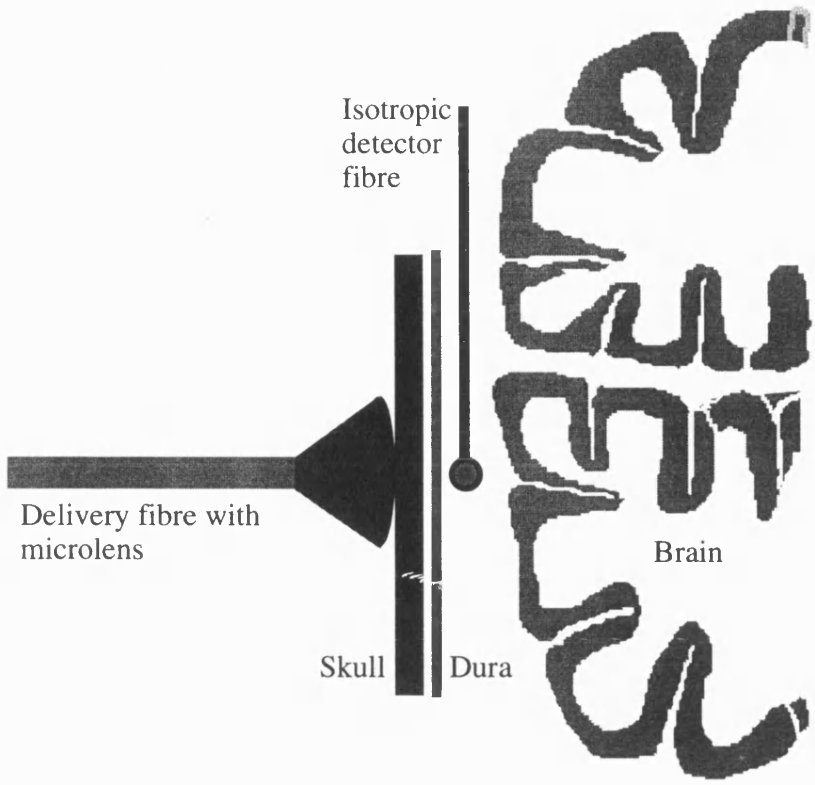


Figure 7.7: Experimental set up for temporal bone studies

7.3.6 Theoretical Model

The calculation of light propagation through tissue is a complex process. However, this is currently an active area of research and several mathematical models have been established which provide satisfactory solutions. For this study a published model (Wang *et al.*, 1995) was used. The model uses a Monte Carlo simulation to predict the transport of light through single or multi-layered tissues. The Monte Carlo method is a mathematical technique for determining the probability of a given process by constructing a model using empirical techniques of simulation and sampling. From this simulation the following optical properties can be determined for a given type of tissue/s:

- *Specular Reflectance:-* This occurs where a boundary or interface exists between two materials of different refractive index. A small percentage of light is reflected in the opposite direction to the incident light.

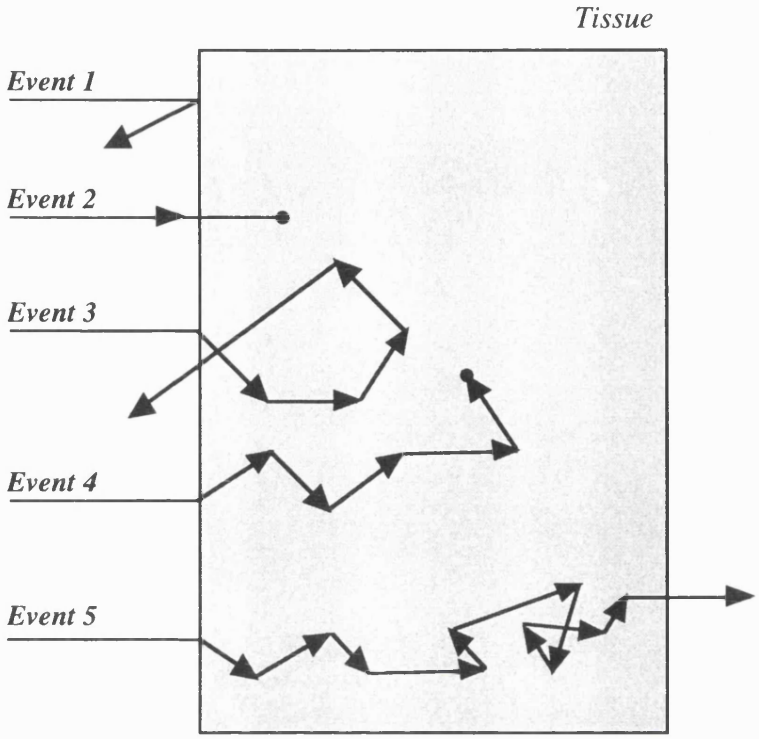
- *Absorption:-* This gives a measure of how much light is absorbed by chromophores present in the tissue. The absorbed light is dissipated as thermal energy, which for low irradiance levels produces negligible heating effects. In the presence of photosensitisers, some absorbed light is re-emitted as fluorescence.

- *Diffuse Reflectance:-* Light which enters the tissue undergoes multiple scattering events, (especially at cell boundaries) and some of the light can be re-emitted from the tissue in the direction of the incident light. This will decrease the amount of light transmitted directly through the tissue.

- *Transmitted Light:-* This term describes light that continues to propagate through the tissue, "surviving" both absorption and scattering, and emerges through the opposite interface.

The above properties are illustrated schematically in Figure 7.8. This shows light being reflected from the tissue surface (specular reflectance), absorption by a chromophore (e.g. haemoglobin), light exiting tissue due to multiple scattering (diffuse reflectance) and transmitted light that has traversed the full thickness of the tissue (and scattered in the process). An example of light that has undergone scattering and subsequent absorption is also shown. With a knowledge of the absorption and scattering coefficients at a particular wavelength of interest, along with thickness of tissue, a Monte Carlo model can be used to provide a statistical prediction of the above optical properties. This is particularly useful and relevant for the base of the skull which is composed of several layers of different tissues, e.g. mucosa, bone and meninges.

Figure 7.8 Schematic representation of the various optical properties of tissue.



Event 1 'Specular Reflectance'.
2 'Absorption'.
3 'Diffuse Reflectance'.
4 A combination of scattering and absorption.
5 'Scattering and Transmission'.

7.4 RESULTS AND DISCUSSION

7.4.1 Skull base

The results from the base of skull experiments are displayed in Figures 7.9-7.13. These illustrate the relative light intensity measured at the base of the cranial cavity, along the midline and laterally into the left and right cerebral hemispheres. In two of the rabbits (rabbits II and III Figures 7.10-7.11) the relative quantity of light transmitted through the base of skull in the midline and on the left and right sides follows a similar trend. The graphs in Figures 7.9 to 7.11, demonstrate that higher PMT values were seen along the centre/midline points of the skull base. The relative values of light transmission are likely to be associated with the thickness of the underlying bone. From these graphs two trends

can be identified. Firstly, the ratio between the maximum and minimum intensity is approximately the same for all five cases, which suggests that the amount of light transmitted through the nasopharynx into the base of the skull depends directly upon the thickness of the skull base. This information cannot be readily translated into a real value for the intensity because the isotropic detector requires calibration for the specific tissue (Marijnissen et al 1996). The second trend concerns the position in the base of the skull where the most light is transmitted. Three of the samples (cases I-III) indicate that most light is transmitted through the midline of the skull base, which is directly above the nasopharynx. However, in the remaining samples (cases IV & V) the exact position of the fibre detector relative to reference anatomical sites is very difficult to determine. This could be due to either individual anatomical variations within the test subjects, or the fibre detector becoming displaced from its anticipated position, possibly by following the path of least resistance through the brain. In all the rabbits, the ratio of maximum to minimum light intensity in the most important regions was 5:1 (rabbit III) i.e. sites closest to the light source. There did not appear to be any areas where the light intensity was particularly high. The is reassuring since a high intracranial intensity, particularly close to the brain stem, would be a cause of great concern.

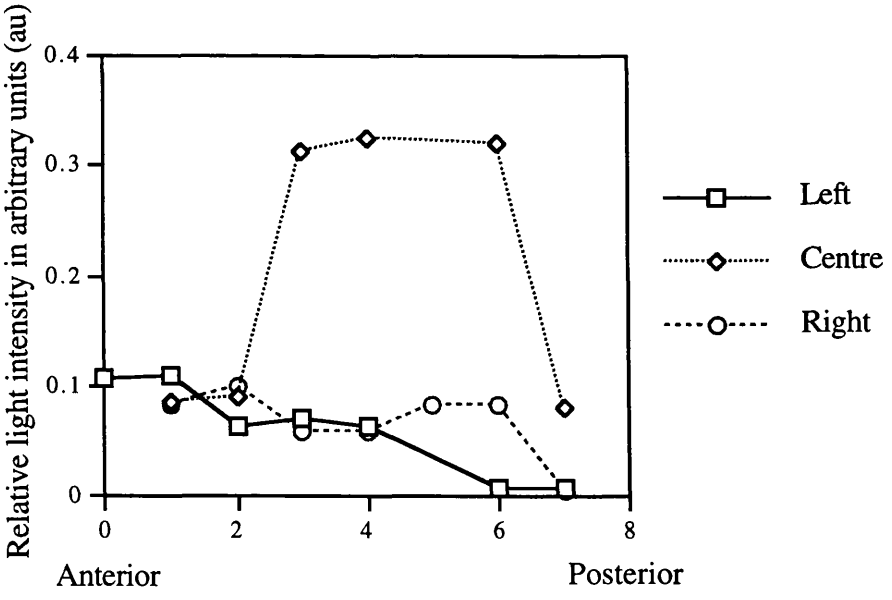


Figure 7.9: Relative light intensity along the base of skull (Rabbit I). Each point represents a single reading from the isotropic fibre.

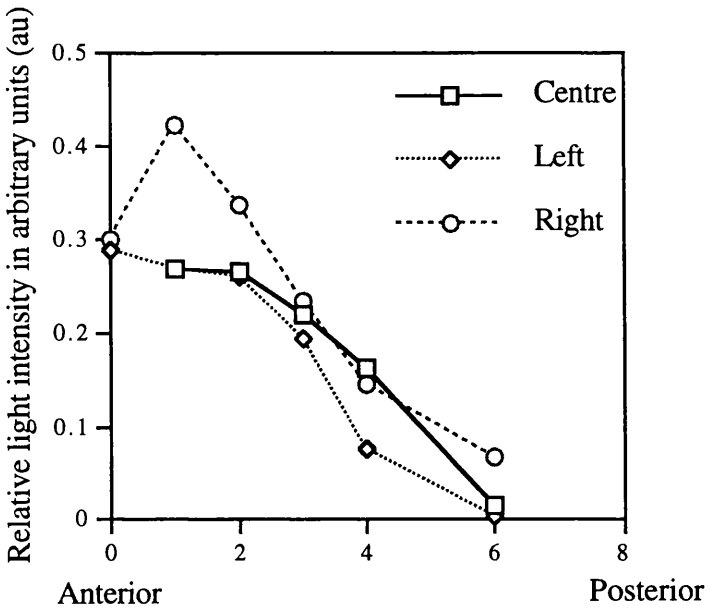


Figure 7.10: Relative light intensity along the skull base (Rabbit II).

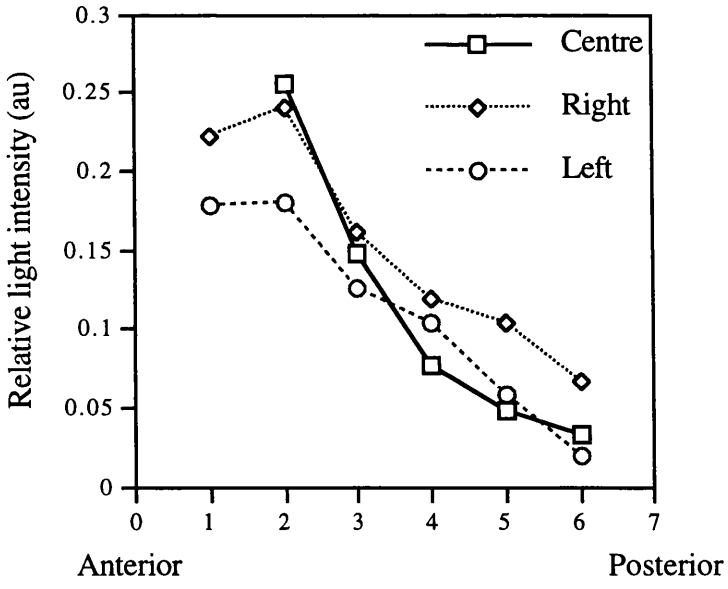


Figure 7.11: Relative light intensity along the base of skull (Rabbit III).

Note: Each point represents a single reading from the isotropic fibre.

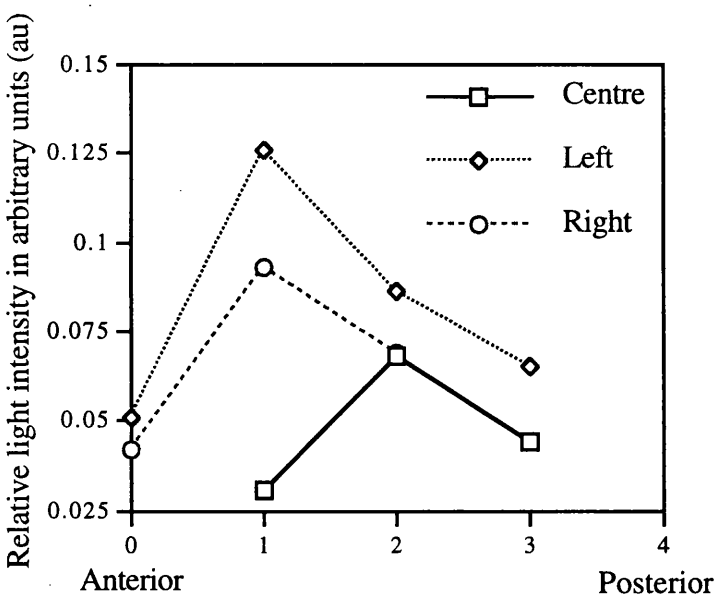


Figure 7.12: Relative light intensity along the base of skull (Rabbit IV)

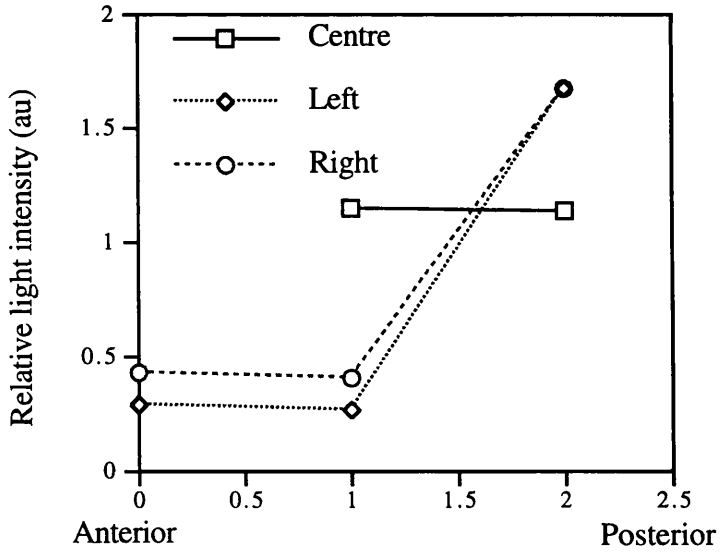


Figure 7.13: Relative light intensity along the skull base (Rabbit V)

Note: Each point represents a single reading from the isotropic fibre.

The change in the relative light intensity (RLI) with increasing distance from the base of skull and within the brain has been plotted for the maximal and minimal values (at the base of the skull) for each rabbit (Figures 7.14-7.18). With increasing distance from the light source the transmitted light was seen to decrease. The curves seen in Figures 7.14-7.16 display a rapid decay, with depths from base of brain, at both sites of maximal and minimal transmission. A step is seen in relative amount of light detected in Figure 7.18, which may correspond to an anatomical feature. Graphs 7.15 and 7.16 both exhibit a linear relationship between the RLI and distance from the light source (for the maximal). This may be the result of saturation of the highly sensitive photomultiplier. At the positions of minimal light transmission at the base of skull, the signal:noise ratio of the PMT is low and this may account for the flat plot observed in both graphs.

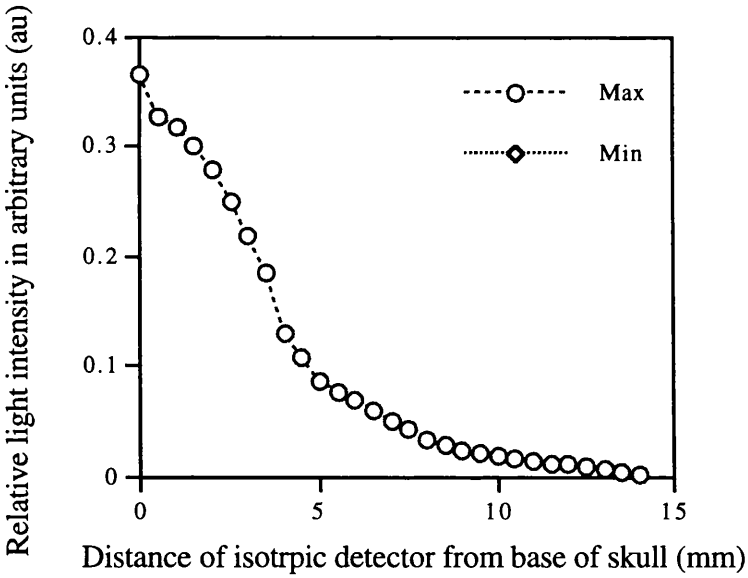


Figure 7.14: Change in relative light intensity with increasing distance from the base of skull (Rabbit I)

Note: Each point represents a single reading from the isotropic detector.

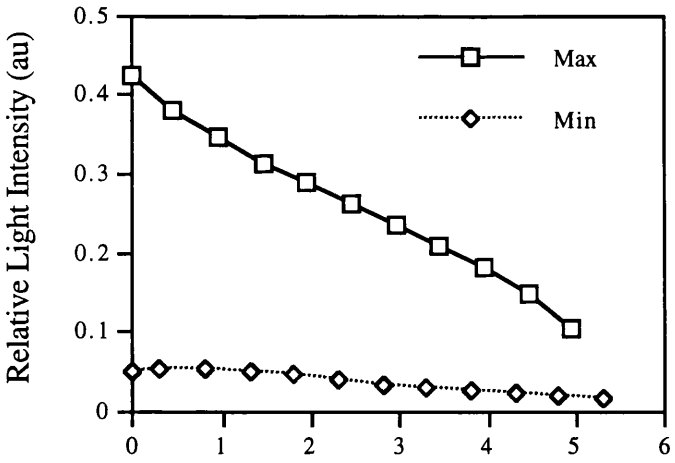


Figure 7.15 : Change in relative light intensity with increasing distance from the skull base (Rabbit II)

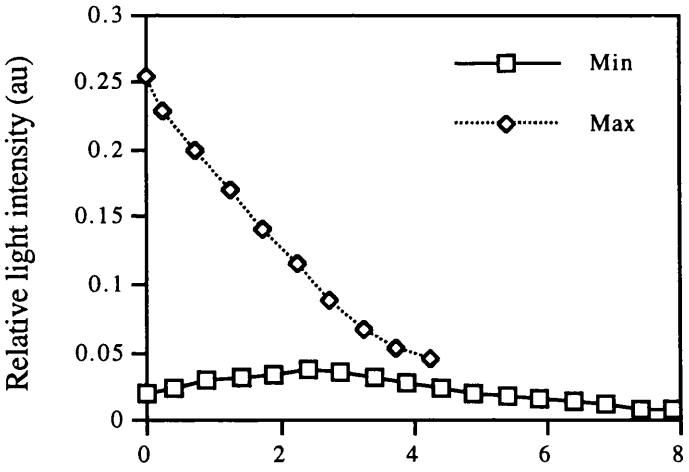


Figure 7.16: Change in relative light intesity with increasing distance from base of skull (Rabbit III)

Note: 'Max' corresponds to the maximum and 'Min' to the minimum relative light intensity measured at the base of skull. Each point represents a single reading from the isotropic detector.

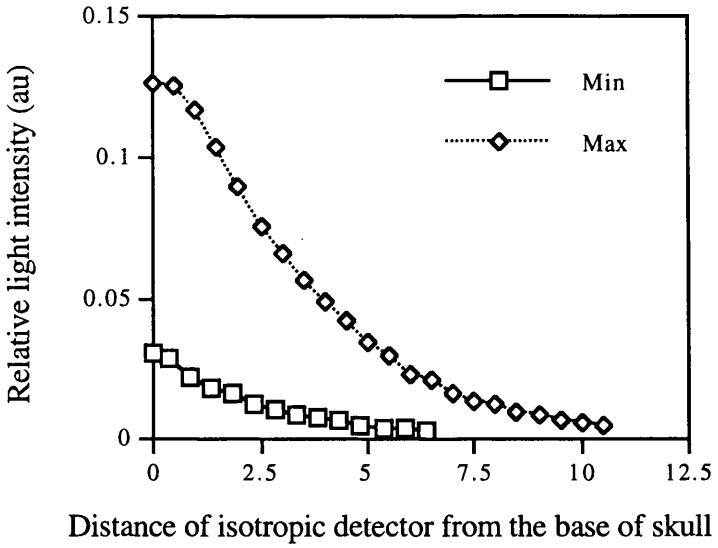


Figure 7.17 : Change in relative light intensity with increasing distance from the skull base (Rabbit IV)

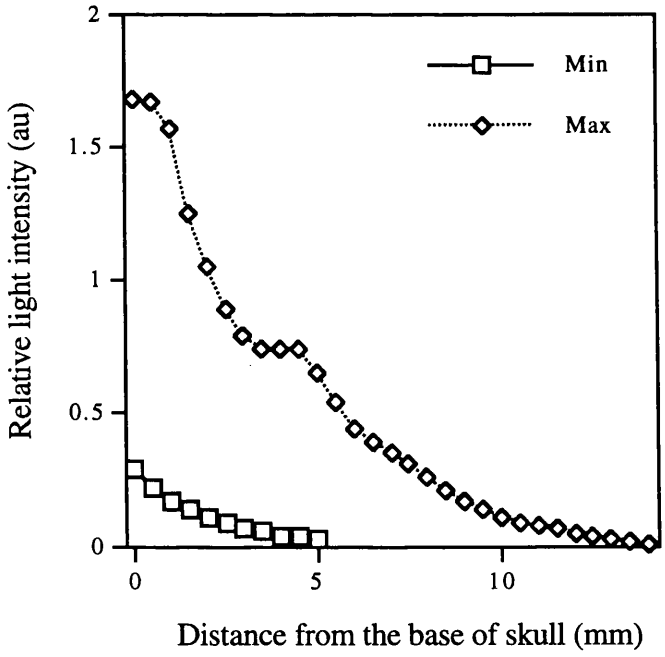


Figure 7.18: Change in relative light intensity with increasing distance from the skull base (Rabbit IV)

Note: 'Max' corresponds to the maximum and 'Min' to the minimum relative light intensity measured at the base of skull.

'Each point represents a single reading from the isotropic detector.

7.4.2 Temporal bone study

The results of the 4 experiments on relative light transmission through the temporal bone shows that a higher signal is observed when the bone has been removed leaving just the underlying dura mater as protection against the incident light (Figure 7.19). The relative light intensity is highest at the centre of the irradiated spot. The shape of the curve is due to the multiple scattering that occurs as well as the redistribution of the light. Light transmission was found to be less in all the samples with intact temporal bone when compared to samples when the bone was removed. The maximum increase in the amount of light transmitted following bone removal was 52%, and corresponded to the central position of the illuminating light spot from the optical fibre. In view of the difference in thickness between the dura (400μm) and bone (0.5-1.2mm) the percentage increase is less than expected and suggest that bone transmits light at this wavelength very well. A higher increase in the relative light transmission was observed when thicker bone was removed, demonstrating that more protection is found with an increasing thickness of bone.

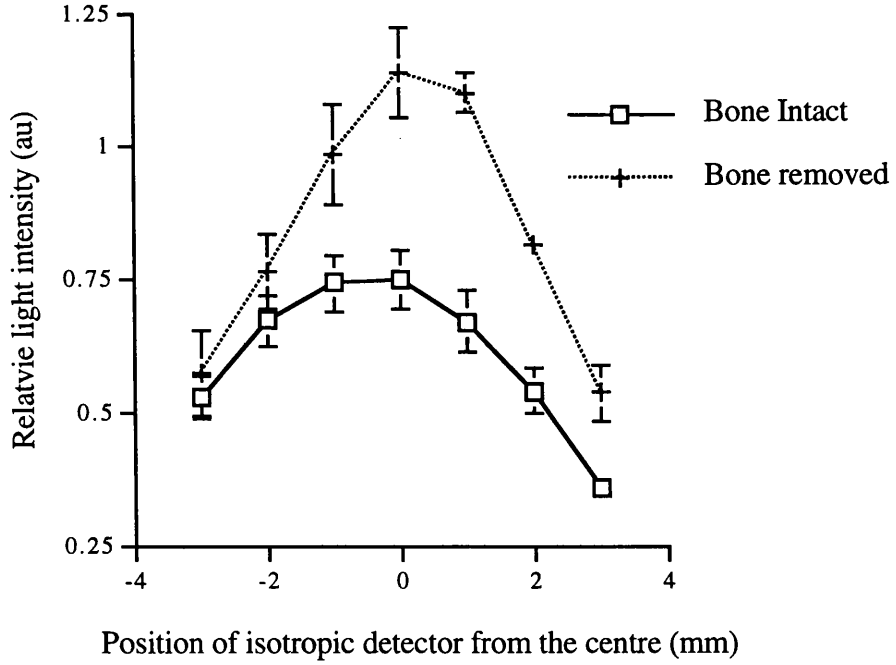


Figure 7.19: Relative light intensity studies on the rabbit temporal bone. Each point represents the mean of 4 samples

7.4.3 Monte Carlo Simulation

The Monte Carlo simulations were carried out in collaboration with Dr. P. Ripley. In order to use a Monte Carlo model which predicts light transport through different layers of tissue several parameters were required. The optical coefficients used for the simulation were obtained from published data or measured directly from clinical samples. Bone data was obtained from previous work on the porcine skull (Firbank *et al.*, 1993). The mucosal data was determined from fresh clinical specimens (Ripley et al, personal communication). Information concerning the meninges was unavailable at the time of this study. The optical properties discussed in section 7.3.6 were obtained using the Monte Carlo program, and are shown in Table 7.1.

	BONE	MUCOSA
Refractive Index (n)*	1.64	1.38
Absorption Coefficient (μ_a)*cm^{-1}	0.17	0.81
Scattering Coefficient (μ_s)*cm^{-1}	150	67
Anisotropic Scattering Factor (g)*	0.93	0.93
Specular Reflectance (Rsp)	5.9%	2.6%
Diffuse Reflectance (R_{diff})	42.6%	18.7%
Absorption (Abn)	32.7%	65.2%
Transmission (Tn)	18.9%	13.8%

Table 7.1: Predicted values of tissue optical properties and their respective coefficient as determined by Monte-Carlo simulation setting the tissue thickness at 0.5cm (λ=652mn).

** indicate data obtained from published work or by personal communication.*

A simulation was performed with 0.5cm mucosal and bone thickness. This was chosen to permit a direct comparison of the tissue optical properties. The optical coefficients for bone and mucosa at the wavelength of interest (652nm) are displayed in Table 7.1. From the table it can clearly be seen that mucosa is highly absorbing with four times greater absorption coefficient than bone. This strong absorption is likely to be due to oxy/deoxy

haemoglobin and cytochrome C oxidase. Nevertheless, the absorption of bone is still reasonably high at 33% (for 0.5cm thickness). Bone, however, has more than twice the scattering coefficient of mucosa. Scattering takes place at cell boundaries, but bone also contains high concentration of hydroxyapatite (scattering centres) which may account for the higher scattering observed relative to mucosa. Increased diffuse reflectance, is often a direct consequence of scattering. Therefore, the higher the scattering, the greater the probability of photons being bounce off cell boundaries and becoming redistributed within the tissue. The percentage transmission of the incident light through 0.5cm thickness of bone and mucosa are comparable (19 and 14%). This suggests that in clinical situations where the underlying bone is thick, few photons will reach the brain as a result of the high diffuse reflectance and scattering potential of bone. Conversely when the bone is thin, but is associated with thick mucosa or even bulky tumour, high absorption of the light is likely to occur, hence reducing the incident light on the brain. Although, nasopharyngeal tumour optical properties have yet to be characterised, absorption is likely to be closer in it's characteristics to mucosa than bone.

Further simulations were carried out to evaluate the effect on light transmission of varying thickness of bone and mucosa using the same Monte Carlo model (Figure 7.20). The bone thickness was varied from 0-2cm and mucosa thickness from 0-0.5cm. The trend seen in the simulated results is in general agreement with the measured data (Figures 7.14-7.16). All sets of data show a rapid decay of light transmission with increasing thickness of bone. The data shows that for 0.5cm bone and 0.2cm thickness of mucosa, in the region of 10% of the incident light will be transmitted. This is reduced to 4% when the mucosa is 0.5cm thick (Figure 7.20).

Results of such simulations may be used to estimate the light dose entering the brain. The threshold of normal brain damage has been determined for both Photofrin® (12.5mg/kg, 48 hour interval) and aluminium phthalocyanine tetrasulphonate (2.5mg/kg, 24 hour interval) to be 1.47J/cm^2 and 7.85J/cm^2 respectively (Dereski *et al.*, 1991)(Leach et al., 1993). These results are encouraging, as the Photofrin dose is much higher than the

clinical dose used. If we assume that there is no integrating sphere effect, and there is no protective effect from the meninges, then for an incident light dose of $20J/cm^2$, the predicted light dose reaching the brain would be $2J/cm^2$ (10%). This value is below the threshold dose for phthalocyanine but above the threshold for Photofrin®. The threshold dose for damage to normal brain using mTHPC has yet to be determined but nasopharyngeal tumour necrosis has been observed clinically with less than $20J/cm^2$. In addition determination of the optical characteristics of the meninges is necessary to take account of its protective effect.

Certain problems have been highlighted in these studies. The anatomical variation between different animals is likely to account for the differences of the light intensity measured at the base of the skull. In addition the results shown only describe trends and calibration of the isotropic light detector is required before absolute values can be determined. Calibration is critical because the light detector is only able to pick up a percentage of the light available. Once calibration has been performed then the data described would enable determination of the amount of light that penetrates through a known thickness of multi-layered tissues such as bone and mucosa.

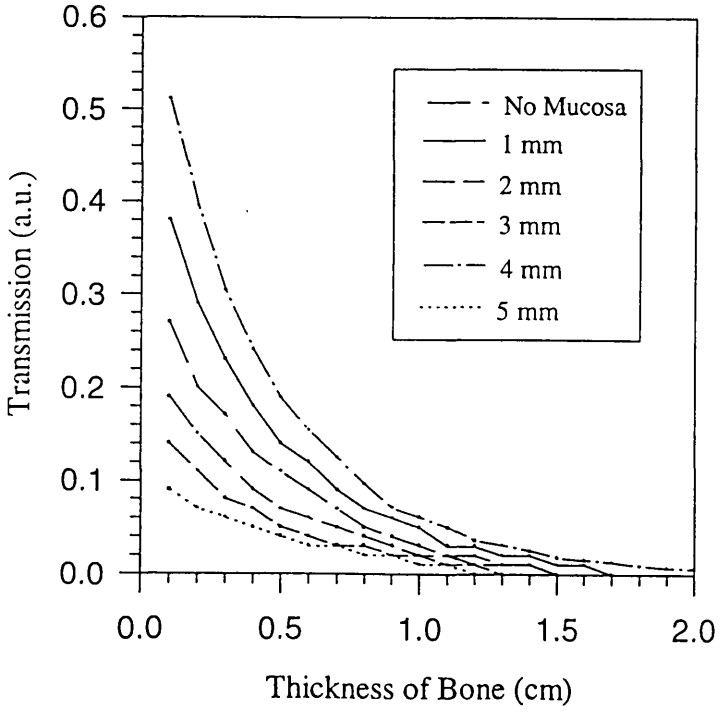


Figure 7.20: Predicted trends of light transmission with varying thickness of bone and mucosa as determined by Monte Carlo simulation (λ=652nm)

The thickness of the base of skull superior to the nasopharyngeal region ranges between 5-10mm (measured with a micrometer) in humans, whilst that in the rabbit is between 2-12mm (measured with a microscope graticule). Therefore, the thinnest part of the normal human skull is still thicker than the rabbit skull base. In view of the little low percentage of light transmission, even in the worse case scenario, the quantity transmitted through the normal human skull should not be of clinical concern. However, in patients with advanced NPC, complete erosion of the bone may have occurred, though the bulk of the tumour would also reduced the amount of light transmitted.

7.5 CONCLUSION

The ultimate aim is the ability to estimate the light distribution within the brain during PDT of the nasopharynx for each individual clinical situation and thereby determine the PDT damage threshold. The ability to predict the likelihood of neurological complications resulting from PDT, will enable clinicians to use this modality with confidence. The only feasible method in which this may be carried out non invasively is by estimation using a combination of both experimental and theoretical models. Validation of this will require the agreement between *ex-vivo* (post mortem) experiments and Monte Carlo data. The optical properties of all the structures of interest, such as dura mater, are not currently available and need to be determined. From knowledge of the known optical characteristics of various tissues and the known thickness of the various structures (as determined by MRI or CT imaging) estimation of light transmission may be determined. Brain threshold studies will also need to be carried out for various sensitisers, to enable the use of predictive simulations for light penetration in tissue.

From this preliminary study it was observed that little light transmits through the rabbit skull base and implies that the brain should be spared from unselective PDT damage. However, more work needs to be carried out in vivo in order to ascertain this conclusively.

7.6 SUGGESTIONS FOR FUTURE STUDIES

Much research still needs to be carried out to correlate local light doses with histological damage, the ultimate aim being accurate prediction of PDT damage. Such studies would require a calibrated detector to be used along with an optimal light dose regimen. The clinicians dream is to know how much of the delivered light dose is absorbed within adjacent tissue structures. This should be performed in conjunction with the determination of the therapeutic threshold of PDT damage for each photosensitiser in normal brain. This has already been established for Photofrin® and AlS4Pc, however these were in animal models (Chen *et al.*, 1996; Dereski *et al.*, 1991; Leach *et al.*, 1993), and to date this has not been carried out with mTHPC, or AlS2Pc.

A more suitable anatomical model is also required i.e. an animal that has a nasopharynx which is comparable to the human. Having established the most appropriate model for the nasopharynx, the effect of this new geometry on the fluence within the cavity should also be investigated. The expected increase in the light fluence due to the integrating sphere effect, may result in a reduction of the actual treatment time and thus reduce the degree of PDT damage in other tissues.

The optical properties of all the tissues of interest, i.e. bone, mucosa, dura and brain may need to be determined from human samples. This is significant where complete erosion of the skull base has occurred by invasion of the carcinoma, and the bone layer has been compromised. Methods of estimating the quantity or percentage of light transmission along with the known PDT thresholds for vital structures could lead to effective treatment of NPC without the concerns of serious intracranial complications.

Clinical Sections

In the earliest studies of the application of PDT in the head and neck region, the aims were to assess the feasibility of the technique and tumour response to treatment and to determine whether any undesired toxic reactions would occur in patients. These early studies, all of which were carried out using haematoporphyrin derivative (HpD), were predominantly carried out on patients with advanced tumours which had failed, or were not amenable to conventional therapy (Carruth, 1985; Gluckman, 1991a; Schuller *et al.*, 1985; Wile *et al.*, 1984). Later investigations suggested that PDT had a more appropriate role in the treatment of early/superficial lesions and field cancerization - the latter being extremely difficult to treat using conventional modalities (Gluckman, 1991a; Grant *et al.*, 1993b; Schweitzer, 1990). Although there were wide variations from study to study in drug and light dosimetry and drug-light interval, which make it difficult to draw truly general conclusions, the results of these early studies have been generally encouraging. With the decrease of enthusiasm for this technique in the upper aerodigestive tract, it is now essential to determine the realistic indications and long term outcome of this modality.

The following chapters of this thesis looks at the way in which three different photosensitisers work in the clinical situation. In view of the low prevalence of oral cancer in England and Wales, (2400 new cases each year; (OPCS, 1994), it was not possible to find patients with disease of identical size, differentiation and anatomical site. The large proportion of patients with multifocal disease or 'field cancerization' adds further difficulties to this heterogenous group of patients. There are obvious limitations in these trials as it was not possible to control all the patients variables. However only patients with either dysphasia or squamous cell carcinoma of the oral cavity were included in these studies which are essentially phase II clinical trial. Any differences observed between patients with isolate or multifocal disease will be of special interest.

The treatment and follow up of patients were carried out over a four year period. At the start of this thesis the only clinically accepted photosensitiser was Photofrin® which was the reason this drug was used initially. It soon became apparent that the prolonged skin photosensitivity was a problem. The next drug which became available was aminolaevulinic acid (ALA) which has the advantage of much shorter period of cutaneous photosensitivity. However, further evaluation of ALA revealed that it could only produce superficial necrosis which was inadequate in certain clinical situations. A more powerful and deep acting photosensitiser was required; mTHPC became available for clinical evaluation and was therefore used in our Phase II study.

All patients in this study had histologically confirmed squamous cell carcinoma or dysplasia of the oral cavity and had refused or could not tolerate conventional therapy. None had regional nodes or distant metastasis at the time of treatment. Prior to photodynamic therapy, all patients had a full routine assessment and diagnostic "work up". Staging of the disease (UICC, 1987) was determined after complete clinical examination of the head and neck region along with imaging techniques to assess the nodal status (X-ray, CT or MRI). The limitations of conventional imaging techniques in the detection of superficial lesion has already been discussed (section 1.6.2). Serum haematological (Hb and platelets) and biochemical (electrolytes, urea and liver function) evaluation were also performed.

Inclusion criteria

- Histologically confirmed SCC or dysplasia of the oral cavity or oropharynx.
- All lesions were clearly visible and accessible for illumination.
- Surface area of the lesions were not thought to be a limiting factor, but large lesions were irradiated with multiple treatments.
- Informed written consent was obtained in all patients.

Approval was obtained from the ethical committee of the University College London Hospitals prior to commencement of the clinical studies.

Chapter 8

PHOTODYNAMIC THERAPY USING PHOTOFRIN® IN THE ORAL CAVITY

8.1 INTRODUCTION

At the start of this thesis, Photofrin® was the only photosensitiser that was easily available for clinical use. In this first clinical chapter, the application of PDT using Photofrin® to treat oral cancers and dysplasias in a total of 22 patients is described. Of these patients, 13 received PDT treatment prior to the start of work on this thesis and 11 of these were included in a previous report by Grant et al (Grant *et al.*, 1993b). Four of these 13 patients have had further PDT treatments undertaken by the author. Thus 13 of the patients reported here have been followed up by this researcher with a further nine first presenting for the first time.

8.2 AIM

The aims of the study presented in this chapter were to assess the efficacy of photodynamic therapy using di-haematoporphyrin ether/ester (DHE/Photofrin®, Quadralogic Technology, QLT Vancouver, Canada), to treat squamous cell carcinoma and dysplasia in the oral cavity.

8.3 MATERIALS AND METHODS

8.3.1 Case selection

Twenty-two patients, with mean age of 61 years (range 33 - 88 years) at presentation, were studied: 12 were female and 10 were male. Sixteen presented with field change disease and the remaining six had isolated lesions. Fourteen of the patients had received previous treatment for their disease or an associated condition (Tables 8.1 and 8.2). Twelve had undergone previous surgery, of whom six had had multiple procedures. One patient had received multiple laser treatments and another had residual disease following photodynamic therapy with 5-aminolaevulinic acid (ALA).

Fourteen patients presented with at least one T1 squamous cell carcinoma (SCC) of the oral cavity, six presented with dysplasia, one with carcinoma *in situ* and another with T2 SCC.

8.3.2 Photosensitiser

The photosensitising agent used was Photofrin®, which is commercially available in a freeze dried formulation requiring reconstitution with 5% dextrose to give a final concentration of 2.5 milligrams per millilitre of Photofrin®. Approval was obtained for this study from the Ethical Committee of University College London Hospital. Careful explanation of the protocol along with verbal and written instructions on cutaneous photosensitivity and the precautions required were given to all patients. Following this informed consent, all patients received Photofrin® at the dose of 2mg/kg body weight by slow intravenous administration, over 20 minutes. All treatments were carried out on an outpatient basis whereby patients went home one hour after administration of the photosensitising agent with careful instructions on avoidance of light exposure. A drug-light interval of 48 hours was used for all patients as the optimal therapeutic ratio has been reported to be between 24-96 hours (section 2.3.1.1).

8.3.3 Light dosimetry

Laser irradiation was carried out using either 628nm light from a gold vapour laser (VGM-2, Dynamic Light, Hornsby, Australia) or 630 ± 2nm light from a copper vapour pumped dye laser (CU25/DL10, Oxford Lasers Ltd., Abingdon, UK). The light was delivered to the treatment site using optical fibres: the earliest cases were treated using a bare, plane-cleaved optical fibre of 400 μm core diameter, which was mode scrambled to give a nearly uniform light dose over the irradiated spot, and later cases were treated using a microlens fibre (QLT Phototherapeutics, Vancouver, Canada). Advancement in laser technology and the unreliability of the earlier lasers was the reason for the different light delivery systems used.

Generally, the laser output power was adjusted to maintain a fluence rate of 250 mW/cm^2 or less at the tissue surface in order to avoid thermal effects. The power from the optical fibre was measured immediately before and after each treatment, using a calibrated power meter. With the copper/dye laser either a Model 212 or Model 210 Power Meter, Coherent Ltd, Cambridge, UK or an "in-house" integrating sphere power meter was used; with the gold laser, the integrating sphere power meter built into the VGM-2 laser control unit was used. The total delivered light fluence was either 50 J/cm^2 or 100 J/cm^2 (Tables 8.1 and 8.2).

In all cases, the area to be treated was illuminated using one or more circular spots to encompass the whole lesion including a border of normal tissue. The spot size was adjusted by altering the perpendicular height of the fibre above the area to be irradiated: in early treatments this height was checked frequently during irradiation using a depth probe and in later treatments the area to be irradiated was marked prior to treatment using dots of crystal violet dye. Multiple overlapping spots were used to treat lesions which were too large to illuminate with a single spot: this was the case with all but one lesion. For short irradiations, the fibre was held "free-hand", with the aid of a handpiece. For longer irradiations, the fibre was clamped in place, using a jointed articulated arm to obtain accurate positioning and to allow easy repositioning if the target area moved. No shielding of normal tissue was required unless in the direct beam of the laser light.

All lesions were photographed before and after PDT and treatment sites were recorded diagrammatically. Biopsies were carried out on all treated sites after healing, irrespective of whether the mucosa appeared clinically normal or there was evidence of residual disease. Two patients required treatment on two separate occasions due to the extent of the disease. In one case excessive swelling caused by the irradiation meant that the patient was unable to keep her mouth open and the treatment had to be aborted.

TABLE 8.1: CLINICAL OUTCOME OF PATIENTS WITH ISOLATED DISEASE

Patient No	Age/ sex	Stage of Disease	Site of malignancy	Site of premalignancy	Previous treatment	Light dose J/cm^2	Early outcome 6-12 weeks	Longer follow up (months)	Latest Follow up (months)
1 MC	65/F	moderate dysplasia		Lower lip	Nil	50	Mild dysplasia		no change (29)
2 LD	82/M	Severe dysplasia		Lower lip	Surgery and RT	100	PD	moderate dysplasia (4)	no change (30)
3 JA	57/M	Severe dysplasia		L tongue	Excision	100	CR	CR	CR (16)
4 RP	51/M	Severe dysplasia		Lip	Nil	100	CR	NCD moderate dysplasia (4)	CR (12)
5 JR	60/F	T1	L Tongue		Surgery	100	mild dysplasia	normal mucosa (12)	CR (20)
6 HG	33/F	T1	Tongue		Nil	100	CR	normal (16)	CR (35)

Key to tables 8.1 and 8.2:

CR= No evidence of abnormal mucosa, either histologically or macroscopically
PD= persistent disease, reduction of lesion by 50% of surface area, but some residual disease

TABLE 8.2 PHOTOFRIN: CLINICAL OUTCOME OF PATIENTS WITH FIELD CHANGE DISEASE

Patient No	*Age/ sex*	*Stage of Disease*	*Site of malignancy*	*Site of pre-malignancy*	*Previous treatment*	*Light dose J/cm²*	*Early outcome 6-12 weeks*	*Longer follow up (months)*	*Latest Follow up (months)*
7 MW	73/F	Severe dysplasia		R buccal mucosa/ R & L mandibular alveolus	Multiple surgery	100	PD	CO_2 excision for residual disease	Recurrence (17) further PDT
8 EB	45/F	Severe Dysplasia	R alveolus R buccal mucosa		Surgery	100	CR hyperkratosis	CR at treated site (26)	Metachronous tumour (19) for further PDT
9 PV	71/F a) 1st PDT	Tis & OSF	L bucco-gingival sulcus	L buccal mucosa	Nil	50	CR	Severe dysplasia (18)	Metachronous tumour (30) further PDT
	b) 2nd PDT	Tis & OSF	L buccal mucosa			100	PD	Refuse further treatment	
10 LF	59/M a) 1st PDT	T1	R buccal mucosa / 1 site	R buccal mucosa / 2 sites	Nil	100	CR scar tissue & hyperplasia	Synchronous tumour (6)	For 2nd PDT
	b) 2nd PDT	T1	R buccal mucosa			100	CR	NCD	NCD(6)
11 PL	72/F a) & b) 1st & 2nd PDT	T1	Palate R alveolus L buccal mucosa	R tongue & buccal mucosa	Multiple surgery & cryosurgery	50	CR	Synchronous tumour (3), for RT	Residual tumour post RT, for PDT
	c) 3rd PDT	T4	Palate			100	PD	Died of metastatic disease (8)	
12 HT	67/F	T1	L buccal mucosa 2 sites	L buccal mucosa 2 sites	Multiple surgery	100	CR	NCD	NCD (40)
13 PD	40/M	T1	Lip	Lip	Surgery	50	CR	CR (18)	CR (26)
14 HO	40/M	T1	R tongue	R tongue base L buccal mucosa	Excision	100	CR	Moderate dysplasia (5)	CR (38) metachronous tumour (39)
15 MWI	88/F	T1	L buccal mucosa	L buccal mucosa 3 sites	Nil	100	CR	No dysplasia (6)	
16 JW	62/M	T1	Floor of mouth		ALA PDT	100	CR hyperplasia	CR	CR (8)

TABLE 8.2 PHOTOFRIN: CLINICAL OUTCOME OF PATIENTS WITH FIELD CHANGE DISEASE

Patient No	*Age/ sex*	*Stage of Disease*	*Site of malignancy*	*Site of pre-malignancy*	*Previous treatment*	*Light dose J/cm²*	*Early outcome 6-12 weeks*	*Longer follow up (months)*	*Latest Follow up (months)*
17 KC	62/M a) & b) 1st & 2nd PDT	Multiple T1	Tongue tip	Dorsum and lateral tongue	Multiple surgery	50	CR	Moderate dysplasia (13)	Further PDT
	c)3rd PDT	Moderate dysplasia		Tongue		100	PD mild dysplasia		Metachronous tumour (14) for further PDT
	d)4th PDT	Multiple T1	Anterior & posterior tongue	Dorsum & lateral tongue		100	CR, hyperkeratosis ant. tongue severe dysplasia post.tongue	Residual leukoplakia	No change at 20 months
18 CH	82/F	T1	R alveolar ridge	R buccal mucosa, floor of mouth	Multiple surgery	100	CR	CR normal mucosa (4)	
19 BE	44/M	T1	R bucco-gingival sulcus	R & L buccal mucosa	Nil	100	CR (12) normal mucosa		
20 LN	54/M	T1 & moderate dysplasia	SCC excised	R & L buccal mucosa	Nil	50	CR	NCD	NCD (49)
21 JS	63/F a) 1st PDT	T1	L alveolar ridge	R buccal mucosa, alveolus	Multiple surgery	50	CR	NCD (7)	Metachronous tumour (14)
	b) 2nd PDT	Multiple T1	R & L alveolus			100	CR right alveolus, PD left alveolus	CR right alveolus	CR right alveolus (24), L alveolus for further PDT
22 PB	70/F	T2	L buccal mucosa	L mandibular alveolus/ L palate	Multiple laser excision & vaporisation	100	PD	CO_2 excision	Recurrence (3) further PDT

8.4 RESULTS

The 22 patients underwent a total of 30 treatment sessions using (Tables 8.1 and 8.2). PDT was generally well tolerated, and was carried out without local anaesthetic or sedation in all except one patient. However, due to the low power output from the laser (150-200 mW), the treatment times were long in some cases. Although the longest irradiation time was 71 minutes, the overall procedure included marking the lesion, positioning the fibre as well as PDT irradiation and time for the patient to have breaks. This produced a total time of up to 4.5 hours. The longer treatments were more tiring for the patients and in one case (patient 22), significant swelling of the left cheek occurred during the latter part of the irradiation time. This made it increasingly difficult for the patients to keep their mouths wide open, even with the aid of a bite block.

8.4.1 Tumour Response

Over the first 24 hours swelling became evident at the treatment site, with ulceration developing within 72 hours. The amount of pain experienced by the patients varied considerably. The majority only required simple analgesia such as Co-codamol, benzydamide mouthwash or spray (Difflam), but a few required stronger analgesia using opiates. Patients were given detailed instructions on oral hygiene using chlorhexidine mouthwash to prevent infection around the necrotic site. The time taken for the necrosis to slough and the ulceration to heal depended on the size of the treatment site, but was typically 3-6 weeks. From clinical observations carried out at regular intervals, especially within the first week following irradiation, there was no obvious selectivity in PDT effect between the tumour and the adjacent normal tissue: the extent of ulceration matched that of the irradiated spot. All patients were reviewed weekly until the area had healed. Patients who had residual disease subsequently had either conventional excision, radiotherapy or further photodynamic therapy (Tables 8.1 and 8.2). All the PDT lesions healed with little or no functional changes and no cosmetic changes. Scarring was present only in patients who had undergone other forms of previous treatment, and in the single patient with oral submucous fibrosis- as is characteristic of the condition.

8.4.2 Patient Response

The follow up times range from 3 - 49 months with a mean of 19 months. At the early follow up stage (6-12 weeks), 17 patients (77%) were successfully cleared of their disease (Tables 8.1 and 8.2). Seven of these 17 patients (41%) subsequently developed synchronous (two) or metachronous (five) tumours in the region of the PDT treated site (patients 8, 9, 10, 11, 14, 17 and 21). In this context, synchronous is used to describe tumours diagnosed within 6 months of identification of one another on the grounds that such lesions are likely to have been present at the time of the initial evaluation (Dhooge *et al.*, 1996; Gluckman & Crissman, 1983; Maisel & Vermeersch, 1981). The time of diagnosis of the subsequent neoplasm was 3, 6, 14, 17, 19, 30 and 38 months post Photofrin® PDT. Following further treatment with PDT to six of these patients, only three (Patient 10, 17 and 21), were cleared of their tumour. However with patient 21 only one of the two irradiated sites was rendered disease free and the other site underwent further treatment. Patient 11 had very advanced T4 disease at this stage, which had been resistant to radiotherapy. All these recurrent lesions were found in patients with field change disease. Seven of the twelve field change patients (58%) with no evidence of disease following PDT developed a second primary tumour within 4 years.

Patient 17 presented a particular therapeutic challenge, as a partial glossectomy had been carried out previously. The first two PDT treatments covered almost the entire surface of the tongue as the patient presented with multiple early SCC and dysplasia which essentially extended over the entire area. Following PDT the patient's SCC reverted to moderate dysplasia. The third treatment down-graded the dysplasia from moderate to mild, but later biopsies (31 months after 2nd PDT) showed early SCC at two sites. The fourth Photofrin® PDT treatment, encompassing once again the majority of the tongue, led to stabilisation of the condition on the anterior tongue with only hyperkeratosis found on follow up biopsy. However the posterior tongue showed residual severe dysplastic changes. Nevertheless this patient's disease has remained stable for the last 20 months.

In total, the 22 patients experienced 30 PDT treatment sessions using Photofrin® (Table 8.3). Eighteen of the treatments (60%) cleared the patient of disease with a minimum review period of 3 months. Nineteen of the treatments (63%) were successful in clearing the patient of disease locally. These were predominantly in patients with T1 SCCs particularly those with field change disease.

Table 8.3
Result after each completed course of Photofrin® PDT (after up to 2 PDT)

Stage of Disease		**CR Free of disease**	**CR Disease elsewhere**	**PD Alternative therapy undertaken or planned**
Dysplasia	Isolated	1	-	3 (downgrading)
Dysplasia	Field change	2	-	2 (size reduction)
Tis	Field change	1	-	1
T1	Isolated	2	-	-
T1	Field change	12	1	3
T2	Isolated	-	-	-
T2	Field change	-	-	1
T3	Isolated	-	-	-
T4	Isolated	-	-	1

CR: Complete response
PD: Persistent disease
30 treatments in 22 patients. Two patients required PDT on 2 separate occasions to cover the entire involved tissue

Partial Response Lesions

Five patients had persistent disease following PDT (patients 1, 3, 5, 7 and 22), although all showed improvement in their condition. Three of these patients had reduced severity of their dysplasia (patient 1, 2 and 5), whilst two (patient 7 and 22) had a reduction in the size of the lesion, from 18.2 to 1.8 cm^2 and from 17.7 to 1.8cm^2 respectively. The latter two patients had subsequent carbon dioxide laser excision of the remaining area of disease. The irradiation was aborted early with patient 22, due to the previously described swelling. Thus the area of residual disease which was subsequently found was not wholly unexpected (Figure 8.1)

Long Term Follow up

Eight patients are alive and disease free with a mean follow up of 28 months (range 16-49 months). Four of these patients had field change disease, whilst the remaining four had isolated tumours. Two patients have died since their treatment, one (patient 11) as a consequence of the disease. The other (patient 16) died 8 months after PDT of an unrelated medical condition. Five further patients who were cleared of their disease at the last appointment are now lost to follow up (patients 2,10,15,18 and 19). Six patients are alive with disease and the remaining patient (14) was free of tumour at 9 months after surgery for a metachronous tumour. The history of the clinical disease and treatment carried out on all 22 patients are illustrated diagrammatically in Figure 8.1-8.3.

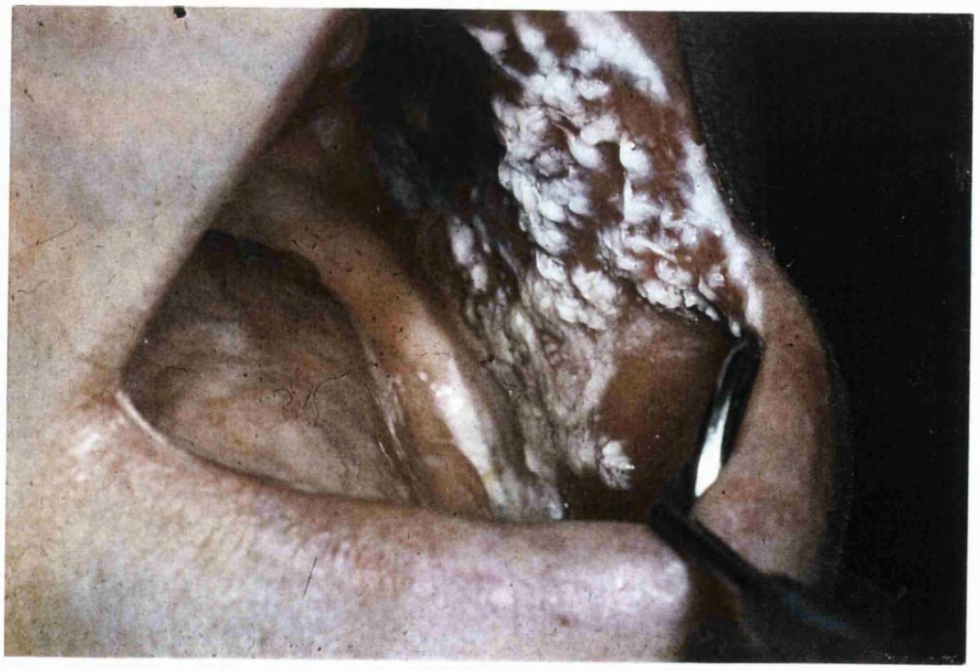


Figure 8.1: **(A)** Patient 22 before PDT.

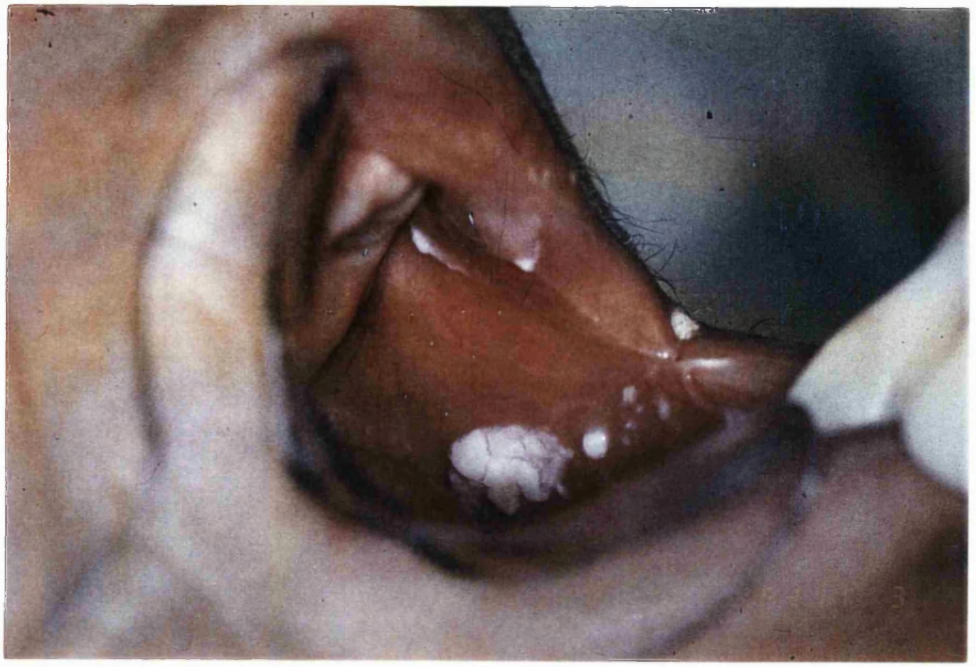


Figure 8.1: **(B)** 4 weeks post Photofrin® PDT, residual disease.

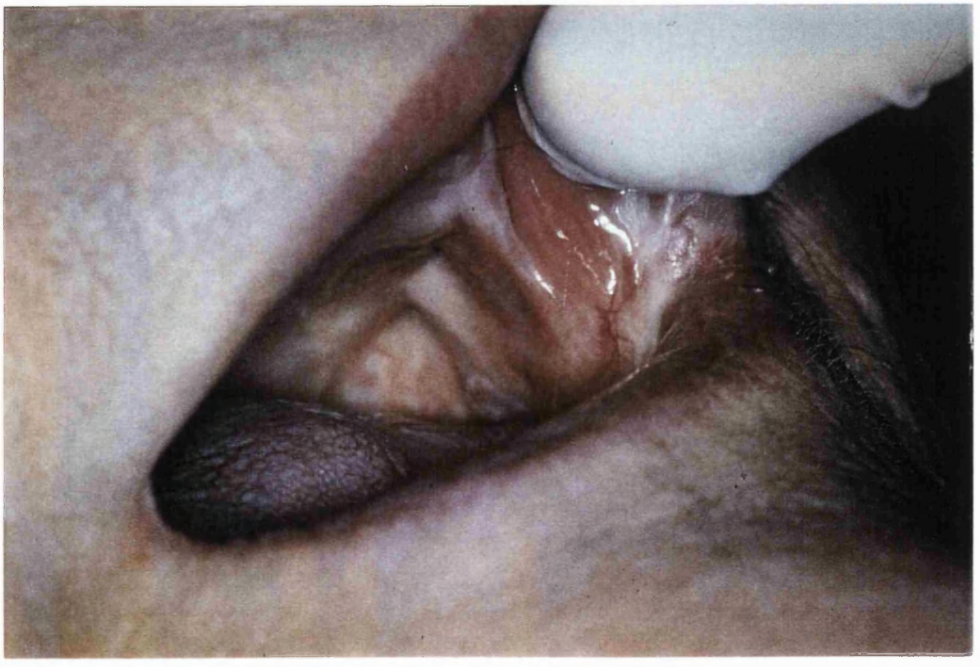


Figure 8.1: **(C)** After further PDT with mTHPC.

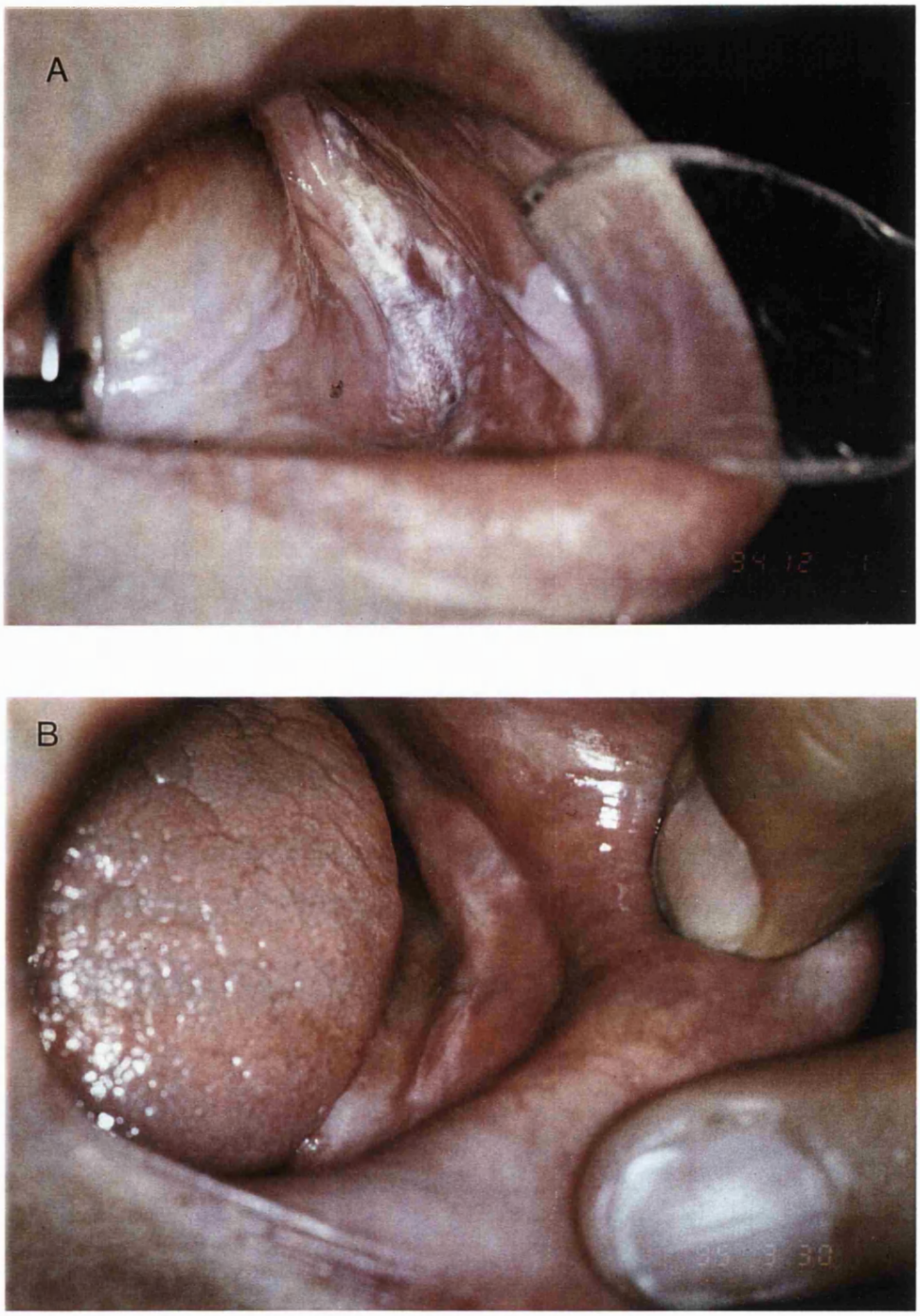


Figure 8.2: Patient with severe dysplasia before (A) and 3 months (B) after Photofrin® PDT.

8.5 Complications

None of the patients exhibited any adverse reaction to the intravenous injection of Photofrin®. One patient developed severe skin burns, with blister formation on the neck, arms and legs, following two hours of intense sunlight exposure five weeks after sensitisation with Photofrin® despite advice to avoid the sun. Hypertrophic scars were evident following healing. Two further patients reported residual increased sensitivity to sunlight up to one year following Photofrin® administration.

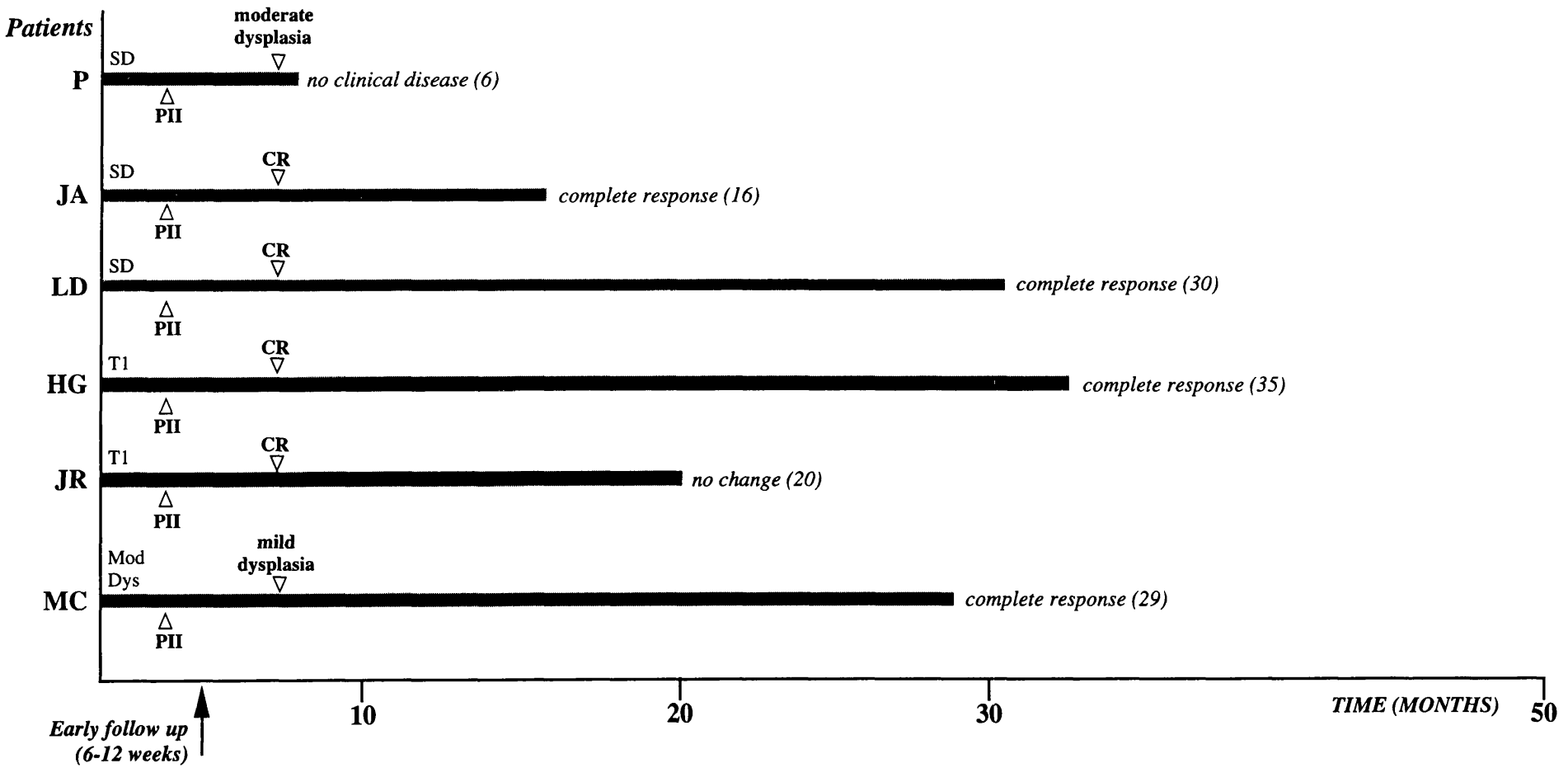
Key to Figures 8.3 & 8.4

Length of line ≡ time from presentation.
Figure in bracket ≡ time from last PDT.

These schematic diagrams illustrate the difference in the nature of disease between those patients with isolated tumours and those with multifocal (field change) disease who often require further treatment for subsequent disease.

PII:	Photodynamic therapy using Photofrin®.
mTHPC:	Photodynamic therapy using mTHPC.
ALA:	Photodynamic therapy using ALA.
S:	Surgery
RT:	Radiotherapy
SD:	Severe dysplasia
Mod Dys:	Moderate dysplasia
Mild Dys:	Mild dysplasia
RA	Right mandibular alveolus.
LA	Left mandibular alveolus.
T1:	T1 squamous cell carcinoma.
• M:	Metachronous/synchronous tumour.
R:	Recurrence.
o	Further dysplasia.
†:	Patient dead.
NCD:	No clinical disease (no recent biopsy).
CR:	Complete response (clinical & histological).
PD:	Persistent disease.

Figure 8.3: History of patients with isolated disease treated with Photofrin® PDT



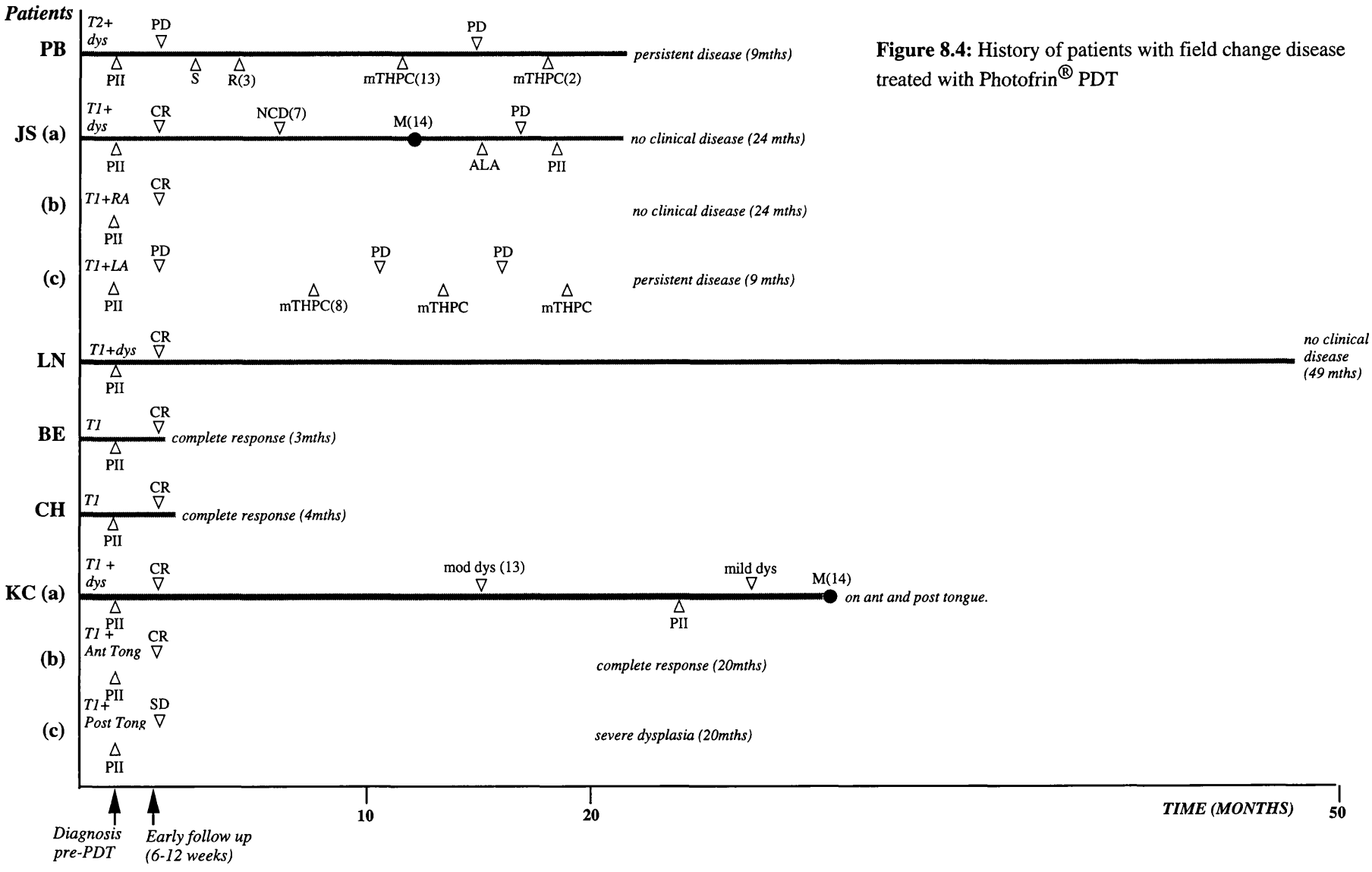


Figure 8.4: History of patients with field change disease treated with Photofrin® PDT

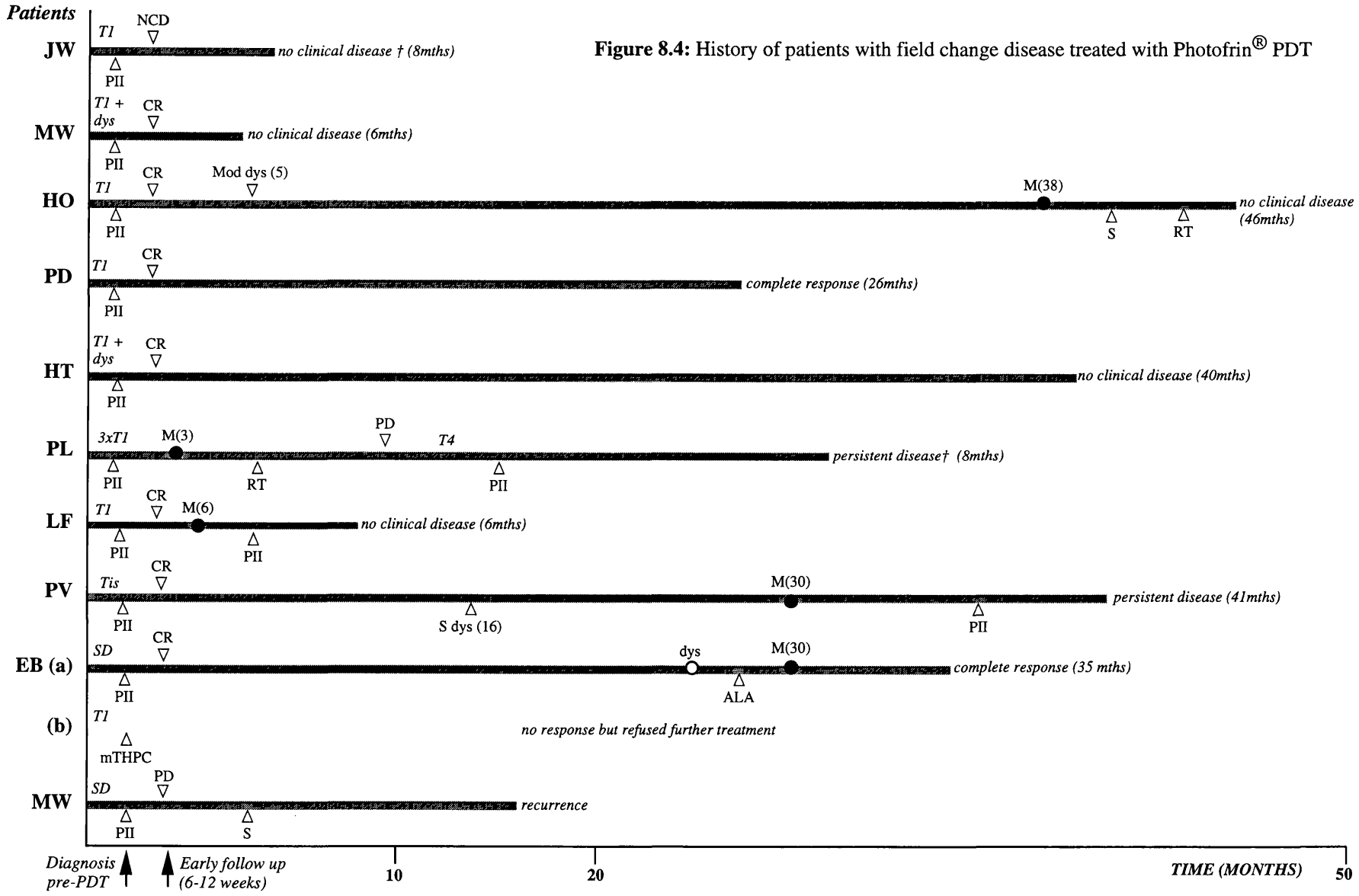


Figure 8.4: History of patients with field change disease treated with Photofrin® PDT

8.6 DISCUSSION

This study provides evidence that photodynamic therapy using Photofrin® is capable of causing necrosis in tumour and normal tissues within the oral cavity. The principles and applications of photodynamic therapy are relatively straightforward. If an appropriate laser is available then the treatment can be carried out simply with support from a physicist. However with the development of "user friendly" diode lasers the necessity for even this support may be eliminated. Such systems are very attractive to clinicians and scientific investigators and may have a major impact on current oncology practice (Dougherty, 1993). Photofrin® PDT induced necrosis at the irradiation site in all the patients treated, and all showed improvement in their disease. Of the patients treated, 77% (17 patients) were tumour free at the short follow up stage (up to 3 months) with the longest "tumour free" follow up period being 49 months. Seven of these patients (including one with only dysplasia) subsequently developed synchronous or metachronous tumours within the upper aerodigestive tract over a period of 38 months. In the five patients with persistent disease the surface area of the affected tissue was reduced in size in two cases, and became amenable to local excision by CO_2 laser, thus avoiding more extensive surgery. The remaining three dysplastic lesions had down-grading in the level of dysplasia.

Seven patients with initial complete response later developed a second malignant tumour over a mean review period of 18 months (range 3 to 38 months) after PDT, which is a normal pattern for this disease. Therefore 41% of patients (7 out of 17) with no evidence of their tumour following PDT developed further cancer over this period of review. The proportion of patients in this present study who developed a second cancer is higher than reported in previous studies (Dhooge *et al.*, 1996; Tepperman & Fitzpatrick, 1981). Figures in the region of 10-30% has been quoted for the proportion of patients with head and neck cancer who develop a second cancer within the same area (Licciardello *et al.*, 1989; Lippman & Hong, 1989; McGuirt *et al.*, 1982; Winn & Blot, 1985) : over an

average period of 3.4 years, 10.6% of patient with cancers in this region develop a second primary.

Four of the seven patients with further primary cancers already had previously successful treatment treated prior to the PDT. In one patient who had multiple T1 tumours of the tongue, the original cancers were successfully treated, although some residual leukoplakia remained. The metachronous tumours which subsequently arose were also successfully treated using PDT. Had this treatment failed, the patient would have required debilitating surgery (total glossectomy) with the associated morbidity. At 20 months since the last PDT application the disease remains stable with some residual leukoplakia.

The results of this present study are comparable to those previously reported in similar groups of patients. Gluckman et al (1991) obtained a complete response in 11 of 13 patients with early cancers of the oral cavity and pharynx, although recurrence was noted in four cases between 8 and 12 months (Gluckman, 1991a). As in the present study, patients with "condemned mucosa" demonstrated excellent results in the short term. Biel performed PDT with Photofrin® on 7 patients with T1 SCC in the oral cavity, and two patients with leukoplakia or severe atypia (Biel, 1995). Complete response was reported for all patients but, within one year, one oral SCC and one leukoplakia recurred and a local regional failure was also noted. Another study of 55 patients treated with PDT with haematoporphyrin derivative for oral cancer demonstrated 69% complete response with an average follow up of 26 months, although half of this group consisted of patients with lip cancer which has a better prognosis. If these patients are removed from the analysis, the complete response rate drops to 38% (Zhao *et al.*, 1987).

Twelve of the patients in our study had received treatment for a previous cancer in the upper aerodigestive tract. Only one of the seven patients, who developed a synchronous or metachronous neoplasm over the duration of this study, died with the disease within 1 year of the diagnosis of the third cancer (patient 11). The remaining six patients are well with up to 38 months follow up. Shons and McQuarrie (Shons & McQuarrie, 1985)

found that 73% of their patients who developed a metachronous tumour locally survived less than 2 years. Gluckman et al (1983) reported 5 year survivals following the diagnosis of a second neoplasm to be 22.3%. The results from this present study is therefore encouraging.

Because of the cumulative toxicity of radiotherapy and the disfigurement caused by surgery, current therapeutic options are limited for patients who develop a second malignant tumour in the head and neck region, following successful treatment of the first. PDT does not cause such cumulative toxicity, and may therefore prove to be a very useful treatment modality in such cases.

8.6.1 Depth of PDT Damage

One limitation of PDT is its relatively shallow depth of effect. This means that the induced necrosis may not penetrate to some of the deeper tumour islands, as was observed in one patient in this study (patient 11) who had advanced (stage IV) disease with tumour involvement of the palatal bone. Photofrin® PDT using surface illumination did not control the local disease. Until recently, no published study had attempted to correlate the depth of PDT damage to the delivered light dose. Grant et al (1995) investigated the depth of necrosis produced in a group of 11 patients who had received standardised drug and light doses (2mg/kg Photofrin®, 50J/cm^2). Even under these treatment conditions, the absolute depth of necrosis was found to vary between 1.1 and 4mm. While this depth is likely to be an underestimate, as a result of sloughing, the four patients in whom viable tumour was found below the necrotic volume were of more concern. This may have been due to inadequate light penetration into the deeper tumour. A number of authors have used interstitial irradiation with the more advanced tumours, in an attempt to increase the depth of necrosis (Gluckman, 1991a; Schuller *et al.*, 1985). Nevertheless, the response following the treatment of advanced head and neck cancers has generally been disappointing with Photofrin®.

8.6.2 Non-Selectivity

Most sensitisers studied to date are retained in both the normal and the neoplastic tissue (Henderson & Dougherty, 1992). However, a degree of selectivity in uptake/retention in tumour tissue with respect to surrounding normal tissue can be found (Gomer & Dougherty, 1979b). This selective concentration of sensitiser in the tumour and the claimed associated selective tumour destruction is often quoted as a potential advantage of PDT (Biel, 1995; Biel *et al.*, 1995; Henderson & Dougherty, 1992; Schweitzer, 1990). However Grant observed no preferential PDT effect in tumour, compared with normal tissue, (Grant, 1995). In the present study I also found no such selectivity in effect was noted. However, since the normal tissue heals very well, with little evidence of scarring, this lack of selectivity may not be a problem.

8.6.3 Disadvantages of Photofrin® PDT

Although effective, PDT using Photofrin® has a number of disadvantages. The 630nm wavelength used to activate the photosensitiser represents a compromise: while Photofrin® absorption is much higher between 400nm and 500nm, the very strong natural absorption in tissue of light in this wavelength range severely limits the penetration depth and therefore the depth of PDT induced necrosis (Berenbaum *et al.*, 1986). As Photofrin® has a subsidiary absorption maximum at 630nm, where light penetration into tissue is greater, this wavelength is used for illumination. A number of "second generation" photosensitisers have significantly longer activation wavelengths and may therefore induce deeper necrosis due to the increased penetration of light (Ris *et al.*, 1991).

The prolonged cutaneous photosensitivity also proves troublesome. Two of the patients in this study experienced increased sensitivity even one year following Photofrin® administration. Similar observations have been reported by other groups (Dougherty *et al.*, 1990b; McCaughan, 1987). In patients who are likely to require multiple courses of treatment, the prolonged avoidance of bright light would represent a significant treatment morbidity. Newer sensitisers, such as ALA, which has a significantly reduced period of

cutaneous photosensitivity (Fan *et al.*, 1996; Grant *et al.*, 1993a; Kennedy & Pottier, 1992; Regula *et al.*, 1995) are attractive alternatives, although the efficacy of these newer drugs needs to be fully assessed.

8.6.4 Improvements Required

For optimisation of PDT in the oral cavity, improvements are required, both in light delivery and dosimetry. The undulating anatomy in the oral cavity renders uniform and accurate irradiation very difficult, particularly in areas such as the posterior ventral tongue, buccal sulcus and retromolar trigone. Movement is a further hindrance to delivery of an accurate light dose, making it necessary to reposition the optical fibre frequently.

There is still a need for a sensitiser with a greater depth of effect if PDT is to be used to treat deeper tumours. Meta tetrahydroxyphenyl chlorin (mTHPC), a second generation photosensitiser, which has been shown to be capable of up to 10 mm depth of necrosis in certain circumstances, may provide some of the answers (Ris *et al.*, 1991). Finally, treatment times with Photofrin® are often prolonged and more active drugs, such as mTHPC, significantly reduce treatment times. They may, therefore, prove to be more acceptable to the patient.

8.7 CONCLUSION

This study has shown that PDT using Photofrin® with surface illumination can effectively destroy early squamous cell carcinomas and dysplasias in the oral cavity. The treatment is simple and safe to instigate and the only significant morbidity is prolonged skin photosensitivity. Unlike conventional therapies, PDT has no cumulative toxicity and can be repeated several times in one patient, thus making it an ideal modality for patients with field change disease. With the treatment parameters used in this study the PDT effect has been shown not to be specific to tumour tissue, as irradiated normal tissue will also under necrosis. This may not necessarily be a disadvantage since successful treatment of

tumours requires that a surrounding cuff of normal tissue be removed (Bown, 1990). In preclinical studies, PDT treated normal tissue has been shown to heal or regenerate with normal mucosa (Barr *et al.*, 1987b; Meyer *et al.*, 1991).

There is plenty of room for improvement in photosensitiser characteristics, such as increased tumour selectivity, reduced cutaneous photosensitivity and increased absorption at longer wavelengths to enhance the depth of light penetration. A closer examination of the use of ALA and mTHPC as photosensitisers in the oral cavity will be described in the following chapters.

Chapter 9

ALA PDT IN THE ORAL CAVITY

9.1. INTRODUCTION

The use of dihaematoporphyrin ether (Photofrin®, Vancouver, Canada) has already been discussed in Chapter 8. Photofrin® has been shown to be beneficial in certain clinical situations (Gluckman, 1991b) and was the first sensitiser to be evaluated in this thesis. However it has an ill defined composition with poor tumour selectivity and causes prolonged cutaneous photosensitivity (6-12 weeks) which persists over several months in some cases (Dougherty *et al.*, 1990a). This problem has prompted the search for new photosensitising agents which may overcome this disadvantage. 5-aminolaevulinic acid (ALA) is one such second generation agent.

ALA is unusual in that it is not itself a photosensitiser but is metabolised to a photoactive substance in vivo via the haem biosynthetic pathway. The systemic administration of ALA overcomes the feedback inhibition of ALA synthetase leading to the overproduction and accumulation of porphyrin precursors to haem, in particular protoporphyrin IX (PpIX) (Pottier *et al.*, 1986). PpIX is the main photoactive substance produced following sensitisation with ALA (Grant *et al.*, 1993a; Mustajoki *et al.*, 1992; Pottier *et al.*, 1986) .

PDT using topical ALA has already been shown to be of value in the treatment of basal cell carcinomas of the skin. ALA has the major advantages that, even after systemic administration, cutaneous photosensitivity only lasts 1-2 days (Mustajoki *et al.*, 1992) and PDT treated tissues heal remarkably well so making it feasible to treat extensive superficial lesions which often pose problems for conventional management in the mouth. Further, in our preclinical and clinical studies, PpIX has been shown to accumulate more in the epithelium than in the underlying muscle (Grant *et al.*, 1993a; Loh *et al.*, 1993a) with the consequent possibility of selective damage to this layer following PDT.

9.2 AIM

The purpose of the present study was to investigate the efficacy of PDT using ALA in the management of malignant and premalignant lesions in the mouth. Two different irradiation regimens were used in order to optimise the PDT effect.

In previous studies in this centre the effect of PDT using ALA was found to be very superficial, even with long treatment times (Grant *et al.*, 1993a; Loh *et al.*, 1993a; Regula *et al.*, 1995). Recent reports from several sources have suggested that PDT effects might be enhanced by fractionating the light dose (Foster *et al.*, 1991; Messmann *et al.*, 1995; Pe *et al.*, 1994; van der Veen *et al.*, 1994). In particular, using ALA, Van den Veen et al (1994) found that a 75 minute period between light applications enhanced the effect (although the total light dose was higher in the fractionated dose experiments), and Messmann et al (1995) found that a single break of 150 seconds during a 250s exposure was capable of increasing the area of a PDT induced ulcer in normal rat colon by a factor of 5 compared with the same total light dose administered without a break. In view of these findings, we studied fractionated light therapy using a long and a short break during treatment to see if deeper effects could be produced than were reported in our first clinical article (Grant *et al.*, 1993a).

9.3. MATERIALS AND METHODS

9.3.1 Case Selection

Eighteen patients were included in this study, 11 male and 7 females, with a mean age of 66 years (range 46-87 years). However, in addition to the criteria outline on page 168 patients with active liver disease were excluded from this study for reasons that will be discussed later. In those patients who had previous treatment with the carbon dioxide laser (Tables 9.1-9.3), PDT was undertaken because histology showed persistent disease at the margins. Details of all treatments are given in Tables 9.1-9.3.

The treatment for the earlier patients were carried out as in-patients, whilst the latter patients were all managed on an out-patient basis.

9.3.2 Photosensitiser

ALA was supplied for this project by DUSA Pharmaceuticals as the hydrochloride in powder form. For clinical use, this was dissolved in orange juice and given by mouth in 3 separate doses of 20mg/kg, at 0, 1, and 2 hours (total dose 60mg/kg) using the regimen established by Regula et al (1995). The rationale for this has already been discussed in Chapter 5.

9.3.3 Laser and Light Delivery

The light source used was a gold vapour laser giving red light at 628nm (Dynamic Light Ltd., Milton Keynes, UK). This was delivered to the patient using a mode scrambled 400 μm core flexible optical fibre with a bare plane cleaved tip which gave a circular spot of light up to 2.5 cm in diameter on the target tissue. The area to be treated was defined as the target lesion together with a minimum margin of 5 mm of surrounding normal tissue. Before treatment started appropriate fibre positions were chosen and treatment times were calculated to give the desired light doses using one of the two dose regimens described below (Study Groups 1 and 2). Whenever possible the entire lesion was treated from a single fibre position but, due to the complex shape of the mouth, this was often not practical and up to 7 different fibre positions had to be used in one patient. The power density was kept below 250mW/cm^2 to avoid thermal effects. As many of the total exposure times were quite long (up to 143 minutes) the fibre was positioned using a multijointed arm to keep its tip in the correct place over the area to be treated. In addition, the area to be irradiated was marked with dots of crystal violet dye. The patients were given systemic analgesia and topical anaesthetic with occasional sedation if necessary. If required, injected local anaesthetic was limited to the site of biopsy.

9.3.4 Irradiation Regimen

Study Group 1 (7 patients) received a total light fluence of 200J/cm^2 in two equal fractions. The first fraction was delivered 2.5 hours after the first dose of ALA and the second at 4 hours, a similar regimen to that described by van der Veen et al (1994).

Study Group 2 (11 patients) received a total fluence of 100J/cm^2 in two fractions with a minimum of one 5 minute interval between fractions. With the long treatment times required, it was sometimes uncomfortable for patients to keep their mouth open continuously, so in these cases there were further breaks during treatment.

The patients were kept in a dark room for 24 hours after receiving ALA with careful testing of skin photosensitivity following this time period. Routine haematological and biochemical investigations were carried out before, and 1 to 2 days following, ALA ingestion (longer if any abnormalities were found).

9.3.5 Evaluation of PDT

In addition to the diagnostic biopsy taken before patients were considered for inclusion in this study, further biopsies were taken immediately prior to PDT (for fluorescence microscopy to determine the tissue level of PpIX) and at 2-8 days after treatment to assess the depth of necrosis. Biopsies were also taken when healing was complete and at long term follow up to assess the results of treatment.

Biopsy specimens taken for fluorescence microscopy were prepared, examined and the data processed as described in section 6.3.4. Fluorescence was excited at 632.8nm using an 8mW Helium Neon laser with subsequent detection over the range of 670-700nm. Background correction was made using control biopsies taken before ALA administration under the same excitation and detection conditions. The sections were then fixed in formalin and stained with haematoxylin and eosin for comparative light microscopy studies. The follow up biopsy specimens, which were not required for fluorescence microscopy, were fixed directly in 10% neutral buffered formalin and processed routinely

in paraffin wax. Sections, 5 μm thick, were cut and stained with haematoxylin and eosin. With the aid of an eyepiece graticule the depths of necrosis and inflammation were measured from the surface of the lesion. The depth of necrosis was defined as the deepest point at which there was necrosis of any tissue layer. The depth of inflammation was measured to the deepest point at which extravasated inflammatory cells were seen in the tissue.

Key to Tables 9.1-9.3

A	alveolus
BM	buccal mucosa
FM	floor of mouth
P	palate
RM	retromolar trigone
T	tongue

Full thickness epithelial necrosis and sloughing present in all cases.

*	Subsequent PDT with a different sensitiser.
**	All 3 patients with field change disease had had multiple excisions of dysplastic areas and carcinomas over a period of 5-12 years prior to PDT.
+	indicates inflammatory changes extend through full thickness of biopsy
NA	not applicable
N	no clinical disease locally, but biopsy refused.
NM	not measurable.
m	developed metastatic neck disease.
H	hyperplasia seen, but no dysplasia
Prev Tx	previous treatment

1-4, 8-10 & 15-18 received 100J/cm^2.

5-7, 11-12 & 13-14 received 200J/cm^2.

Table 9.1: CLINICAL OUTCOME OF PATIENTS WITH MODERATE DYSPLASIA

Patient	*Age/Sex*	*Site*	*Previous Treatment*	*Maximum Necrosis mm*	*Maximum Inflammation mm/layer*	*Histology (healed/weeks)*	*Size of lesion before/healed*	*Histology (longest f/u months)*
1	73/M	BM	nil	0.9	1.2/submucosa	mild(5)	$2.6cm^2/1.7cm^2$	mild (20)
2	69M	FM	CO_2 laser	0.8	1.5/submucosa	normal (5)	$6.2cm^2/0cm^2$	normal (13)
3	52/M	FM	nil	1.1	6^+/muscle	mild (4)	$7.1cm^2/0\ cm^2$	N(18)
4	79/M	T	nil	0.6	2.5^+/submucosa	mild (6)	$3.1cm^2/0cm^2$	mild (23)
5	67/M	BM	nil	0.5	1.1/submucosa	$normal^H(5)$	$1.8cm^2/0cm^2$	moderate (27)
6	69/F	T	nil	NM	NM	mild(5)	$3.1cm^2/0.8cm^2$	mild (23)
7	46/M	A	CO_2 laser	0.2	0.9/submucosa	N(5)	$1.0cm^2/0cm^2$	normal(23)

Table 9.2: CLINICAL OUTCOME OF PATIENTS WITH SEVERE DYSPLASIA

Patient	*Age /Sex*	*Site*	*Previous Treatment*	*Maximum Necrosis mm*	*Maximum Inflammation mm/layer*	*Histology (healed/weeks)*	*Size of lesion before/healed (cm^2)*	*Histology (longest f/u months)*
8	51/M	FM	nil	0.1	1.0/submucosa	normal (5)	$6.3cm^2/0cm^2$	normal (24)
9	75/M	BM	CO_2 laser	0.6	1.5/mucosa	moderate (2)	$7.1cm^2/0cm^2$	moderate(24)
10	70/M	BM/A	nil	0.3	1.2/submucosa	$normal^H(8)$	$8.8cm^2/0cm^2(5)$	N (3) lost to follow up
11	49/M	T	CO_2 laser	0.3	0.8/muscle	N (12)	$8.0cm^2/0cm^2(5)$	normal(30)
12	59/F	T	CO_2 laser	0.6	2.5/muscle	N (12)	$4.8cm^2/0cm^2$	normal (32)

TABLE 9.3: CLINICAL OUTCOME OF PATIENTS WITH SQUAMOUS CELL CARCINOMA

Patient	*Age/ Sex*	*Site*	*Previous Treatment*	*Maximum Necrosis mm*	*Maximum Inflammation mm/layer*	*Histology (healed/weeks)*	*Size of lesion before/healed (cm^2)*	*Histology (longest f/u months)*
13	52/F	FM	CO_2 laser	NM	NM	normal (5)	$1.8cm^2/0cm^2$	normal (29)
14	85/F	P	field change**	NM	NM	mild (4)	$4.6cm^2/0.8cm^2$	mild (31)
15	87/F	T	field change**	0.5	1.2/muscle	N	$4.0cm^2/0cm^2$	NA^m
16	81/F	A	mandibular resection	0.5	1.0/mucosa	NA	$16.7cm^2/0.6cm^2$	NA^{*m}
17	62/M	FM	nil	1.3	NM	SCC (5)	$8.0cm^2/1.0cm^2$	NA
18	65/F	BM/A P/RM	field change**	0.7	1.3/submucosa	SCC (12)	$8.5cm^2/8.5cm^2$	NA*

9.4 RESULTS

9.4.1 Tissue Response

After PDT the treated area became inflamed within a few hours. This was followed by sloughing of the superficial layers after 1-2 days leaving a shallow ulcer.

Oral analgesics were usually required from the first or second day after PDT for about a week. Excellent healing was found in all areas irrespective of the size of the original lesion, though larger lesions often took longer to heal (3-5 weeks). There was no evidence of scarring in those lesions which had not received previous surgery, other than at the site where biopsies were taken. Once healed, patients did not perceive any changes in function or sensation within the oral cavity.

9.4.2 Depth of PDT damage

Study Group 1 consisted of 3 male and 4 female patients, with an age range from 46-85 years (mean 61 years). Four had previously undergone surgery. There were two early or microinvasive squamous cell carcinomas (SCC), two severe dysplasias and three moderate dysplasias. This group received a total light dose of 200J/cm^2 with a 90 minute break between light fractions. The depth of necrosis found in the four assessable early post PDT biopsies varied from 0.2 to 0.6mm (mean 0.4mm), although it was difficult to measure the absolute depths of necrosis as sloughing was present in all cases. The figures are therefore likely to be underestimates. However complete necrosis of the epithelial layer was found in all cases. The depth of inflammatory response varied from 0.8-2.5mm (mean 1.3mm) and in two of the four cases extended into the underlying muscle. In three cases it was not possible to measure the exact extent of damage due to the orientation of the specimen. Details are given in Tables 9.1-9.3.

Study Group 2 consisted of 8 male and 3 female patients, with an age range of 51-87 years (mean 69). Five had previously undergone surgery. There were four squamous cell carcinomas (two invasive, 15 & 16, and two microinvasive, 17 & 18), three severe

dysplasias and four moderate dysplasias. In this group one of the 11 early post PDT biopsies could be assessed. This group received a total dose of 100J/cm^2 with at least one short 5 minute break in light delivery at the midpoint. The depth of necrosis in this group varied from 0.1-1.3mm (mean 0.7mm) but, as in Group 1, all cases showed complete necrosis of the epithelial layer. The depth of inflammatory response ranged from 1-6mm (mean 1.8mm). In three cases this extended into the muscle layer, in 6 into the submucosa and for the remaining 3 just into the mucosa. Details are given in Tables 9.1-9.3.

The numbers in each group were small, but there were no obvious differences and there was no statistically significant difference between the depths of necrosis or inflammatory response between the two groups (based on a Wilcoxon matched-pairs signed-rank test- (see appendix to Chapter 9). In view of this, for further analysis, the results of the two groups are considered together.

9.4.3 Clinical Outcome

Seventeen of the 18 patients were reassessed when healing was complete and 13 had further biopsies at this stage (2-12 weeks after PDT). Long term follow up data was available on all 18 patients, 11 of whom had a late follow up biopsy. Those who refused repeat biopsies usually did so because the treated areas looked macroscopically normal. All except 2 patients had at least one biopsy after healing, and these were the two patients with invasive carcinomas who developed metastatic neck disease. Details of the specimens taken and the histological findings are given in Tables 9.1-9.3.

All 7 patients with moderate dysplasia improved after treatment i.e. the mucosa became normal or only mildly dysplastic. However in the case of one patient who showed only hyperplasia just after healing moderate dysplasia was seen again 48 weeks later (Table 9.1). In 4 of the 5 patients with severe dysplasia there was no evidence of residual disease after treatment with up to 32 months follow up, (one patient refused biopsy at long term follow up). In the other patient moderate dysplasia was seen at early and late

follow up (Table 9.2). It is notable that in three of the patients with regression but not clearance of dysplasia (patients 1, 3 & 9), there was no macroscopic evidence of disease and the mucosal surfaces appearing clinically normal once the PDT treated area had healed.

In two of the 6 cases of squamous cell carcinoma (cases 13 & 14), no evidence of tumour was found following treatment although one exhibited persistent mild dysplasia at 1 year. Case 15 had local control of disease but subsequently developed metastatic neck disease. This patient had a 12 year history of recurrent oral cancer and precancer requiring multiple surgical procedures. Case 16 initially had a local marginal mandibular resection and was treated with PDT when the specimen showed tumour at the margins. After PDT only small nodules of tumour were visible locally, but she developed metastatic neck disease which was treated with radiotherapy and further surgery. Some months later she had further local recurrence, treated with PDT using meta tetrahydroxyphenyl chlorin (mTHPC), but she then developed further tumour in the neck and died soon after. Patient 17 had an initial pre-PDT biopsy showing just severe dysplasia. It was only following ALA PDT, when 88% of the mucosal lesion had cleared that the residual lesion was biopsied and found to be microinvasive SCC with changes extending down the salivary ducts (Figure 9.1 C & D). This suggested that PDT using ALA was able to deal with the severe dysplasia in this case but not the deeper invasive disease. As this patient was not suitable for surgery, further PDT was carried out using Photofrin® with no evidence of tumour at 11 months follow up. In the last patient (case 18), there was no reduction in the tumour size. This patient also had a 10 year history of field change disease with recurrent SCC, so received further PDT using Photofrin®. This controlled the disease for a while, but further local recurrence has been treated recently with PDT using mTHPC.

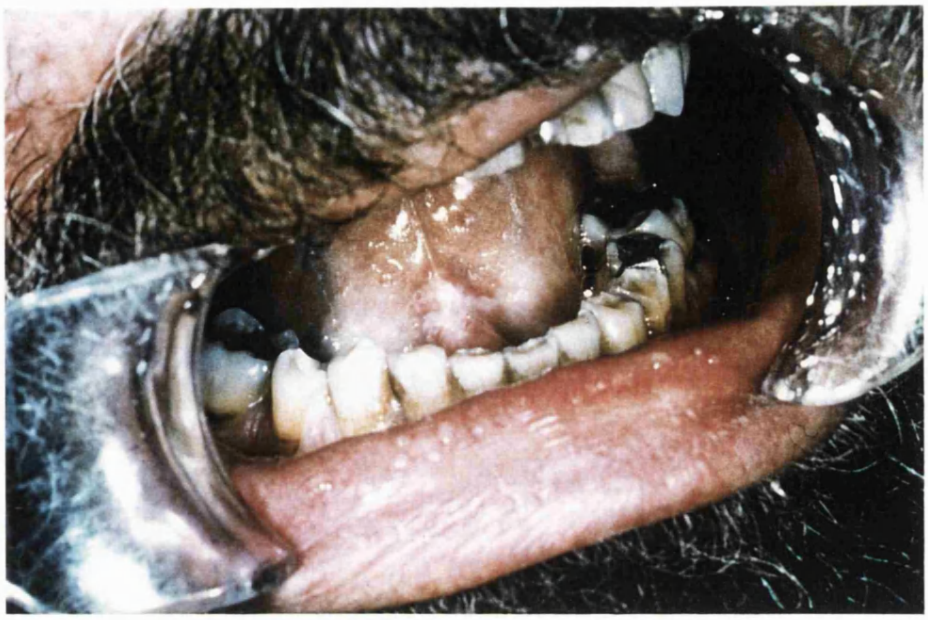


Figure 9.1 (A): Patient 8, severe dysplasia of ventral tongue and floor of mouth.

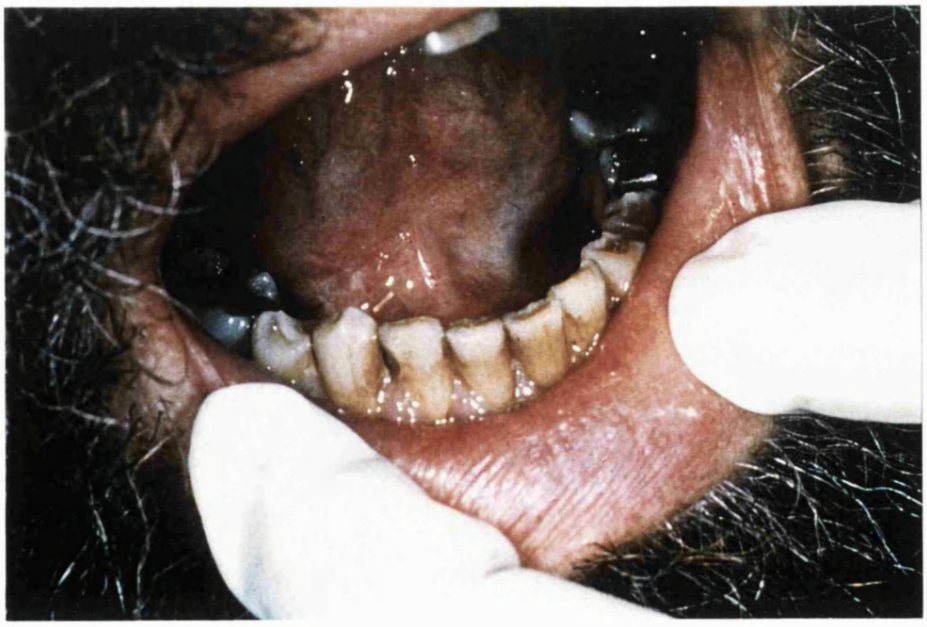


Figure 9.1 (B): Patient 8, three weeks post photodynamic therapy with ALA, with normal mucosa.

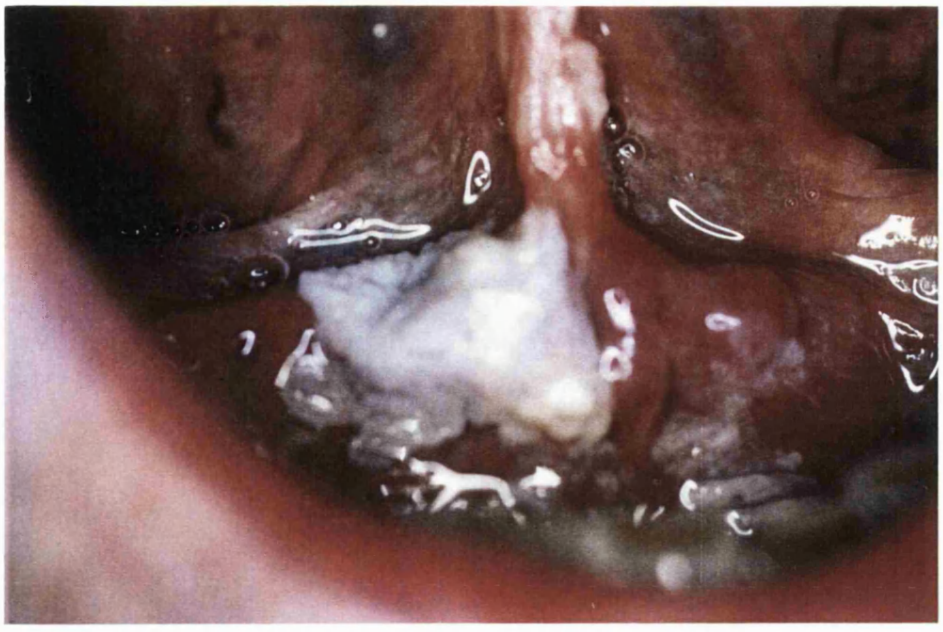


Figure 9.1 (C): Patient 17. Before PDT, with severely dysplastic lesion.

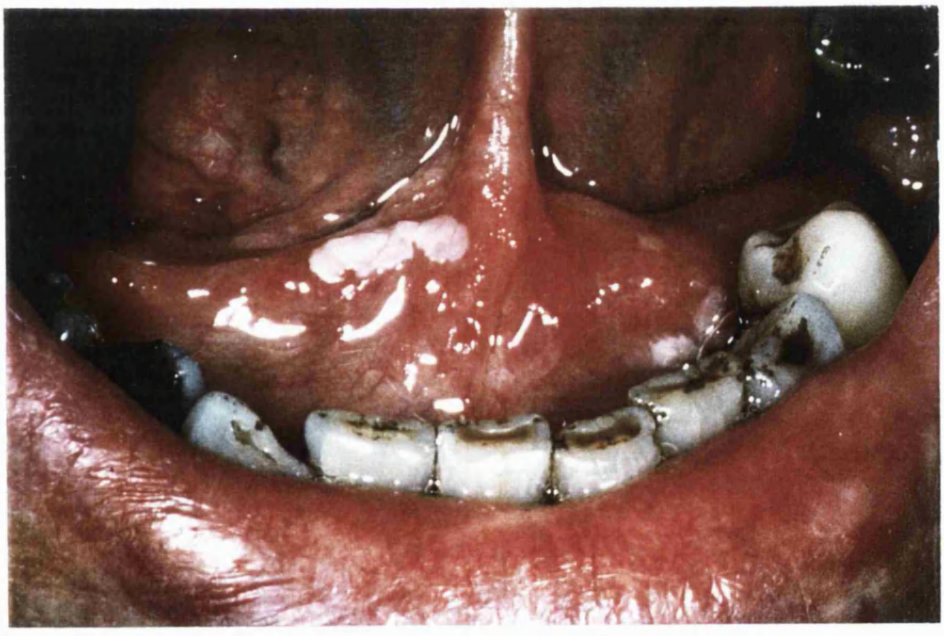


Figure 9.1 (D): Patient 17. Four weeks after PDT with ALA, residual patch found to be microinvasive disease.

9.4.5 Complications

Patients reported experiences ranging from mild discomfort to severe pain during the time of irradiation, in some cases requiring more analgesia than had been anticipated. Significant discomfort was experienced by 8 of the 18 patients (44%). Although it was improved by intravenous pethidine and topical lignocaine, it was generally not eliminated - even in the few cases where a local anaesthetic block was administered.

Nausea and vomiting was experienced by six patients. The nausea was often an early feature presenting during the period of drug administration, whilst vomiting started several hours following ALA ingestion and resolved by 24 hours. Only one patient had cutaneous photosensitivity lasting beyond 24 hours, but this resolved by 48 hours.

No changes were noted in the haematological indices, although in 9 cases liver enzymes were elevated compared with baseline values obtained prior to ALA ingestion. The enzyme most commonly affected was aspartate transaminase (AST). The AST level rose above the normal range in only 5 cases, (maximum 3.5 times the upper limit of normal), with bilirubin elevated in only two (Figure 9.2). Monitoring of liver function was only continued more than 2 days after ALA ingestion in patients with elevated AST levels. In all cases this elevation was asymptomatic and returned to normal levels within 10 days, with the exception of one patient with a history of excessive alcohol intake, where liver enzymes were elevated for 30 days.

No correlation was seen between those patients with transient elevation in liver enzymes and the occurrence of nausea and vomiting. The delayed onset of vomiting suggests that the cause is unlikely to be direct stimulation of the gastric mucosa and may be the result of central stimulation (Dr A. Gorchein-personal communication). One patient developed a pruritic rash a week after treatment, which on biopsy was diagnosed as a cutaneous lichenoid reaction. This gradually resolved uneventfully over the following three weeks.

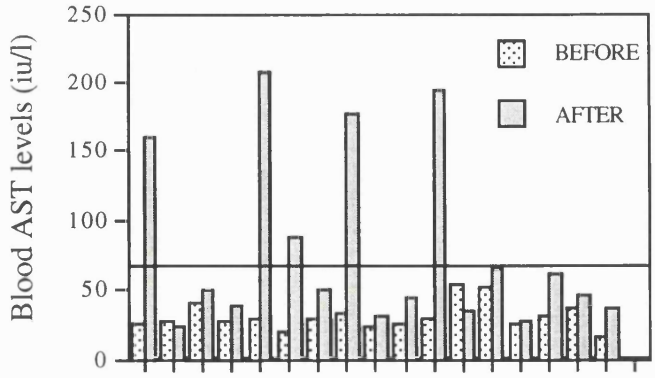


Figure (9.2): Aspartate Transaminase (AST) levels before and After ALA administration (60mg/kg). The values reached after ALA administration illustrates the highest levels reached. Horizontal line denotes upper limits of normal AST level.

9.4.6 Fluorescence microscopy

All biopsies taken following ALA administration, but prior to laser irradiation, revealed maximum fluorescence in the epithelial layer (Figure 9.3 a-d). In histological sections containing normal and abnormal tissue, no difference could be detected in the fluorescence between normal and abnormal areas of epithelium. The fluorescence was, however, higher in these areas than in the underlying subcutaneous tissue with a ratio of approximately 2-3:1. This correlated well with the histological results which showed that the PDT effect was essentially limited to the epithelium.

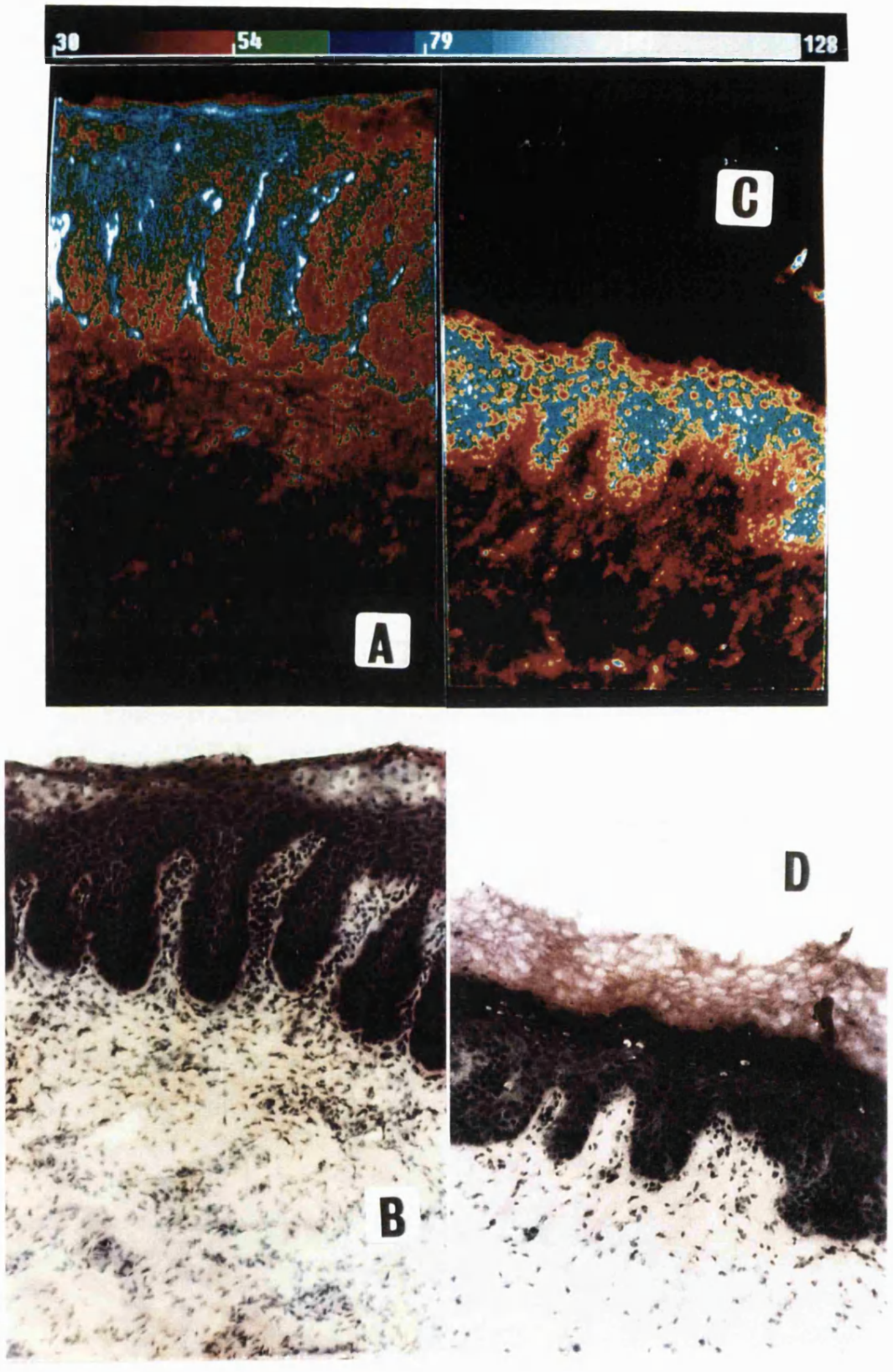


Figure 9.3: CCD picture immediately before PDT showing accumulation of fluorescence in the epithelium (A and C) and corresponding H&E (B&D).

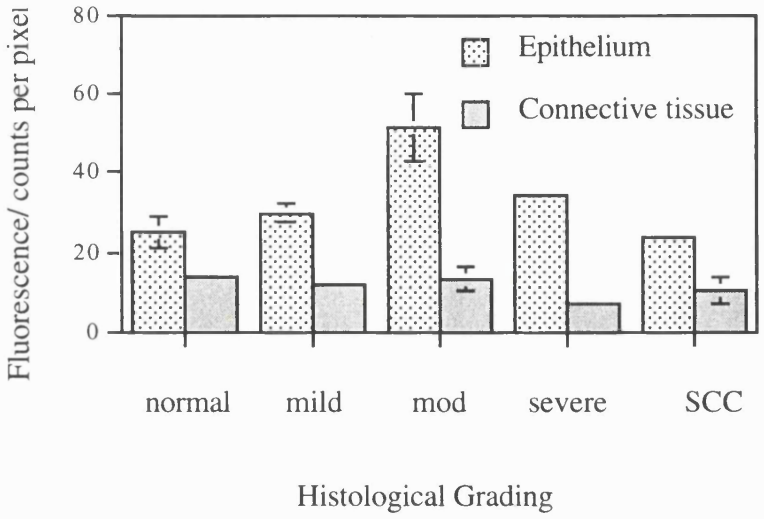


Figure 9.4 : Results of fluorescence microscopy following sensitisation with systemic ALA at 60 mg/kg. All biopsies taken immediately before irradiation

Figure 9.4 shows the combined results (mean ±SEM) following analysis of 71 fluorescence images obtained from 17 patients. A comparison of the fluorescence observed by quantitative microfluorimetric imaging, after ALA sensitisation, to chemical extraction with HPLC confirm that PpIX is the predominant porphyrin which accumulates in both normal and abnormal tissues (Loh *et al.*, 1993b). The histological grading determined was that of the area which correlated to a specific fluorescence image and not to the whole biopsy specimen. There were between 4-43 images in each group, normal (13), mild (45), moderate (6), severe (3) and SCC (4). The biopsy specimens were taken immediately prior to laser irradiation. The results suggest that the fluorescence intensity is higher for moderate dysplastic changes than for normal or mildly dysplastic specimens. The relative fluorescence intensity of the areas showing severe dysplastic changes or frank carcinoma did not demonstrate significantly higher fluorescence when compared to normal or mild atypia. However, the numbers of patients (and therefore samples), with these changes were much smaller than the number of samples showing normal or dysplastic changes. It is likely that the higher relative fluorescence seen with the moderately dysplastic lesion is the result of having too small a number of samples. This would be consistent with the observation that there was no significant demarcation in fluorescence intensity between normal and abnormal tissue when both were on the

same image. However, further study would be required to confirm this. Ideally the comparison of different grades of disease should be assessed in the same patient to avoid inter-patient variability. Nevertheless the relative fluorescence intensity of the underlying connective tissue was found to be constant irrespective of the extent of epithelial atypia and always much lower than seen in the overlying epithelium. Even within the epithelium there was variation in the level of relative fluorescence intensity, with higher levels seen in the more actively proliferating basal cells.

9.4.7 Histology

The biopsies taken during the ulcerated stages demonstrate the characteristic epithelial necrosis and replacement by an inflamed fibrinopurulent slough. The whole of the epithelium was extensively transformed by granulation tissue and densely infiltrated by acute and chronic inflammatory cells. Prominent within the infiltrate were eosinophils. At the histological level no significant vascular damage was observed with patent venules evident (Figure 9.5).

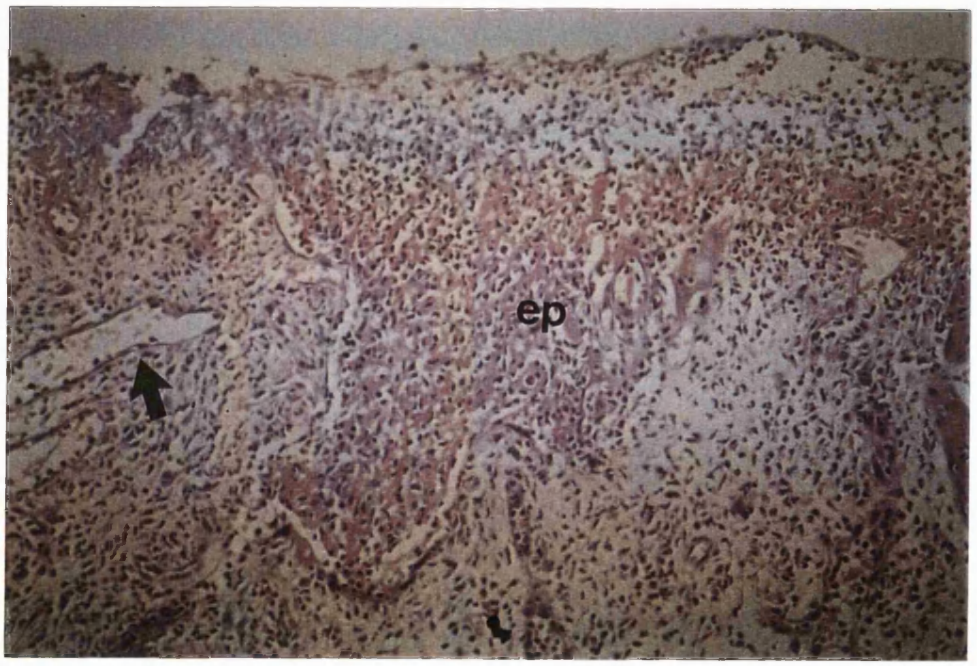


Figure 9.5: Necrotic epithelium, after ALA PDT, showing patent venules (arrow).

9.5 DISCUSSION

PDT using ALA has considerable attractions over treatment using first generation, sensitisers for treating premalignant and early malignant lesions of the mouth. The significantly shorter cutaneous photosensitivity makes this drug eminently more acceptable to patients and clinicians alike.

9.5.1 Depth of Damage

In the present study using ALA, the depths of necrosis (0.1-1.3mm) and of inflammation (0.8-6mm) were quite variable and considerably smaller than those seen with Photofrin®. Grant et al (1992) measured the depth of PDT necrosis in a series of 11 patients with malignant and premalignant disease of the oral cavity (Grant *et al.*, 1992) and showed that, despite using identical treatment parameters, (2mg/kg Photofrin® and 50J/cm^2 red light 48 hours later), the depth of microvascular damage and inflammatory response ranged from 4-12mm.

The depth of necrosis was often difficult to measure accurately as the time from PDT to biopsy varied. In most cases, some sloughing of necrosed tissue had taken place and a few lesions were partly healed at the time of biopsy. Nevertheless, two important conclusions could be reached. Firstly there was complete epithelial necrosis in every case and secondly no necrosis was seen in muscle.

9.5.2 Selectivity

The other important observation was that the area of necrosis always corresponded to the area exposed to the light, whether that area was normal or abnormal (ulceration was sometimes also noticed in sites distant from the lesion, which was thought to be due to scattering or reflection of light from the primary target site). Thus there was no selectivity of necrosis between abnormal and normal epithelium. In contrast there was selectivity of necrosis between epithelium and the underlying muscle. This is consistent with our findings on fluorescence microscopy which showed the same level of active PpIX in

normal and the adjacent abnormal epithelium, but much lower levels in the underlying connective tissue and muscle.

9.5.3 Clinical Response

Reviewing all the results in the present series, the patients with dysplasia clearly did better than those with carcinomas. All 12 patients with dysplasia showed improvement after PDT. Ten of the patients (83%) showed clinically (macroscopically) normal mucosa when healing was complete, although of the 9 that were biopsied at this time one showed moderate and four showed mild dysplasia histologically. It was of some concern that persistent dysplasia was seen in lesions that macroscopically had reverted to normal, although the changes in these areas were never more than moderate dysplasia. Even patients in whom the biopsy is normal after PDT cannot be regarded as cured, but the risk of malignancy should have been reduced and they can be given PDT with ALA again if necessary. One of our patients with moderate dysplasia had a normal biopsy after PDT but subsequently converted to moderate dysplasia again on check biopsy a year later. This is not surprising as one is dealing with a field cancerization process and because premalignant lesions of the mouth have a high recurrence rate after all forms of treatment (Hong *et al.*, 1995; Shklar, 1981). Nevertheless, the risks are lower with milder degrees of dysplasia, and oral leukoplakia associated with mild dysplasia has only a 3% risk of malignant transformation (Maerker & Burkhardt, 1978). PDT seems to down regulate the severity of dysplasia in these patients and, as it can be repeated if necessary, it is reasonable to hope that their disease can be kept under control for many years with minimal morbidity.

Five of the six patients with SCC had some benefit following ALA PDT, though the response was not as promising as with the more superficial dysplastic lesions. Only three of the patients became clinically free of tumour at the treatment site. This was confirmed histologically in two patients, but the third patient developed neck nodes shortly after PDT and the treated site did not have a repeat biopsy.

9.5.4 Optimisation of ALA PDT

The maximum depth of necrosis seen in tumours was 1.3mm, comparable to the results found by Regula et al in gastrointestinal tumours (Regula *et al.*, 1995). The implications of this limited depth of necrosis were highlighted by case 17 in whom the PDT cleared the areas of severe dysplasia, but revealed the part of the lesion where an invasive carcinoma had developed. Clinically this was valuable, as it meant that the area requiring further treatment with a different photosensitiser was much smaller than the original lesion. However it would have been be preferable if the PDT with ALA could have produced a greater depth of necrosis.

9.5.5 Intravenous Preparation and Larger ALA Dose

There are various ways in which optimisation of ALA PDT might be achieved. Lofgren et al (Lofgren *et al.*, 1995) found necrosis up to 12mm deep in a rabbit papilloma model and Regula et al (Regula *et al.*, 1995) found 8mm of damage in a tumour transplanted into the hamster pancreas. The main difference between these experiments and our clinical studies was the dose of ALA (50-200mg/kg given intravenously for the papilloma model and up to 400mg/kg orally, equivalent to 200mg/kg IV, for the pancreas work (Loh *et al.*, 1993a). The maximum dose that patients can tolerate orally is 60mg/kg (Regula *et al.*, 1995) which is equivalent to 30mg/kg IV (Loh *et al.*, 1993a). Recent work by Messmann et al (Messmann *et al,* 1995) in normal rat colon showed that it was difficult to obtain any PDT effect with 25mg/kg ALA IV (equivalent to 50mg/kg orally). With 50mg/kg IV or above, the PDT effect initially increased with increasing light dose and then reached a plateau at about 150 J/cm^2. It is difficult to extrapolate between rats and humans with regard to doses, particularly when discussing an agent that has to be metabolised to the active derivative in situ, but the doses are likely to be roughly comparable which suggests that the threshold dose for a PDT effect is between 50 and 100mg/kg by mouth. Thus the maximum dose of ALA tolerated by mouth (60mg/kg) may be only just above the threshold dose for producing a PDT effect. This strongly suggests that it would be worth preparing a formulation of ALA that can be given intravenously. Not only would this halve the dose required to achieve specific tissue levels, but also the first pass metabolism

in the liver which leads to increased transaminase levels associated with oral administration (Regula *et al.*, 1995) would be avoided and the maximum tolerable dose may be higher. If this led to higher tissue levels of PpIX, a greater depth of necrosis might be possible. Another possible way of increasing tissue levels of PpIX is to pretreat with iron chelators, which slow down the conversion of PpIX to haem (Chang *et al.*, 1994).

9.5.6 Modification of Irradiation Regimen

The depth of PDT necrosis may also be enhanced by fractionating the light dose (Foster *et al.*, 1991; Gibson *et al.*, 1990a; Hua *et al.*, 1995; Messmann *et al.*, 1995; van der Veen *et al.*, 1994). There are two ways in which this can be done, depending on the duration of the break between fractions. van der Veen et al (1994) showed in a rat mammary carcinoma model, that PDT damage was increased after two fractions of 100J/ cm^2 with a recovery period of 75 minutes between irradiations, compared with a single treatment of 100J/cm^2. They suggested that the break permitted more PpIX to be synthesised from ALA, but this work has the problem that the total light dose was greater when two fractions were used, and this may also explain the enhanced effect. Messmann et al used a much shorter break (Messmann *et al,* 1995) and suggested that the break permitted reoxygenation of the tissue so when the light was applied for the second fraction, more oxygen was available to provide the PDT effect. The time at which the break was made markedly influenced the effect (greater effect with an earlier break), but there was no advantage in having more than one break. It was not clear from this work whether fractionating the light increased the maximum area of necrosis achievable or just made it possible to achieve the same area of necrosis with a smaller total light dose. Another option is to reduce the fluence rate, which Gibson (1990) and Hua (1995) showed could increase tumour doubling time in a rat mammary carcinoma model (Gibson *et al.*, 1990a; Hua *et al.*, 1995), but this would increase already long treatment times. Hua (1995) was able to produce ALA PDT damage comparable to Photofrin® damage in the same model by administering the ALA drug dose in 2 equal portions with a 90 minute time interval (total dose 600mg/kg) with a 30s on and 30s off "pulsed" light delivery.

The light fractionation regimens used in the present study were chosen on the basis of these two possible mechanisms (resynthesis and reoxygenation) for enhancing the PDT effect after the work of Grant *et al* (Grant, Hopper, MacRobert 1993) and Regula *et al* (Regula, MacRobert, Gorchein 1995) showed such superficial necrosis. The number of cases treated was small but it is clear that there were no major differences between our results with either the short 5 minute break or the longer 1.5 hour break and the previous studies in which light was delivered without a break.

9.6 SUMMARY

- Photodynamic therapy with ALA is capable of producing consistent epithelial
- All 12 patients with dysplasia showed improvement after PDT.
- Results on invasive cancers are less satisfactory, mainly because the PDT effect is too superficial with current treatment regimens. This may be improved by using different regimens with ALA or using other, more powerful photosensitising agents . A deeper tissue effect may be produced than with ALA, but the price to pay may be scarring in the underlying muscle.
- The convenience of the short cutaneous photosensitivity (1-2 days) with systemic ALA means that PDT can be repeated at short intervals if necessary.
- Transient changes in liver enzymes were seen in 28% of patients. All cases resolved uneventfully nevertheless ALA should be used with caution in patients with active liver disease.

Chapter 10

PHOTODYNAMIC THERAPY USING mTHPC FOR DISEASE IN THE ORAL CAVITY

10.1 INTRODUCTION

Second generation sensitisers have advantages over Photofrin® and ALA in having major absorption peaks at wavelengths greater than 650nm, with consequently deeper penetration of light into tissue.

The two previous chapters have explored photodynamic therapy using Photofrin® and ALA induced PpIX as a photosensitising agents. PDT with these two sensitisers has been shown to be a useful modality in the oral cavity. However neither conform to the ideal characteristics of a photosensitiser which would conform to the following criteria:

- a tumour sensitiser which is more active than HpD
- a stable, single compound,
- absorption at a longer wavelength in the red part of the spectrum leading to greater tissue penetration
- favourable pharmacokinetic properties with improved tumour selectivity (Berenbaum *et al.*, 1993);
- reduced cutaneous photosensitivity (Savary *et al.*, 1993).

These criteria led to the development of meta tetrahydroxyphenyl chlorin (mTHPC, Foscan® Scotia Pharmaceuticals, Guildford, UK) at Queen Mary and Westfield College and St Mary's Hospital Medical School, London (Bonnett, 1995).

Early clinical work on the tumour bed after resection of mesotheliomas showed that an intravenous dose of 0.3mg/kg body weight mTHPC, activated with red light at 650nm with an energy density of 10J/cm^2 produced 10mm depth of tumour necrosis (Ris *et al.*, 1991). Savary et al (1993) carried out mTHPC-PDT on a group of 12 patients, the majority of whom had early stage oesophageal and tracheobronchial tumours,. However two patients in this series had oral cancer. They observed promising results using an mTHPC dose of 0.15-0.3mg/kg with irradiation at 514 and 652nm, depending on the location of the tumour. Seven out of nine treatment sites evaluated at the 3 month stage were found to be tumour free. Both patients with early oral tumours showed no residual

disease at the three month stage. The use of this photosensitiser with a variety of tumours in the head and neck region has been reported in a group of 11 patients (Dilkes *et al.*, 1995) with encouraging results.

The aim of this study was to assess the efficacy of photodynamic therapy using mTHPC as photosensitising agent in a larger group of patients all with lesions in the oral cavity. A range of light doses (5-20J/cm^2) was used to evaluate the response of oral squamous cell carcinomas and dysplasia with standard drug doses.

10.2 MATERIALS AND METHODS

10.2.1 Case Selection

Twenty patients were included in this study with a mean age of 61 years (range 30-82 years). There were 16 males and four females. Ten of the patients had received previous treatment for the tumour or associated disease. Seven patients had already undergone surgery, two of whom had had removal of regional neck nodes. Four patients had received radiotherapy to the head and neck region prior to their PDT treatment. Three patients had undergone photodynamic therapy with either Photofrin® or 5-aminolaevulinic acid. Nineteen patients had histologically confirmed oral squamous cell carcinomas, in addition to areas of dysplasia in some, and only one had severe epithelial dysplasia alone. Twelve patients had single lesions and eight exhibited field change disease. The total number of lesions in the field change group was 16 (1-5 lesions per patient). Of the 28 lesions, there was one carcinoma in-situ, eight T1 carcinomas, ten T2 tumours, one T3 and six T4 tumours. In addition there were two isolated dysplastic lesions. The majority of the lesions treated were either T1 or T2 squamous cell carcinomas.

10.2.2 Photosensitiser

The photosensitising agent used was meta tetrahydroxyphenyl chlorin (mTHPC, Foscan® Scotia Pharmaceutical Plc. Guildford). The mTHPC was supplied as crystals

(92% w/w pure) which were dissolved in a solvent consisting of polyethylene glycol 400 (1.5g), water and ethanol BP (96%v/v) 1g, producing a solution with a concentration of 4mg mTHPC per ml. To ensure complete dissolution of the mTHPC, the powder/diluent mixture was vigorously shaken for two minutes.

The intravenous dose (0.15mg/ kg body weight) was administered over 5 minutes, through a 0.25 μm filter (Ministart, Sartorius GmbH. Model No SM16534) to remove any undissolved particles. this drug was given 72 to 96 hours prior to laser irradiation, with special care to avoid extravasation. Patients were kept in a dark room after receiving mTHPC and given verbal and written instructions on avoidance of bright light during the 2-3 weeks for which they were photosensitive. The majority of treatments were carried out on an in-patient basis, although in later cases, some were allowed home after drug administration.

10.2.3 Laser and light delivery

The light source used was a copper vapour laser pumping a dye laser containing Rhodamine 590 and 652 tuned to give red light at 652nm (Visiray, Hornsby, Australia). The laser was coupled to a 0.4 mm core diameter flexible fibre attached to a microlens (PDT Systems Inc. Santa Barbara, CA) to facilitate even light distribution throughout the treatment field. A customised fibre holder with an angled end was constructed to allow ease of positioning of the fibre. This enabled the difficult regions of the oral cavity to be irradiated with near perpendicular incidence. The power output of the microlens was measured with a calibrated integrating sphere immediately before and after the irradiation of each spot. The irradiance was kept below 250mW/cm^2, to avoid thermal effects, with typical irradiances in the range 50- 150mW/cm^2.

Treatments were carried out with the patient conscious in all except one case (patient 14), where a general anaesthetic was administered to improve access for a lesion on the soft palate. The treatment times were short, ranging from 113 to 480 seconds, and were generally well tolerated by the patients. The area to be treated was defined as the lesion

with a 1-2 cm margin of normal tissue. This area was marked with small dots of crystal violet blue dye to facilitate repositioning or resizing of the irradiated spot when these were made necessary by patient movement. The treatment times were calculated to give the chosen light dose (5-20J/cm^2) and when possible the whole lesion was irradiated using one spot. Frequently the site of the lesion did not allow for this and up to 10 different fibre positions were used.

10.2.4 Evaluation of PDT

Biopsies were taken (when tolerated by patients) on four separate occasions: initially at diagnosis, immediately prior to irradiation, during the ulcerated stage and after PDT-when healing was complete. The local anaesthetic applied prior to light activation was limited to the site of biopsy to avoid possible alteration in the PDT effect. All specimens, except those taken for fluorescence microscopy were directly fixed in 10% neutral buffered formalin and processed routinely in paraffin wax. Five micrometer sections were cut and stained with haematoxylin and eosin. The specimens taken for fluorescence microscopy were oriented on cork discs using OCT medium (Tissue Tek 11 embedding compound) and, frozen and stored in liquid nitrogen prior to study. Ten micrometer sections were prepared as previously described in Chapter 9 (section 9.3.5). The distribution of mTHPC in the tissue was quantified by fluorescence microscopy using an inverted microscope, with epifluorescence and phase contrast (Olympus IMT-2, Hamburg, Germany), attached to a high resolution cryogenically cooled charged couple device (CCD) camera model 1, Wright, Cambridge, UK). These same sections were then fixed in formalin and stained with haematoxylin and eosin for comparative light microscopy studies.

10.3 RESULTS

10.3.1 Clinical Course

Twenty-eight lesions were treated over 26 PDT treatment sessions. The (details are given in Tables 10.1-10.4.

Tumour Response- Early

Within a few hours of PDT the treated area exhibited erythema, oedema and sometimes even blister formation. This was followed by a phase characterised by vascular stasis and congestion leading to necrosis, the formation of a fibrinopurulent slough and ultimately ulceration. The ulceration typically took 5-8 weeks to heal with some scarring in 5 patients.

Tumour Response- Late

The follow up of the patients with no clinical disease after treatment ranged from 24-94 weeks with a mean of 63 weeks. In Tables 10.1-10.6 persistent disease (PD) signifies clinically apparent residual disease. A complete response (CR) indicates the disappearance of all disease clinically or on biopsy, determined by two observations not less than 4 weeks apart. In this series the minimum length of follow up with complete response lesions was 5 months. This was in a patient in whom a synchronous tumour was identified and subsequently managed with radiotherapy such that further follow up of the initial lesion could no longer be considered as solely the response to PDT.

Table 10.1 summarises the results for the twelve patients with isolated disease. Five J/cm^2 was successful in the treatment of the dysplasia and the T1 and T2 SCC's, whilst $20J/cm^2$ was adequate for the T3 lesion. In the T4 tumours local control was achieved in 1 of 3 treated with $5J/cm^2$ and 2 of 3 treated with $20J/cm^2$, but all later developed distant disease.

Table 10.2 summarises the results for the eight patients with field change in the oral cavity. The low light dose of $5J/cm^2$ was adequate for the one dysplastic lesions, but only 2 of the 7 T1 and T2 SCC's treated with this low light dose were clear. Four of the other 5 had a second PDT treatment with a higher light dose but only one was cleared of the disease. One then underwent surgery and radiotherapy and the other two had a third

PDT treatment. To date, there is no disease in one patient but, the other still has persistent disease.

In the patients with field change disease the results were better with higher light doses. 10 J/cm^2 resulted in control of disease in the Tis lesion and in two of the five T1 tumours treated with this dose. Both T1 lesions treated with 20J/cm^2 were cleared when they had not responded to lower doses. Results with T2 lesions in these patients showed a similar trend. Two of three T2 lesions treated with 5J/cm^2 showed a complete response, but the third only cleared with 20J/cm^2. Only one of five T2 lesions was successfully treated with 10J/cm^2, the two failures treated with a higher dose were cleared. It appears that residual disease following PDT in field change patients requires a higher light dose to be used. Table 10.4 summaries the results in relation to the initial light doses used for tumours of each stage whilst Table 10.3 shows the results in relation to the light doses used for each stage of tumour. Table 10.5 illustrates the changes in the size of lesions requiring more than one PDT treatment and Table 10.6 summaries the final outcome for each lesion.

Table 10.1 & 10.2 Key

S:	surgery	**ALA:**	photodynamic therapy with 5-aminolaevulinic acid
RT:	radiotherapy	**PII:**	photodynamic therapy with Photofrin®
C:	chemotherapy	**NCD**	No clinical disease, but refused biopsy
Cryo:	cryotherapy	**NA:**	not appropriate, as persistent disease
mod:	moderate	**NR:**	No response
max:	maxillary	**FOM:**	floor of mouth

*	control of local disease, but neck nodes, subsequently died
2*	radical surgery, radiotherapy & chemotherapy.
3*	for surgery & radiotherapy
4*	scarring +++ no evidence of local recurrence 3 months after PDT, but evidence of cutaneous metastases, died 8 months after PDT.
5*	2nd primary Ca found on GI endoscopy. Radiotherapy for GI tumour.
6*	developed 2nd primary for radiotherapy
7*	planned surgery died of bronchopneumonia

Table 10.1: CLINICAL OUTCOME OF PATIENTS WITH ISOLATED LESIONS

Patients	Age/Sex	Site	TNM Stage	Previous Treatment	Light dose J/cm^2	Histology healed (weeks)	Size of lesion before/healed (cm^2)	Histology longest f/u (weeks)
(1)	73/M	tongue	T0/ dysplasia	nil	5	NCD	3.1/0	refuse Bx NCD (92)
(2)	53/M	tongue /FOM	T1	nil	5	hyperplasia (7)	24/0	mild dysplasia (94)
(3)	80/M	tongue	T2	nil	5	hyperkeratosis (12)	6/0	NCD (88)
(4)	56/M	palate	T2	nil	5	mod dysplasia (16)	12.6/0	no macroscopic change (74)
(5)	58/M	retromolar buccal mucosa	T2	nil	5	mild/mod dysplasia(4)	3.1/0	mild dysplasia (92)
(6)	51/M	tongue	T3	CO2 laser	20	NCD	4.7/0	moderate dysplasia(37)
(7)	80/F	mandible	T4	S, RT, ALA	5	NCD	12.2/0	no local disease*
(8)	49/M	tongue	T4	RT	5	persistent disease	9.4/9.4 debulking only area unchanged	Further PDT
(9)	60/M	alveolus	T4	nil	5	NR	3.9/3.9	surgery & RT^2*
(10)	42/M	tongue	T4	nil	20	hyperplasia (13)	1.3/0	recurrence at 26^3*
(11)	79/M	palate & max alveolus	T4	RT	20	NCD	32/0	no local disease^4*
(12)	82/F	alveolus	T4	nil	20	persistent disease	2.4/1.2	surgery (8)

Table 10.2 : CLINICAL OUTCOME OF PATIENTS WITH FIELD CHANGE DISEASE

Patients	Age/Sex	Site	Staging (TNM)	Previous Treatment	Light dose /cm^2	Histology healed (weeks)	Size of lesion before/healed(cm^2)	Histology /longest f/u (weeks)
(13)	30/M	tongue	T1	S	5	persistent disease	1.5/0.1	for surgery & RT(12)
(14) [5]*	54/M a)	L tongue	T2	nil	5	hyperkeratosis (26)	6.1/0	NCD (88)
	b)	L buccal mucosa	T2	nil	5	normal (26)	12.6/0	NCD(88)
	c)	soft palate	T2	nil	5		1.2/0.6	
					10	persistent disease	0.6/0.3	
					20	NCD	2.4/0	NCD (21)
	d)	R buccal mucosa	T2	nil	10	persistent disease	12.6/ 7.1	PR with 1st PDT
					10	negligible ulceration	7.1/7.1	NR with 2nd PDT
	e)	R. mand alveolus	T1	nil	5	persistent disease	3.1/1.2	NA
					10	negligible ulceration	1.2/1.2	NA
(15)	61/F a)	buccal mucosa	T2	S	10	persistent disease	4.9/0.8	
					20	NCD	0.8/0	NCD (35)
	b)	L alveolus	T2	S	10	NCD	2.0/0	NCD (44)
	c)	palate	T1	S	10	persistent disease	1.2/0.03	
					20	NCD	0.03/0	NCD(35)
(16)	59/M a)	Buccal mucosa/ alveolus	dysplasia	S	5	NCD	24.3/0	NCD (89)
	b)	alveolus	T1	S	10	hyperplasia(15)	0.3/0	mild dysplasia (64)
(17)	65/F a)	palate	T1	S, C	5	persistent disease	6.2/3.1	
				PII/ALA	20	NCD	3.1/0	NCD(52)
	b)	alveolus	T1	S, C	5	persistent disease	2/1.3	
				PII/ALA	10	persistent disease	1.3/0.6	
					15	persistent disease	0.8/0.3	for further PDT
(18)	60/M	(L)FOM	Tis	S	10	fibrosed oral mucosa(8)	9.6/0	NCD (24)[6]*
(19)	64/M	tongue	T1	nil	10	mild dysplasia (32)	1.1/0	no change(84)
(20)	59/M	retromolar	T2	S, cryo, RT	10	persistent disease	1.17/0.8	data not available lost to follow up[7]*

Table 10.3
Lesion response in relations to delivered energy

Light Dose	Patient Group	Dysplasia	Tis	T1	T2	T3	T4
5J/cm^2	Isolated disease	1 CR		1 CR	3 CR		1CR 1PD 1NR
5J/cm^2	Field change	1 CR		4 PD	2 CR 1 PD		
10J/cm^2	Field change		1CR	2 CR 2 PD 1 NR	1 CR 4 PD 1 NR		
15J/cm^2	Field change			1 PD			
20J/cm^2	Isolated disease					1 CR	2 CR 1PD
20J/cm^2	Field change			2 CR	2 CR		

Note: response is related to initial pathology, Not all lesions healed with normal mucosa as some had residual mild or moderate dysplasia as shown in Tables 1 & 2

Twenty-one lesions received one treatment; 5 lesions received two treatment; 2 lesions received 3 PDT treatment

CR: complete local response PD: Persistent disease NR: No response

Table 10.4
Response of lesions after a single treatment with PDT

Light Dose	Patient Group	Dysplasia	Tis	T1	T2	T3	T4
5J/cm^2	Isolated disease	1 CR		1 CR	3 CR		1CR 1PD 1NR
5J/cm^2	Field change	1 CR		4 PD	2 CR 1 PD		
10J/cm^2	Field change		1CR	2 CR 1 PD	1 CR 3 PD		
15J/cm^2	Field change						
20J/cm^2	Isolated disease					1 CR	2 CR 1PD
20J/cm^2	Field change						

Note: response is related to initial pathology, Not all lesions healed with normal mucosa.

Some had residual mild or moderate dysplasia as shown in Tables 1 & 2

CR: complete local response PD: Persistent disease NR: No response

Table 10.5: Lesions treated more than once with PDT- all were in patients with field change disease. Four of 7 lesions failing initial PDT were cleared by repeating PDT with a higher light dose.

Patient and lesion reference	Stage	Light dose 1st PDT (J/cm^2)	Size before/after (cm^2)	Light dose 2nd PDT (J/cm^2)	Size before/after (cm^2)	Light dose 3rd PDT (J/cm^2)	Size before/after (cm^2)
14 (e)	T1	5	3.1/1.2	10	1.2/1.2		
15 (c)	T1	10	1.2/0.03	20	0.03/0		
17 (a)	T1	5	6.2/3.1	20	3.1/0		
17 (b)	T1	5	2/1.3	10	1.3/0.6	15	0.8/0.3
14 (c)	T2	5	1.2/0.6	10	0.6/0.3	20	2.4/0
14 (d)	T2	10	12.6/7.1	10	7.1/7.1		
15 (a)	T2	10	4.9/0.8	20	0.8/0		

Table 10.6: Final Result (after up to 3 PDTs) for each lesion treated

Stage of Disease		CR Free of disease	CR Disease elsewhere	PD Alternative therapy undertaken or planned
Dysplasia	Isolated	1	-	-
Dysplasia	Field change	1	-	-
Tis	Field change	1	-	-
T1	Isolated	1	-	-
T1	Field change	3	2	2
T2	Isolated	3	-	-
T2	Field change	2	3	2
T3	Isolated	1	-	-
T4	Isolated	1	2	3

CR: Complete response

PD: Persistent disease

10.4 Complications

10.4.1 Pain Control

All except 2 patients reported discomfort at the injection site at the time of intravenous administration of mTHPC but this settled within minutes in all cases. Laser irradiation was well tolerated in most patients with, mild discomfort being the most commonly described sensation. Three experienced more significant pain. Analgesia was required by all patients in the early ulcerative phase which started, starting 1-2 days post PDT. Oral opiates had to be prescribed for 1-2 weeks for most patients.

10.4.2 Scarring

Scarring was seen in 5 patients, (patients: 2,3,5,11,18). This generally improved with time (Figure 10.1) although two patients developed complications (patients: 11, 18). One had a very large T4 tumour on the palate ($32cm^2$) irradiated with $20J/cm^2$ which resulted in ulceration and necrosis of the normal oral tissue remote from the target lesion due to reflected and scattered light within the mouth. This was avoided in subsequent cases by shielding the normal tissues (Figure 10.2). The other problem was significant tethering of the tongue after treatment of a carcinoma in situ in the floor of the mouth adjacent to a skin graft placed following resection of a previous tumour.

10.4.3 Cutaneous Photosensitivity

Five patients experienced mild cutaneous photosensitivity due to inadvertent exposure to sunlight within the first 2 weeks post sensitisation, but all recovered without long-term effects. One patient experienced pain behind the eyes two weeks post sensitisation, following sudden exposure to bright light without eye protection, but this rapidly settled when the eyes were covered. There were no changes in either haematological or biochemical indices following administration of mTHPC.

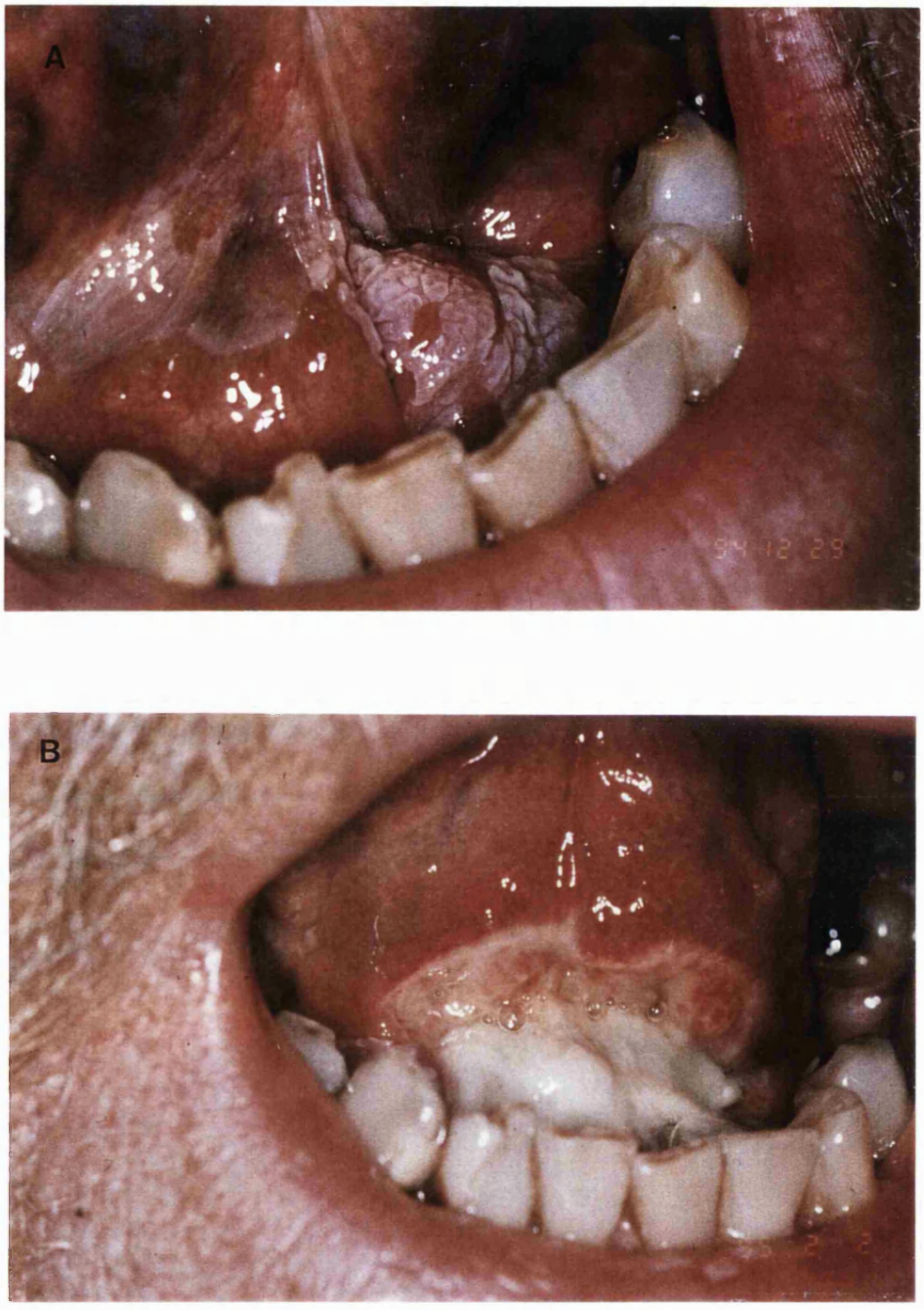


Figure 10.1: (A) Patient 2 before PDT with mTHPC. (B) 13 days after PDT, showing ulceration with the presence of a thick fibrinopurulent slough of the entire irradiated site.

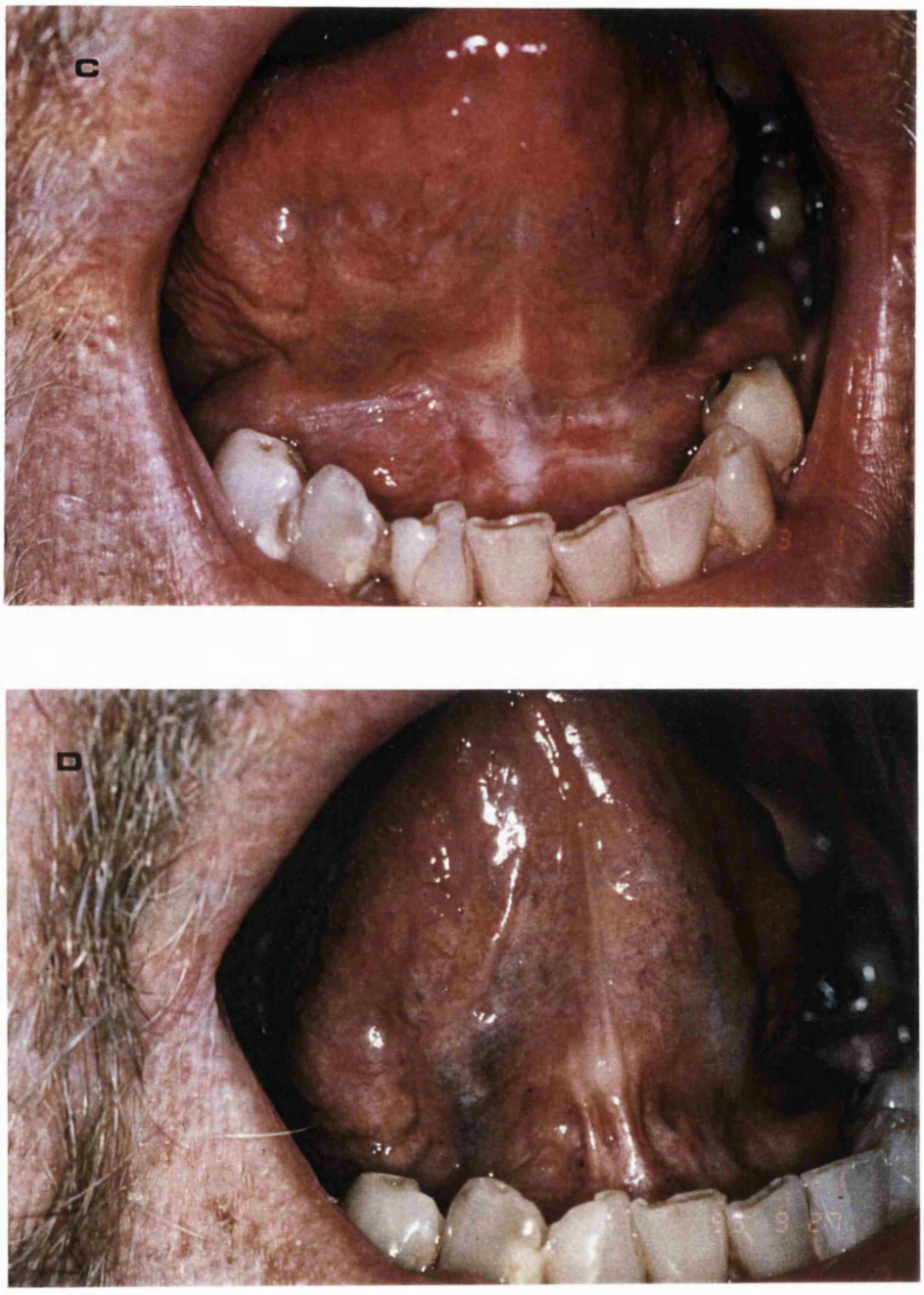


Figure 10.1: (C) Patient 2, seven weeks after PDT with complete healing of the treatment site. Scarring is present (hyperplasia was seen on post treatment biopsy). (D) 8 months after PDT showing disappearance of the scarring at treatment site. At 23 months after treatment, the patient is still free of disease clinically, though mild dysplasia was seen on histology.

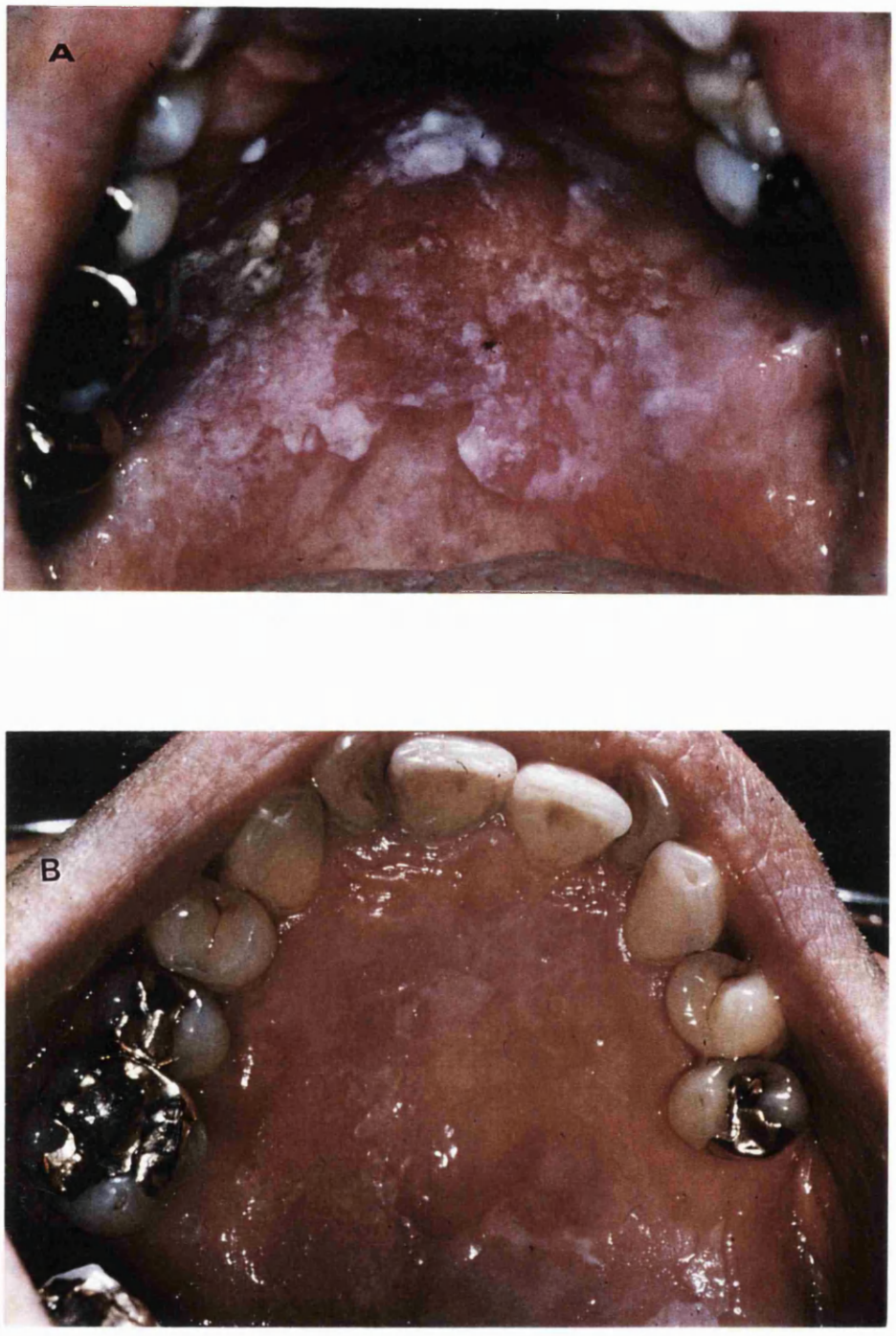


Figure 10.2: (A) Patient 4, with disease involving the entire palate. (B) 2 months after PDT. The patient was free of disease, but moderate dysplasia was present on biopsy.

10.4.4 Pathological Changes

Biopsies taken during the ulcerated stage show characteristic histopathological changes. In the early stages (3-7 days) lesions were characterised by massive acute inflammation, vascular damage and oedema (Figure 10.3 and 10.4). This was associated with ulceration of the overlying epithelium and necrosis which frequently extended to the submucosa and occasionally into muscle. The mucosa was replaced by a dense oedematous fibrinous purulent slough, with a massive exocytosis of neutrophils and occasional eosinophils. Small blood vessels, arterioles and venules showed loss of endothelial cells and prominent pavementation of polymorphonuclear leukocytes. The acute inflammatory infiltrate and oedema extended deep into the underlying submucosa and muscle when present. There was prominent separation of muscle fibres by inflammatory cells and oedema fluid with scattered infiltrates of eosinophils.

In later biopsies at 10-12 days there was still evidence of widespread ulceration and mucosal necrosis with acute inflammatory cells, although infiltrates of lymphocytes and macrophages were surprisingly few at this stage. At the margins of the ulcerated area, growth of new epithelium was frequently seen below the slough. Deep below the mucosa, and often within muscle, there was deposition of immature granulation tissue with proliferating endothelial cells, fibroblasts and chronic inflammatory cells. This process of organisation suggest that the end result would be scarring, which was consistent with the clinical findings and was seen in specimens taken after healing was complete (Figure 10.5).

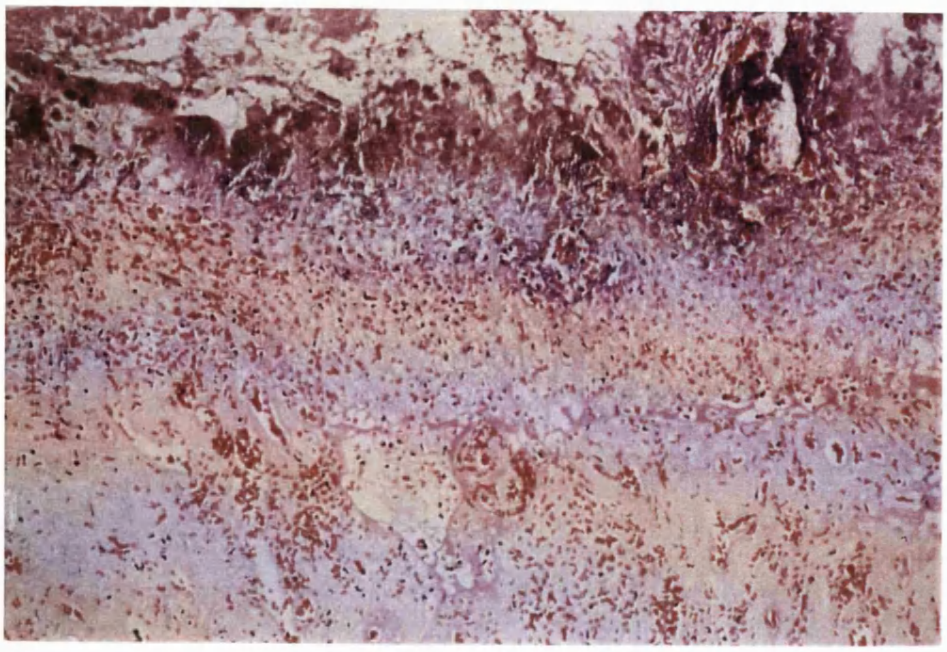


Figure 10.3: Characteristic findings three days after PDT. The epithelium is completely replaced by necrotic ulcer slough and the connective tissue is oedematous and shows inflammation and haemorrhage.

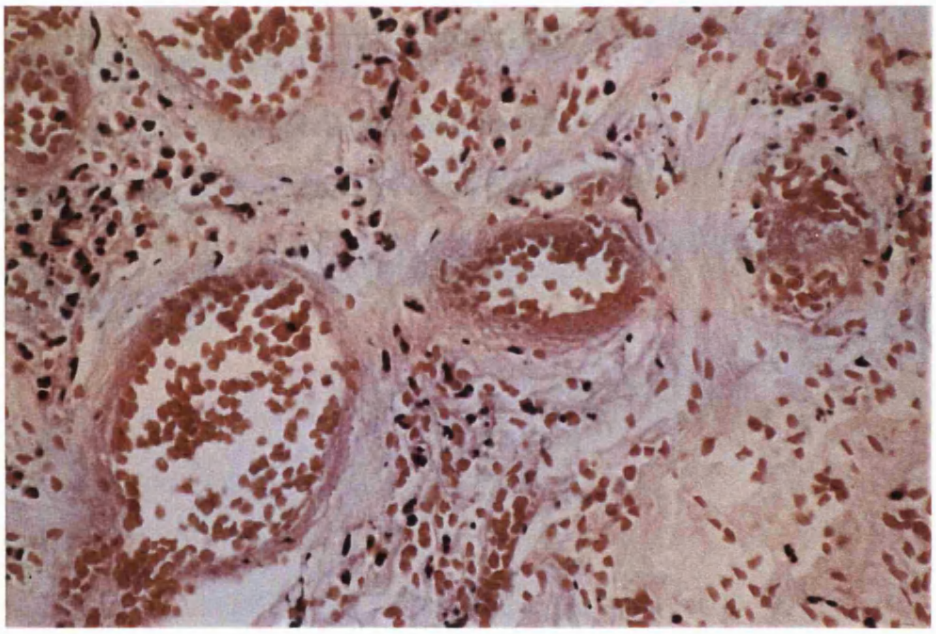


Figure 10.4: Characteristic changes in blood vessels three days after PDT. There is extensive vascular damage with loss of endothelial cells and resulting oedema, extravasation of red blood cells and inflammation. Thrombus formation is prominent.

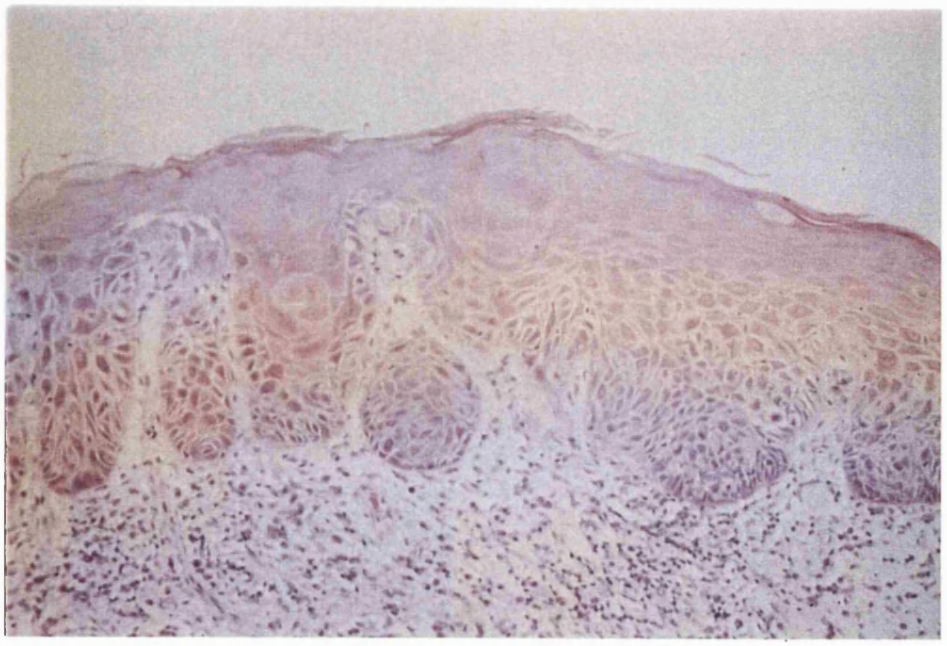


Figure 10.5: (A) Biopsy showing severe epithelial dysplasia with evidence of microinvasion before PDT.

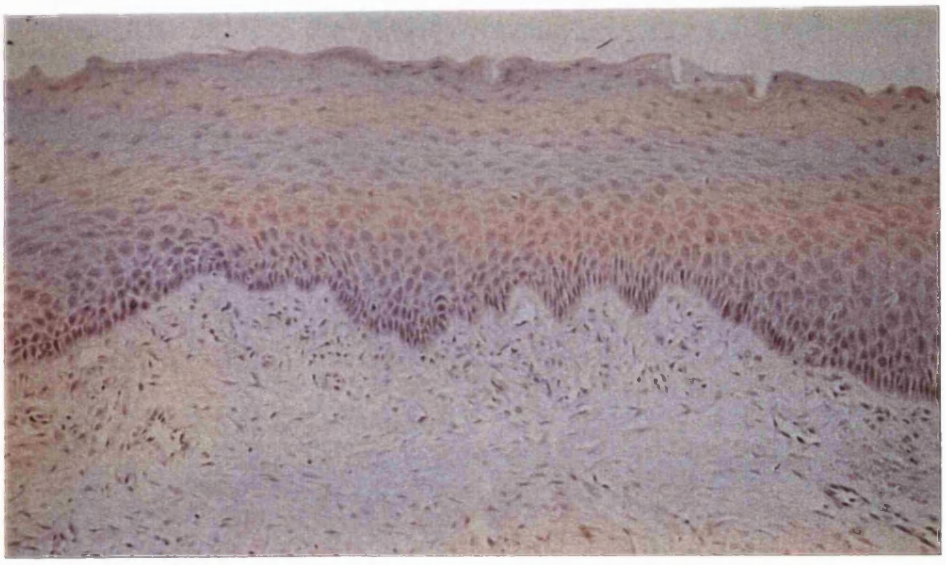


Figure 10.5: (B) Biopsy of the same site 3 months after PDT showing healing with well ordered epithelium overlying fibrous scar tissue.

10.4.5 Fluorescence Microscopy

The fluorescence images obtained by quantitative fluorescence microscopy analysis, showed slightly higher levels of mTHPC in the connective tissue relative to the tumour or epithelium immediately before PDT (Figure 10.6). Nine biopsy samples were obtained. The tumour/epithelium to underlying connective tissue ratios are 1:2 (range 1-3) see appendix. No correlation was observed between the fluorescence signal obtained by fluorescence microscopy and tumour staging in contrast to that observed by fluorescence spectroscopy (Braichotte *et al.*, 1995a) or the outcome of the PDT treatment.

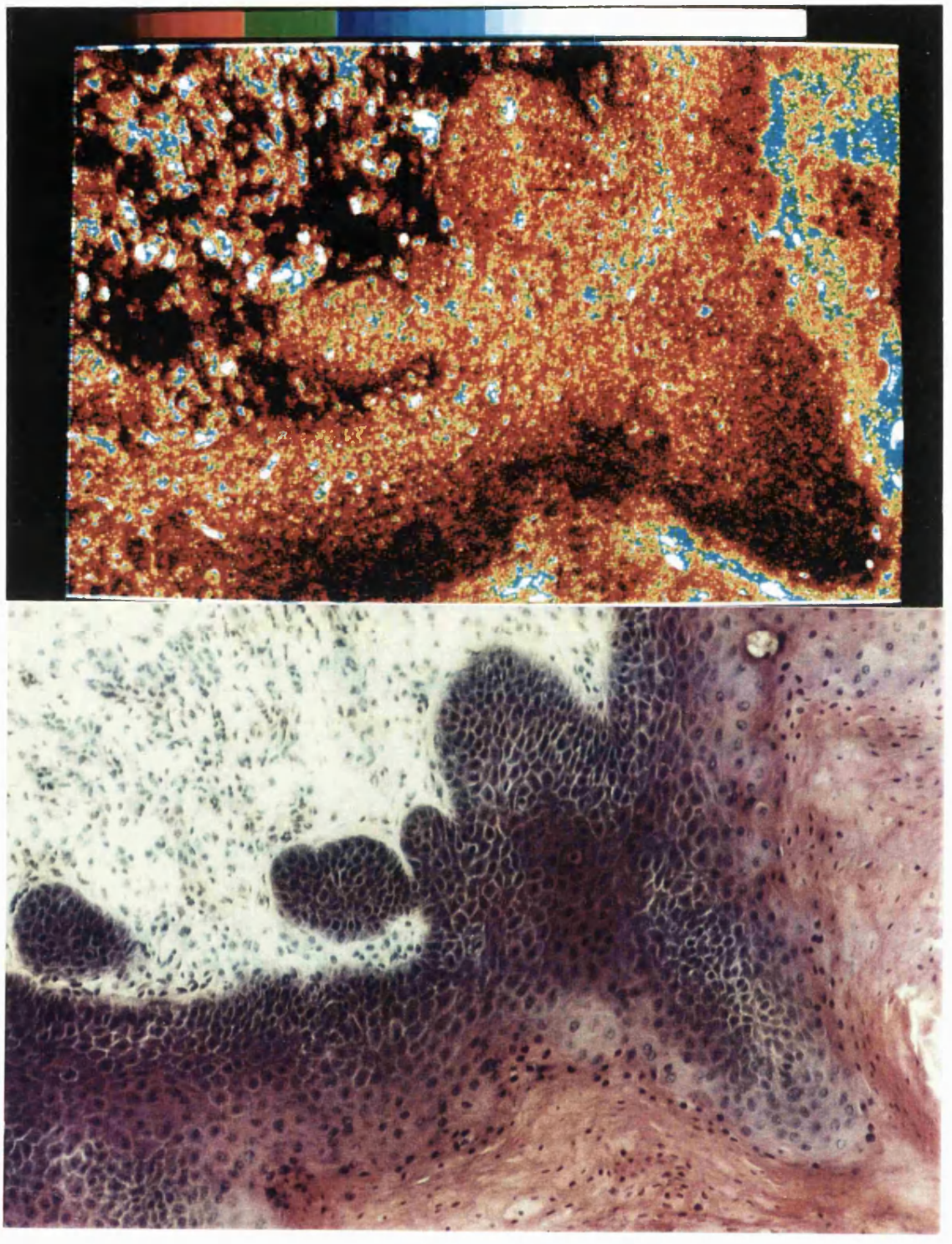


Figure 10.6: CCD image 4 days after sensitisation with mTHPC (immediately before PDT) and corresponding H&E.

10.5 DISCUSSION

These results show that photodynamic therapy using mTHPC is a promising treatment for patients with oral cancer which is not only well tolerated but also simple and quick to execute. Though some scarring may occur the healing is generally better than with other forms of local tissue destruction and so there are less functional changes. These factors need to be balanced against the draw back of cutaneous photosensitivity, which can last up to 6 weeks but usually clears in about 3 weeks (Savary *et al.*, 1993).

10.5.1 Depth of PDT Damage

Fundamental to the success of photodynamic therapy is that the depth of PDT necrosis must be greater than the depth of each individual tumour. A concern is that surface irradiation of deeper tumours may leave residual viable tumour, even if the surface heals with regeneration of normal mucosa, as the red light used to activate the photochemical reaction only penetrates a few millimetre. The depth of light penetration and hence sensitiser activation is important whether the mechanism of cell kill is direct cellular cytotoxicity or destruction of the tumour vasculature. From the histopathology, vascular damage plays an important role in the patients described in this study (Figure 10.4). There are a number of ways in which the depth of tumour damage may be enhanced. Interstitial PDT may improve results in the T4 tumours, as the fibre can be implanted deep into the lesion and subsequently withdrawn during treatment thus enabling the whole depth of the cancer to be uniformly irradiated. The extent of PDT damage has also been shown in preclinical studies to be enhanced either by fractionating the light dose (Messmann *et al.*, 1995; Mlkvy *et al.*, 1996; van der Veen *et al.*, 1994) or reducing the power density (Gibson *et al.*, 1990a). Since irradiation time with mTHPC is short, reducing the power density or the inclusion of dark periods would not elongate the treatment unacceptably.

10.5.2 PDT of Oral Dysplasia

Although PDT with mTHPC was successful in the treatment of the oral dysplasias, the use of a shorter acting photosensitising agent such as ALA may adequately eradicate this superficial disease as shown in previous studies (Chapter 9). ALA has the advantage of only 1-2 days of cutaneous photosensitivity, although it may cause elevations in liver enzymes and its depth of effect is probably inadequate for treating invasive cancers (Grant *et al.*, 1993a; Regula *et al.*, 1995).

10.5.3 Response in field change disease versus single tumours

Our results showed a better response in those with isolated lesions than in those with field change disease (Table 10.5). With isolated disease all lesions up to stage T3 could be cleared in one treatment whereas with field change disease we could only clear 10 of 14 T1 and T2 lesions. Even so 4 of these required more than one treatment, and most required higher light doses than the isolated lesions.

10.5.4 Light Dose Relative to Response

From our results a very low light dose, 5J/cm^2, is capable of eliminating some cancers in the oral cavity (50%, 7/14) Table 10.3. However inadequate responses were noted in 7 lesions. The overall success with 10J/cm^2 was 36%. In one case when the same lesion was irradiated at a second PDT treatment with the same light dose no response was seen. This would suggest that the residual disease was deeper than had been thought or the problem may have been a feature of the optical properties of the tumour. The number of lesions treated with the higher light dose, 20J/cm^2, was smaller but 7 of the 8 lesions showed no disease following the PDT.

On analysis of the patient response it was found that 75% (6/8) of the isolated lesions had a CR with 5J/cm^2 whilst only 38% (3/8) of lesions in field change patients showed no evidence of disease following PDT with this dose (one of these was a dysplasia). The reason why isolated lesions fail to respond in patients with coexistent field change disease is not clear, especially since these patients tended to have a lower stage (4 T1 and T2)

than patients with isolated lesions (3 T4). Overall 75% (9/12) of the isolated lesions showed no clinical disease following a single PDT treatment (5-20J/cm^2) Table 10.4. In the field change group 75% eventually showed no clinical disease but 7 lesions received more than one treatment, with control of disease in only four Tables 10.5 and 10.6. The response after 1 treatment was 50%. As patients with isolated lesions generally had more advanced tumours, malignancy in field change disease may impart a certain resistance to PDT.

10.5.5 Fluoresce Microscopy

The fluorescence distribution seen in the "immediately" pre-PDT biopsy specimens, under fluorescence microscopy showed slightly higher signal in the underlying connective tissue (tumour:connective tissue ratio 1:2). This localisation of mTHPC in the lamina propria was also observed by Savary et al in tumour samples (1993). The range is from unity up to three times the fluorescence in the corium immediately adjacent to lesion. This distribution is quite different to that seen with ALA, where the fluorescence is well localised to the epithelium (Fan *et al.*, 1996; Grant *et al.*, 1993a; Loh *et al.*, 1993b). In the limited numbers of specimens obtained, no relationship was observed between the stage of disease and the fluorescence or fluorescence ratio of tumour to connective tissue. In a group of 4 patients with oral SCC, Braichotte (Braichotte *et al*, 1995) observed a higher fluorescence ratio the more advanced the lesion, with the use of non-invasive fluorescence spectroscopy. This highlights the necessity the for differences in interpretation of fluorescence measured from individual cells and from the surface of tumours.

10.5.6 Partial Response Lesions

Six lesions persisted after PDT; two were T4 tumours and the most likely explanation is that adequate light doses did not reach all parts of the lesions. The other four were in three patients with field change disease. Three of these were exophytic white masses in patients with proliferative verrucous leukoplakia (PVL) (Zakrzewska *et al.*, 1996), a condition often quoted as being particularly difficult to treat with conventional modalities.

These lesions are often thicker than other SCC's and it is probable that the dense white fronds reflected more light, so a lower light dose than anticipated reached the base of each lesion. This can lead to PDT necrosis in other parts of the mouth if normal areas are not adequately shielded - as happened in case 11. In vivo monitoring of the absorbed light dose, perhaps by monitoring photobleaching at the surface of and within the tumour, may improve the dosimetry for these lesions and also allow for inter patient variability. Another approach would be the delivery of light by insertion of optical fibres directly into these masses which, may also help with bulkier tumours.

10.5.7 Non Selectivity

The non-selectivity with mTHPC-PDT has been also reported by Savary et al (1993), who reported 11 patients who received PDT to the healthy mucosa of the mouth and bronchi following sensitisation with either 0.3mg/kg or 0.15 mg/kg mTHPC. At 20 hours post sensitisation, irradiation with 6J/cm^2 at 652nm to the oral mucosa resulted in erythema and fibrin formation. In one patient irradiated at 218 hours petechial erythema was still observed at the treated site.

10.6 SUMMARY

- Photodynamic therapy using mTHPC has been shown to be well tolerated and simple with short treatment times.
- Shielding of normal tissue is required with this sensitiser.
- Isolated lesions up to T2 can be controlled with a light dose as low as 5J/cm^2.
- Patients with field change disease require more aggressive treatment.
- Repetition of mTHPC PDT treatment has been shown to be of value in the management of patients with field change disease as there is no cumulative toxicity.
- Scarring was observed in some patients but generally improved with time.
- Larger numbers of patients are required with longer follow up to allow full evaluation of mTHPC. A large multicentric trial has been set up to do this and is currently under way.

Chapter 11

SUMMARY AND FUTURE DIRECTION

11.1 PRECLINICAL EXPERIMENTS

This thesis has involved both preclinical and clinical studies to evaluate the potential and safety of photodynamic therapy (PDT) for treating premalignant and malignant disease of the mouth and nasopharynx. The preclinical section of this thesis consisted of three Chapters , 5, 6 and 7. The animal studies looked at the effect of tumouricidal PDT on normal tissues to see if there is any unacceptable damage, whilst the clinical studies have tried to identify which lesions are most suitable for treatment with each photosensitiser.

Chapter 5 consisted of two experiments, involving a total of 58 rabbits. The first part of the study was to evaluate the effect of PDT on normal rabbit mandibular bone. PDT with Photofrin®, ALA and mTHPC, was observed to cause no more than slight initial retardation in the rate of bone healing after tooth extraction. No damage to normal bone was seen. The second part of the study confirmed that the regimen used in the bone PDT section caused necrosis in soft tissues (mucosa and muscle) and enabled the determination of early differences in the histological response with each photosensitiser. An interesting pathological observation was the difference in the nature of the inflammatory response with the different photosensitisers. The reason for this is uncertain, but may be related to the difference in depths of damage with each Photosensitiser or the difference in the mechanism of damage. Vascular damage has been shown in both the Photofrin and mTHPC sections whilst direct cell kill is believed to be more important with ALA.

The resistance of bone to PDT is encouraging in view of the potential role of PDT in the treatment of tumours which have invaded or are adjacent to bone. Conventional therapies such as surgery would advocate the removal of substantial quantities of bone, whilst radiotherapy can led to bone necrosis. One of the limitations of this study was that the bony defect created by removal of the rabbit incisor tooth only produced a small opening exposed to the oral environment. It is possible that a more extensive area of exposed bone may heal differently and an appropriate model for this needs to be determined.

Supporting this difference is the finding of sequestration of bone in two patients who have recently undergone PDT treatment of extensive areas over the mandible and maxilla. Both treatment sites ultimately healed without further problems. Unfortunately, there was no opportunity to carry out comprehensive clinical evaluation of PDT in the treatment of tumours invading bone. In such studies, it would be essential to know the extent of tumour invasion and treat along potential pathways of tumour spread e.g. perineural invasion and along the periodontal ligament.

Chapters 6 and 7 investigated the effect of PDT on normal nasopharyngeal mucosa and the likelihood of PDT damage to normal brain resulting from light transmitted through the base of skull during irradiation in the nasopharynx. The first part of Chapter 6 was to determine the biodistribution of mTHPC and AlS2Pc in the nasopharyngeal (NP) tissues and brain of both the rat and rabbit. Subsequently, nasopharyngeal PDT was carried in the rabbit model. Both sensitisers were found to distribute to the nasopharyngeal mucosa following IV administration, but most significant was the lack of sensitiser accumulation in the brain. The nasopharyngeal PDT studies showed greater damage with AlS2Pc using one hour drug-light interval, relative to 24 hours, whilst the unpredictable response following 0.3 mg/kg mTHPC and 80J/cm^2 irradiation suggests this is close to the threshold dose. The absence of neurological deficit and brain necrosis, even in the excessively irradiated rabbits, was extremely encouraging.

Chapter 7 consists of two experimental set-ups. The first part was to determine the light transmitted through the base of skull during PDT in the nasopharynx, whilst the second part looked at light transmission through a known thickness of bone. The finding of only a 52% increase in light transmission adjacent to the brain following removal of the overlying bone (0.5-1.2mm thick) leaving only dura mater intact is pleasantly surprising. Although the potential of PDT damage to the brain is increased when there is tumour extension into the middle cranial fossa, if the dura is intact then the risk of damage to the brain still appears to be low. The reasons for this include the negligible accumulation of photosensitiser in the normal brain when the blood-brain barrier is intact, as well as the

protective role played by the dura mater. However, the additive influence of the cerebral spinal fluid, pia and arachnoid mater will further reduce the small amount of light reaching the brain. The potential of repetitive treatment without cumulative toxicity, as well as modification of irradiation regimen, may further reduce any likely PDT damage to the brain by remaining below the threshold level for brain necrosis. With the current knowledge of PDT in the upper aerodigestive tract, PDT treatment of early NPC would be very exciting. However in view of the high response rates to radiotherapy, it is more likely in the early stages of clinical evaluation, that this modality will be used in the treatment of recurrent or residual NPC after radiotherapy.

11.2 CLINICAL STUDIES

The purpose of Chapters 8, 9 and 10 was the clinical evaluation of three photosensitisers. A summary of the findings is given below.

11.2.1 Photofrin® PDT (22 patients in the study)

- At the early follow up stage (up to 3 months), a 77% complete response rate was observed in patients with predominantly T1 tumours. Further disease developed in 41% of the patients (over a 38 months period), but 55% of patients in this study presented with field change disease. The large percentage of patients with field cancerization is due to our special interest in this subgroup.
- Healing without scarring was seen in all patients except those who had received previous therapy other than PDT.
- The complex nature of patients with field cancerization was exemplified in this study and the encouraging results will be described later.

11.2.2 ALA PDT (18 patients)

- The necrosis following ALA PDT was limited to the epithelium.
- Fluorescent microscopy showed preferential accumulation of PpIX fluorescence in the epithelium (2-3:1 epithelium:connective tissue).
- Either eradication or improvement of disease was observed in all patients with moderate or severe dysplasia. Control of disease in patients with invasive carcinomas was less successful, as only 2 out of 6 patients were tumour free post PDT.

Numerous animal studies have shown various light fractionation regimens to improve the extent of PDT response (Chapter 9 Section 9.5.6), but there is not enough data to know if this can be exploited clinically. At the present stage, ALA is best suited for epithelial lesions, dysplasia and carcinoma in situ.

11.2.3 mTHPC PDT (20 patients)

- mTHPC is a powerful photosensitising agent. Extremely low energy densities (5 J/cm^2) were capable of producing tumour necrosis.
- The 9 complete response lesions in patients with isolated disease consists of one dysplasia, one T1, three T2, one T3 and three T4 lesion. Residual disease was seen in the remaining three T4 lesions, suggesting that the depth of PDT damage was not sufficient, but two of these lesions were irradiated with only $5J/cm^2$. Twelve out of 16 lesions in patients with field cancerization had no local disease following PDT (one dysplasia, one Tis, five T1 and five T2 lesions). Persistent disease was found in two T1 and two T2 lesions, the problem of inadequate light penetration throughout the lesion is a likely reason for the lack of response in these lesion, which were all exophytic white masses. The response in patients with field cancerization, was less successful than in those with isolated lesion when compared at each stage of disease. Although overall response rates (i.e. absence of clinical

disease) were similar, 75% complete response in both patients with isolated lesions and multiple lesions. Seven lesions in the field change group required more than one treatment compared with single treatment in all patients with isolated tumours.

- Scarring was observed in 5 patients after PDT, but generally improved with time and avoided with careful shielding of normal tissue.

11.2.4 Field Cancerization

The results observed in patients with field change disease was most interesting and have been grouped together. A total of 26 patients with field cancerization received PDT, 18 had previous treatment. Twenty-one patients (81%) had a complete response for a minimum period of 3 months (median 19 months). Eighteen of the patient presented with simultaneous, synchronous/metachronous tumours over a period of 60 months. To date, 16 patients (73%) are alive and 11 are disease free. One of the most remarkable cases was a patient with a total of 6 SCC, three prior to embarking PDT, 13 years after the initial diagnosis he is disease free. These result compare favourably with previous reports, Cohn and Peppard (1980) noticed that 75% of patients died within 12 months of the second diagnosis. Whilst, Carr and Langdon (1989) observed 7 months to be the average survival of patients after diagnosis of the second tumour. The majority of patients in this study were seen after the second diagnosis. The management of patients with field change disease continues to be a problem with conventional methods. A modality which is tissue conserving and without cumulative toxicity would be ideal. It is feasible that PDT in the treatment of field cancerization may be the first to gain approval.

Comparison of three photosensitisers

The differences in the three photosensitisers evaluated clinically are show in Table 11.1.

Table 11.1 Comparison of Photofrin, ALA and mTHPC

	Photofrin	ALA	mTHPC
No of patients	**22**	**18**	**20**
Photosensitiser dose (mg/kg)	2	60	0.15
Route of administration	Intravenous (infusion)	Oral	Intravenous (bolus)
Drug-light interval (hours)	48	2.5-4	72-96
Activating wavelength (nm)	630	630	652
Light dose (J/cm^2)	100	100-200	5-20
Nausea & vomiting	No	Occasionally	No
Pain on irradiation	Rare	moderate-severe	mild
Pain after PDT	mild-moderate	mild	moderate-severe
Changes in LFT	None	Yes	None
Photosensitivity	6-12 weeks	1-2 days	2-4 weeks

LFT: liver function test

The above table summarises the differences between the three photosensitisers. The difference in the depth of necrosis produced is shown in Figure 11.1. The main advantage of ALA induced PpIX is the significantly shorter cutaneous photosensitivity compared with the other two agents. The disadvantages of ALA includes the more superficial effect produced, pain at the time of irradiation and potential changes in liver enzymes. The low energy density required for activation of mTHPC means a significant

reduction in treatment time and the period of cutaneous photosensitivity appears to be shorter than seen with Photofrin®, but formal skin testing needs to be carried out.

The differences in the mean depths of necrosis produced between the three photosensitisers were determined histologically. The data for the 11 Photofrin® patients were obtained from the published work of Grant et al (1997) and were predominantly excisional specimens. The data for ALA (14 patients) and mTHPC (8 patients) were from biopsies taken during the ulcerated stages of the patients reported in Chapters 9 and 10. The depths of necrosis of two different light fractionation ALA groups, were combined for the following analysis as no difference in PDT effect had been observed between them. The depth of necrosis was defined as the distance from the surface of the slough to the deepest point of necrosis as measured by an eyepiece graticule (ALA and mTHPC) or by computer aided image analysis (Photofrin®).

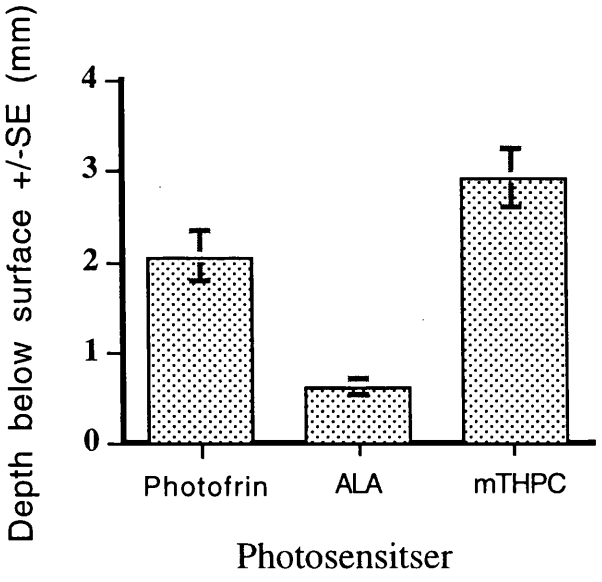


Figure 11.1: Mean depth of necrosis produced clinically with Photofrin, ALA and mTHPC (+/- SEM) ($P<0.05$)

11.2.5 Analysis of outcome in patients with early disease.

Table 11.3 combines the data concerning patients with dysplasia through to T1 tumours treated with all three photosensitisers. As shown in the table a number of patients received more than one course of PDT treatment.

Dysplastic disease

At the early follow up stage mean 9 weeks (2-18 weeks), of the 23 cases of dysplasia:

- seven had downgrading after PDT
- 13 had complete response
- two had persistent disease but both showed reduction in the size of the lesion.

At the later follow up, mean 23 months, (range 3-49 months) of the 20 patients who had either complete response or downgrading of disease previously, there were:

- 5 cases of downgrading
- 13 cases of complete response
- one patient developed a metachronous tumour at 14 months
- one reverted to moderate dysplasia

Early tumours

At the early follow up, mean of 11 weeks, (range 3-32 weeks) of patients with Tis and T1 disease, there were:

- 23 cases of complete response
- 5 cases of persistent disease

With extended follow up, at about 18 months (range 4-38):

- 20 showed a complete response
- 2 patients developed synchronous tumours at 3 and 6 months following treatment
- one patient was lost to follow up (after 12 weeks)

Three of the patients with complete response developed metachronous tumours at 14, 30 and 39 months after PDT.

Despite the heterogeneous group of patients treated it is possible to see that PDT shows promise in the treatment of dysplastic disease and early tumours.

TABLE 11. 3: CLINICAL OUTCOME OF PATIENTS WITH DYSPLASIA Tis AND T1 SCC

Patient No	Age/ sex	Stage of Disease	Site of disease	Photosensitser	Light dose J/cm^2	Early outcome weeks	Latest Follow up (months)
1	65/F	moderate dysplasia	Lower lip	Photofrin	50	mild dysplasia (16)	no change (29)
2	62/M	moderate dysplasia	Tongue	Photofrin	100	mild dysplasia (10)	metachronous tumour (14)
3	54/M	moderate dysplasia	R & L buccal mucosa	Photofrin	50	CR (16)	NCD (49)
4	73/M	moderate dysplasia	buccal mucosa	ALA	100	mild dysplasia (5)	mild dysplasia (20)
5	69M	moderate dysplasia	floor of mouth	ALA	100	normal (5)	normal (13)
6	52/M	moderate dysplasia	floor of mouth	ALA	100	mild dysplasia (4)	NCD(18)
7	79/M	moderate dysplasia	tongue	ALA	100	mild dysplasia (6)	mild dysplasia (23)
8	67/M	moderate dysplasia	buccal mucosa	ALA	200	normal(5)	moderate dysplasia (27)
9	69/F	moderate dysplasia	tongue	ALA	200	mild dysplasia (5)	mild dysplasia (23)
10	46/M	moderate dysplasia	alveolus	ALA	200	NCD(5)	normal(23)

TABLE 11. 3: CLINICAL OUTCOME OF PATIENTS WITH DYSPLASIA Tis AND T1 SCC

Patient No	Age/ sex	Stage of Disease	Site of disease	Photosensitser	Light dose J/cm^2	Early outcome weeks	Latest Follow up (months)
11	82/M	severe dysplasia	Lower lip	Photofrin	100	persistent disease	
12	57/M	severe dysplasia	L tongue	Photofrin	100	CR (12)	CR (16)
13	51/M	severe dysplasia	Lip	Photofrin	100	CR (12)	CR (12)
14	73/F	severe dysplasia	R buccal mucosa/ R & L mandibular alveolus	Photofrin	100	PD	
15	45/F	severe dysplasia	R alveolus R buccal mucosa	Photofrin	100	CR (12)	CR at treated site (26) metachronous tumour (19)
16	51/M	severe dysplasia	floor of mouth	ALA	100	normal (5)	normal (24)
17	75/M	severe dysplasia	buccal mucosa	ALA	100	moderate dysplasia (2)	moderate dysplasia (24)
18	70/M	severe dysplasia	buccal mucosa and alveolar	ALA	100	normal(8)	NCD (3) lost to follow up
19	49/M	severe dysplasia	tongue	ALA	200	NCD (12)	normal(30)
20	59/F	severe dysplasia	tongue	ALA	200	NCD (12)	normal (32)
21	73/M	severe dysplasia	tongue	mTHPC	5	NCD (12)	NCD (22)

TABLE 11. 3: CLINICAL OUTCOME OF PATIENTS WITH DYSPLASIA Tis AND T1 SCC

Patient No	Age/ sex	Stage of Disease	Site of disease	Photosensitser	Light dose J/cm^2	Early outcome weeks	Latest Follow up (months)
22	59/M a)	severe dysplasia	Buccal mucosa/ alveolus	mTHPC	5	NCD (18)	NCD (21)
23	71/F	Tis	L bucco-gingival sulcus	Photofrin	50	CR	Severe dysplasia (18)/metachronous tumour (30)
23	71/F	Tis	L buccal mucosa	Photofrin	100	persistent disease (14)	
24	60/M	Tis	(L)floor of mouth	mTHPC	10	fibrosed oral mucosa (8)	NCD (6)
25	60/F	T1	L Tongue	Photofrin	100	mild dysplasia (13)	CR (20)
26	33/F	T1	Tongue	Photofrin	100	CR (14)	CR (35)
27	59/M	T1	R buccal mucosa	Photofrin	100	CR (12)	synchronous tumour (6)
27	59/M	T1	R buccal mucosa	Photofrin	100	CR (10)	NCD(6)
28	72/F	T1	Palate R alveolus L buccal mucosa	Photofrin	50	CR (8)	synchronous tumour (3)
29	67/F	T1	L buccal mucosa	Photofrin	100	CR (3)	NCD (40)
30	40/M	T1	Lip	Photofrin	50	CR (6)	CR (26)

TABLE 11. 3: CLINICAL OUTCOME OF PATIENTS WITH DYSPLASIA Tis AND T1 SCC

Patient No	Age/ sex	Stage of Disease	Site of disease	Photosensitser	Light dose J/cm^2	Early outcome weeks	Latest Follow up (months)
31	40/M	T1	R tongue	Photofrin	100	CR (9)	CR (38) metachronous tumour (39)
32	88/F	T1	L buccal mucosa	Photofrin	100	CR (9)	no dysplasia (6)
33	62/M	T1	Floor of mouth	Photofrin	100	CR (16)	CR (8)
34	62/M	multiple T1	Tongue tip	Photofrin	50	CR (10)	moderate dysplasia (13)
2	62/M	Multiple T1	Anterior & posterior tongue	Photofrin	100	CR (6)	residual leukoplakia (20)
34	82/F	T1	R alveolar ridge	Photofrin	100	CR (16)	CR (4)
35	44/M	T1	R bucco-gingival sulcus	Photofrin	100	CR (12)	
36	63/F	T1	L alveolar ridge	Photofrin	50	CR (6)	NCD (7) metachronous tumour (14)
36	63/F	multiple T1	R alveolus	Photofrin	100	CR right alveolus (8)	CR right alveolus (24)
36	63/F	multiple T1	L alveolus	Photofrin	100	persistent disease left alveolus	

TABLE 11. 3: CLINICAL OUTCOME OF PATIENTS WITH DYSPLASIA Tis AND T1 SCC

Patient No	Age/ sex	Stage of Disease	Site of disease	Photosensitser	Light dose J/cm^2	Early outcome weeks	Latest Follow up (months)
37	52/F	T1	floor of mouth	ALA	200	normal (5)	normal (29)
38	53/M	T1	tongue floor of mouth	mTHPC	5	hyperplasia (7)	mild dysplasia (22)
39	30/M	T1	tongue	mTHPC	5	persistent disease	
40	54/M	T1	R. mand alveolus	mTHPC	5-10	persistent disease	
16	59/M	T1	alveolus	mTHPC	10	hyperplasia(15)	mild dysplasia (15)
41	65/F	T1	palate	mTHPC	5-20	NCD (8)	NCD(12)
41	65/F	T1	alveolus	mTHPC	5-15	persistent disease	
42	64/M	T1	tongue	mTHPC	10	CR (32)	mild dysplasia (20)

Table 11.3: Depth of PDT induced necrosis

	Photofrin	ALA	mTHPC
No. of patients	**11**	**14**	**11**
Light dose (J/cm^2)	50	100-200	5-10
Mean depth of necrosis (mm+/-SE)	2.06 (0.9)	0.61 (0.3)	2.9 (0.9)
Range (mm)	1.1-4.1	0.1-1.3	2.0-5.0

The difference in depths of necrosis produced with the three photosensitisers was found to be significant ($p<0.05$) Nonparametric Analysis of Variants, Nanostat, Alphabridge, London). Data from patient irradiated with the light doses (20/cm^2 for mTHPC) currently used in the formal clinical trial are required. This data suggest that mTHPC is capable of a greater depth of necrosis compared with Photofrin® and ALA.

11.3 FUTURE DIRECTION

Photodynamic therapy has been under evaluation as a therapeutic modality for many years. The recent approval for the clinical use of Photofrin® in a number of countries suggest that PDT is beginning to gain acceptance. However a number of problems exist which has delayed general acceptance of this modality. This thesis has gone some way towards answering these problems for oral tumours, but many questions remain and require further assessment. The morbidity of prolonged cutaneous photosensitivity has been overcome with the endogenous photosensitiser, ALA induced PpIX. However modifications are required to overcome the superficial nature of necrosis. The inclusion of different dark intervals with a fractionated irradiation regime have been reported to enhance the PDT effect produced. The two different treatment protocols used in Chapter 9 still resulted in superficial necrosis. The timing of this dark period may be significant and this remains to be determined. The shift of the activating wavelength from 630 nm to

635 nm has been shown initially to be associated with better response in an animal model, but the clinical significance of this is not known.

The tolerated dose of ALA used (60 mg/kg) may be only just above the therapeutic level for producing a PDT effect. A move towards an intravenous preparation may lead to higher tissue levels of PpIX as well as higher tolerated dose due to the avoidance of first pass metabolism in the liver. At the present time PDT with ALA is only suitable in the treatment of epithelial conditions such as dysplasia. There are suggestions that skin photosensitivity may be less of a problem with AlS2Pc compared with Photofrin. In view of the preclinical results phthalocyanines may be a more appropriate sensitiser for the treatment of deeper disease especially in countries where cutaneous photosensitivity might prove to be a major issue.

The other second generation sensitiser evaluated in this thesis was mTHPC which has been shown to produce deeper necrosis which can extend into the muscle. Further studies are required to determine the depth of necrosis produced using the higher light doses ($20J/cm^2$).

The recent appearance of the "black box" or diode laser should make PDT more widely amenable, as such devices are more user friendly and cheaper to purchase relative to the dye lasers. As yet a suitable 630 nm semiconductor laser does not exist, but enthusiasm is mounting with non laser light sources. These LED (light emitting diode) and other non laser devices need to assessed, and if they prove to be suitable will further reduce the cost of the light source.

Improvements are required in the light delivery currently available. Microlens have an average divergence of approximately 30^o but increasing the divergence would enable the larger surface areas such as the buccal mucosa to be irradiated using a single spot. The role of PDT with bulky tumours remains questionable. Further studies are required into

the role of PDT as an adjunct to surgical debulking and the use of interstitial fibres for treating more solid lesions.

Current imaging facilities are unable to image tumours which are only a few millimetres deep. Unless improvements occur the knowledge of the depth of effect achieved by various photosensitisers would not alleviate concerns of the possibility of undertreatment. Further investigations may enable more accurate determination of the depth of thin tumour.

As the presence of oxygen is a fundamental requirement of PDT, the potential of real-time monitoring of the oxygen status during irradiation might prove to be very useful. The availability of such devices for clinical PDT would enable treatment parameters to be adjusted according to the oxygen status of the tumour e.g. reduction of the fluence or inclusion of a dark interval when the oxygen tension is low.

The PDT effect on normal tissue has been evaluated to a degree in this thesis, but greater knowledge is still needed. Especially for PDT in the nasopharynx, further scrutiny is needed to determine the threshold of damage to the normal brain, concurrently with more light transmission studies.

In Conclusion

PDT continues to be a field of research, with more questions raised as experience is obtained. PDT has been shown to have a potentially significant role in the treatment of head and neck cancer and should cease to be regarded as just another experimental modality. However, before PDT can be widely accepted, such treatment has to be able to produce equivalent or better success rates relative to conventional modalities. In order for this to occur formal clinical trials with adequate follow up are required. Several are currently in progress.

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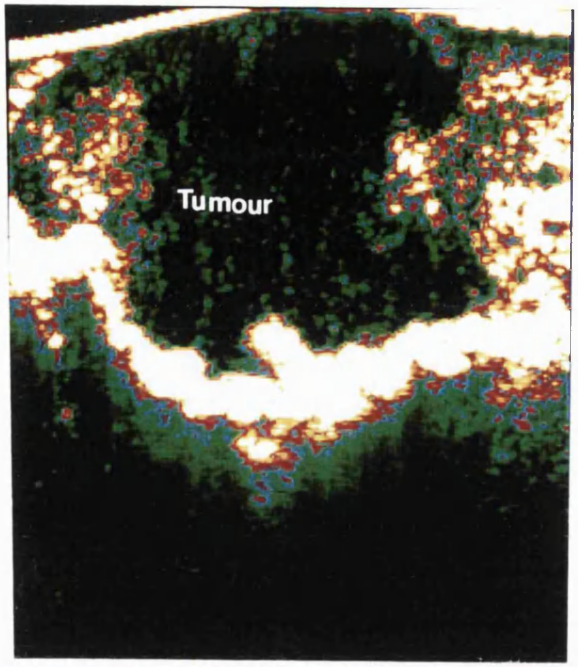
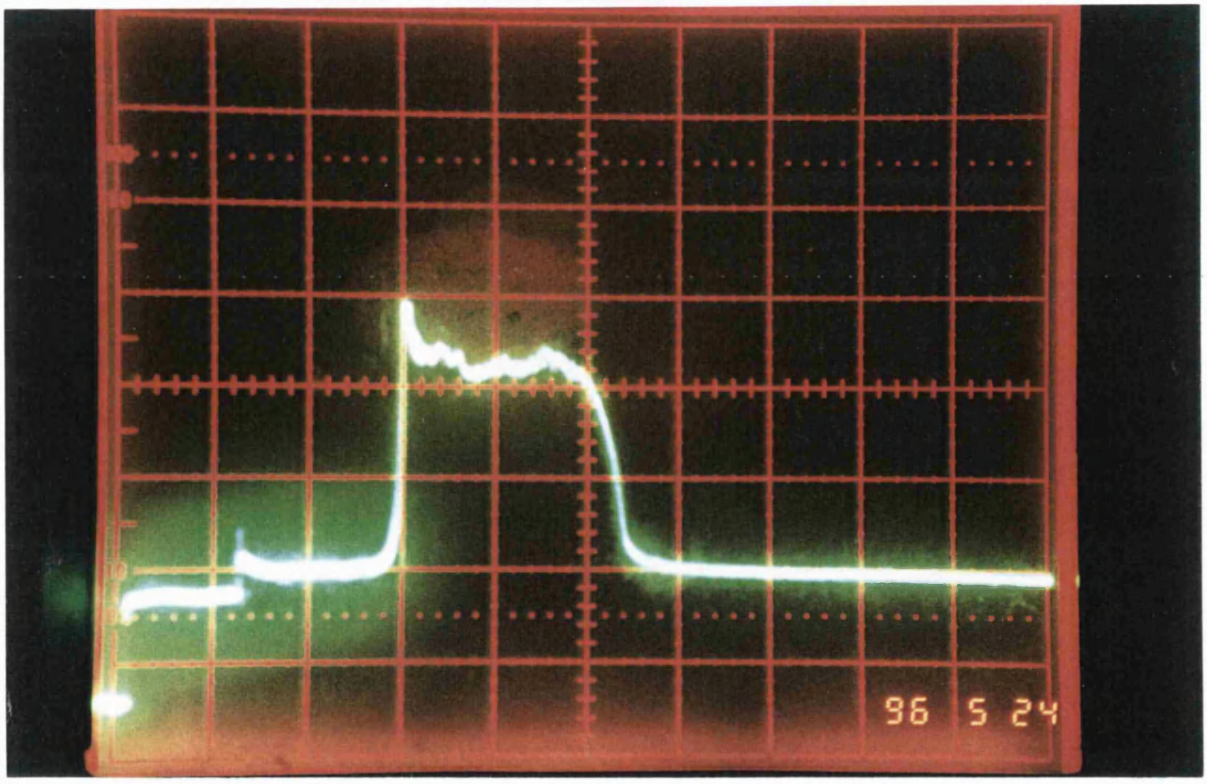
Appendix to chapter 1



Colour enhanced ultrasound image of tongue tumour. Depths of lesions can be measured from the image obtained using a high frequency probe, bandwidth centre frequency 20MHz (photograph scaled up from 10mm horizontal and 3mm vertical).

Personal communication, Dr A Sahota



Video scan of the intensity profile along a 1cm cylindrical diffuser showing good uniform emitted light. A similar fibre was used in Chapters 5 and 6.

Personal communication, Dr P Ripley

Appendix to Chapter 5

Surface area of rabbit incisor teeth

Mandibular Incisior (cm^2)	Maxillary Incisior (cm^2)
0.79 0.81 0.85 0.84 0.91 0.83 (mean 0.84 $\pm$0.04)	0.84 0.76 0.78 0.78 0.80 0.83 (mean 0.8 $\pm$0.03)

Appendix to Chapter 5

Woven bone Score

Group	Day 3	Day 10	Day 21
Photofrin PDT (1 H)	+ 0 0	+++ ++ ++	+++ ++ +++
Photofrin PDT (48 H)	0 0 0	++ ++ +++	+++ +++ ++
Photofrin Drug control	+ + +	+++ +++ ++	+++ +++ +++
ALA PDT	++ ++ +	++ ++ +++	+++ +++ +++
ALA drug control	+ + 0	+ +++ +++	+++ +++ +++
mTHPC PDT	0 0 0	+++ +++ +++	+++ +++ +++
mTHPC drug control	+ + 0	+++ ++ +++	+++ +++ +++
Light 630nm	+ +	+++ +++	+++ +++
Light 650nm	0 0	++ ++	+++ +++
Extraction only	+ +	++ ++	+++ +++

Key:

0: absence or minimal presence of woven bone (score =0)

+: presence of woven bone along the socket margin (score =1)

++: socket half filled with new bone (score =2)

+++: socket was at least 3/4 filled with bone (score =3)

Appendix to Chapter 6

Fluorescence studies with mTHPC (1mg/kg) in the nasopharynx.
As illustrated in Figure 6.2 and 6.3 (pages 116 & 117)

Connective Tissue

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	19.88	6.74	1.74
1 hour	20.44	16.97	3.9
24 hours (1 day)	74.12	38.68	10.73
48 hours (2 days)	46.12	21.69	4.34
72 hours (3 days)	40.29	11.63	2.6
96 hours (4 days)	32.03	11.62	2.74
144 hours (6 days)	35.77	18.99	3.88
192 hours (8 days)	27.33	13.58	2.96

Appendix to Chapter 6

Respiratory Epithelium

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	19.88	6.74	1.74
1 hour	21.34	11.59	4.73
24 hours (1 day)	91.46	49.29	14.86
48 hours (2 days)	42.09	12.39	3.10
72 hours (3 days)	53.22	11.33	3.03
96 hours (4 days)	47.34	13.40	4.47
144 hours (6 days)	61.49	26.44	6.83
192 hours (8 days)	31.55	18.03	5.21

Appendix to Chapter 6

Brain

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	3.61	1.14	0.29
1 hour	3.56	1.16	0.24
24 hours (1 day)	5.37	3.02	0.63
48 hours (2 days)	3.25	1.46	0.25
72 hours (3 days)	3.85	1.71	0.36
96 hours (4 days)	3.15	1.18	0.23
144 hours (6 days)	4.21	1.57	0.33
192 hours (8 days)	6.17	2.83	0.58

Appendix to Chapter 6

Fluorescence studies with mTHPC (0.3mg/kg) in the nasopharynx
As illustrated in Figures 6.1 and 6.3 (pages. 116 & 117)

Connective Tissue

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	19.88	6.74	1.74
1 hour	21.94	9.78	2.37
24 hours (1 day)	51.45	17.66	4.28
72 hours (3 days)	23.39	8.01	1.94
96 hours (4 days)	36.01	14.47	3.08

Respiratory Epithelium

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	19.88	6.74	1.74
1 hour	23.44	6.35	2.01
24 hours (1 day)	34.27	11.23	3.97
72 hours (3 days)	23.73	3.06	1.08
96 hours (4 days)	43.06	14.91	8.61

Appendix to Chapter 6

Brain

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	3.61	1.14	0.29
1 hour	3.75	1.80	0.39
24 hours (1 day)	2.80	1.76	0.32
72 hours (3 days)	4.28	1.45	0.40
96 hours (4 days)	2.83	1.48	0.31

Appendix to Chapter 6

Fluorescence studies with AlS2Pc in the nasopharynx
As illustrated in Figure 6.6 (page 120)

Connective Tissue

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	0.98	1.09	0.27
30 minutes	53.76	19.02	3.66
1 hour	42.94	15.51	2.99
4 hours	42.81	11.41	2.77
6 hours	47.67	13.15	2.94
24 hours	57.53	13.7	3.33
72 hours	29.17	10	1.7

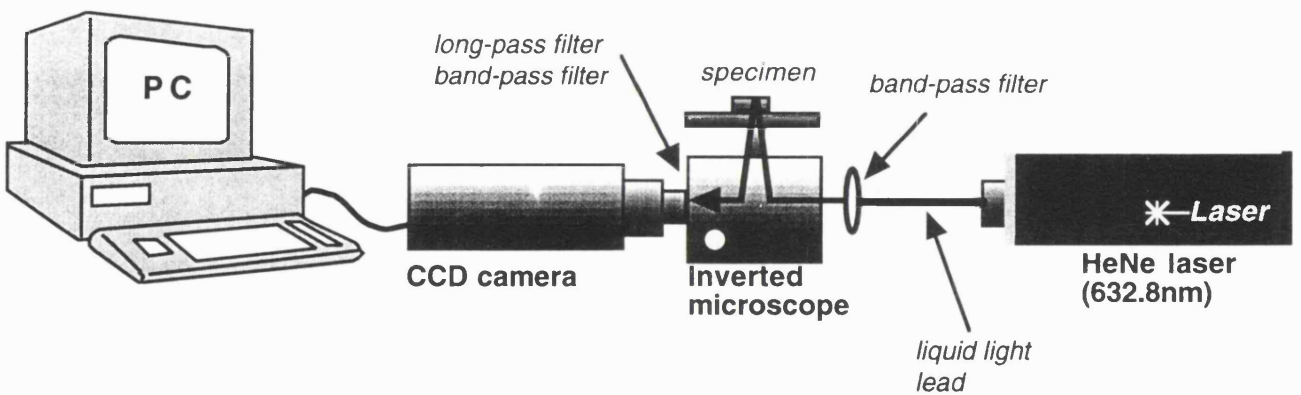
Respiratory Epithelium

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	1.24	1.51	.36
30 minutes	28.32	8.42	2.54
1 hour	30.39	4.97	2.22
4 hours	52.98	15.52	5.489
6 hours	40.81	11.93	4.51
24 hours	57.83	9.82	2.53
72 hours	25.87	11.92	4.22

Brain

Time of sacrifice after photosensitser administration	Mean	Standard Deviation	Standard Error
Control	.99	.59	0.12
30 minutes	2.58	2.46	0.48
1 hour	3.04	2.21	0.50
4 hours	1.93	0.82	0.21
6 hours	1.73	1.42	0.29
24 hours	1.51	1.04	0.19
72 hours	1.69	1.83	0.43

DIAGRAM OF APPARATUS FOR FLUORESCENCE MICROSCOPY STUDIES



Appendix to Chapter 6

Data from fluorescence microscopy (CCD) study

Photosensitiser: mTHPC
Drug dose: 1mg/kg
Each value is the mean fluorescence of the tissue for an area of the specimen in counts per pixel. The background fluorescence of each slide has been subtracted.

Key:

Time: indicates the interval between photosensitiser administration and sacrifice.

For 1mg/kg mTHPC
0 = control
1H= 1 hour
1D= 1 Day (24 hours)
2D= 2 Day (48 hours)
3D= 3 Day (72 hours)
4D= 4 Day (96 hours)
6D= 6 Day (144 hours)
8D= 8 Day (192 hours)

CT-B: connective tissue
RE-B: respiratory epithelium
B-B: brain
G-B: salivary gland. Although this was not plotted some areas imaged only contained glandular tissue.

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
1	0	26.37	•	•	•
2	0	•	•	2.20	•
3	0	23.86	•	•	•
4	0	15.19	•	•	•
5	0	•	•	3.70	•
6	0	•	•	5.10	•
7	0	•	•	4.80	•
8	0	•	•	5.00	•
9	0	14.10	•	•	•
10	0	•	•	3.55	•
11	0	•	•	4.40	•
12	0	•	•	4.59	•
13	0	•	•	1.90	•
14	0	•	15.19	•	•
15	0	•	26.37	•	•
16	0	•	14.10	•	•
17	0	•	23.86	•	•
18	0	14.30	14.30	•	•
19	0	13.10	13.10	•	•
20	0	15.10	15.10	•	•
21	0	14.50	14.50	•	•
22	0	18.70	18.70	•	•
23	0	34.80	34.80	•	•
24	0	17.20	17.20	•	•
25	0	23.90	23.90	•	•
26	0	23.40	23.40	•	•
27	0	•	•	3.80	•
28	0	•	•	4.60	•
29	0	•	•	3.00	•
30	0	•	•	3.30	•
31	0	29.80	29.80	•	•
32	0	13.90	13.90	•	•
33	0	•	•	2.20	•
34	0	•	•	2.00	•
35	1D	•	•	4.70	•
36	1D	•	•	•	•
37	1D	•	•	0.00	•
38	1D	•	•	0.00	•
39	1D	•	•	4.50	•
40	1D	58.34	•	•	•
41	1D	•	98.16	•	•
42	1D	•	108.22	•	•
43	1D	38.32	•	•	•
44	1D	•	•	4.40	•
45	1D	•	•	5.80	•
46	1D	•	•	•	•
47	1D	•	•	3.40	•
48	1D	•	•	2.00	•
49	1D	•	•	3.10	•
50	1D	•	•	6.80	•
51	1D	•	•	8.20	•
52	1D	•	•	8.40	•
53	1D	•	•	1.50	•
54	1D	•	•	8.70	•
55	1D	•	•	6.90	•
56	1D	•	•	2.50	•
57	1D	•	•	6.20	•
58	1D	•	•	7.90	•

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
59	1D	•	•	6.80	•
60	1D	144.90	•	•	•
61	1D	•	•	5.70	•
62	1D	41.10	•	•	•
63	1D	116.30	•	•	•
64	1D	•	•	11.40	•
65	1D	•	•	4.90	•
66	1D	•	•	9.70	•
67	1D	•	45.00	•	•
68	1D	•	133.80	•	•
69	1D	•	175.90	•	•
70	1D	143.40	•	•	•
71	1D	•	•	•	•
72	1D	83.30	•	•	•
73	1D	58.80	•	•	•
74	1D	46.94	•	•	•
75	1D	56.23	•	•	•
76	1D	40.90	•	•	•
77	1D	•	•	•	•
78	1D	•	129.37	•	•
79	1D	•	37.75	•	•
80	1D	•	82.02	•	•
81	1D	91.94	•	•	•
82	1D	•	129.60	•	•
83	1D	43.06	•	•	•
84	1D	•	30.99	•	•
85	1D	•	35.45	•	•
86	1H	•	•	•	11.440
87	1H	•	•	•	8.020
88	1H	•	•	•	•
89	1H	•	•	•	7.440
90	1H	•	•	•	•
91	1H	•	•	•	2.920
92	1H	•	•	•	3.540
93	1H	•	•	•	4.890
94	1H	•	•	•	2.272
95	1H	•	•	•	•
96	1H	29.48	•	•	•
97	1H	16.34	•	•	•
98	1H	13.04	•	•	•
99	1H	9.45	•	•	•
100	1H	9.22	•	•	•
101	1H	20.08	•	•	•
102	1H	•	•	•	•
103	1H	13.63	•	•	•
104	1H	10.72	•	•	•
105	1H	17.72	•	•	•
106	1H	9.73	•	•	•
107	1H	•	•	•	•
108	1H	5.50	•	•	•
109	1H	•	•	•	•
110	1H	9.41	•	•	•
111	1H	•	•	•	•
112	1H	•	•	•	•
113	1H	•	•	3.71	•
114	1H	8.67	•	•	•
115	1H	•	•	1.60	•
116	1H	•	•	•	•

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
117	1H	•	•	•	•
118	1H	•	•	•	•
119	1H	•	•	•	•
120	1H	•	23.86	•	•
121	1H	11.91	•	•	•
122	1H	10.98	•	•	•
123	1H	•	•	•	•
124	1H	•	20.08	•	•
125	1H	•	35.18	•	•
126	1H	•	•	5.19	•
127	1H	•	•	4.38	•
128	1H	•	•	.23	•
129	1H	•	39.56	•	•
130	1H	•	•	•	•
131	1H	•	•	•	•
132	1H	•	•	•	•
133	1H	•	•	•	•
134	1H	•	•	3.88	•
135	1H	•	•	2.79	•
136	1H	•	13.74	•	•
137	1H	•	2.29	•	•
138	1H	•	26.44	•	•
139	1H	•	•	•	•
140	1H	•	•	•	•
141	1H	•	•	10.81	•
142	1H	•	•	3.40	•
143	1H	•	•	3.33	•
144	1H	•	•	•	•
145	1H	•	26.50	•	•
146	1H	•	42.79	•	•
147	1H	•	37.52	•	•
148	1H	•	•	4.03	•
149	1H	•	•	3.50	•
150	1H	•	72.61	•	•
151	1H	•	•	4.76	•
152	1H	•	•	3.55	•
153	1H	•	•	3.63	•
154	1H	•	•	3.56	•
155	1H	•	•	3.55	•
156	1H	•	•	4.44	•
157	1H	•	•	4.75	•
158	1H	•	•	2.69	•
159	1H	•	•	3.28	•
160	1H	•	•	3.85	•
161	1H	•	•	3.32	•
162	1H	•	•	5.95	•
163	1H	•	•	4.04	•
164	1H	•	•	2.49	•
165	1H	•	•	•	•
166	2D	•	•	7.70	•
167	2D	•	•	3.55	•
168	2D	•	40.70	•	•
169	2D	•	•	•	•
170	2D	•	•	2.60	•
171	2D	•	•	2.10	•
172	2D	•	•	5.10	•
173	2D	•	•	1.50	•
174	2D	•	•	•	•

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
175	2D	•	36.10	•	•
176	2D	•	68.40	•	•
177	2D	•	53.80	•	•
178	2D	•	•	5.60	•
179	2D	•	37.40	•	•
180	2D	•	31.90	•	•
181	2D	•	•	2.50	•
182	2D	•	•	2.00	•
183	2D	•	64.10	•	•
184	2D	•	•	3.60	•
185	2D	•	•	2.20	•
186	2D	•	•	5.70	•
187	2D	•	•	2.70	•
188	2D	•	•	3.00	•
189	2D	•	•	3.60	•
190	2D	•	•	4.50	•
191	2D	•	•	3.50	•
192	2D	•	•	4.10	•
193	2D	•	•	4.30	•
194	2D	90.00	•	•	•
195	2D	•	•	2.70	•
196	2D	•	•	3.70	•
197	2D	•	•	3.10	•
198	2D	•	•	1.50	•
199	2D	•	29.80	•	•
200	2D	44.70	•	•	•
201	2D	26.60	•	•	•
202	2D	33.00	•	•	•
203	2D	63.30	•	•	•
204	2D	20.50	•	•	•
205	2D	•	34.10	•	•
206	2D	21.50	•	•	•
207	2D	24.70	•	•	•
208	2D	25.20	•	•	•
209	2D	19.50	•	•	•
210	2D	27.80	•	•	•
211	2D	20.40	•	•	•
212	2D	57.30	•	•	•
213	2D	31.70	•	•	•
214	2D	56.40	•	•	•
215	2D	77.90	•	•	•
216	2D	42.80	•	•	•
217	2D	60.40	•	•	•
218	2D	•	•	•	•
219	2D	68.50	•	•	•
220	2D	79.10	•	•	•
221	2D	44.10	•	•	•
222	2D	40.40	•	•	•
223	2D	•	•	1.40	•
224	2D	•	•	2.70	•
225	2D	•	•	0.00	•
226	2D	•	•	2.50	•
227	2D	•	•	3.20	•
228	2D	•	•	2.90	•
229	2D	•	•	3.60	•
230	2D	•	•	3.70	•
231	2D	•	•	2.50	•
232	2D	•	•	5.00	•

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
233	2D	•	•	2.10	•
234	2D	•	•	•	•
235	2D	•	50.30	•	•
236	2D	36.20	•	•	•
237	2D	75.70	•	•	•
238	2D	65.80	•	•	•
239	2D	•	43.90	•	•
240	2D	•	45.10	•	•
241	2D	•	38.40	•	•
242	2D	•	23.50	•	•
243	2D	•	29.70	•	•
244	2D	•	46.30	•	•
245	3D	•	77.70	•	•
246	3D	•	40.00	•	•
247	3D	•	•	•	•
248	3D	•	38.30	•	•
249	3D	•	49.10	•	•
250	3D	17.10	•	•	•
251	3D	•	•	6.80	•
252	3D	47.00	•	•	•
253	3D	36.80	•	•	•
254	3D	56.80	•	•	•
255	3D	•	•	4.50	•
256	3D	35.20	•	•	•
257	3D	•	•	5.20	•
258	3D	•	•	•	•
259	3D	•	49.60	•	•
260	3D	•	•	4.90	•
261	3D	•	•	•	•
262	3D	•	50.20	•	•
263	3D	•	49.40	•	•
264	3D	27.30	•	•	•
265	3D	•	57.20	•	•
266	3D	•	56.60	•	•
267	3D	•	•	3.00	•
268	3D	•	71.50	•	•
269	3D	•	•	•	•
270	3D	•	50.70	•	•
271	3D	50.10	•	•	•
272	3D	•	41.20	•	•
273	3D	•	•	4.70	•
274	3D	•	•	2.00	•
275	3D	•	•	•	•
276	3D	•	•	2.30	•
277	3D	•	•	4.00	•
278	3D	•	•	8.00	•
279	3D	•	•	1.70	•
280	3D	•	•	3.00	•
281	3D	•	50.40	•	•
282	3D	•	•	3.00	•
283	3D	•	•	5.20	•
284	3D	•	•	3.25	•
285	3D	•	•	2.20	•
286	3D	•	•	4.80	•
287	3D	•	•	•	•
288	3D	•	63.20	•	•
289	3D	•	•	2.60	•
290	3D	•	•	3.10	•

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
291	3D	46.70	•	•	•
292	3D	47.30	•	•	•
293	3D	48.50	•	•	•
294	3D	•	•	6.69	•
295	3D	53.00	•	•	•
296	3D	58.30	•	•	•
297	3D	42.40	•	•	•
298	3D	40.70	•	•	•
299	3D	45.60	•	•	•
300	3D	17.80	•	•	•
301	3D	29.60	•	•	•
302	3D	•	•	3.10	•
303	3D	•	•	2.50	•
304	3D	30.20	•	•	•
305	3D	•	•	2.00	•
306	3D	37.50	•	•	•
307	3D	•	•	•	•
308	3D	•	•	•	•
309	3D	37.80	•	•	•
310	4D	29.60	•	•	•
311	4D	•	•	3.30	•
312	4D	•	•	2.90	•
313	4D	23.50	•	•	•
314	4D	•	•	2.80	•
315	4D	•	•	2.80	•
316	4D	•	•	3.50	•
317	4D	•	•	2.60	•
318	4D	•	•	6.70	•
319	4D	•	•	2.70	•
320	4D	29.10	•	•	•
321	4D	•	•	3.80	•
322	4D	•	•	2.10	•
323	4D	•	•	3.00	•
324	4D	•	•	2.80	•
325	4D	•	•	4.30	•
326	4D	•	54.30	•	•
327	4D	•	56.40	•	•
328	4D	•	•	3.30	•
329	4D	•	•	2.10	•
330	4D	•	•	3.20	•
331	4D	•	•	2.60	•
332	4D	•	51.20	•	•
333	4D	26.60	•	•	•
334	4D	•	51.20	•	•
335	4D	•	18.50	•	•
336	4D	45.20	•	•	•
337	4D	32.20	•	•	•
338	4D	36.10	•	•	•
339	4D	34.40	•	•	•
340	4D	31.60	•	•	•
341	4D	20.20	•	•	•
342	4D	•	40.70	•	•
343	4D	•	59.30	•	•
344	4D	•	35.90	•	•
345	4D	28.60	•	•	•
346	4D	16.60	•	•	•
347	4D	•	58.60	•	•
348	4D	31.70	•	•	•

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
349	4D	•	•	3.10	•
350	4D	•	•	1.50	•
351	4D	•	•	1.80	•
352	4D	•	•	5.60	•
353	4D	•	•	4.70	•
354	4D	•	•	4.30	•
355	4D	•	•	2.11	•
356	4D	•	•	2.40	•
357	4D	16.50	•	•	•
358	4D	61.10	•	•	•
359	4D	24.80	•	•	•
360	4D	•	•	•	•
361	4D	•	•	3.30	•
362	4D	•	•	1.60	•
363	4D	53.00	•	•	•
364	4D	35.80	•	•	•
365	6D	•	37.00	•	•
366	6D	•	•	5.40	•
367	6D	•	•	6.60	•
368	6D	•	55.50	•	•
369	6D	•	66.10	•	•
370	6D	•	41.80	•	•
371	6D	•	35.00	•	•
372	6D	•	•	7.50	•
373	6D	•	•	2.10	•
374	6D	•	•	5.60	•
375	6D	•	•	3.40	•
376	6D	•	•	1.60	•
377	6D	•	•	4.50	•
378	6D	•	•	3.90	•
379	6D	•	•	3.80	•
380	6D	33.86	•	•	•
381	6D	•	•	4.70	•
382	6D	•	•	5.00	•
383	6D	23.07	•	•	•
384	6D	•	•	•	•
385	6D	•	•	•	•
386	6D	•	34.00	•	•
387	6D	•	•	•	•
388	6D	•	•	•	•
389	6D	•	72.90	•	•
390	6D	•	42.20	•	•
391	6D	•	108.40	•	•
392	6D	•	95.80	•	•
393	6D	•	•	•	•
394	6D	•	69.70	•	•
395	6D	•	•	•	•
396	6D	20.00	•	•	•
397	6D	24.92	•	•	•
398	6D	81.40	•	•	•
399	6D	31.10	•	•	•
400	6D	73.00	•	•	•
401	6D	•	44.31	•	•
402	6D	18.90	•	•	•
403	6D	•	•	•	•
404	6D	17.00	•	•	•
405	6D	54.30	•	•	•
406	6D	•	59.10	•	•

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
407	6D	15.60	•	•	•
408	6D	•	•	•	•
409	6D	•	•	•	•
410	6D	•	114.40	•	•
411	6D	21.00	•	•	•
412	6D	14.20	•	•	•
413	6D	38.90	•	•	•
414	6D	66.70	•	•	•
415	6D	•	•	•	•
416	6D	•	•	5.20	•
417	6D	•	•	3.40	•
418	6D	•	•	1.00	•
419	6D	•	•	3.80	•
420	6D	•	•	5.50	•
421	6D	•	•	5.00	•
422	6D	•	•	3.80	•
423	6D	•	•	2.40	•
424	6D	•	•	5.60	•
425	6D	•	•	3.20	•
426	6D	•	•	3.80	•
427	6D	33.70	•	•	•
428	6D	38.90	•	•	•
429	6D	60.60	•	•	•
430	6D	•	46.11	•	•
431	6D	45.70	•	•	•
432	6D	23.30	•	•	•
433	6D	32.10	•	•	•
434	6D	41.30	•	•	•
435	6D	19.50	•	•	•
436	6D	29.40	•	•	•
437	8D	•	27.70	•	•
438	8D	•	•	•	•
439	8D	•	25.70	•	•
440	8D	•	•	•	•
441	8D	•	•	10.80	•
442	8D	•	•	•	•
443	8D	•	•	•	•
444	8D	•	•	4.70	•
445	8D	•	•	3.10	•
446	8D	•	•	4.30	•
447	8D	•	34.00	•	•
448	8D	•	19.70	•	•
449	8D	17.60	•	•	•
450	8D	22.00	•	•	•
451	8D	•	•	•	•
452	8D	•	19.38	•	•
453	8D	•	•	•	•
454	8D	•	•	•	•
455	8D	•	18.90	•	•
456	8D	•	60.90	•	•
457	8D	•	•	•	•
458	8D	•	•	8.00	•
459	8D	•	•	7.30	•
460	8D	•	•	10.90	•
461	8D	•	•	11.20	•
462	8D	•	65.20	•	•
463	8D	•	17.92	•	•
464	8D	•	17.00	•	•

	Time	CT-B(1)	RE-B (1)	B-B (1)	G-B (1)
465	8D	12.70	•	•	•
466	8D	•	•	0.00	•
467	8D	18.50	•	•	•
468	8D	•	•	9.10	•
469	8D	•	•	8.20	•
470	8D	•	•	5.30	•
471	8D	20.30	•	•	•
472	8D	36.06	•	•	•
473	8D	15.99	•	•	•
474	8D	•	•	6.20	•
475	8D	•	•	7.80	•
476	8D	•	•	2.70	•
477	8D	10.50	•	•	•
478	8D	•	•	6.40	•
479	8D	•	•	2.40	•
480	8D	•	•	3.60	•
481	8D	•	•	6.90	•
482	8D	•	•	4.40	•
483	8D	•	•	6.10	•
484	8D	24.60	•	•	•
485	8D	•	•	7.50	•
486	8D	•	•	•	•
487	8D	•	•	•	•
488	8D	15.80	•	•	•
489	8D	22.70	•	•	•
490	8D	•	•	•	•
491	8D	•	•	•	•
492	8D	33.10	•	•	•
493	8D	62.30	•	•	•
494	8D	•	54.00	•	•
495	8D	•	•	•	•
496	8D	40.90	•	•	•
497	8D	14.50	•	•	•
498	8D	53.60	•	•	•
499	8D	28.87	•	•	•
500	8D	20.60	•	•	•
501	8D	32.00	•	•	•
502	8D	•	•	4.50	•
503	8D	29.80	•	•	•
504	8D	41.60	•	•	•
505	8D	•	•	6.60	•

Appendix to chapter 6

Data from fluorescence microscopy (CCD) study

Photosensitiser: mTHPC
Drug dose: 0.3 mg/kg
Each value is the mean fluorescence of the tissue for an area of the specimen in counts per pixel. The background fluorescence of each slide has been subtracted.

Key:

Time: indicates the interval between photosensitiser administration and sacrifice.

For 0.3mg/kg mTHPC
0 = control
1H= 1 hour
24H= 24 hours
72= 72 hours
96= 96 hours

CT-B: connective tissue
RE-B: respiratory epithelium
B-B: brain
G-B: salivary gland. Although this was not plotted some areas imaged only contained glandular tissue.

	Time	RE-B	CT-B	G-B	B-B
1	H1	17.53	11.89	•	•
2	H1	•	•	•	3.72
3	H1	•	•	•	4.38
4	H1	•	•	•	2.86
5	H1	•	15.55	3.55	•
6	H1	•	18.44	•	•
7	H1	•	45.10	•	•
8	H1	•	•	•	1.89
9	H1	•	15.64	•	•
10	H1	26.09	41.41	•	•
11	H1	•	•	5.76	•
12	H1	•	•	•	1.45
13	H1	14.20	•	•	•
14	H1	•	•	•	•
15	H1	•	•	•	1.23
16	H1	•	•	•	2.72
17	H1	•	15.18	•	•
18	H1	•	•	•	6.18
19	H1	•	•	•	•
20	H1	•	•	•	•
21	H1	32.27	15.58	•	•
22	H1	•	•	•	•
23	H1	26.42	20.88	•	•
24	H1	•	•	•	4.89
25	H1	27.43	22.12	•	•
26	H1	29.85	21.68	•	•
27	H1	20.28	18.27	•	•
28	H1	•	•	•	4.48
29	H1	•	•	•	6.56
30	H1	•	•	•	2.10
31	H1	•	•	•	.35
32	H1	•	•	•	6.48
33	H1	•	•	•	5.46
34	H1	•	11.30	•	•
35	H1	•	18.52	•	•
36	H1	•	•	•	3.91
37	H1	•	•	•	3.79
38	H1	•	•	•	4.09
39	H1	•	•	•	4.62
40	H1	•	•	•	5.59
41	H1	•	•	•	2.07
42	H1	•	33.72	•	•
43	H1	14.68	28.79	•	•
44	H1	25.63	18.95	•	•
45	H24	•	•	•	4.86
46	H24	•	•	•	3.93
47	H24	•	•	•	.09
48	H24	•	•	•	•
49	H24	•	•	•	3.38
50	H24	•	•	•	2.78
51	H24	•	•	•	2.80
52	H24	•	•	•	1.13
53	H24	•	•	•	1.43
54	H24	•	•	•	6.42
55	H24	•	•	•	4.45
56	H24	•	•	•	4.87
57	H24	•	•	•	.13
58	H24	•	49.12	•	•

	Time	RE-B	CT-B	G-B	B-B
59	H24	•	•	•	2.17
60	H24	•	•	•	2.57
61	H24	•	•	•	4.19
62	H24	•	•	•	.02
63	H24	•	•	•	.09
64	H24	•	•	•	.82
65	H24	•	•	•	3.71
66	H24	•	•	•	2.13
67	H24	28.00	35.52	•	•
68	H24	•	•	6.08	•
69	H24	52.92	•	•	•
70	H24	•	46.92	•	•
71	H24	20.21	•	•	•
72	H24	•	•	•	•
73	H24	•	38.62	•	•
74	H24	•	•	2.29	•
75	H24	•	•	3.52	•
76	H24	48.81	•	•	•
77	H24	32.41	37.73	•	•
78	H24	•	•	•	3.85
79	H24	•	•	•	1.15
80	H24	•	•	•	2.05
81	H24	•	•	•	3.95
82	H24	•	•	•	.02
83	H24	•	•	•	•
84	H24	•	•	6.90	•
85	H24	•	•	4.27	•
86	H24	•	•	12.17	•
87	H24	•	•	4.34	•
88	H24	•	•	4.19	•
89	H24	•	75.96	•	•
90	H24	•	43.91	•	•
91	H24	•	84.43	•	•
92	H24	•	84.32	•	•
93	H24	•	•	•	5.55
94	H24	•	43.20	•	•
95	H24	•	•	•	3.58
96	H24	•	•	•	3.17
97	H24	•	•	•	3.95
98	H24	28.68	79.87	•	•
99	H24	27.23	44.09	•	•
100	H24	•	31.09	•	•
101	H24	•	•	•	4.07
102	H24	•	•	7.22	•
103	H24	•	43.64	•	•
104	H24	•	•	•	3.38
105	H24	35.92	44.77	•	•
106	H24	•	48.38	•	•
107	H24	•	43.12	•	•
108	H72	25.51	26.36	•	•
109	H72	•	15.68	•	•
110	H72	•	•	•	5.81
111	H72	•	•	•	4.23
112	H72	21.86	32.62	•	•
113	H72	29.65	27.80	11.20	•
114	H72	20.51	25.14	•	•
115	H72	23.12	26.57	7.88	•
116	H72	•	•	•	5.07

	Time	RE-B	CT-B	G-B	B-B
117	H72	•	•	•	6.24
118	H72	•	•	•	5.04
119	H72	•	•	•	4.28
120	H72	•	•	•	5.89
121	H72	•	•	•	•
122	H72	•	•	•	5.56
123	H72	•	•	•	2.25
124	H72	21.38	16.82	•	•
125	H72	•	13.42	•	•
126	H72	•	•	3.81	•
127	H72	•	•	•	2.80
128	H72	•	18.21	6.67	•
129	H72	•	16.10	•	•
130	H72	•	21.79	•	•
131	H72	•	34.24	4.74	•
132	H72	•	10.11	2.49	•
133	H72	•	40.05	6.28	•
134	H72	•	21.75	•	•
135	H72	25.84	21.42	•	•
136	H72	21.94	29.47	•	•
137	H72	•	•	•	2.41
138	H72	•	•	•	•
139	H72	•	•	•	2.53
140	H72	•	•	•	3.50
141	H96	•	•	•	1.45
142	H96	•	•	•	4.33
143	H96	•	•	•	3.89
144	H96	•	•	•	2.75
145	H96	•	•	•	•
146	H96	•	•	•	.42
147	H96	•	•	•	2.38
148	H96	•	22.76	•	•
149	H96	•	58.87	•	•
150	H96	•	•	•	2.93
151	H96	•	•	•	4.29
152	H96	•	49.25	•	•
153	H96	•	•	•	2.77
154	H96	•	30.25	•	•
155	H96	•	46.97	•	•
156	H96	•	34.70	•	•
157	H96	•	55.31	•	•
158	H96	28.56	•	•	•
159	H96	•	41.31	1.72	•
160	H96	•	•	•	3.28
161	H96	•	•	•	1.11
162	H96	•	•	•	2.59
163	H96	42.27	15.58	•	•
164	H96	•	20.82	2.13	•
165	H96	•	45.54	•	•
166	H96	•	40.72	•	•
167	H96	•	21.08	•	•
168	H96	•	22.02	•	•
169	H96	•	41.13	•	•
170	H96	•	26.85	•	•
171	H96	•	•	•	•
172	H96	•	•	•	•
173	H96	•	46.97	•	•
174	H96	•	•	•	•

	Time	RE-B	CT-B	G-B	B-B
175	H96	•	•	•	2.47
176	H96	•	•	•	3.44
177	H96	•	35.76	•	•
178	H96	•	•	•	1.87
179	H96	•	•	•	1.89
180	H96	•	24.74	•	•
181	H96	58.34	67.76	•	•
182	H96	•	25.23	•	•
183	H96	•	•	•	4.49
184	H96	•	•	•	5.90
185	H96	•	•	•	3.44
186	H96	•	•	•	1.32
187	H96	•	•	•	2.35
188	H96	•	•	•	5.48
189	H96	•	18.53	•	•
190	H96	•	•	•	.21
191	H0	29.80	•	•	•
192	H0	17.20	•	•	•
193	H0	13.90	•	•	•
194	H0	15.10	•	•	•
195	H0	23.40	•	•	•
196	H0	23.90	•	•	•
197	H0	23.90	23.86	•	•
198	H0	18.70	15.19	•	•
199	H0	17.20	14.10	•	•
200	H0	18.70	•	•	•
201	H0	34.80	•	•	•
202	H0	•	•	•	4.40
203	H0	14.10	•	•	•
204	H0	•	•	•	3.55
205	H0	•	•	•	4.59
206	H0	•	•	•	1.90
207	H0	23.86	•	•	•
208	H0	14.30	•	•	•
209	H0	14.50	•	•	•
210	H0	13.10	•	•	•
211	H0	15.19	•	•	•
212	H0	26.37	•	•	•
213	H0	34.80	26.37	•	•
214	H0	•	•	•	3.80
215	H0	•	•	•	3.30
216	H0	•	•	•	4.60
217	H0	•	•	•	2.00
218	H0	•	•	•	2.20
219	H0	•	•	•	3.00
220	H0	•	•	•	4.80
221	H0	•	•	•	2.20
222	H0	•	•	•	5.00
223	H0	•	•	•	5.10
224	H0	•	•	•	3.70
225	H0	•	15.10	•	•
226	H0	•	23.40	•	•
227	H0	•	14.50	•	•
228	H0	•	13.10	•	•
229	H0	•	14.30	•	•
230	H0	•	13.90	•	•
231	H0	•	18.70	•	•
232	H0	•	34.80	•	•

	Time	RE-B	CT-B	G-B	B-B
233	H0	•	23.90	•	•
234	H0	•	29.80	•	•
235	H0	•	17.20	•	•

Appendix to chapter 6

Data from fluorescence microscopy (CCD) study

Photosensitiser: AlS2Pc
Drug dose: 1mg/kg
Each value is the mean fluorescence of the tissue for an area of the specimen in counts per pixel. The background fluorescence of each slide has been subtracted.

Key:

Time: indicates the interval between photosensitiser administration and sacrifice.

For 1mg/kg AlS2Pc

T0 = control
T 30 min = 30 minutes
T1= 1 hour
T4 = 4 hours
T6 = 6 hours
T24 = 24 hours
T72 = 72 hours

CT-B: connective tissue
RE-B: respiratory epithelium
B-B: brain
G-B: salivary gland. Although this was not plotted some areas imaged only contained glandular tissue.

	Time	RE-B	CT-B	G-B	B-B
1	T1	•	•	•	•
2	T1	•	•	•	2.70
3	T1	•	•	•	•
4	T1	•	•	•	.85
5	T1	•	•	•	.60
6	T1	•	•	•	1.70
7	T1	•	•	•	2.10
8	T1	•	•	•	1.80
9	T1	•	•	•	2.70
10	T1	•	•	•	5.46
11	T1	•	•	•	1.80
12	T1	•	•	•	•
13	T1	•	•	•	•
14	T1	•	•	•	4.35
15	T1	28.21	49.95	•	•
16	T1	38.25	42.91	10.98	•
17	T1	•	•	•	•
18	T1	•	•	•	•
19	T1	•	•	•	1.19
20	T1	•	•	•	6.11
21	T1	•	•	•	1.13
22	T1	•	•	•	8.26
23	T1	•	•	•	7.08
24	T1	•	•	•	•
25	T1	•	•	•	1.86
26	T1	•	•	•	1.08
27	T1	•	•	•	•
28	T1	•	•	•	2.32
29	T1	•	•	•	4.79
30	T1	•	•	•	2.88
31	T1	•	40.43	•	•
32	T1	•	72.33	•	•
33	T1	28.55	61.94	•	•
34	T1	25.20	57.14	•	•
35	T1	31.72	40.34	•	•
36	T1	•	36.02	•	•
37	T1	•	54.48	•	•
38	T1	•	36.06	•	•
39	T1	•	76.05	•	•
40	T1	•	52.68	•	•
41	T1	•	59.06	13.24	•
42	T1	•	31.10	•	•
43	T1	•	28.27	•	•
44	T1	•	•	•	•
45	T1	•	•	•	•
46	T1	•	20.74	•	•
47	T1	•	18.58	•	•
48	T1	•	•	20.98	•
49	T1	•	29.69	9.50	•
50	T1	•	55.15	•	•
51	T1	•	34.35	24.42	•
52	T1	•	•	22.06	•
53	T1	•	36.93	28.33	•
54	T1	•	29.51	•	•
55	T1	•	56.60	14.83	•
56	T1	•	20.36	•	•
57	T1	•	•	•	•
58	T1	•	28.39	•	•

	Time	RE-B	CT-B	G-B	B-B
59	T1	•	52.77	•	•
60	T1	•	37.66	•	•
61	T30 min	•	69.05	•	•
62	T30 min	•	•	•	7.41
63	T30 min	•	•	•	3.85
64	T30 min	•	26.24	•	•
65	T30 min	19.14	39.96	•	•
66	T30 min	•	•	19.68	•
67	T30 min	•	•	•	•
68	T30 min	34.50	50.74	•	•
69	T30 min	•	60.75	•	•
70	T30 min	•	•	19.93	•
71	T30 min	22.88	61.61	•	•
72	T30 min	•	36.20	12.51	•
73	T30 min	27.30	81.00	•	•
74	T30 min	•	84.56	•	•
75	T30 min	•	•	•	2.94
76	T30 min	•	•	•	.31
77	T30 min	•	•	•	2.39
78	T30 min	•	•	•	2.41
79	T30 min	•	•	•	.47
80	T30 min	•	•	•	.30
81	T30 min	•	•	•	1.14
82	T30 min	•	•	•	2.76
83	T30 min	•	•	•	1.93
84	T30 min	•	•	•	7.52
85	T30 min	•	•	•	5.23
86	T30 min	•	•	•	3.75
87	T30 min	•	•	•	6.03
88	T30 min	•	65.61	16.14	•
89	T30 min	•	•	•	1.32
90	T30 min	•	•	•	8.62
91	T30 min	•	32.72	•	•
92	T30 min	•	60.20	•	•
93	T30 min	•	•	•	.26
94	T30 min	•	•	•	.65
95	T30 min	•	•	•	1.21
96	T30 min	•	•	•	1.19
97	T30 min	•	•	•	.68
98	T30 min	•	•	•	2.01
99	T30 min	•	•	•	.20
100	T30 min	•	•	•	1.47
101	T30 min	•	69.20	•	•
102	T30 min	40.76	69.86	•	•
103	T30 min	37.60	24.02	•	•
104	T30 min	18.27	•	•	•
105	T30 min	•	•	•	.92
106	T30 min	•	32.69	8.63	•
107	T30 min	18.14	25.83	•	•
108	T30 min	•	62.24	•	•
109	T30 min	•	64.04	•	•
110	T30 min	36.82	27.11	•	•
111	T30 min	32.41	44.87	3.03	•
112	T30 min	•	76.08	•	•
113	T30 min	•	37.27	•	•
114	T30 min	•	43.70	•	•
115	T30 min	•	53.35	•	•
116	T30 min	•	73.21	•	•

	Time	RE-B	CT-B	G-B	B-B
117	T30 min	23.64	79.49	•	•
118	T4	•	•	•	2.25
119	T4	•	30.19	•	•
120	T4	•	•	•	2.86
121	T4	•	•	•	1.40
122	T4	•	40.25	•	•
123	T4	•	•	•	1.79
124	T4	•	•	•	2.19
125	T4	•	•	•	.37
126	T4	•	45.36	•	•
127	T4	•	50.17	•	•
128	T4	•	•	•	2.34
129	T4	•	•	•	2.57
130	T4	•	•	•	2.15
131	T4	•	29.51	•	•
132	T4	•	45.37	•	•
133	T4	•	40.26	•	•
134	T4	•	•	•	3.27
135	T4	•	55.43	•	•
136	T4	•	35.45	•	•
137	T4	•	•	•	.78
138	T4	•	•	•	2.00
139	T4	•	56.11	•	•
140	T4	•	•	•	•
141	T4	•	•	•	1.12
142	T4	22.39	53.14	•	•
143	T4	•	•	•	2.81
144	T4	•	•	•	•
145	T4	•	•	•	•
146	T4	56.34	•	•	•
147	T4	•	47.42	•	•
148	T4	70.00	49.28	•	•
149	T4	49.09	64.40	•	•
150	T4	•	28.95	•	•
151	T4	47.61	24.94	•	•
152	T4	64.12	31.53	13.51	•
153	T4	68.27	•	•	•
154	T4	46.00	•	•	•
155	T4	•	•	•	•
156	T4	•	•	•	•
157	T4	•	•	•	•
158	T4	•	•	•	•
159	T4	•	•	•	.84
160	T4	•	•	•	2.11
161	T24	•	•	•	.92
162	T24	•	•	•	.17
163	T24	•	•	•	1.31
164	T24	•	•	•	1.10
165	T24	•	•	•	.32
166	T24	•	•	•	1.09
167	T24	•	•	•	1.13
168	T24	•	•	•	1.02
169	T24	•	•	•	.09
170	T24	•	67.23	•	•
171	T24	65.75	52.90	•	•
172	T24	•	36.48	•	•
173	T24	•	66.73	•	•
174	T24	•	88.52	•	•

	Time	RE-B	CT-B	G-B	B-B
175	T24	48.78	•	•	•
176	T24	68.92	65.01	•	•
177	T24	•	49.47	•	•
178	T24	•	72.20	•	•
179	T24	•	•	•	2.30
180	T24	•	45.80	17.05	•
181	T24	•	•	•	2.41
182	T24	•	•	•	1.92
183	T24	•	43.97	13.45	•
184	T24	•	•	14.02	•
185	T24	•	53.29	16.96	•
186	T24	52.33	55.59	•	•
187	T24	•	•	•	1.67
188	T24	•	•	•	1.57
189	T24	•	•	•	1.39
190	T24	•	•	•	1.44
191	T24	•	•	•	1.51
192	T24	•	•	•	1.69
193	T24	•	•	•	1.38
194	T24	•	•	•	1.41
195	T24	•	•	•	2.40
196	T24	•	•	•	4.54
197	T24	•	63.35	•	•
198	T24	•	•	•	1.56
199	T24	•	•	•	.53
200	T24	•	•	•	2.45
201	T24	•	•	•	1.64
202	T24	•	41.18	8.46	•
203	T24	•	•	•	.11
204	T24	•	•	•	1.70
205	T24	•	74.80	15.63	•
206	T24	•	46.72	23.08	•
207	T24	•	54.68	•	•
208	T24	•	•	•	4.36
209	T24	•	•	•	.24
210	T24	•	•	•	•
211	T6	•	•	•	2.33
212	T6	•	•	•	1.36
213	T6	•	48.86	22.07	•
214	T6	•	•	•	2.25
215	T6	•	•	•	5.70
216	T6	•	•	•	1.89
217	T6	•	72.89	•	•
218	T6	•	•	•	5.26
219	T6	•	•	17.19	•
220	T6	•	•	•	.88
221	T6	•	•	•	•
222	T6	•	46.79	17.36	•
223	T6	•	•	•	.20
224	T6	•	•	•	.63
225	T6	•	•	•	.72
226	T6	•	•	•	2.97
227	T6	•	57.21	33.09	•
228	T6	•	•	•	1.73
229	T6	•	•	•	.47
230	T6	•	•	•	1.21
231	T6	•	•	•	.63
232	T6	•	•	•	1.19

	Time	RE-B	CT-B	G-B	B-B
233	T6	•	•	•	1.70
234	T6	•	•	•	2.00
235	T6	•	55.46	•	•
236	T6	•	48.04	•	•
237	T6	65.22	48.70	•	•
238	T6	32.54	34.35	•	•
239	T6	•	65.16	31.84	•
240	T6	•	•	33.69	•
241	T6	43.71	61.17	•	•
242	T6	35.56	33.57	•	•
243	T6	•	27.89	•	•
244	T6	•	35.65	20.85	•
245	T6	•	38.97	•	•
246	T6	•	51.50	•	•
247	T6	•	•	28.85	•
248	T6	39.10	•	•	•
249	T6	•	•	•	.78
250	T6	•	•	•	.51
251	T6	•	•	•	.71
252	T6	•	•	•	3.46
253	T6	•	•	•	1.93
254	T6	•	39.18	•	•
255	T6	28.55	33.51	•	•
256	T6	•	46.25	•	•
257	T6	•	•	•	1.02
258	T6	40.98	72.09	•	•
259	T6	•	36.08	25.22	•
260	T72	•	•	•	•
261	T72	•	•	•	•
262	T72	•	•	•	•
263	T72	•	25.06	•	•
264	T72	•	36.65	•	•
265	T72	•	•	•	•
266	T72	•	•	•	•
267	T72	•	•	•	•
268	T72	•	•	•	•
269	T72	•	•	•	•
270	T72	•	28.84	•	•
271	T72	•	24.70	•	•
272	T72	•	•	7.67	•
273	T72	•	18.14	•	•
274	T72	•	28.25	•	•
275	T72	•	25.68	•	•
276	T72	•	22.32	•	•
277	T72	•	31.49	•	•
278	T72	•	30.02	•	•
279	T72	•	•	•	•
280	T72	•	•	•	•
281	T72	•	•	•	1.19
282	T72	•	•	•	.93
283	T72	•	•	•	7.42
284	T72	•	•	•	3.93
285	T72	•	•	•	1.06
286	T72	•	•	•	.02
287	T72	•	•	•	.78
288	T72	•	•	•	2.11
289	T72	•	36.22	•	•
290	T72	•	24.86	•	•

	Time	RE-B	CT-B	G-B	B-B
291	T72	•	•	•	•
292	T72	•	42.38	10.35	•
293	T72	•	30.50	•	•
294	T72	•	25.19	10.57	•
295	T72	•	16.07	•	•
296	T72	•	15.41	6.85	•
297	T72	•	•	•	.22
298	T72	•	•	•	1.68
299	T72	•	•	•	1.58
300	T72	•	•	•	1.70
301	T72	•	•	•	.27
302	T72	•	•	•	•
303	T72	•	•	•	•
304	T72	•	•	•	1.59
305	T72	•	•	•	•
306	T72	•	•	•	1.51
307	T72	•	•	•	.29
308	T72	•	•	•	.10
309	T72	•	•	•	4.06
310	T72	29.20	27.96	•	•
311	T72	•	18.96	•	•
312	T72	•	29.58	7.91	•
313	T72	•	17.50	•	•
314	T72	•	31.59	•	•
315	T72	20.51	28.49	10.47	•
316	T72	•	19.62	•	•
317	T72	•	69.71	•	•
318	T72	8.16	•	4.04	•
319	T72	19.58	31.54	•	•
320	T72	19.25	30.65	•	•
321	T72	•	46.22	•	•
322	T72	27.59	32.14	•	•
323	T72	•	22.22	•	•
324	T72	47.56	27.72	•	•
325	T72	35.09	36.73	•	•
326	T72	•	30.05	•	•
327	T72	•	29.31	•	•
328	T0	•	•	•	.42
329	T0	.02	.02	•	.66
330	T0	•	•	•	•
331	T0	.05	.05	3.19	•
332	T0	1.00	1.00	•	.92
333	T0	.40	.40	•	.08
334	T0	1.10	1.10	•	•
335	T0	2.01	2.01	•	.62
336	T0	2.35	2.35	•	.01
337	T0	•	•	•	1.39
338	T0	2.27	2.27	•	•
339	T0	•	•	3.86	•
340	T0	5.52	•	•	•
341	T0	•	•	1.00	•
342	T0	•	•	•	1.92
343	T0	•	•	•	1.92
344	T0	1.16	1.16	•	•
345	T0	•	•	•	1.93
346	T0	•	•	•	1.49
347	T0	•	•	•	1.82
348	T0	3.80	3.80	•	•

	Time	RE-B	CT-B	G-B	B-B
349	T0	.42	.42	•	•
350	T0	0.00	0.00	•	•
351	T0	•	•	.40	•
352	T0	•	•	•	•
353	T0	0.00	0.00	•	•
354	T0	0.00	0.00	•	•
355	T0	•	•	•	1.13
356	T0	.10	.10	•	•
357	T0	•	•	•	.17
358	T0	•	•	•	.85
359	T0	•	•	•	1.69
360	T0	•	•	•	1.05
361	T0	1.50	1.50	•	•
362	T0	•	•	•	.38
363	T0	•	•	•	.69
364	T0	•	•	•	.67
365	T0	•	•	•	1.03
366	T0	.55	.55	•	•
367	T0	•	•	•	1.11
368	T0	•	•	•	1.18
369	T0	•	•	•	.67

Appendix to Chapter 6

Method for determination of 5, 10, 15, 20-tetra (m-Hydoxyphenyl) chlorin in plasma and tissues by HPLC

Personal communication with Dr C K Lim

Apparatus

Pye Unicam PU4010 pump.
Linear UVIS-204 detector set at 423nm.
Perkin-Elmer LS-3 flurometer set at ex. 423nm, em. 653nm.
Rheodyne injector (200µl loop).
Hypersil-ODS column, 250mm X 5mm i.d., 5µm particle size.
Dounce homogenizer.
Eppendorf microcentrifuge.

Materials and reagents

5,10,15,20-tetra(m-hydroxyphenyl)chlorin (m-THPC)
Acetonitrile (HPLC grade, Rathburn).
Methanol (PCLC grade, Rathburn).
Dimethyl sulphoxide (DMSO, AR grade, BDH).
Trifluoroacetic acid (TFA, 1ml ampoule, Piercee)

Extraction of m-THPC in plasma

For the extraction of m-THPC in human plasma, a vortex-mix 200µl of plasma with 400µl of methanol-DMSO (4:1 v/v) in a 1ml Eppendorf microcentrifuge tube for 30sec. Cool on ice for 30min. Centrifuge at 8800xg for 20min. Inject 200µl of the supernatant.

For mouse plasma, vortex-mix 50µl of plasma with 250µl of methanol-DMSO (4:1 v/v). Cool, centrifuge and inject 50-100µl of the supernatant In cases where plasma m-THPC levels are high, dilute 50µl of the supernatant with 100-250µl of a diluent containing 1ml of water and 2ml of methanol-DMSO (4:1 v/v). Inject 50µl.

The diluent must contain water to make it compatible with the HPLC mobile phase. Otherwise peak distortion will be observed.

Extraction of m-THPC in tissues.

The homogenising medium consisted of 8 parts methanol-DMSO (4:1 v/v) and 1 part water. Tissue (100-300mg) was homogenized in 2ml of the tube, cool on ice for 30 min

and centrifuged at 2600xg for 10min. 400µl of the supernatant was mixed with 200µl of water and 200µl of the solution was injected.

The correct proportion of water in the homogenizing medium and in the final solution is important for satisfactory chromatography. Tissues contain much less water than plasma. Injection of tissue extract with methanol-DMSO (4:1 v/v) as the extractant in the absence of water resulted in peak distortion. The above mentioned is suitable for all tissues, including tumour, liver, spleen, kidney, brain, muscle and skin.

High performance liquid chromatography (HPLC)

- Column: Hypersil-ODS (5µm) or any other C_{18} column.
- Mobile phase: Acetonitrile - 0.1% TFA (77:23 v/v)
- 0.1% TRA was made by diluting 1ml (1 ampoule) of TFA to 1 litre with water.
- Flow rate: 1ml/min.
- Detector: vis, 423nm; fluorescence, ex. 423nm, em. 653nm.
- Columns from a different manufacturer may behave differently and a slight adjustment of the acetonitrile content in the mobile phase may be necessary.

Calibration Curves.

These were constructed by plotting a suitable range of m-THPC concentractions in accurately spiked control plasma or tissue homogenates against peak areas, using the same extraction and HPLC separation procedures described above for plasma or tissues.

Surface area of the rabbit nasopharyx

(As measured using callipers)

Surface Area (cm^2)	
4.31	Mean:3.95
3.88	SD: 0.3
3.65	SEM: 0.2

Appendix to Chapter 9

Wilcoxon Two-Sample Rank Test

1. Necrosis

n=13

100J/cm	Rank
0.5	8
0.65	13
0.5	8
0.1	1
0.9	14
1.2	15.5
0.6	11.5
0.4	5.5
0.37	4
1.3	17
2.1	18
0.6	11.5
0.4	5.5
Total	132.5

200 J/cm	Rank
0.5	8
0.3	3
0.2	2
1.2	15.5
0.55	10
Total	38.5 = t

Critical t for n_1=5, n_2=13: 27
therefore the difference is not significant.

2. Inflammation

n=9	
1.2	8.5
1.25	10
1.0	4
1.0	4
1.2	8.5
4.1	14
1.5	11
1.1	6.5
1.0	4
Total	70.5

n=5	
1.1	6.5
0.8	1
0.9	2
4.0	13
2.5	12
Total=	34.5 = t

Critical t for n_1=5, n_2=13: 22
therefore the difference is not significant

Kathleen Fan

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Publication de l'Union Internationale Contre le Cancer

PHOTODYNAMIC THERAPY USING mTHPC FOR MALIGNANT DISEASE IN THE ORAL CAVITY

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Photodynamic therapy (PDT) produces local tumor necrosis, on activation of a previously administered sensitizer with non-thermal light of an appropriate wavelength. It is attractive for treating tumors of the mouth as tissue healing is particularly good. We describe the use of the photosensitizing agent meta tetrahydroxyphenyl chlorin (mTHPC, Foscan®) for PDT of oral cancer, including patients with field cancerization. Nineteen patients with histologically confirmed oral cancer (8 with field change disease) and one with severe dysplasia, were sensitised with mTHPC intravenously. Activation was carried out 72–96 hr later with laser light at 652 nm using a range of light doses. The results were assessed clinically and histologically. Multiple biopsies were taken during the ulcerative stages to look at the effects of PDT and after healing to assess the overall treatment result. All single lesions up to stage T3 cleared after one PDT treatment (total of 6 patients). Three out of 6 T4 tumours were also cleared. Lesions in patients with field change disease did less well, only 9 of 14 T1 and T2s clearing, including 4 that required extra treatments with a higher light dose. Most healed very well, but tongue tethering was seen in 1 patient and another had necrosis in normal areas due to light scattering within the mouth. PDT using mTHPC is a promising new treatment for patients with oral cancer. *Int. J. Cancer* 73:25–32, 1997.

The worldwide annual incidence of cancers in the oral cavity and pharynx is estimated to be 412,000 (Parkin *et al.*, 1993). The majority of these tumors are found in the mouth and tongue, where treatment is conventionally a combination of surgery and radiotherapy. However, the overall survival rates have not improved substantially in the last 2 decades (Lippman *et al.*, 1994). Furthermore, the attendant morbidity (Fuller, 1987) in terms of function and cosmesis after surgery, along with radiation-induced changes to the mucous membrane, salivary glands and facial bones, is poorly tolerated by many patients.

Among those with tumors of the upper aerodigestive tract, there is a subgroup of patients who pose a particularly difficult therapeutic challenge. These patients have multiple primary cancers, which develop from independent premalignant foci described by Slaughter *et al.* (1953) as "field carcinogenesis" or "field cancerization". The magnitude and severity of this problem in patients with oral cancers has been demonstrated in several studies in which the rate of development of second primary tumors was found to be in the region of 3–4% per year, with 10.6% of patients developing a new primary over an average 3.4-year follow-up (Day and Blot, 1992; Jovanoic *et al.*, 1994).

Photodynamic therapy (PDT) involves the administration of a photosensitizing agent, allowing time for its accumulation in the target tissue (tumor). The sensitizer is then activated by non-thermal light of a wavelength matched to its absorption characteristics. In the presence of oxygen, a photochemical interaction results in the production of highly reactive species, in particular singlet oxygen, which are toxic to the cells in which they are produced (Foote, 1990). Due to a degree of preferential accumulation of the photosensitizing agent in tumor tissue, coupled with the targeting afforded by highly directional illumination, some selectivity in PDT effect can be obtained (Bonnett, 1995) although it is seldom possible to get selective tumor necrosis when both tumor and adjacent normal tissue are exposed to the same light dose. PDT has been used as an experimental clinical modality for some years and has recently been approved for clinical use in Japan, Canada, The Netherlands, United States and France in specific malignant and premalignant conditions. Most experience has been gained with the photosensitizing agent di-hematoporphyrin ether/ester (Photofrin, Quadralogic Technology, Vancouver, Canada), which has been shown to be of benefit in a range of clinical situations (Grant *et al.*, 1993*a* and *b;* Wenig *et al.*, 1990). This first generation photosensitizer has, however, a number of limitations. It is composed of a complex and variable mixture of porphyrins, with a weak absorption band in the red region of the spectrum (630 nm) and causes cutaneous photosensitivity for up to 3 months.

Considerable effort has been put into the development of new photosensitizers, with the aim of overcoming some of these disadvantages. The ideal photosensitizer should be a single compound (Bonnett, 1995), and should have improved tumor selectivity (Berenbaum *et al.*, 1993) and increased absorption in the red region for deeper tissue penetration of light and minimal skin photosensitivity to sunlight (Savary *et al.*, 1993). Meta tetrahydroxyphenyl chlorin (mTHPC, Foscan® Scotia Pharmaceuticals, Guildford, UK) is a promising new compound. Early clinical work using PDT on the tumor bed after resection of mesotheliomas showed that a dose of 0.3 mg/kg body weight mTHPC, activated with red light at 650 nm with a dose of 10J/cm^2, produced 10-mm depth of tumor necrosis (Ris *et al.*, 1991) with safe healing. The use of this photosensitizer to treat a variety of tumors in the head and neck region has been reported in a diverse group of 11 patients (Dilkes *et al.*, 1995) with encouraging results.

The aim of our study was to determine the efficacy of mTHPC PDT using a range of light doses, in a group of patients with oral squamous cell carcinomas or severe epithelial dysplasia.

MATERIAL AND METHODS

Twenty patients were included in this study. Nineteen had histologically confirmed oral squamous cell carcinomas, in addition to areas of dysplasia in some, and one just had severe epithelial dysplasia. Twelve patients had single lesions and eight exhibited field change disease (multicentric neoplasms). The patients in the field change group conform to the criteria originally described by Warren and Gates (1932): (1) histologically confirmed malignant neoplasm; (2) each tumor "geographically" distinct; (3) none of the lesions are metastases. The total number of lesions in the 8 field change patients was 16 (1–5 lesions per patient at the time of treatment). Ten patients had had previous treatment for the tumor or associated disease, including 2 who had had removal of regional neck nodes. The remaining 11 patients had either refused or were

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not suitable for conventional therapies. The study was approved by the Ethics Committee of the University College London Hospitals and all treatments were carried out with fully informed patient consent.

Photodynamic therapy

The mTHPC was supplied as crystals, which were dissolved in a solvent consisting of polyethylene glycol, water and ethanol, producing a solution with a concentration of 4 mg mTHPC per ml. The intravenous dose (0.15 mg/kg body weight) was administered through a filter 72 to 96 hr prior to laser irradiation, with special care taken to avoid extravasation. The light source used was a copper vapour pumped dye laser emitting red light at 652 nm (Dynamic Light, Hornsby, Australia), with the beam delivered to the patient via a 0.4-mm core diameter flexible microlens fiber (PDT Systems, Santa Barbara, CA) to obtain even light distribution

TABLE I – CLINICAL OUTCOME OF PATIENTS WITH ISOLATED LESIONS[1]

Patients	Age/sex	Site	TNM stage	Previous treatment	Light dose (J/cm²)	Histology healed (weeks)	Size of lesion before/healed (cm²)	Histology longest f/u (weeks)
(1)	73/M	Tongue	T0/dysplasia	Nil	5	NCD	3.1/0	Refuse biopsy NCD (92)
(2)	53/M	Tongue/FOM	T1	Nil	5	Hyperplasia (7)	24/0	Mild dysplasia (94)
(3)	80/M	Tongue	T2	Nil	5	Hyperkeratosis (12)	6/0	NCD (88)
(4)	56/M	Palate	T2	Nil	5	Mod. dysplasia (16)	12.6/0	No macroscopic change (74)
(5)	58/M	Retromolar buccal mucosa	T2	Nil	5	Mild/mod. dysplasia (4)	3.1/0	Mild dysplasia (92)
(6)	51/M	Tongue	T3	CO_2 laser	20	NCD	4.7/0	Moderate dysplasia (37)
(7)	80/F	Mandible	T4	S, RT, ALA	5	NCD	12.2/0	No local disease[2]
(8)	49/M	Tongue	T4	RT	5	Persistent disease	9.4/9.4 Debulking only area unchanged	Further PDT
(9)	60/M	Alveolus	T4	Nil	5	NR	3.9/3.9	Surgery and RT[3]
(10)	42/M	Tongue	T4	Nil	20	Hyperplasia (13)	1.3/0	Recurrence at (26)[4]
(11)	79/M	Palate and max. alveolus	T4	RT	20	NCD	32/0	No local disease[5]
(12)	82/F	Alveolus	T4	Nil	20	Persistent disease	2.4/1.2	Surgery (8)

[1]Abbreviations used: S: surgery; max: maxillary; mod: moderate; RT: radiotherapy; FOM: floor of mouth; NR: no response; NCD: no clinical disease, but refused biopsy; ALA: photodynamic therapy with 5-aminolaevulinic acid.–[2]Control of local disease, but neck nodes, subsequently died.–[3]Radical surgery, radiotherapy and chemotherapy.–[4]For surgery and radiotherapy.–[5]Scarring +++ no evidence of local recurrence 3 months after PDT, but evidence of cutaneous metastases, died 8 months after PDT.

TABLE II – CLINICAL OUTCOME OF PATIENTS WITH FIELD CHANGE DISEASE[1]

Patients	Age/sex	Site	Staging (TNM)	Previous treatment	Light dose/cm²	Histology healed (weeks)	Size of lesion before/healed (cm²)	Histology/longest f/u (weeks)
(13)	30/M	Tongue	T1	S	5	Persistent disease	1.5/0.1	For surgery and RT (12)
(14)[2]	54/M a)	L tongue	T2	Nil	5	Hyperkeratosis (26)	6.1/0	NCD (88)
	b)	L buccal mucosa	T2	Nil	5	Normal (26)	12.6/0	NCD (88)
	c)	Soft palate	T2	Nil	5		1.2/0.6	
					10	Persistent disease	0.6/0.3	
					20	NCD	2.4/0	NCD (21)
	d)	R buccal mucosa	T2	Nil	10	Persistent disease	12.6/7.1	PR with 1st PDT
					10	Negligible ulceration	7.1/7.1	NR with 2nd PDT
	e)	R. mand alveolus	T1	Nil	5	Persistent disease	3.1/1.2	NA
					10	Negligible ulceration	1.2/1.2	NA
(15)	61/F a)	Buccal mucosa	T2	S	10	Persistent disease	4.9/0.8	
					20	NCD	0.8/0	NCD (35)
	b)	L alveolus	T2	S	10	NCD	2.0/0	NCD (44)
	c)	Palate	T1	S	10	Persistent disease	1.2/0.03	
					20	NCD	0.03/0	NCD (35)
(16)	59/M a)	Buccal mucosa/ alveolus	dysplasia	S	5	NCD	24.3/0	NCD (89)
	b)	Alveolus	T1	S	10	Hyperplasia (15)	0.3/0	Mild dysplasia (64)
(17)	65/F a)	Palate	T1	S, C	5	Persistent disease	6.2/3.1	
				PII/ALA	20	NCD	3.1/0	NCD (52)
	b)	Alveolus	T1	S, C	5	Persistent disease	2/1.3	
				PII/ALA	10	Persistent disease	1.3/0.6	
					15	Persistent disease	0.8/0.3	For further PDT
(18)	60/M	(L)FOM	Tis	S	10	Fibrosed oral Mucosa (8)	9.6/0	NCD (24)[3]
(19)	64/M	Tongue	T1	Nil	10	Mild dysplasia (32)	1.1/0	No change (84)
(20)	59/M	Retromolar	T2	S, cryo, RT	10	Persistent disease	1.17/0.8	Data not available lost to follow-up[4]

[1]Abbreviations used: FOM: floor of mouth; S: surgery; C: chemotherapy; RT: radiotherapy; Cryo: cryotherapy; NR: no response; NA: not appropriate, as persistent disease; NCD: no clinical disease, but refused biopsy; PII: photodynamic therapy with Photofrin; ALA: photodynamic therapy with 5-aminolaevulinic acid.–[2]2nd primary Ca found on GI endoscopy. Radiotherapy for GI tumour.–[3]Developed 2nd primary for radiotherapy.–[4]Planned surgery died of bronchopneumonia.

TABLE III – RESPONSE OF LESIONS AFTER A SINGLE TREATMENT WITH PDT[1]

Light dose (J/cm^2)	Patient group	Dysplasia	Tis	T1	T2	T3	T4
5	Isolated disease	1 CR		1 CR	3 CR		1 CR 1 PD 1 NR
5	Field change	1 CR		4 PD	2 CR 1 PD		
10	Field change		1 CR	2 CR 1 PD	1 CR 3 PD		
15	Field change						
20	Isolated disease					1 CR	2 CR 1 PD
20	Field change						

[1]Response is related to initial pathology. Not all lesions healed with normal mucosa. Some had residual mild or moderate dysplasia as shown in Tables I and II. CR: complete local response. PD: Persistent disease. NR: No response.

TABLE IV – LESIONS TREATED MORE THAN ONCE WITH PDT[1]

Patient and lesion reference	Stage	Light dose 1st PDT (J/cm^2)	Size before/after	Light dose 2nd PDT (J/cm^2)	Size before/after	Light dose 3rd PDT (J/cm^2)	Size before/after
14 (e)	T1	5	3.1/1.2	10	1.2/1.2		
15 (c)	T1	10	1.2/0.03	20	0.03/0		
17 (a)	T1	5	6.2/3.1	20	3.1/0		
17 (b)	T1	5	2/1.3	10	1.3/0.6	15	0.8/0.3
14 (c)	T2	5	1.2/0.6	10	0.6/0.3	20	2.4/0
14 (d)	T2	10	12.6/7.1	10	7.1/7.1		
15 (a)	T2	10	4.9/0.8	20	0.8/0		

[1]All were inpatients with field change disease.

throughout the treatment field. A customised fiber holder with an angled end was constructed to allow ease of positioning of the fiber. This enabled the difficult regions of the oral cavity to be irradiated with near perpendicular incidence.

Treatments were carried out with the patient conscious in all except one case (patient 14), where a general anesthetic was administered to improve access for a lesion on the soft palate. The treatment times were short, ranging from 113 to 480 sec, and treatment was generally well tolerated by the patients. The treatment area was defined as the lesion with a 1–2 cm margin of normal tissue. This area was marked with small dots of crystal violet blue dye to allow repositioning or resizing of the irradiated spot, made necessary by patient movement. The incident irradiance was kept at or below 250 mW/cm^2 to avoid thermal effects in all cases. The treatment times were calculated to give the chosen light dose (5–20 J/cm^2) and when possible the whole lesion was irradiated using one spot. Frequently, the site of the lesion did not allow for this and up to 10 different fiber positions were used. In this situation, the light dose would have been higher in the small areas where 2 spots overlapped. This was considered to be acceptable, as it was important to deliver at least the prescribed dose to the whole lesion.

Patients were kept in a dimmed room after receiving mTHPC, and given verbal and written instructions on avoidance of bright light during the 2–3 weeks for which they were likely to be photosensitive.

Evaluation of PDT effects

When tolerated by patients, biopsies were taken on 4 separate occasions: at initial diagnosis, immediately prior to irradiation (for cases treated or re-treated a long time after initial diagnosis), during the ulcerated stage and after PDT, when healing was complete. The application of local anesthetic was limited to the site of biopsy to avoid any possible alteration in the PDT effect. The specimens were directly fixed in 10% neutral buffered formalin and processed routinely in paraffin wax. Five-micrometer sections were cut and stained with hematoxylin and eosin.

TABLE V – FINAL RESULT (AFTER UP TO 3 PDT'S) FOR EACH LESION TREATED[1]

Stage of disease		CR (free of disease)	CR (disease elsewhere)	PD (alternative/ further therapy undertaken or planned)
Dysplasia	Isolated	1	—	—
Dysplasia	Field change	1	—	—
Tis	Field change	1	—	—
T1	Isolated	1	—	—
T1	Field change	3	1	3
T2	Isolated	3	—	—
T2	Field change	2	3	2
T3	Isolated	1	—	—
T4	Isolated	1	2	3

[1]CR: complete response; PD: persistent disease.

Routine hematological and biochemical investigations were carried out before and after mTHPC administration.

RESULTS

Clinical course

Twenty-eight lesions were treated in 26 PDT treatment sessions (summarised in Tables I to V). Four patients (14–17) underwent more than one PDT treatment (3 for repeat treatment on lesions that had not responded adequately and 1 for a new lesion). All except 2 patients reported discomfort at the injection site at the time of i.v. administration of mTHPC but this settled within minutes in all cases. Laser irradiation was well tolerated in most patients, mild discomfort being the most commonly described sensation, but 3 patients experienced significant pain. Within a few hours of PDT, the treated area exhibited erythema, edema and in some cases blister formation. This was followed by a phase characterized by vascular stasis and congestion, leading to necrosis, the formation of a fibrino-purulent slough and ultimately ulceration. The ulceration

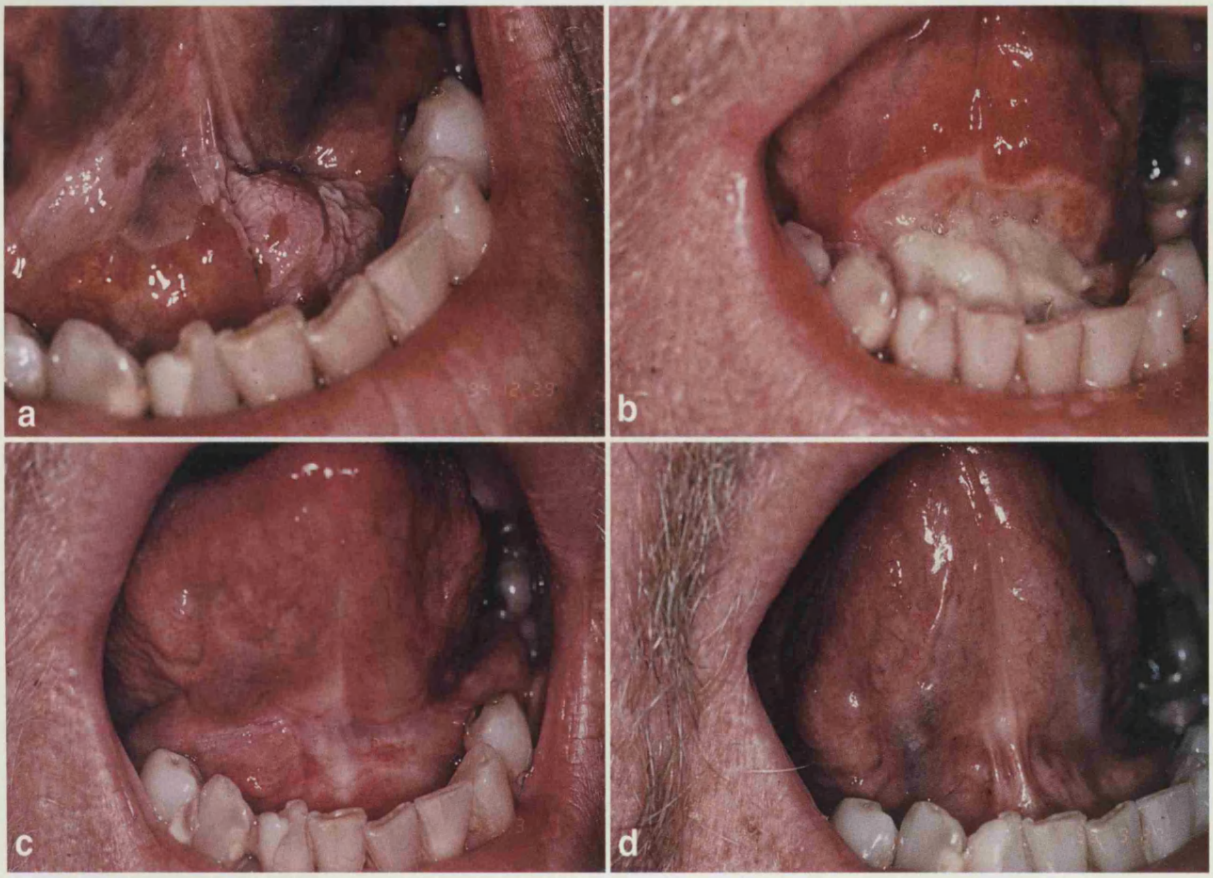

FIGURE 1 – *(a)* Patient 2: T1 squamous cell carcinoma and surrounding leukoplakia of the ventral tongue/floor of mouth before photodynamic therapy. *(b)* Patient 2: 13 days after photodynamic therapy showing ulceration with the presence of a thick fibrino-purulent slough of the entire irradiated site which included normal and abnormal regions. *(c)* Patient 2: 7 weeks after PDT with complete healing of the treatment site. Scarring is present (hyperplasia was seen on the post treatment biopsy). *(d)* Patient 2: 8 months after PDT, showing disappearance of the scarring at the treatment site. At 20 months after treatment, the patient is still free of disease clinically though mild dysplasia was seen on histology.

typically took 5–8 weeks to heal, with some scarring in 5 patients (patients 2, 3, 5, 11, 18). These effects generally improved with time (Fig. 1*a–d*), although 2 patients developed complications (patients 11, 18). One of these had a very large T4 tumor on the palate (32 cm^2), irradiated with 20 J/cm^2, which resulted in ulceration and necrosis of the normal oral tissue well away from the target lesion due to reflected and scattered light within the mouth. This occurrence was avoided in subsequent cases by shielding the normal tissues (Fig. 2*a,b*). The second problem was significant tethering of the tongue after treatment of a carcinoma *in situ* in the floor of the mouth adjacent to a skin graft placed following resection of a previous tumor.

Analgesia was required by all patients in the early ulcerative phase, starting 1–2 days post PDT, with oral opiates being prescribed for 1–2 weeks for most patients. Five patients experienced mild cutaneous photosensitivity due to inadvertent exposure to sunlight within the first 2 weeks post sensitization, but all recovered without long-term effects. One patient experienced pain behind the eyes 2 weeks post sensitisation, following sudden exposure to bright light without eye protection, but this rapidly settled when the eyes were covered. All patients had a full blood count, serum electrolytes and liver function tests, before and up to 3 months after administration of mTHPC. There were no changes in either the hematological or biochemical indices following PDT.

The follow-up of patients with no clinical disease after treatment ranged from 24–94 weeks with a mean of 63 weeks. In Tables I and II persistent disease (PD) signifies residual disease, which could be seen clinically. A complete response (CR) indicates the disappearance of all disease clinically or on biopsy, determined by 2 observations not less than 4 weeks apart. In this series the minimum length of follow-up with complete response lesions is 4 months.

Table I summarizes the results for the 12 patients with isolated disease. A dose of 5 J/cm^2 was successful in the treatment of the dysplasia and the T1 and T2 SCCs, whilst 20 J/cm^2 were adequate for the T3 lesion (Tables I, III). In the T4 tumors, local control was achieved in 1 of 3 treated with 5 J/cm^2 and 2 of 3 treated with 20 J/cm^2, but all later developed distant disease.

Table II summarizes the results for the 8 patients with field change disease in the oral cavity. The low light dose of 5 J/cm^2 was adequate for the 1 dysplastic lesion, but only 2 of the 7 T1 and T2 SCCs treated with this low light dose were cleared (Table III). Four of the other 5 had a second PDT treatment with a higher light dose but only 1 was cleared of the disease (Table II). One then underwent surgery and radiotherapy, and the other 2 had a third PDT treatment. There is no disease currently present in 1 of these 2, while the other still has persistent disease.

In the patients with field change disease, the results were better with higher light doses. 10 J/cm^2 resulted in control of disease in the Tis (Carcinoma *in situ*) lesion and in 2 of the 5 T1 tumors treated with this dose (Table II). Both T1 lesions treated with 20 J/cm^2 were cleared when they had not responded to lower doses (Table IV). Results with T2 lesions in these patients showed a

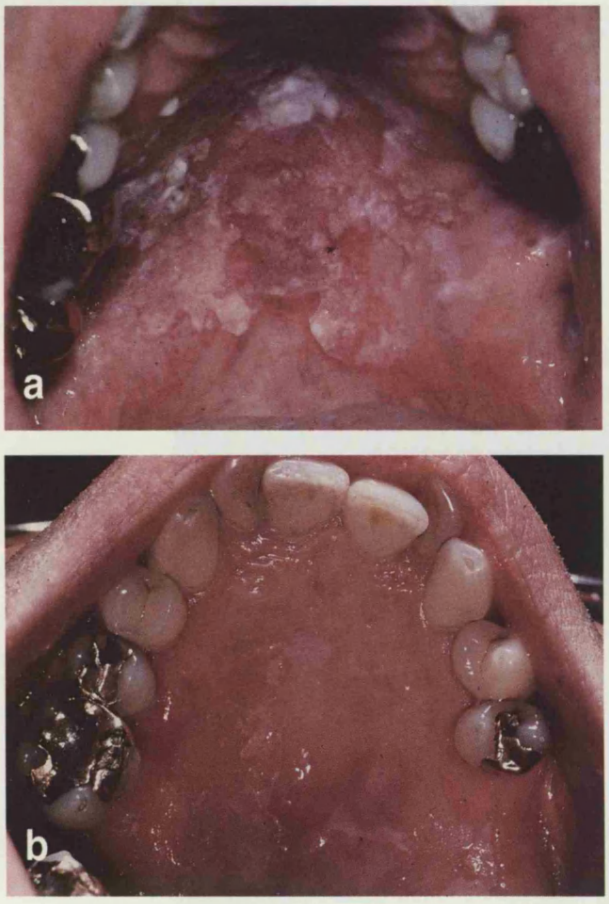

FIGURE 2 – *(a)* Patient 4: with disease involving the entire palatal mucosa. *(b)* Patient 4: 2 months after PDT. The patient was free of tumor, but moderate dysplasia was present on biopsy.

similar trend. Two of 3 T2 lesions treated with 5 J/cm^2 showed a complete response, but the third only cleared with 20 J/cm^2 (Tables III, IV). Only one of 5 T2 lesions was successfully treated with 10 J/cm^2, the 2 failures treated with a higher dose were cleared. It appears that residual disease following PDT in field change patients requires a higher light dose to be used. Table III summarizes the results in relation to the initial light doses used for tumors of each stage.

Pathological changes

Biopsies taken during the ulcerated stage show characteristic histopathological changes. In the early stages (3–7 days), lesions were characterized by massive acute inflammation, vascular damage and edema. This was associated with ulceration of the overlying epithelium and necrosis, which frequently extended to the submucosa and occasionally into muscle. The mucosa was replaced by a dense edematous fibrino-purulent slough, with a massive exocytosis of neutrophils and occasional eosinophils (Fig. 3). Small blood vessels, arterioles and venules showed loss of endothelial cells and prominent pavementation of polymorphonuclear leukocytes (Fig. 4). The acute inflammatory infiltrate and edema extended deep into the underlying submucosa and muscle when present. There was prominent separation of muscle fibers by inflammatory cells and edema fluid with scattered infiltrates of eosinophils.

In later biopsies at 10–12 days there was still evidence of widespread ulceration and mucosal necrosis with acute inflammatory cells, although infiltrates of lymphocytes and macrophages were surprisingly few at this stage. At the margins of the ulcerated area, growth of new epithelium was frequently seen below the fibrino-purulent slough. Deep below the mucosa, and often within muscle, there was deposition of immature granulation tissue with proliferating endothelial cells, fibroblasts and chronic inflammatory cells. This process of organisation suggested that the end result would be scarring, which was consistent with the clinical findings and was seen in specimens taken after healing was complete (Fig. 5*b*).

DISCUSSION

Our results show that photodynamic therapy using mTHPC is a promising treatment for patients with oral cancer. It is not only well tolerated but also simple and quick to execute. Though some scarring may occur, the healing is generally better than with other forms of local tissue destruction and so there are less functional changes (Fig. 1*a–d*). There is also the advantage that PDT does not cause cumulative toxicity, so if responses are incomplete, PDT can be repeated. These factors need to be balanced against the disadvantage of cutaneous photosensitivity, which can last up to 6 weeks (Savary *et al.*, 1993) although usually last about 3 weeks.

It is fundamental to the success of photodynamic therapy that the depth of PDT necrosis is greater than the depth of each individual tumor. There is concern that surface irradiation of deep tumors may leave residual viable tumor even if the surface heals with regeneration of normal mucosa, as the red light used to activate the photochemical reaction only penetrates a few millimeters. The depth of light penetration, and hence sensitizer activation, is important whether the mechanism of cell kill is direct cytotoxicity or destruction of the tumor vasculature. From the histopathology, vascular damage plays an important role in the patients described in this report (Fig. 4). There are a number of ways in which the depth of tumor damage may be enhanced. Interstitial PDT may improve results in the T4 tumors, as the fiber can be implanted deep into the lesion and subsequently withdrawn during treatment, thus enabling the whole depth of the cancer to be irradiated. In preclinical studies the extent of PDT damage has also been shown to be enhanced either by fractionating the light dose (Messmann *et al.*, 1995; van der Veen *et al.*, 1994) or reducing the fluence rate (Gibson *et al.*, 1990), and since irradiation time with mTHPC is short, reducing the fluence rate would not elongate the treatment unacceptably.

Although PDT with mTHPC was successful in the treatment of the oral dysplasias, the use of a shorter acting photosensitizing agent such as ALA may adequately eradicate this superficial disease as shown in previous studies (Fan *et al.*, 1996). ALA has the advantage of inducing only 1–2 days of cutaneous photosensitivity, although it may cause elevated levels of liver enzymes and the depth of effect is probably inadequate for treating invasive cancers (Grant *et al.*, 1993*a*; Regula *et al.*, 1995).

Our results showed a better response in patients with isolated lesions than in those with field change disease (Table V). With isolated disease, all lesions up to stage T3 could be cleared in one treatment whereas with field change disease, we could only clear 9 of 14 T1 and T2 lesions. Even so, 4 of these required more than one treatment, and most required higher light doses than the isolated lesions.

Eight lesions persisted after PDT. Three were T4 tumors and most likely adequate light doses did not reach all parts of the lesions. The other 5 were in 3 patients with field change disease. Three of these were exophytic white masses in patients with proliferative verrucous leukoplakia (PVL) (Zakrzewska *et al.*, 1996), a condition often quoted as being particularly difficult to treat with conventional modalities. These lesions are often thicker than other SCCs and it is probable that the dense white fronds reflected more light, so a lower light dose than anticipated reached

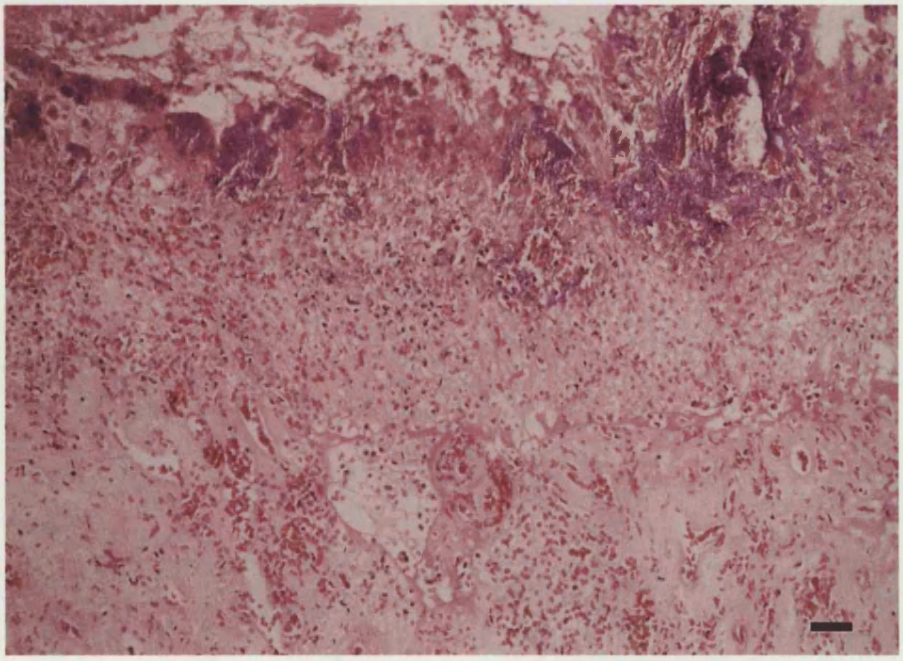

FIGURE 3 – Characteristic findings 3 days after PDT. The epithelium is completely replaced by necrotic ulcer slough and the connective tissue is edematous and shows inflammation and haemorrhage. H&E; bar: 50 μm.

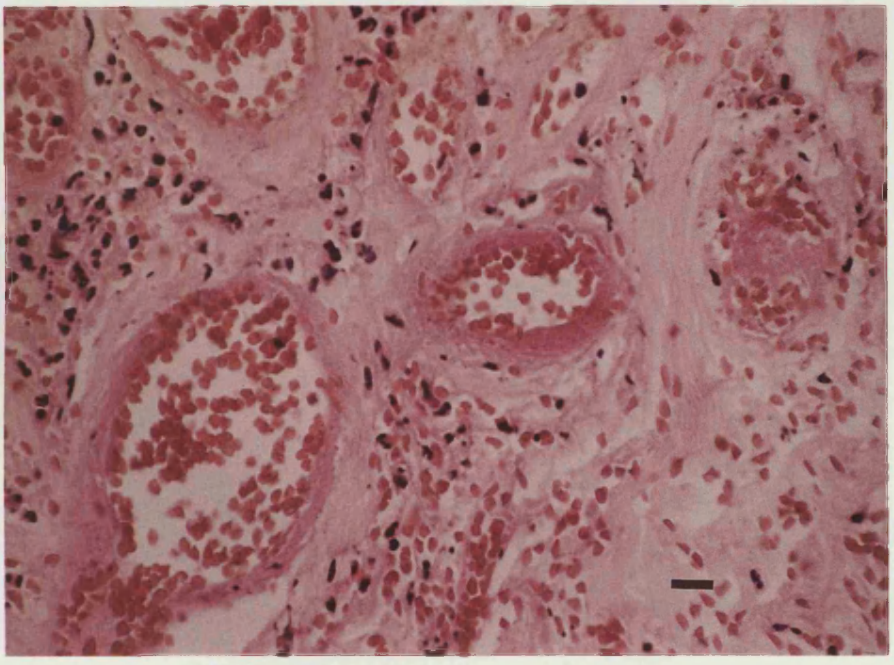

FIGURE 4 – Characteristic changes in blood vessels 3 days after PDT. There is extensive vascular damage with loss of endothelial cells and resulting edema, extravasation of red blood cells and inflammation. Thrombus formation is prominent. H&E; bar: 20 μm.

the base of each lesion. This can lead to PDT necrosis in other parts of the mouth if normal areas are not adequately shielded as happened in one case. *In vivo* monitoring of the absorbed light dose, perhaps by monitoring photobleaching at the surface of and within the tumor, may improve the dosimetry for these lesions and also allow for inter-patient variability. Another approach would be the delivery of light by insertion of optical fibers directly into these white masses, which may also help with bulkier tumors.

The key to successful PDT lies in matching the depth of necrosis to the depth of tumor. Improvements in current imaging techniques may allow more accurate assessment of tumor thickness and enable the tailoring of treatment parameters to the depth of individual lesions. In the meantime, repeated PDT treatment has been shown to be of value in the management of patients with field change disease, as there is no cumulative toxicity. It would, therefore, be reasonable to hope that this type of disease

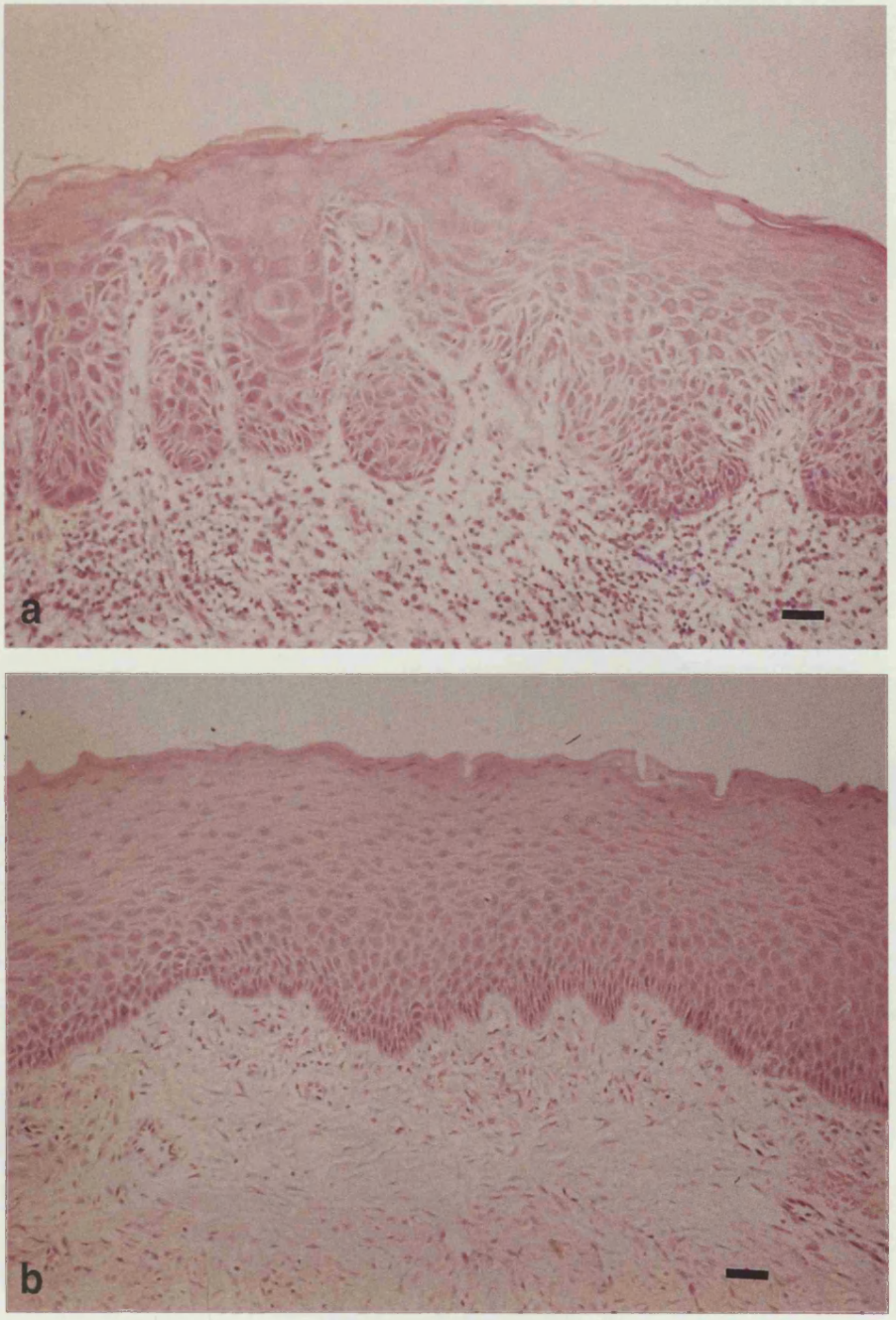

FIGURE 5 – *(a)* Biopsy showing severe epithelial dysplasia with evidence of microinvasion before PDT. H&E; bar: 50 µm. *(b)* Biopsy of the same site 3 months after PDT showing healing with well-ordered epithelium overlying fibrous scar tissue. H&E; bar: 50 µm.

may be kept under control for extended periods with minimal morbidity.

Photodynamic therapy using mTHPC has many benefits. It has been shown to be well tolerated and simple with short treatment times compared with PDT using other photosensitizers. From our study, it would appear that many isolated lesions up to T2 can be controlled with a light dose as low as 5 J/cm^2 although the required dose will vary according to the depth of the lesion to be treated, and, particularly in patients with field change disease, is likely to be up to 20 J/cm^2. This modality has been shown to be of benefit in the management of patients with field change disease and superficial cancers of the oral cavity. However, larger numbers of patients are required with longer follow-up to allow full evaluation of this photosensitizing agent. To this end, a large multicentric trial is currently underway.

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Photodynamic Therapy Using 5-Aminolevulinic Acid for Premalignant and Malignant Lesions of the Oral Cavity

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BACKGROUND. Premalignant changes in the mouth, which are often widespread, are frequently excised or vaporized, whereas cancers are treated by excision or radiotherapy, both of which have cumulative morbidity. Photodynamic therapy (PDT) is another option that produces local tissue necrosis with light after prior administration of a photosensitizing agent. This heals with remarkably little scarring and no cumulative toxicity. This article describes the use of PDT with the photosensitizing agent 5-aminolevulinic acid (ALA) for premalignant and malignant lesions of the mouth.

METHODS. Eighteen patients with histologically proven premalignant and malignant lesions of the mouth were sensitized with 60 mg/kg ALA by mouth and treated with laser light at 628 nanometers (100 or 200 Joules/cm^2). The results were assessed macroscopically and microscopically. Biopsies were taken immediately prior to PDT for fluorescence studies, a few days after PDT to assess the depth of necrosis, when healing was complete, and up to 88 weeks later.

RESULTS. The depth of necrosis varied from 0.1 to 1.3 mm, but complete epithelial necrosis was present in all cases. All 12 patients with dysplasia showed improvement (repeat biopsy was normal or less dysplastic) and the treated areas healed without scarring. Some benefit was observed in five of six patients with squamous cell carcinoma, but only two became tumor free (one with persistent mild dysplasia). No patient had cutaneous photosensitivity for longer than 2 days.

CONCLUSIONS. PDT using ALA for dysplasia of the mouth produces consistent epithelial necrosis with excellent healing and is a simple and effective way to manage these patients. Results in invasive cancers are less satisfactory, mainly because the PDT effect is too superficial with current treatment regimens using ALA as the photosensitizing agent. ***Cancer* 1996; 78:1374–83.**
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KEYWORDS: photodynamic therapy, oral premalignancy, oral cancer, 5-aminolevulinic acid.

Oral cancer encompasses cancer of the lip, tongue, and lining of the mouth and is the sixth most common malignancy worldwide. The number of new cases estimated worldwide for 1985 was 412,000,[1] with 2400 new cases reported every year in the United Kingdom alone.[2] Despite recent advances in conventional management, the prognosis has not improved over the past 30 to 40 years.[3]

The mainstays of treatment at present are surgery and radiotherapy, alone or in combination. The most notable recent advances have been in reconstructive techniques after excisional surgery, but this may still leave debilitating functional and cosmetic defects. Radiotherapy has the advantage of greater tissue conservation but is associated with complications that include xerostomia, mucositis, ulcer-

ation, osteoradionecrosis, and skin or subcutaneous fibrosis. The results of chemotherapy have been most disappointing, and the options available for treating recurrences after surgery or radiotherapy are often severely limited and carry high morbidity.

Oral cancers can arise in clinically normal mucosa but are often preceded by premalignant lesions. The prevalence of such conditions in the general population may be up to 4.6%,[4] with the rate of malignant transformation in oral epithelial dysplasia and leukoplakia with severe dysplasia quoted as 14% and 43%, respectively.[4–6] There are three principal areas of uncertainty associated with premalignancy. First, there are no widely accepted criteria for defining the presence and degree of dysplasia. Second, it is not clear which lesions require treatment, nor how aggressively such lesions should be treated.[7] Third, many such patients exhibit a field change effect, so it may be necessary to treat large areas. In these circumstances, it may not be feasible to treat all affected areas at one time, so re-treatment may be necessary. Consequently, a modality with no cumulative toxicity would be preferable.

Current management includes exclusion of risk factors such as smoking, along with active therapy such as surgical excision, topical cytotoxic therapy, systemic retinoid therapy, cryosurgery, or laser therapy, but none are entirely satisfactory or universally effective.[8,9] Photodynamic therapy (PDT) has been widely investigated as a therapeutic tool, showing particular promise in the local treatment of early malignancies. The treatment is based on the administration of a photosensitizing drug, which ideally will preferentially accumulate in tumor tissue. This is followed by local exposure of the tumor to nonthermal light, usually from a laser, using a wavelength matched to the absorption characteristics of the photosensitizer. This excitation of the photosensitizer in the presence of oxygen leads to the formation of singlet oxygen, a highly reactive molecular species capable of producing a local cytotoxic effect.

A number of photosensitizers are currently under investigation, the most commonly used being dihematoporphyrin ether (Phototherapeutics, Vancouver, Canada). Although Photofrin has been shown to be beneficial in certain clinical situations,[10] it has an ill-defined composition with poor tumor selectivity and causes prolonged cutaneous photosensitivity (6 to 12 weeks). This has prompted the search for new photosensitizing agents such as 5-aminolevulinic acid (ALA). ALA is unusual in that it is converted to a photoactive substance in vivo via the heme biosynthetic pathway. The systemic administration of ALA overcomes the feedback inhibition of ALA synthetase leading to the overproduction and accumulation of porphyrin precursors to heme, in particular protoporphyrin IX (PpIX).[11] PpIX is the main photoactive substance produced following sensitization with ALA,[11–14] and PDT using topical ALA has already been shown to be valuable in the treatment of basal cell carcinomas of the skin.[12] A major advantage of ALA is that, even after systemic administration, cutaneous photosensitivity only lasts 1 to 2 days.[13] In addition, PDT-treated tissues heal remarkably well, making it feasible to treat extensive superficial lesions that often pose problems for conventional management in the mouth.[14] Further, in the authors' preclinical and clinical studies, PpIX has been shown to accumulate more in the epithelium than in the underlying muscle,[14,15] with the consequent possibility of selective damage to this layer following PDT.

The present study investigated the efficacy of PDT using ALA in the management of malignant and premalignant lesions in the mouth. In previous studies performed in this center, the effect of PDT using ALA was found to be very superficial, even with long treatment times.[14–16] Recent reports from several centers have suggested that PDT effects might be enhanced by fractionating the light.[17–20] In particular, using ALA, Van der Veen et al.[17] found that a 75-minute period between light treatments enhanced the effect (although the total light dose was higher in the fractionated dose experiments). Messmann et al.[20] found that a single break of 150 seconds during a 250-second exposure was capable of increasing the area of a PDT-induced ulcer in normal rat colon by a factor of 5 compared with the same total light dose administered without a break. In view of these findings, we studied fractionated light therapy using a long and a short break during treatment to see if deeper effects could be produced than were seen in our first clinical report.[14]

MATERIALS AND METHODS

Patients and Treatment

Eighteen patients were included in this study. All had histologically confirmed premalignant or malignant lesions of the mouth. Details are given in Tables 1–3. None had regional nodes or distant metastasis at the time of treatment. In those patients who had previous treatment with the carbon dioxide laser, PDT was undertaken because the surgical specimen showed persistent disease at the margins. The study was approved by the hospital ethical committee, and all treatment was carried out with fully informed consent from the patients.

ALA was supplied for this project as the hydrochloride in powder form by DUSA Pharmaceuticals (Tarrytown, New York). For clinical use, this was dissolved in orange juice and given by mouth in three separate doses of 20 mg/kg at 0, 1, and 2 hours (total dose, 60 mg/kg) using the regimen established by Regula et al.[16] The light source used was a gold vapor laser giving

TABLE 1
Clinical Outcome of Patients with Moderate Dysplasia

Patient no.	Age (yr)/sex	Site	Previous treatment	Maximum necrosis (mm)	Maximum inflammation (mm/layer)	Histology (healed/wk)	Size of lesion before/healed	Histology (longest follow-up/wk)
1	73/M	BM	Nil	0.9	1.2/Submucosa	Mild (5)	2.6 cm^2/1.7 cm^2	Mild (40)
2	69M	FM	CO_2 laser	0.8	1.5/Submucosa	Normal (5)	6.2 cm^2/0 cm^2	Normal (32)
3	52/M	FM	Nil	1.1	6*/Muscle	Mild (4)	7.1 cm^2/0 cm^2	N (28)
4	79/M	T	Nil	0.6	2.5*/Submucosa	Mild (6)	3.1 cm^2/0 cm^2	Mild (56)
5	67/M	BM	Nil	0.5	1.1/Submucosa	Normal (5)†	1.8 cm^2/0 cm^2	Moderate (48)
6	69/F	T	Nil	NM	NM	Mild (5)	3.1 cm^2/0.8 cm^2	Mild (64)
7	46/M	A	CO_2 laser	0.2	0.9/Submucosa	N (5)	1.0 cm^2/0 cm^2	Normal (64)

BM: buccal mucosa; FM: floor of mouth; N: no clinical disease macroscopically, but biopsy refused; T: tongue; NM: not measurable; A: alveolus.
* Inflammatory changes extend through full thickness of biopsy specimen.
† Hyperplasia seen, but no dysplasia.
Full-thickness epithelial necrosis and sloughing present in all cases.
Patients 1–4 received 100 J/cm^2; patients 5–7, received 200 J/cm^2.

TABLE 2
Clinical Outcome of Patients with Severe Dysplasia

Patient no.	Age (yr)/sex	Site	Previous treatment	Maximum necrosis (mm)	Maximum inflammation (mm/layer)	Histology (healed/wk)	Size of lesion before/healed	Histology (longest follow-up/wk)
8	51/M	FM	Nil	0.1	1.0/Submucosa	Normal (5)	6.3 cm^2/0 cm^2	Normal (60)
9	75/M	BM	CO_2 laser	0.6	1.5/Mucosa	Moderate (2)	7.1 cm^2/0 cm^2	Moderate (54)
10	70/M	BM/A	Nil	0.3	1.2/Submucosa	Normal (8)*	8.8 cm^2/0 cm^2(5)	N (36)
11	49/M	T	CO_2 laser	0.3	0.8/Muscle	N (12)	8.0 cm^2/0 cm^2(5)	Normal (78)
12	59/F	T	CO_2 laser	0.6	2.5/Muscle	N (12)	4.8 cm^2/0 cm^2	Normal (76)

FM: floor of mouth; BM: buccal mucosa; A: alveolus; N: no clinical disease macroscopically, but biopsy refused; T: tongue.
* Hyperplasia seen, but no dysplasia.
Full-thickness epithelial necrosis and sloughing present in all cases.
Patients 8–10 received 100 J/cm^2; patients 11 and 12 received 200 J/cm^2.

TABLE 3
Clinical Outcome of Patients with Squamous Cell Carcinoma

Patient no.	Age (yr)/sex	Site	Previous treatment	Maximum necrosis (mm)	Maximum inflammation (mm/layer)	Histology (healed/wk)	Size of lesion before/healed	Histology (longest follow-up/wk)
13	52/F	FM	CO_2 laser	NM	NM	Normal (5)	1.8 cm^2/0 cm^2	Normal (88)
14	85/F	P	Field change*	NM	NM	Mild (4)	4.6 cm^2/0.8 cm^2	Mild (76)
15	87/F	T	Field change*	0.5	1.2/Muscle	N	4.0 cm^2/0 cm^2	NA†
16	81/F	A	Mandibular resection	0.5	1.0/Mucosa	NA	16.7 cm^2/0.6 cm^2	NA†,‡
17	62/M	FM	Nil	1.3	NM	SCC (5)	8.0 cm^2/1.0 cm^2	NA‡
18	65/F	BM/A/P/RM	Field change*	0.7	1.3/Submucosa	SCC (12)	8.5 cm^2/8.5 cm^2	NA‡

FM: floor of mouth; NM: not measurable; BM: buccal mucosa; P: palate; T: tongue; N: no clinical disease macroscopically, but biopsy refused; NA: not applicable; A: alveolus; SCC: squamous cell carcinoma; RM: retromolar trigone.
* All three patients with field change disease had multiple excisions of dysplastic areas and carcinomas over 5–12 years before photodynamic therapy.
† Developed metastatic neck disease.
‡ Subsequent photodynamic therapy with a different sensitizer.
Full-thickness epithelial necrosis and sloughing present in all cases.
Patients 15–18 received 100 J/cm^2; patients 13 and 14 received 200 J/cm^2.

red light at 628 nanometers (nm) (Dynamic Light, Milton Keynes, UK). This was delivered to the patient using a single mode scrambled, 0.4 mm flexible optical fiber with a bare flat-cleaved tip that gave a circular spot of light up to 2.5 cm in diameter on the target tissue. The area to be treated was defined as the target lesion together with a margin of 5 mm of surrounding normal tissue. Appropriate fiber positions and treatment times were calculated to give the desired light doses before treatment started, using one of the two dose regimens described below (Study Groups 1 and 2). Whenever possible, the entire lesion was treated from a single fiber position. However, due to the difficult shape of the mouth, this was often not practical, and up to seven different fiber positions had to be used in each patient. The power density was kept below 250 mW/cm^2 to avoid thermal effects. As many of the total exposure times were quite long (up to 143 minutes), the fiber was positioned in a multijointed arm to keep its tip in the correct place over the area to be treated. Patients were given systemic analgesia and topical anesthetic with occasional sedation if necessary. Injected local anesthetic was limited to the site of biopsy.

Study Group 1 (seven patients) received a total light fluence of 200 J/cm^2 in two equal fractions. The first fraction was delivered 2.5 hours after the first dose of ALA, and the second was delivered at 4 hours.

Study Group 2 (11 patients) received a total fluence of 100 J/cm^2 in two fractions, with a minimum of one 5-minute interval between fractions. With the long treatment times required, it was sometimes uncomfortable for patients to keep their mouth open continuously, so in these cases there were further breaks during treatment.

In addition to the diagnostic biopsy specimen taken before patients were considered for inclusion in this study, further biopsies were performed immediately before PDT (for fluorescence microscopy to look at the tissue level of PpIX), 2 to 5 days after treatment to assess the depth of necrosis (slightly later in three patients because the treatment site was still too sore at 5 days, but before the treated area had healed), and subsequently when healing was complete and at long term follow-up to assess treatment results.

Patients were kept in a dark room for 24 hours after receiving ALA. Routine hematologic and biochemical investigations were performed before and 1 to 2 days after ALA ingestion (longer if any abnormalities were found).

Tissue Analysis

Biopsy specimens taken for fluorescence microscopy were oriented on cork discs using OCT medium (Tissue Tek 11 embedding compound, Miles Inc, Elkhart, Indiana), frozen, and stored in liquid nitrogen before study. Sections, 10 μm, were prepared using a Slee MHR cryostat (Mainz, Germany) and examined on an inverted microscope (IMT-2 Olympus, Olympus, Hamburg, Germany) with epifluoresence and phase contrast attachments.[21] Fluorescence was excited at 632.8 nm using an 8 mW Helium Neon laser (Aerotech Inc., Pittsburgh, PA) and detected using a highly sensitive cryogenically cooled, charged coupled device camera (Wright Institute, London, UK). A combination of bandpass (Omega Optical, Albany, VT) and longpass (Schott RG665) filters (Glenn Spectra, UK) was used to select a wavelength range of 670 to 700 nm for detection. The signal was then processed by an IBM PC computer into a false color-coded image, allowing quantification of the fluorescence intensity at the site of interest, in counts per pixel. Background correction was made using control biopsy specimens obtained before ALA administration under the same excitation and detection conditions. The sections were then fixed in formalin and stained with hematoxylin and eosin for comparative light microscopy studies. The follow-up biopsy specimens that were not required for fluorescence microscopy were fixed directly in 10% neutral buffered formalin and processed routinely in paraffin wax. Sections, 5 μm, were cut and stained with hematoxylin and eosin. With the aid of an eyepiece graticule, the depths of necrosis and inflammation were measured from the surface of the lesion. The depth of necrosis was defined as the deepest point at which there was necrosis of any tissue layer. The depth of inflammation was measured to the deepest point at which extravasated inflammatory cells were seen in the tissue.

RESULTS

Early Results and Depth of PDT Damage

Patients reported experiences ranging from mild discomfort to severe pain during the time of irradiation, in some cases requiring more analgesia than had been anticipated. Significant discomfort was experienced by 8 of 18 patients. Nausea and vomiting was experienced by six. Nausea was often an early feature presenting during the period of drug administration, whereas vomiting started several hours after ALA ingestion and resolved by 24 hours. Only one patient had cutaneous photosensitivity lasting longer than 24 hours, but this resolved by 48 hours. After PDT, the treated area became inflamed within a few hours. This was followed by sloughing of the superficial layers after 1 to 2 days, leaving a shallow ulcer. Oral analgesics were usually required from the first or second day after PDT for approximately 1 week. Excellent healing was found in all areas irrespective of the size of the original lesion, although larger lesions often took longer to heal (3 to 5 weeks). There was no evidence of scarring in those

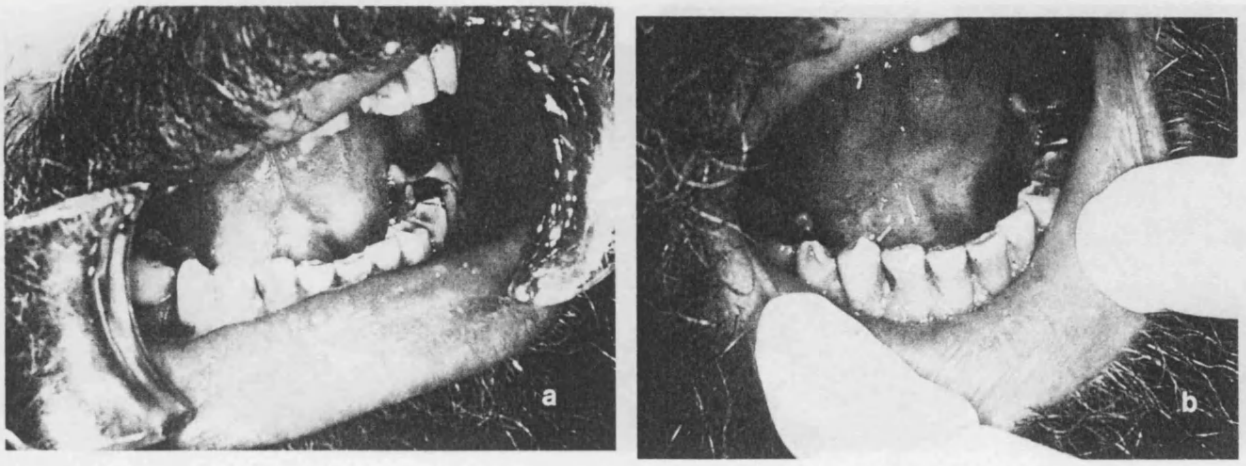

FIGURE 1. (A) Patient 8. Severe dysplasia of ventral tongue and floor of mouth. (B) Patient 8. Three weeks after photodynamic therapy with ALA. with normal mucosa.

lesions that had not undergone previous surgery, other than at the site where biopsy specimens were taken (Fig. 1). Once healed, patients did not perceive any changes in function or sensation within the oral cavity.

Study Group 1 (200 J/cm^2, 90 minutes between light fractions) consisted of three male and four female patients, with an age range from 46 to 85 years (mean, 61 years). Four had previously undergone surgery. There were two early or microinvasive squamous cell carcinomas (SCC), two severe dysplasias, and three moderate dysplasias. The depth of necrosis found in the four assessable early post PDT biopsy specimens varied from 0.2 to 0.6 mm (mean, 0.4 mm), although it was difficult to measure the absolute depths of necrosis because sloughing was present in all cases. Thus, the figures are likely to be underestimates. However, complete necrosis of the epithelial layer was found in all cases. The depth of inflammatory response varied from 0.8 to 2.5 mm (mean, 1.3 mm) and extended into the underlying muscle in two of four cases. In three cases it was not possible to measure the exact extent of damage due to the orientation of the specimen. Details are given in Tables 1–3.

Study Group 2 (100 J/cm^2, 5 minutes between light fractions) consisted of eight male and three female patients, with an age range from 51 to 87 years (mean, 69 years). Five had previously undergone surgery. There were four SCC (two invasive [patients 15 and 16] and two microinvasive [patients 17 and 18]), three severe dysplasias, and four moderate dysplasias. In this group, only 1 of 11 early post PDT biopsy specimens was not assessable. The depth of necrosis in this group varied from 0.1 to 1.3 mm (mean, 0.7 mm), but as in Group 1, all cases showed complete necrosis of the epithelial layer. The depth of inflammatory response ranged from 1 to 6 mm (mean, 1.8 mm). In three cases this extended into the muscle layer, in six into the submucosa, and for the remaining three, just into the mucosa. Details are given in Tables 1–3.

The numbers in each group were small, but there were no obvious differences and there was no statistically significant difference between the depth of necrosis or inflammatory response between the two groups (Wilcoxon matched-pairs signed-rank test). In view of this, the results of the two groups are considered together for further analysis.

Clinical Outcome

Seventeen of 18 patients were reassessed when healing was complete, and 13 underwent further biopsies at this stage (2 to 12 weeks after PDT). Long term follow-up data were available on all 18 patients, 12 of whom underwent a late follow-up biopsy. Those who refused repeat biopsies usually did so because the treated areas looked macroscopically normal. All except two patients underwent at least one biopsy after healing, and these were the two patients with invasive carcinomas who developed metastatic neck disease. Details of the specimens taken and the histologic findings are given in Tables 1–3.

All seven patients with moderate dysplasia improved after treatment, i.e., the mucosa became normal or only mildly dysplastic. However, in one case that only showed hyperplasia just after healing, moderate dysplasia was seen again 48 weeks later (Table 1). In four of five patients with severe dysplasia, there was no evidence of residual disease after treatment with 36 to 78 weeks of follow-up, although one refused biopsy at the long term follow-up. In the other patient, moderate dysplasia was seen at early and late follow-up (Table 2). It is notable that in three of the patients with regression but not clearance of dysplasia (Pa-

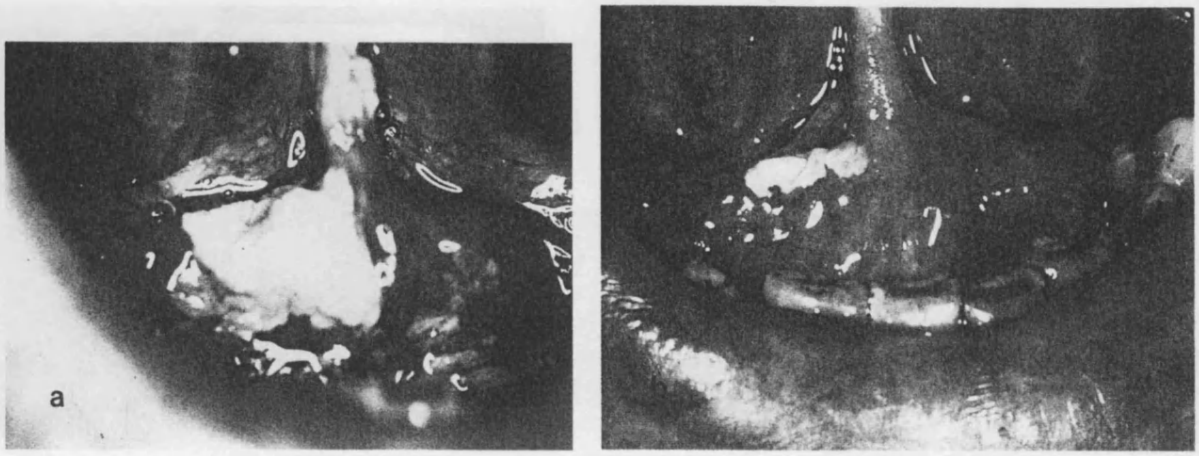

FIGURE 2. (A) Patient 17. Before PDT, with severely dysplastic lesion. (B) Patient 17. Four weeks after PDT with ALA, residual patch found to be microinvasive disease.

tients 1, 3, and 9), there was no macroscopic evidence of disease, the mucosal surfaces appearing clinically normal once the PDT-treated area had healed.

In two of six patients with SCC (Patients 13 and 14), no evidence of tumor was found following treatment, although one exhibited persistent mild dysplasia at 1 year. Patient 15 had local control of disease but subsequently developed metastatic neck disease. This patient had a 12-year history of recurrent oral cancer and precancer requiring multiple surgical procedures. Patient 16 initially had a local marginal mandibular resection and was treated with PDT when the specimen showed tumor at the margins. After PDT, only small nodules of tumor were visible locally, but she developed metastatic neck disease that was treated with radiotherapy and further surgery. Some months later, the patient had further local recurrence that was treated with PDT using metatetrahydroxyphenyl chlorin (mTHPC), but she then developed further tumor in the neck and died soon after. Patient 17 underwent an initial pre-PDT biopsy that showed just severe dysplasia. It was only after ALA PDT, when 88% of the mucosal lesion had cleared, that a biopsy was performed on the residual lesion and it was found to be microinvasive SCC with changes extending down the salivary ducts (Fig. 2). This suggested that PDT using ALA was able to deal with the severe dysplasia in this case but not the deeper invasive disease. As this patient was not suitable for surgery, further PDT was carried out using Photofrin with no evidence of tumor at 11 months follow-up. In the last patient (Patient 18), there was no reduction in tumor size. This patient also had a 10-year history of field change disease with recurrent SCC, so she received further PDT using Photofrin. This controlled the disease for a while, but further local recurrence has been treated recently with PDT using mTHPC.

No changes were noted in the hematologic indices, although elevation of liver enzymes was observed in nine cases compared with baseline values obtained before ALA ingestion. The enzyme most commonly affected was aspartate transaminase (AST). The AST level rose above the normal range in only five cases, (maximum, 3.5 times the upper limit of normal), with bilirubin elevated in only two. Monitoring of liver function was only continued more than 2 days after ALA ingestion in cases with elevated AST levels. In all cases this was asymptomatic and returned to normal levels within 10 days, with the exception of one patient in whom liver enzymes were elevated for 30 days—this patient had a history of excess alcohol intake. One patient developed a pruritic rash 1 week after treatment, which on biopsy was diagnosed as a cutaneous lichenoid reaction. This gradually resolved uneventfully over the following 3 weeks.

Fluorescence Microscopy

All biopsy specimens taken after ALA administration but before laser irradiation revealed maximum fluorescence in the epithelial layer (Fig. 3). In histologic sections containing normal and abnormal tissue, no difference could be detected in the fluorescence between normal and abnormal areas of epithelium. The fluorescence was, however, higher in these areas than in the underlying subcutaneous tissue, with a ratio of approximately 2–3:1. This correlated well with the histologic results which showed that the PDT effect was essentially limited to the epithelium.

DISCUSSION

PDT has considerable attractions for treating premalignant and early malignant lesions of the mouth. It is

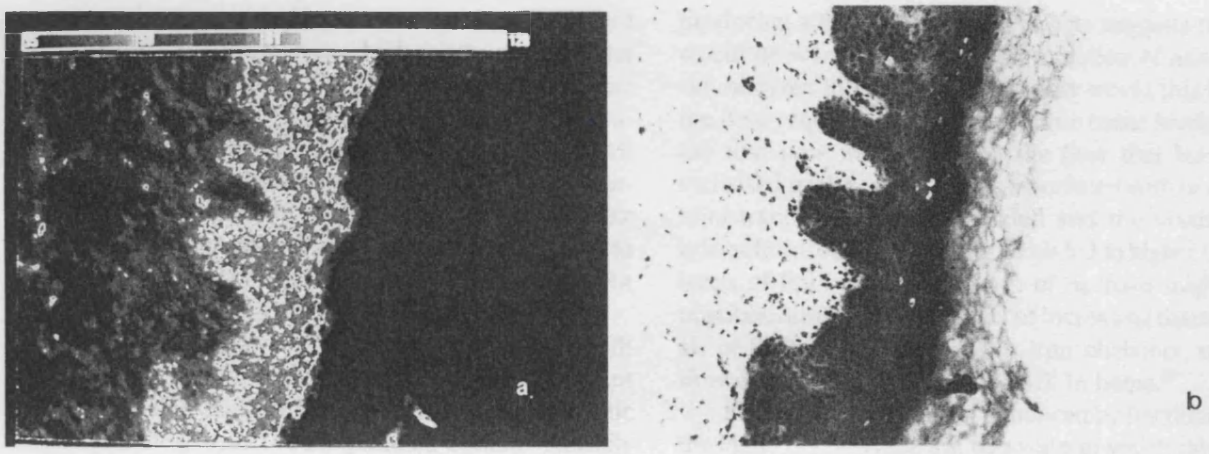

FIGURE 3. (A) Charged coupled device. False color-coded image showing maximal fluorescence in epithelium immediately before laser irradiation of lesion on the alveolus. (B) Corresponding H & E section showing moderate dysplasia.

relatively easy to apply, there is no cumulative toxicity, and healing is superior to other forms of local tissue destruction as the epithelium and lamina propria heal mainly with regeneration and only necrosed muscle is likely to scar. Against this must be balanced the problems of general skin photosensitivity and the fact that the red light used for treatment only penetrates a few millimeters into tissue, so only a small depth of tissue can be treated. To date, most studies have used the first-generation photosensitizer dihematoporphyrin ether (Photofrin),[10,22] and the results are encouraging, particularly for early cancers of the upper aerodigestive tract. Nevertheless, the data are largely empiric. The treatment parameters reported vary widely (drug dose, 1 to 5 mg/kg; light fluence, 50 to 1650 J/cm^2; and time from sensitization to irradiation, 48 to 72 hours), and few real attempts have been made to correlate the treatment parameters used with the depth of PDT necrosis produced and the depth of the lesion being treated. However, this is not an easy task. Grant et al.[23] measured the depth of PDT necrosis in a series of 11 patients with malignant and premalignant disease of the oral cavity. They showed that, despite using identical treatment parameters (2 mg/kg Photofrin and 50 J/cm^2 red light 48 hours later), the depth of microvascular damage and inflammatory response ranged from 4 to 12 mm.

In the present study using ALA, the depths of necrosis (0.1 to 1.3 mm) and of inflammation (0.8 to 6 mm) were also quite variable and considerably less than those seen with Photofrin. The depth of necrosis was often difficult to measure accurately as the time from PDT to biopsy varied. In most cases, some sloughing of necrosed tissue had taken place and a few lesions were partly healed at the time of biopsy. Nevertheless, two important conclusions could be reached. First, there was complete epithelial necrosis in every case and no necrosis was seen in muscle, although inflammation was seen in muscle in some cases. Second, the area of necrosis always corresponded to the area exposed to the light, whether that area was normal or abnormal (ulceration was sometimes also noticed in sites distant from the lesion, thought to be due to scattering or reflection of light from the primary target site). Thus, there was no selectivity of necrosis between abnormal epithelium and the normal epithelium from which the diseased tissue arose. In contrast, there was selectivity of necrosis between epithelium and the underlying muscle. This is consistent with our findings on fluorescence microscopy that showed the same level of active photosensitizer in normal and abnormal epithelium, but much lower levels in the underlying muscle.

Reviewing all results in the present series, the patients with dysplasia clearly did better than those with carcinomas. All 12 patients with dysplasia showed improvement after PDT. Ten patients (83%) showed clinically (macroscopic) normal mucosa when healing was complete, although of the nine that underwent biopsy at this time, one showed moderate and four showed mild dysplasia. It was of some concern that persistent dysplasia was seen in lesions that macroscopically had reverted to normal, although the changes in these areas were never more than moderate dysplasia. Even patients in whom the biopsy specimen was normal after PDT cannot be regarded as cured, but the risk of malignancy should have been reduced and they can be given PDT with ALA again if necessary for regression. One of our patients with moderate dysplasia had a normal biopsy specimen after PDT but subsequently regressed to moderate dysplasia again on check biopsy 1 year later. This is not surprising as one is dealing

with a field cancerization process and premalignant lesions of the mouth have a high recurrence rate after all forms of treatment.[24,25] Nevertheless, the risks are lower with milder degrees of dysplasia, and oral leukoplakia associated with mild dysplasia only has a 3% risk of malignant transformation.[6] PDT seems to downregulate the severity of dysplasia in these patients; as it can be repeated if necessary, it is reasonable to hope that their disease can be kept under control for many years with minimal morbidity.

Five of six patients with SCC had some benefit following ALA PDT, although the response was not as promising as with the more superficial dysplastic lesions. Only three of the patients became clinically free of tumor at the treatment site. This was confirmed histologically in two patients. The third developed neck nodes shortly after PDT, and a repeat biopsy was not performed at the treated site.

The maximum depth of necrosis seen in tumors was 1.3 mm, comparable to the results found by Regula et al.[16] in gastrointestinal tumors. The implications of this limited depth of necrosis were highlighted by Patient 17, in whom the PDT cleared the areas of severe dysplasia but revealed the part of the lesion where an invasive carcinoma had developed. Clinically, this was valuable because it meant that the area requiring further treatment with a different photosensitizer was much smaller than the original lesion, but it would be preferable if the PDT with ALA could produce a greater depth of necrosis.

There are various ways in which this might be achieved. Lofgren et al.[26] found necrosis up to 12 mm deep in a rabbit papilloma model, and Regula et al.[27] found 8 mm of damage in a tumor transplanted into the hamster pancreas. The main difference between these experiments and our clinical studies was the dose of ALA (50 to 200 mg/kg given intravenously [i.v.] for the papilloma model and up to 400 mg/kg orally, equivalent to 200 mg/kg i.v., for the pancreas study[15,27]). The maximum dose that patients can tolerate orally is 60 mg/kg,[16] which is equivalent to 30 mg/kg i.v. Recent work by Messmann et al.[20] in normal rat colon showed that it was difficult to get any PDT effect with 25 mg/kg ALA i.v. (equivalent to 50 mg/kg orally). With 50 mg/kg or more i.v., the PDT effect initially increased with increasing light dose and then reached a plateau at approximately 150 J/cm^2. It is difficult to extrapolate between rats and humans with regard to doses, particularly when discussing an agent that has to be metabolized to the active derivative in situ, but the doses are likely to be roughly comparable. This suggests that the threshold dose for obtaining a PDT effect is between 50 and 100 mg/kg by mouth. Thus, the maximum dose of ALA tolerated by mouth (60 mg/kg) may be only just above the threshold dose for producing a PDT effect. This strongly suggests that it would be worth preparing a formulation of ALA that can be given intravenously. Not only would this halve the dose required to achieve specific tissue levels, but the first pass metabolism in the liver that leads to increased transaminase levels associated with oral administration[16] would be avoided and the maximum tolerable dose may be higher. If this led to higher tissue levels of PpIX, a greater depth of necrosis might be possible. Another possible way of increasing tissue levels of PpIX is to pretreat with iron chelators, which slow down the conversion of PpIX to heme.[28]

PDT effects may also be enhanced by fractionating the light.[17,18,20,29] There are two ways in which this can be done, depending on the duration of the break between fractions. van der Veen et al.[17] showed, in a rat mammary carcinoma model, that PDT damage was increased after two fractions of 100 J/cm^2 with a recovery period of 75 minutes between irradiations, compared with a single treatment of 100 J/cm^2. They suggested that the break permitted more PpIX to be synthesized from ALA. However, this work has the problem that the total light dose was greater when two fractions were used, so that could have been the explanation of the enhanced effect. The other option is to use a much shorter break, as shown by Messmann et al.[20] They suggested that the break permitted reoxygenation of the tissue; therefore, when the light was applied for the second fraction, there was more oxygen available for a PDT effect. The time at which the break was made markedly influenced the effect (greater effect with an earlier break), but there was no advantage in having more than one break. It was not clear from this work whether fractionating the light increased the maximum area of necrosis achievable or just made it possible to achieve the same area of necrosis with a smaller total light dose. Another option is to reduce the fluence rate, which Gibson et al.[29] showed could increase tumor doubling time in a rat mammary carcinoma model, but this would increase already long treatment times.

The light fractionation regimens used in the present study were chosen on the basis of two possible mechanisms for enhancing the PDT, after the work of Grant et al.[14] and Regula et al.[16] showed such superficial necrosis. The number of patients treated was small and the depth of necrosis quite variable, but it is clear that there were no major differences between our results with either the short 5-minute break or the longer 1.5-hour break and the previous studies in which light was delivered without a break. From the Messmann et al studies,[20] it appears possible that we could have achieved the same clinical results with shorter treatment times using a 5-minute break. In view of the long treatment times required for some patients, this could

be a considerable advantage and will be tried in future patients.

The synthesis and evaluation of new photosensitizers continues to be an active area of research. Clinical trials of PDT with other second-generation photosensitizers have begun, with drugs such as mTHPC, mono-L-aspartyl chlorin e6 sodium, tin-ethyl etiopurin, and aluminum disulfonated phthalocyanine. These have the advantage over Photofrin and ALA of major absorption peaks at wavelengths greater than 650 nm, with consequently deeper penetration of light into tissue. With mTHPC, the light dose required may be as low as 10 J/cm,^{2} with resultant shorter irradiation times—a definite clinical advantage.[30] With these other photosensitizers, the effect produced can go deeper into tissue than with ALA, but the price to pay is possible scarring in the underlying muscle, as they do not show the selectivity between epithelium and muscle that is seen with ALA. Skin photosensitivity may also be less severe or clear faster than with Photofrin, although none clears the skin within 2 days as is seen with ALA. The convenience of the short cutaneous photosensitivity using ALA means that PDT can be repeated at short intervals if necessary.

Conventional treatments for leukoplakias and other premalignant lesions of the mouth include excision by scalpel, diathermy knife, or carbon dioxide laser.[31,32] However, these may require general anaesthesia, possibly leave some scarring, and are no more curative than PDT. Local therapy cannot cure multiple extensive lesions or treat field carcinogenesis,[33] and patients remain at risk of developing further disease. Chemoprevention with vitamin A and analogues has been investigated over the past 30 years, but although studies have shown regression during treatment, systemic toxicity and recurrence after cessation of therapy are problems.[9] PDT using ALA can deal with superficial lesions with healing by regeneration of normal mucosa while preserving underlying tissue and function. These results need collaboration in larger series of patients, but as the method has minimal systemic toxicity and no known cumulative toxicity, it is looking very promising in this field. The more powerful photosensitizers such as mTHPC should be seen as complimentary to ALA for treating more invasive tumors, especially if such lesions are revealed after treatment of more extensive areas of dysplasia with ALA PDT.

CONCLUSION

We conclude that PDT using ALA produces consistent epithelial necrosis with excellent healing in dysplasia of the mouth and is a simple and effective way of managing these patients. Results in invasive cancers are less satisfactory, mainly because the PDT effect is too superficial with current treatment regimens. This may be improved by using different regimens or other, more powerful photosensitizing agents.

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Photodynamic therapy on normal rabbit mandible

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ABSTRACT

Photodynamic therapy has been proposed as an intra-operative adjunct to surgical resection of tumours invading bone. To assess this, we studied the effects of PDT in normal bone. Forty-four rabbits were sensitised with Photofrin 3mg/kg, 5-aminolaevulinic acid[ALA] 400mg/kg, or meso-tetrahydroxyphenylchlorin [mTHPC] 0.3mg/kg. A mandibular incisor was removed and the socket irradiated with a cylindrical diffusion fibre (630 nm Photofrin and ALA, 650 nm mTHPC, 100J per treatment. Irradiation was given 1 or 48 hours after Photofrin , 72 hours after mTHPC, whilst 2 doses were given 2.5 and 4 hours after the 1st fractionated dose of ALA. The socket of the ipsilateral maxillary incisor was used as a non irradiated control to assess healing without PDT. Other controls assessed healing after irradiation of unsensitised animals. Rabbits were killed 3,10,21 days after treatment. Tooth socket healing appeared to be the same in all groups of animals with evidence of woven bone formation by 10 days. We conclude that PDT is unlikely to have any effect on healing in normal bone, which makes it suitable for treating tumours invading bone.

Keywords: photodynamic therapy, ALA, Photofrin, mTHPC, bone

1. INTRODUCTION

Carcinomas of the head and neck account for approximately 4% of all cancers and are the 6th most common cancer worldwide[1]. Despite recent advances in the management of head and neck cancer, survival rates have remained poor. Local control of the disease is a prerequisite for successful management since dissemination is generally a late feature. In addition, general health of this group of patients is often compromised making intensive therapy difficult.

The use of PDT in the management of head and neck cancer has been investigated[3,4], and has been suggested as an intra-operative adjunct to surgical resection of head and neck cancer, since it offers the potential advantage of selectively destroying tumour whilst preserving the adjacent normal tissue. If PDT can be used to eradicate carcinomas of the head and neck, it is important to determine its effect on uninvolved tissues.

The aim of this study was to examine the effects of PDT on bone healing in an animal model, using photosensitisers currently used in clinical trials and with treatment values known to produce tumour necrosis.

2. MATERIALS AND METHODS.

Forty-four adult female New Zealand white(NZW) rabbits were divided in to four treatment groups and controls. Thirty-six rabbits were sensitised with Photofrin (3mg/kg), 5-aminolaevulinic acid [ALA] (400mg/kg), or meso-Tetrahydroxyphenylchlorin [mTHPC](0.3mg/kg). The ALA was fractionated into three equal doses and given via gavage over two hours, Photofrin and mTHPC were administered intravenously. A mandibular incisor tooth was removed and the socket irradiated using a cylindrical diffusion fibre. Light wavelengths of 650 nm were used with mTHPC, 630 nm with both Photofrin and ALA . Irradiation was given at 1 or 48 hours after Photofrin, 72 hours after mTHPC, whilst 2 doses were given at 2.5 and 4 hours after the 1st fractionated dose of ALA .The total energy given per irradiation dose was 100J. The socket of the ipsilateral maxillary incisor tooth was used as the non irradiated control for the effect of sensitiser along with tooth extraction. Eight further controls assessed for healing after irradiation of unsensitised animals and for extraction only. The rabbits were then sacrificed at 3, 10 and 21 days after treatment.

Following sacrifice the maxillae and mandibles were harvested from the animals. Decalcified tissue sections were

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obtained using longitudinal sections taken through the tooth socket followed by routine histological processing and staining with haematoxylin and eosin .The microscopic tissue sections were analysed for the presence of haematoma, granulation tissue, inflammatory response, osteoid and woven bone formation. Each category was semi-quantitatively scored on a 0-3 scale. For woven bone formation a score of '0' indicated the absence or minimal presence of woven bone, a score of '1' indicated the presence of woven bone along the socket margin, a score of '2' indicated that the socket was half filled with new bone and a score of '3' indicated that the socket was at least 3/4 or more filled with bone.

3. RESULTS

Histological examination of decalicified tissue sections showed initial bone healing was retarded in the PDT treated groups as compared to the groups exposed to sensitiser alone. At ten days bone formation was similar for both PDT treated groups using all three sentistisers and in the group receiving the sensitiser alone. By 21 days there was complete filling of tooth sockets in all groups. Control tissues for bone healing using laser light at 630 nm and 650 nm and in the group that underwent tooth extraction alone were similar showing early bone formation within sockets by three days.

4. DISCUSSION

Despite advances in surgical and radiotherapeutic management of head and neck cancers, survival rates have remained poor for this form of cancer and have not improved over the last three to four decades. One of the principal difficulties associated with the treatment of cancer of the head and neck is control of local disease. Particularly problematic is the management of cancer invasion into hard tissues that often necessitates mutilating surgery.

PDT offers a potentially powerful alternative to the conventional management of head and neck cancers, facilitating selective eradication of tumour whilst preserving adjacent normal tissues. An earlier study from our group found that the photosensitiser Aluminium disulphonated phthalocyanine (Al-S2Pc) coupled with PDT had a negligible effect on bone healing in an animal model[2]. By contrast, this current study has examined the effects of PDT on bone healing using three photosensitisers in current clinical use, along with the use of a cylindrical diffusion fibre thus allowing for a more uniform distribution of light along the socket. We found that the effects of the photosensitisers, Photofrin, ALA and mTHPC were similar causing initial retardation of bone formation by three days followed by normal healing rates by ten days compared with controls. This suggests that the effects of these photosensitisers coupled with PDT are only transiently inhibitory to osteoblastic activity.

Results from this study show that PDT using these photosensitisers has negligible effect on bone , therefore may have a potential role the management of head and neck cancer which are adjacent to or involve bone.

4. ACKNOWLEDGEMENT

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Photodynamic therapy using 5-aminolaevulinic acid in oral malignancy and premalignancy

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ABSTRACT

17 patients with oral cancer and precancer were treated with photodynamic therapy using systemic 5-aminolaevulinic acid. A brisk PDT response was noted in all patients but damage was confined to the epithelium on histological evaluation.

Keywords photodynamic therapy, aminolaevulinic acid, oral cancer.

2.INTRODUCTION

Photodynamic therapy using the naturally occurring photosensitizer 5-aminolaevulinic acid (ALA) as a precursor for protoporphyrin IX (PPIX) is a promising treatment for early squamous cell carcinoma of the oral cavity. Previously we have shown that PPIX fluorescence builds up in the epithelial layer making this an ideal treatment for intraepithelial disease (1). This study was undertaken to evaluate the histological effects of PDT in the treatment of oral dysplasia and early invasive squamous cell carcinoma.

3.PATIENT POPULATION

Seventeen patients (8 male 9 female, age range 31-84 years) were included in the study. There were 9 patients with squamous cell carcinoma, 7 with dysplasia and 1 other tumour.

4.METHOD

All patients received oral ALA, 60 mgs/kg in three divided doses and were then photoirradiated at 2.5 and 4 hours after the first dose with monochromatic red light of 628nm. The light dose was 100 J/sq cm for each treatment period delivered by a gold vapour laser. Patients were monitored clinically and biochemically and follow up biopsies were performed at 2-4 days post treatment.

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5.RESULTS

A characteristic PDT effect was seen of swelling at 12 hours, ulceration within 72 hours and healing with no detectable scarring by 4 weeks. Nausea was seen in approximately half the patients and although liver enzymes were commonly elevated, these returned to normal in 16 patients within 3 days and within 3 weeks in the remaining patient.

Histological features showed consistent full thickness epithelial necrosis in all cases and where it was possible to measure the depth of effect, this was 0.86mms (range 0.15 - 1.8mms). There was no evidence of any vascular damage and no suggestion of selectivity in the PDT effect.

6.DISCUSSION

It is a little disappointing that a greater depth of effect could not be obtained although, given the concentration of fluorescence in the epithelium, it is hardly surprising.

It seems likely that the oral route of administration is one limiting factor, although a fall in tissue oxygen tension and photobleaching may also be important. The treatment parameters may also require further investigation, especially the time of treatment and possibly the light wavelength.

7.CONCLUSION

From these investigations it would appear that PDT using systemic ALA is of value only in the treatment of intraepithelial disease.

8.ACKNOWLEDGEMENTS

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Short Communication

Photodynamic Therapy and Lip Vermilion Dysplasia: a Pilot Study

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PHOTODYNAMIC THERAPY (PDT) selectively destroys dysplastic skin or mucosa without scarring [1], and may be the ideal treatment for early tumours of the lips. One patient with a T_1 lip carcinoma and 3 patients with lip vermilion dysplasia were sensitised by infusion of Photofrin (2 mg/kg)*.

After 48 h, target lesions were illuminated by timed and measured pumped-dye laser light (wavelength = 628 nm). The first patient received 50 j/sq cm with a 100 mW beam, whilst the patients with dysplasia were treated with 400 mW, to give 100 j/sq cm in a shorter period, i.e.

$$\text{Energy(j/sq cm)} = \frac{\text{Beam power(j/s or watts)} \times \text{t(s)}}{\text{Spot area (sq cm)}}.$$

Postoperative discomfort was controlled with oral ibuprofen, and topical chloramphenicol ointment applied to prevent infection. Microbiopsy specimens were obtained postoperatively in the 3 cases of vermilion dysplasia. The same pathologist (PMS) examined the dysplastic lesions before and after PDT, and the results were collated (see Table 1). Natural porphyrin photosensitisers are "accumulated in tumor tissue after exogenous administration or endogenous synthesis" [2]. Prospective second generation sensitisers are activated by longer wavelength light than Photofrin (over 650 nm), conferring a gain in treatment depth with shorter duration of photosensitivity [3, 4]. PDT has direct tumoricidal action and a potent indirect effect on microvasculature causing rapid ischaemic necrosis [2, 4]. PDT photosensitisers cause dysplastic tissue to fluoresce when illuminated, and be more accurately targeted for phototoxic ablation [3] (Spectraphos CCD camera†). Although experimental in head and neck oncology, Photofrin/PDT is licensed in Canada for the prophylactic treatment of bladder cancer recurrence.

There is no evidence that PDT is mutagenic [5], although the mechanism of primary DNA disruption leading to mutation in solar cheilitis [6, 7], is very likely to be free radicals produced by the interaction of UV irradiation (295–400 nm), and native porphyrin. In PDT, the exposure parameters, both wavelength and porphyrin concentration, exert a lethal effect, rather than a genetic change [3, 4].

With preservation of connective tissue scaffolding, PDT ulceration heals without scar formation, if infection can be avoided. This is important where surgery would cause cosmetic or functional morbidity, such as the lips and periorbital skin. With primary treatment and biopsy monitoring controlled by a photo-detection camera, PDT may revolutionise current treatment.

A tingling sensation rather than pain was felt during PDT treatment of lip vermilion. A beam power of 400 mW illuminated a 16 mm diameter spot and produced a surface irradiance of 189 mW/sq cm. Hyperthermia does not contribute to tissue damage if irradiance is kept below the 200 mW/sq cm threshold [8]. Zhao *et al.* [9] measured the temperature of the lip skin during PDT and found that the surface temperature only began to exceed the core temperature when the irradiance power exceeded 400 mW/sq cm. Zhao's treatment durations were much greater (1800 s). Our patients developed painful ulcers after a few days, but healing without noticeable scarring was delayed up to 4 weeks. Biopsy, after PDT, demonstrated histological improvement in the degree of dysplasia. However, a normal clinical appearance could not be relied upon to exclude dysplasia. This finding agrees with Grant *et al.* [1], who treated intraoral cancers but found the native target mucosa still dysplastic, albeit up-graded histologically. Regular monitoring was necessary to anticipate the need for further intervention.

Presumably, PDT can be repeated, although there is evidence that repeated exposure to the porphyrin photosensitiser induces resistant cells [10, 11]. This might be overcome by using alternative photosensitising agents.

The macroscopic and microscopic appearance (see Figs 1 and 2) of dysplastic vermilion demonstrated a desirable response to PDT. Supporting a proposed mechanism of healing by replenishment from neighbouring epithelium, the normal-looking treated vermilion was shown, histologically, to be dysplastic, although up-graded.

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*Photofrin QLT DP 73—A mixture of porphyrins purified by gel filtration chromotography of haematoporphyrin derivative (Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York 10965, U.S.A.).
†Spectraphos CCD cancer photo-detection camera (Ideon S-223 Lund, Sweden).

Table 1.

History	Pre-biopsy	Dosimetry	Post-biopsy
Male 82 years. Smoker. Chronic lip ulcer (2 years). Radiotherapy for previous SCC (mandible).	Severe dysplasia.	Two spots, 10 mm, 12 mm, 100 j/sq cm, 400 mW, 200 s, 294 s.	Moderate dysplasia (4 months).
Male 51 years. Pipe smoker. Leucoplakia in lip angles. Diabetic.	Severe dysplasia.	Two spots, 16 mm, 16 mm, 100 j/sq cm, 400 mW, 510 s, 510 s.	(R) Angle no dysplasia at 6 months. (L) Angle moderate dysplasia at 4 months.
Female 67 years. Non-smoker. Chronic ulcer of lower lip (1 year).	Moderate dysplasia. Actinic cheilitis.	Four spots, 12 mm each, 100 j/sq cm, 400 mW, 294 s.	Mild dysplasia at 2.5 months.
Male 39 years. Pipe smoker. Keratosis of lower lip (4 years). SCC (lip) excised 10 years ago.	Well-differentiated squamous cell carcinoma (superficial).	One spot, 14 mm, 50 j/sq cm, 100 mW, 769 s.	Clinically cured at 12 months (biopsy refusal).

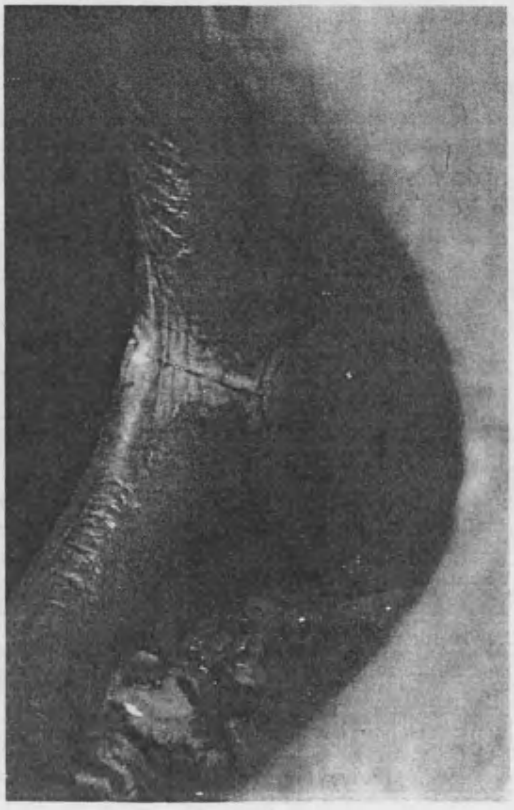

Fig. 1. Severe dysplasia pre-PDT.

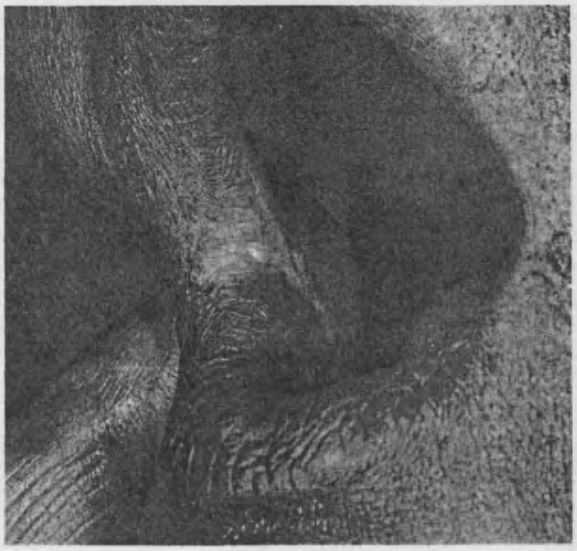

Fig. 2. Normal mucosa post-PDT.

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