ENHANCEMENT OF SELECTIVITY FOR PHOTODYNAMIC THERAPY

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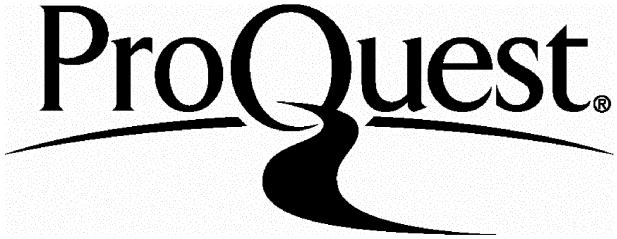

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Abstract

Photodynamic Therapy (PDT) is a technique for producing localised tissue damage with low power light following prior administration of a photosensitising drug. The promise of PDT has been based on the selective retention of photosensitisers by tumours, but this aspect has been over-emphasised with a maximum ratio of photosensitiser concentration of 3:1, tumour to normal, for extracranial tumours and current drugs. This makes selective tumour necrosis difficult to achieve. This thesis explores ways in which selectivity may be improved.

Aluminium sulphonated phthalocyanine (AlSPc) has better photochemical properties than the widely used HpD and Photofrin II, but has the same tumour selectivity, although the ratio was improved marginally using its disulphonated component. However, when used in conjunction with the radioprotective drug W7, in a rat colon cancer model, tumour necrosis was the same as without W7 while there was no damage to adjacent normal colon.

A radical new approach is to give 5-aminolaevulinic acid (ALA) which induces endogenous production of the photosensitiser protoporphyrin IX. This improves selectivity in the rat colon cancer to 6:1 (tumour to normal mucosa), but also sensitises the mucosa selectively compared with the underlying muscle (10:1), giving a tumour to muscle ratio of 60:1. This has enormous potential for treating small tumours or areas of dysplasia in a range of hollow organs. ALA also has the major advantages of a short optimum drug to light time (typically 4-6 hours), short duration of skin sensitivity (approximately 24 hours) and it can be given orally with minimal systemic toxicity.

This work has also shown *in vitro* that PDT with AlSPc sensitisation can kill *helicobacter pylori* at doses unlikely to affect gastric mucosa.

In conclusion, by careful choice of photosensitising agents and treatment regimes, it is possible to limit PDT effects to abnormal tissues, and even if there is some normal tissue damage, in most cases, this heals without significant sequelae. In the gastrointestinal tract, PDT may have a role in the eradication of small tumours unsuitable for surgery, in the treatment of dysplasia and possibly even in the management of conditions related to *helicobacter pylori* infection. There is now sufficient information to start clinical trials with ALA.

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LIST OF ABBREVIATIONS

ALA	Aminolaevulinic acid
AlPc	Aluminium phthalocyanine
AlSPc	Aluminium sulphonated phthalocyanine
AlS_1Pc	Aluminium monosulphonated phthalocyanine
AlS_2Pc	Aluminium disulphonated phthalocyanine
AlS_3Pc	Aluminium trisulphonated phthalocyanine
AlS_4Pc	Aluminium tetrasulphonated phthalocyanine
CCD	Charged-coupled device
CuPc	Copper phthalocyanine
DHE	Dihaematoporphyrin ether
DMH	Dimethylhydrazine
DMSO	Dimethylsulphoxide
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
H & E	Haematoxylin and Eosin
H. influenzae	Haemophilus influenzae
H. mustelae	Helicobacter mustelae
Hp	Haematoporphyrin
HpD	Haematoporphyrin derivative
H. pylori	Helicobacter pylori
HSV	Herpes simplex virus
HVG	Haematoxylin and Van Gieson's
IH	Intimal hyperplasia
LDL	Low density lipoproteins
MIC	Minimum inhibitory concentration
MSPc	Sulphonated metallophthalocyanine
P. acnes	Propionibacterium acnes

Pc	Phthalocyanine
PDT	Photodynamic therapy
PP IX	Protoporphyrin IX
TPPS	Tetraphenylporphinesulphonate
W7	WR-77913
ZnPc	Zinc phthalocyanine
ZnSPc	Zinc sulphonated phthalocyanine

CHAPTER 1 - AN INTRODUCTION TO PHOTODYNAMIC THERAPY

1.1. Basis of photodynamic therapy

Photodynamic therapy (PDT) is an experimental modality that uses the interaction of light and a photosensitising agent to destroy tissue, generally tumour tissue. Destruction of cellular components occurs in the presence of oxygen through the photosensitised production of cytotoxic species within the target tissue. PDT is traditionally considered a method of treating tumours utilising the selective retention of the photosensitiser in the neoplastic tissue and it is this phenomenon which has been considered to offer an advantage over conventional modalities for treating tumours. However, selectivity of this treatment is far from being universal and there are many complicating factors.

1.2. History of PDT

The history of the development of photodynamic therapy can be divided into three main sections. The first covers the initial discovery of the 'photodynamic action' of light; secondly, the localisation and detection of porphyrins in tumours; and, finally the combination of activating light and porphyrin to perform photodynamic therapy of tumours.

1.2.1. The discovery of photodynamic action

The development of PDT is considered to have begun with Niels Finsen who discovered that he could use light from an arc lamp to treat a tubercular condition of the skin called lupus vulgaris. In 1903 he was awarded a Nobel prize for his work. As early as the beginning of this century Raab had noticed the lethal effects of light on paramecia in the presence of acridine (Raab, 1900). Shortly after that, the first use of the photodynamic concept of tumour irradiation following administration of a photosensitising drug was reported by Jesionek and von Tappeiner (1905). They

used topically applied eosin with sunlight exposure to treat skin cancer and this resulted in some improvement in most cases. Hausmann discovered that the addition of haematoporphyrin (Hp) in slightly alkaline solution to cultures of the protozoa Paramaecia rendered them sensitive to light, so they were killed by bright daylight (Hausmann, 1908). Furthermore he found that parenteral administration of Hp to mice was well tolerated if the animals were kept in darkness, but that they succumbed to severe shock in a few hours when exposed to bright light (Hausmann, 1911). In 1913 Meyer-Betz performed this same experiment, but on man. He intravenously injected himself with 200 mg/kg of Hp and reported on the effects. After exposing himself for a short time to the sun he developed pricking and burning of the exposed areas of his skin, followed by erythema, oedema and pain. The photosensitivity remained for over 6 weeks (Meyer-Betz, 1913). These experiments showed that PDT could damage normal tissues.

1.2.2. Tumour detection using porphyrins

A report by Policard (1924), showed spontaneous fluorescence in experimental tumours when exposed to light from a Woods lamp. This fluorescence was attributed to an accumulation of porphyrins in neoplastic tissue from haemolytic bacterial infection. Figge *et al.* (1948) reported the observation of the tendency for i.p. injected Hp to accumulate in neoplastic tissues of mice, in addition to embryonic and traumatised tissue, and investigated this as a means to improve existing methods of tumour detection and therapy. They found that the Hp accumulated in tumour within a period of 24 to 48 hours and that normal tissues did not concentrate the Hp, with the exception of the liver, lymph nodes and the greater omentum. The red fluorescence of the tumour remained high for 10 to 14 days. Mesoporphyrin and coproporphyrin were also studied and shown to concentrate in neoplastic tissue, although the fluorescence was not as great. This group then gave a series of patients an i.v. dose of between 300 and 1000 mg of Hp and demonstrated red fluorescence in lymphatic and cancer tissues; it was suggested that red fluorescence of Hp and its

tendency to accumulate in tumours could be used to assist the surgeon to visualise neoplastic tissue during operations (Rassmussen-Taxdal *et al.*, 1954). It was reported that no toxicity was observed after introduction of the Hp. Winkelman *et al.* (1960) reported the first attempt to quantify the porphyrin in tumour tissue in the rat thigh compared to normal thigh muscle. No porphyrin could be detected in normal thigh muscle at all Hp doses and at all times of sacrifice after porphyrin injection, but no other normal tissues were examined. At 24 hours after an i.p. injection of 160 mg/kg a tumour concentration of up to 10 μg/g was observed. Schwartz *et al.* (1955) pointed out that the Hp product commercially available contained a mixture of porphyrins with varying localising properties that in some cases exceeded those of pure haematoporphyrin. This awareness led Lipson *et al.* (1960) to develop the derivative of Hp that enhanced the amount of tumour localising component in the clinically used drugs. They treated Hp with acetic and sulphuric acids and collected the precipitate after neutralisation with sodium acetate. This precipitated derivative was further alkalinised with sodium hydroxide and neutralised with hydrochloric acid to make it physiologically compatible with the biological system. Utilising this derivative of haematoporphyrin, or HpD, and by greatly improving both activation and detection of Hp fluorescence, using filter systems, Lipson *et al.* (1961a) were able to reduce the dye concentration to 50 mg/kg, thus reducing photosensitivity from the porphyrin. This detection system was then considered sufficiently promising to use in patients undergoing bronchoscopy or oesophagoscopy for known, or suspected, malignancies. After studying 15 patients, given an i.v. dose of 2 mg/kg HpD approximately 3 hours before endoscopy, neither false-positive nor false-negative results developed in patients where enough of the activating light reached the region of involvement (Lipson *et al.*, 1961b). A further series of 35 patients were reported (Lipson *et al.*, 1964) and were given 1.5 mg/kg HpD. It was found that as well as the activating light reaching the involved region in sufficient intensity it was also necessary to avoid bleeding as blood absorbed the activating light and if the bleeding was excessive it was possible that no fluorescence could be observed. They

concluded that due to skin photosensitivity they could not advocate the use of this technique in all endoscopic procedures, but it was of use whenever there was a problem in the detection of malignant disease or whenever there was a question of whether or not a known lesion was too extensive to be helped by an operation. Gregorie *et al.* (1968) using a slight modification of that described by Lipson *et al.* (1961b) administered i.v. HpD to 226 patients to study fluorescence of various lesions. They found that with the state of development, at that time, the technique was unreliable as a means of diagnosis of malignant tumours, although, the technique in some instances proved useful for assessing the margins of squamous cell carcinomas and in locating obscure primary malignant sites. They concluded that further studies were needed to elucidate the mechanisms of the photoreactions and to provide technical refinements in the clinical applicability of HpD fluorescence for identification of malignant disease.

Long *et al.* (1962) when studying the evaluation of radioactive compounds for the external detection of cerebral tumours found the ratio of cobaltous-Co^{57} tetraphenylporphinesulphonate (TPPS) in tumour to normal brain was 60 to 1. TPPS, although of mixed sulphonation, was used due to its stability and its homogeneity, and therefore the variability and impurities encountered in the biological porphyrins such as haematoporphyrin or protoporphyrin were avoided. The specific tumour localisation observed was considered to be due to passive diffusion of TPPS from the blood stream to tumour bed through a breakdown in the blood brain barrier. Winkelman and Hayes (1963) went on to study the distribution of endogenous porphyrins and TPPS administered parenterally 24 hours before sacrifice in tumour transplanted in the rat. Normal liver, kidney and spleen were also investigated. They found that the red fluorescence used to detect the TPPS did not always correlate with the extracted tissue TPPS concentration. The Co^{57} TPPS was then introduced into patients with malignant tumours (Winkelman *et al.*, 1964). Patients with cerebral and visceral tumours were studied by scintillation scanning. It

was found that cerebral tumours could be successfully demonstrated, whereas tumours in other locations could not be observed. They proposed that this failure of the radioisotope to localise in tumours to the same extent as TPPS was due to the dissociation of the radiolabel from the TPPS. Barker *et al.* (1970) investigated the combination of various porphyrins with rat tissues *in vivo*. A number of porphyrins with varying peripheral groups and solubilities were synthesised. By chemically shielding or rearrangement of particular groups, their importance in the localisation in tissues was investigated. The correlation between tissue uptake and the photosensitisation which followed the introduction of the porphyrins was also investigated. It was found that for localisation in tissue there was a requirement for groups capable of ionic or hydrogen bonding at more than one edge of the tetrapyrrole nucleus. All soluble porphyrins tested induced a cutaneous photosensitivity on i.p. injection.

1.2.3. Photodynamic therapy

From the previous studies showing that many neoplasms were capable of taking up and retaining porphyrins it was considered reasonable that accumulation of haematoporphyrin in a malignant tumour would result in sensitisation of the tumour which could then be destroyed by visible light. Diamond *et al.* (1972) demonstrated that a combination of haematoporphyrin and light treatment was lethal to glioma cells in culture and produced massive destruction of porphyrin-containing gliomas transplanted subcutaneously in rats. Dougherty (1974) used fluorescein and visible light as a means of destroying tumour tissue. They found retardation, or temporary arrest in growth of a mouse mammary carcinoma implanted subcutaneously in albino mice. Surrounding normal tissue effects were not documented. However, Dougherty *et al.* (1975) showed 50 % of tumours could be cured by PDT with HpD with essentially no damage to normal skin within the light field. Kelly and Snell (1976) were the first to report treatment of human tumours using PDT. They had previously shown that human bladder carcinoma grown in immunosuppressed mice was more

sensitive to photodynamic therapy than normal bladder tissue (Kelly *et al.*, 1975). Therefore, a study was undertaken to determine whether HpD was taken up preferentially by human bladder carcinoma and to discover whether such tumours could be destroyed selectively by PDT. The distribution of HpD was studied in patients undergoing transurethral resections and total cystectomy for carcinoma of the bladder. Although HpD fluorescence was not confined to malignant tissues with the dose used, there was preferential localisation in malignant and pre-malignant epithelium. Also high intensity illumination of an area of HpD sensitised carcinoma caused tumour destruction. Therefore, it was suggested that HpD could be used as an aid to the diagnosis and treatment of carcinoma of the bladder. Since 1976 many patients with various malignant lesions have been treated with PDT and this is discussed in section 1.4.

1.3. Principles of PDT

The basic biological and photochemical principles are examined in this section. The photobiology is still inadequately understood even though an ever increasing number of patients are being treated with this therapy.

1.3.1. Porphyrins as photosensitisers

The ideal properties of a photosensitiser can be summarised as follows; it should be red or near infrared light absorbing; non-toxic; selectively retained in tumours relative to normal adjacent tissue, with low skin photosensitising potency; an efficient generator of cytotoxic species, usually singlet oxygen; fluorescent for visualisation; of defined chemical composition, and preferably water soluble.

The most commonly used sensitiser in clinical therapy is a complex mixture of porphyrins, derived from haematoporphyrin and termed haematoporphyrin derivative (HpD) (Berenbaum *et al.*, 1982; Kessel, 1984). HpD (also known as Photofrin I) is not an ideal photosensitiser. It has only a weak molecular absorptivity in the red region of the spectrum where tissue penetration is high. Other disadvantages relate to tissue selectivity of photosensitisation. HpD sensitises skin so that patients must avoid strong light for some weeks after treatment (Moan *et al.*, 1987). HpD also sensitises other normal tissues, which compromises its use against tumours of certain sites, especially brain where any normal tissue which undergoes damage will not regenerate. There is also difficulty in obtaining a between batch reproducibility in composition of photosensitiser. HpD is an unsatisfactory mixture of compounds, consisting in a large part of materials inactive *in vivo*, and much work has been directed at isolating the fractions within this mixture that are the active components for PDT. HpD localising components are poorly fluorescent, hydrophobic, high-molecular weight aggregates whose main constituents are considered to be either a dimer or an oligomer between haematoporphyrin units (Berenbaum *et al.*, 1982). Pure Hp is not selectively retained in tumours; however most commercial

preparations of Hp contain high-molecular weight components, probably similar to those in HpD, and these appear to be the effective photosensitisers in PDT, while pure Hp is ineffective (Dougherty, 1983).

Gel filtration for fractionation of HpD yielded a partially purified fraction consisting of the HpD components from the original mixture which were primarily responsible for the photosensitising properties (Dougherty, 1983). This active fraction was characterised as dihaematoporphyrin ether, or DHE (also known as Photofrin II), an aggregated mixture of Hp molecules linked by ether and/or ester bonds (Dougherty, 1984). It is claimed that this preparation is a more potent tumour sensitiser than HpD and in therapeutically equivalent doses has less skin sensitising activity. However, this material shares the main disadvantages of HpD, that is, incompletely defined composition with weak red light absorption.

1.3.1.(A) Proposed mechanisms for selective uptake of porphyrins

Many studies have been performed with various mixtures of haematoporphyrin and its derivatives. There is often some degree of selectivity of uptake of these drugs by tumours over normal tissue and the reasons for this have not been fully elucidated. It has been established that after injection HpD accumulates in tumour cells and the ratio of the concentrations of HpD in tumour and normal cells depends on time. The tumour localising ability of [14C]- and [3H]haematoporphyrin derivative in mice bearing transplantable tumours has been studied (Gomer and Dougherty, 1979). The amount of both forms of HpD which localised in the tumour after an i.p. injection of 10 mg/kg was higher than in skin and muscle tissue but was less than in liver, kidney or spleen. These results were considered to disprove the generalisation that HpD accumulated in malignant tissue to a higher degree than in all normal tissue. After an i.p. injection of 5 mg/kg [^{14}C]PII normal tissues take up the drug within about 7.5 hours after administration, with order of highest concentration being liver, adrenal gland, bladder > pancreas, kidney, spleen > stomach, bone, lung, heart > muscle >>

brain. Only skeletal muscle, brain, and skin located contralaterally to subcutaneously implanted tumour had a peak [^{14}C] activity lower than tumour tissue, with skin overlying tumour showing concentrations not significantly different from tumours. After 75 days all tissues examined retained some of its [^{14}C] activity (Bellnier *et al.*, 1989). Various authors give contradictory data on the time after which the tumour to normal ratio reaches a maximum and this is considered to be due to different authors using different mixtures of porphyrins with varying compositions and different tumour models, as well as differences dependent on porphyrin assay technique. HpD does not only accumulate in tumours but also regenerating tissue. Selman *et al.* (1985a) showed accumulation in injured normal bladder mucosa. Porphyrin fluorescence was detectable in areas of inflammation and hyperplasia around the injury. Selectivity of uptake and retention of HpD by tumours *in vivo* is generally not reproduced by malignant cells *in vitro*. Moan *et al.* (1981) found slightly higher uptake of porphyrins per unit volume of malignant cells, but in spite of this the degree of photosensitisation was comparable for both cell types. Therefore, this suggests that any tumour localising properties of HpD are due to some extracellular difference between tumours and normal tissues. It was shown by Bohmer and Morstyn (1985) that HpD uptake is influenced by serum concentration, pH, temperature and size of the target cells. The pH seems to play a particularly important role as at pH 6 the rate of HpD incorporation was much higher than at pH 7.4. Therefore it was suggested that greater tumour selectivity would be promoted by artificially increasing tumour acidity. A decrease in tumour pH has also been reported to have a role in sensitiser uptake by Pottier and Kennedy (1990). The affinity of porphyrins for circulating lipoproteins may represent a major pathway of tumour localisation of components of HpD. It has been shown that LDLs can inhibit Photofrin II uptake by cells cultured *in vitro* (Korbelik *et al.*, 1990). It is known that neoplastic cells tend to have elevated levels of LDL receptors and other sites of higher dye localisation, such as sites of wound healing, also demonstrate elevated numbers of these receptors. This suggests that growing tissues have a higher affinity for

porphyrins that can bind to LDL. However other types of lipoproteins and plasma proteins also demonstrate an affinity for HpD components and there is no direct evidence that the LDL receptor pathway is the principle element in selective localisation of Photofrin II in tumours (Korbelik, 1992). There is still considerable controversy over the issue of carriers of sensitisers as they seem to differ both with species and nature of sensitiser. The majority of the retained porphyrin appears to be in the extracellular matrix of the tumour. Rapidly proliferating capillaries of the tumour vasculature are 'leakier' than normal allowing the sensitiser to be retained in the extracellular matrix of the tumour. Little porphyrin is found in the malignant cells of the tumour. Bugelski *et al.* (1981) examined the distribution of ^{3}H-HpD and found that the relative amounts of porphyrin in tumour stroma to that in tumour cells was approximately 5:1. There are other possible mechanisms which may have relevance in the tumour localisation of photosensitisers. Bugelski *et al.* (1981) have shown that tumour associated macrophages contain elevated levels of HpD. It has also been demonstrated that macrophages have a considerably greater capacity for Photofrin II uptake compared with tumour cells (Korbelik *et al.*, 1991). Since the tumour environment affects the dye retention, it is likely that any selective tumour retention is dependent upon different types of tumour system.

1.3.1.(B) PDT using porphyrins

The use of porphyrins for photosensitisation depends on the relative affinity of these dyes for neoplastic tissues as compared with normal tissues. It is desirable for there to be minimal photodamage to surrounding tissues. High tumour selectivity can not only yield greater treatment efficiencies due to higher levels of sensitisers in tumours but will also produce lower skin photosensitivity and lower systemic toxicity. Normal tissues can be damaged due to PDT in the same way as tumours. PDT with HpD on normal tissues has received relatively little investigation. PDT resulted in a rapid cessation of intestinal blood flow (Selman *et al.*, 1985b). A study on normal rat jejunum showed upon light microscopy that an area treated with PDT gave extensive

sloughing of the mucosa and submucosa with sparing of the muscular and serosal layers (Chaudhuri *et al.*, 1986). One normal tissue that appears to exhibit an anomaly after PDT treatment is the normal pancreas. Tissue damage could not be produced in hamster normal pancreas whereas pancreatic tumour and surrounding normal tissues exhibited damage (Schroder *et al.*, 1988). This absence of response of normal pancreas has been observed to correspond to a lack of HpD photobleaching, monitored using fluorescence, after light exposure at 630 nm; whereas photobleaching was observed in pancreatic tumour (Mang and Wieman, 1987a).

Therefore, apart from treatments to the pancreas, there will always be some normal tissue damage of tissues surrounding the tumour unless tumour selectivity of porphyrins can be improved. Many studies have been performed to this end with different degrees of success. Intraneoplastic administration of ^{3}H-HpD resulted in 2.5 times higher concentration of sensitiser in tumour and approximately 10 times lower in skin. This selectivity was confirmed using fluorescence microscopy, although the HpD was not distributed uniformly throughout the tumour (Steichen *et al.*, 1986). Intraarterial versus intravenous administration of HpD was also studied as a means of enhancing selectivity of tumour sensitisation. Intraarterial administration was not found to increase the concentration of HpD in tumour relative to the surrounding tissues such as skin and muscle (Elmer *et al.*, 1987).

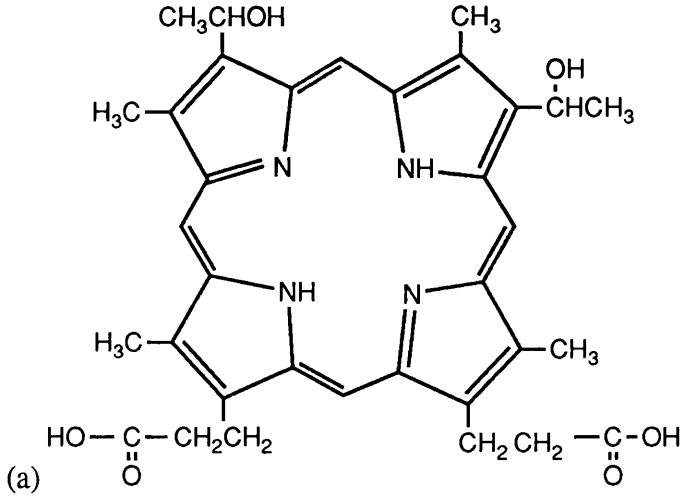
Jori *et al.* (1986) has suggested using sensitisers incorporated in unilamellar liposomes of dipalmitoyl-phosphatidylcholine. This not only provides a vehicle for administering water insoluble porphyrins but also gives more efficient tumour targeting than obtained by administration of the same porphyrins dissolved in homogeneous aqueous solution. An increase of 2 to 3 times the porphyrin level in tumour was observed using liposomes accompanied by no obvious enhancement of skin uptake.

Loss of porphyrin fluorescence (photobleaching) and lowering of intratumoural porphyrin level have been demonstrated both *in vitro* and *in vivo* during illumination with light following exposure to to HpD or DHE. This process is independent of porphyrin tumour level *in vivo* and could lead to tumour protection at low porphyrin levels. However, the photobleaching process, which occurs concurrently with tissue damage due to the photodynamic process, can be exploited to protect normal tissue. By controlling the sensitiser and light dose tumour tissue can be selectively destroyed. This depends on there being a differential between tumour and normal tissue sensitiser uptake. It may be possible to destroy the smaller amount of drug in the normal tissue by the photobleaching process before irreparable photodynamic damage has occurred while at the same time destroying tumour (Mang *et al.*, 1987b). A combination of photobleaching and increased uptake of porphyrin by tumour could lead to considerably more selective treatment.

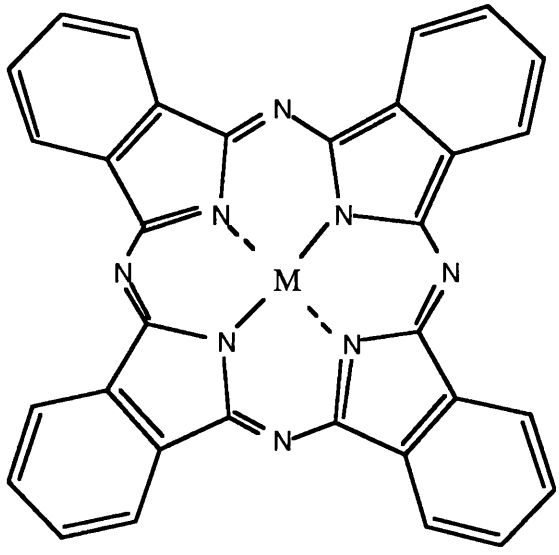
It must be remembered that the sensitiser tumour localisation phenomenon is relative rather than absolute. A particular photosensitiser may be present at a higher level in tumour when compared to surrounding tissue but other organs may have a higher drug level than tumour. The requirement of selectivity depends on the nature of application. As long as there is an adequate difference in photosensitiser concentration to achieve an effective therapeutic ratio and biological response after light the presence of the chromophore may not need to be exclusive to tumour. Principles established using porphyrins have been applied to the discovery and application of new sensitisers as a means of potentially enhancing selectivity and providing better methods of treatment. A class of second generation photosensitisers are the phthalocyanines and these are discussed in section 1.3.2.

1.3.2. Phthalocyanines

Phthalocyanines (Pc) are analogues of porphyrins in that their central macrocycle consists of a cyclic tetrapyrrole unit (Figure 1.1).



(a)



(b)

Figure 1.1
The structures of the (a) haematoporphyrin and (b) metallophthalocyanine molecules.

However, in the Pc's the pyrrole subunits are linked together by nitrogen atoms, whereas in porphyrins the subunits are linked via methylene bridges. Furthermore, Pc conjugation of the macrocycle is extended by benzo rings on the four pyrrole subunits, resulting in strong absorption bands in the clinically useful red region of the spectrum with maxima around 670 nm. They form stable complexes with many metal ions. The photophysical properties of the Pc's are strongly influenced by the presence and nature of the central metal ion which is reflected in the large variations in yield and lifetimes of photoexcited triplet metallophthalocyanines. Since a long-lived triplet state is essential for efficient photosensitisation via the Type II mechanism, a Pc dye containing a dimagnetic metal is better suited for this function than a paramagnetic metal such as copper, cobalt, nickel, iron and lead which enhance the intersystem crossing and shorten the lifetime of the triplet state making the dye photoinactive (Chan *et al.*, 1987). Several studies have shown that metal free Pc molecules are inactive (Chan *et al.*, 1987; Evensen and Moan, 1987; and Brasseur *et al.*, 1987a) and this suggests that a central metal is a prerequisite for a good photosensitiser. The phthalocyanines containing the diamagnetic metal ions aluminium, tin, zinc and gallium have been found to be active sensitisers in cell culture and *in vivo* (Chan *et al.*, 1987; Brasseur *et al.*, 1987a). Ring substitution renders the dye soluble in water which facilitates systemic administration of the sensitiser. Most biological studies on Pc's have been conducted with water soluble sulphonated metallophthalocyanines (MSPc); particularly aluminium sulphonated phthalocyanine (AlSPc) which has the advantage of an extra planar ligand which inhibits aggregation. The introduction of sulphonate groups is relatively easy and sulphonated phthalocyanines are efficient photosensitisers.

Several pharmacokinetic and tumour uptake studies of aluminium sulphonated phthalocyanine have been reported. Using an alkaline extraction assay Tralau *et al.* (1987) observed a peak tumour to normal tissue ratio at 24-48 hours after administration of AlSPc in two autochthonous tumours in the rat colon and the

hamster pancreas and in a transplanted glioma in the rat brain, compared with peaks at 1-3 hours in the normal tissues. Those tumours outside the central nervous system gave a maximal tumour to normal ratio of 2-3 to 1, whereas the tumour transplanted in the brain gave a maximum ratio of 28 to 1. They then compared their results with those of HpD from the literature and found that the results from comparable models were remarkably similar. It was considered that there was unlikely to be any significant difference between the selectivity of uptake of HpD and AlSPc (with a mixture of sulphonated components). The colonic tumour model was examined at the time of maximum differential using fluorescence microscopy and significant photosensitiser accumulation was found in tumour stroma, whereas tumour and normal mucosa contained similar amounts (Barr *et al.*, 1991). Three murine tumours of different histological type (Colo 26 - a colorectal carcinoma, M5076 - a reticulum cell sarcoma, and UV-2237 - a fibrosarcoma) were grown subcutaneously in mice (Chan *et al.*, 1988). All the tumours took up and retained AlSPc to a greater extent than adjacent normal skin and muscle. Maximum uptake in all three tumour types was achieved 24-48 h after dye administration, but differences in uptake were found between the three tumours suggesting that phthalocyanine affinity is markedly dependent on the nature of the tumour. This specific uptake was, in part, a consequence of uptake by individual neoplastic cells, as shown by dye fluorescence sorting and analysis of a suspension of tumour cells prepared after dye administration. Using highly sensitive fluorescence microscopy on cells *ex vivo* it was shown that cells which took up most AlSPc were largely of the monocyte-macrophage series (Chan *et al.*, 1989a). The sensitiser selectivity may also vary for the same tumour growing at different anatomical sites. A murine colorectal carcinoma established in the lung, thorax, kidney and subcutaneously in the flank region of syngenic Balb/c mice, took up and retained greater amounts of sensitiser than did adjacent normal tissue. However, such selective retention was not observed when the tumour was grown in the spleen, where the neoplastic and normal tissue took up and retained the same amounts of sensitiser, or in the liver, where normal tissue took up

more AlSPc than either metastatic or directly implanted tumours (Chan *et al.*, 1989b). Therefore, AlSPc ratios found in tumour/adjacent tissue may vary for a single tumour growing at different anatomical sites and such variability could have a distinct effect on the efficacy of PDT. In a comparative uptake study of different photosensitisers by C3H mammary carcinomas and normal tissues in mice, it was found that AlSPc and AlPc were taken up in large amounts by the spleen, kidney and liver. The AlSPc retention by the tumour was more efficient than that of HpD, while the uptake of the unsubstituted sensitiser was only half of that of HpD (Peng *et al.*, 1987; Moan *et al.*, 1987).

In summary, tumour selectivity of AlSPc may not differ that much from that of HpD; although, this will very much depend on the nature of the tumour and its anatomical location. A fuller discussion of the relative merits of PDT with AlSPc and its components appears in the relevant chapters (see chapters 3 and 4).

1.3.3. Photochemistry

Photosensitised oxidation reactions are the basis for the cytotoxic effect of photodynamic therapy (Figure 1.2). The first step is absorption of light of an appropriate wavelength by a photosensitiser (S) to produce an excited singlet state ($^1S^*$). At this point a number of processes may occur to the $^1S^*$ state including fluorescence decay ($h\nu_F$). This reaction is of particular interest for PDT since fluorescence detection may be used to monitor sensitisers both *in vitro* and *in vivo*. The most important process is conversion of the short-lived singlet to the long-lived triplet state ($^3S^*$). In the presence of oxygen, two competing reactions of the excited sensitiser with tissue can occur (Foote, 1968). These processes are called Type I and Type II reactions. The excited singlet state, or usually the longer lived excited sensitiser state the triplet state can either react with the substrate or solvent (Type I) or with oxygen (Type II). The Type I reaction results in either hydrogen atom or electron transfer, yielding radicals or radical ions which can then react with oxygen to

produce reactive peroxide species or hydroxyl radicals. The Type II reaction leads mainly to singlet molecular oxygen by energy transfer ($^1O_2$*). Singlet oxygen is the lowest energy electronically excited state of molecular oxygen and has a relatively long intrinsic lifetime. It is highly reactive toward many biomolecules including lipids, proteins and DNA, and is therefore toxic to all cells, not just tumour cells. In the Type II process the sensitiser molecule then returns to its ground state and is recycled. It is thought that the Type II reaction is the mechanism for the oxygen dependent cytotoxicity of the photosensitiser in PDT. Evidence for this Type II mechanism is based on the *in vitro* studies of cellular photosensitisation in the presence of singlet oxygen scavengers which were found to suppress the photocytotoxic effect (Valenzeno, 1987). The importance of oxygen was demonstrated *in vitro* when comparing cytotoxicity which revealed rapid cell death in an oxygenated environment but hardly any effect in an oxygen free environment (Mitchell *et al.*, 1985). Induction of tumour hypoxia *in vivo* by clamping normal rodent liver sensitised with AlSPc immediately before laser irradiation suppressed the PDT damage (Bown *et al.*, 1986). It is possible, therefore, that pre-existing tumour hypoxia or rapid oxygen depletion during treatment would possibly account for treatment failures attributed to inadequate quantities of light or photosensitiser.

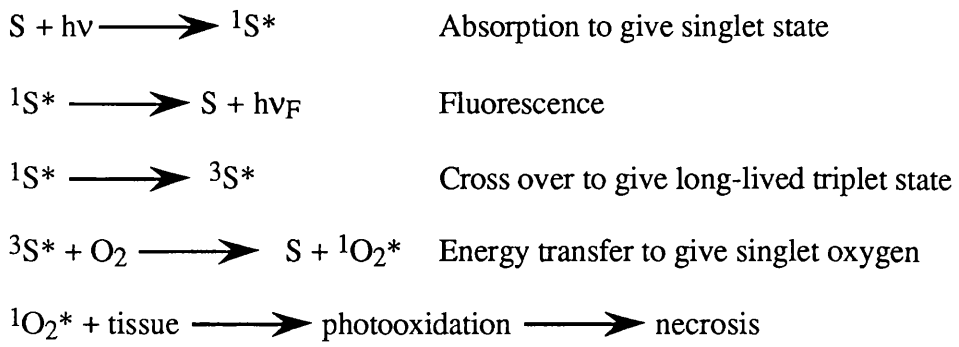


Figure 1.2
Photochemistry of sensitiser S for PDT.

Photodegradation of the sensitiser molecule, generally photobleaching can also occur and this may proceed by either a Type I, or a Type II mechanism. In the first instance, the excited photosensitiser triplet state can react with tissue components to form radical or ionic products and in the latter mechanism singlet oxygen can react with the sensitiser resulting in an oxidised photoproduct. Photobleaching is a permanent loss of the chromophore used in PDT and this may be beneficially exploited *in vivo* to improve selectivity (Barr *et al.*, 1990b).

1.3.4. Light and lasers

1.3.4.(A) Light delivery

Light is required for a photodynamic effect. In order to obtain the desired PDT effect a light source must be delivered externally, or interstitially, to the target tissue. Dosimetry in phototherapy is complicated because it involves interactions between light and endogenous and exogenous chromophores. The liver, for example, exhibits high light absorption and therefore especially poor light penetration due to its high haemoglobin content. On average the penetration depth of light into tissue is about 1-2 mm at 630 nm, the wavelength used for clinical treatment with Photofrin II, while penetration is approximately twice as much at 700-850 nm (Wilson, 1989). It is this increase in penetration depth of longer wavelength light that has provided the incentive for development of sensitisers absorbing at these wavelengths. An example of such a group of sensitisers is the naphthalocyanines. Due to their extra benzene ring which is condensed to the periphery of the phthalocyanine molecule they absorb strongly at 760 nm (McCubbin and Phillips, 1986). The absorption of light by the photosensitiser itself can limit tissue light penetration and is particularly pronounced with sensitisers which absorb very strongly at the treatment wavelength (Wilson *et al.*, 1986).

Light may be described as " directed" or " diffuse". The rays of directed light have a definite spatial relationship, e. g. collimated, diverging, or converging. " Specular"

reflection of light at a polished surface and refraction of light by passage through a transparent medium change the path of light rays, but a high degree of "directionality" is retained. The rays in uniformly diffuse light move in random directions. Directed light is converted to diffuse light by propagating in a turbid material or after reflection by a matt surface. Figure 1.3 depicts the basic phenomena that occur when a collimated light beam enters a tissue layer.

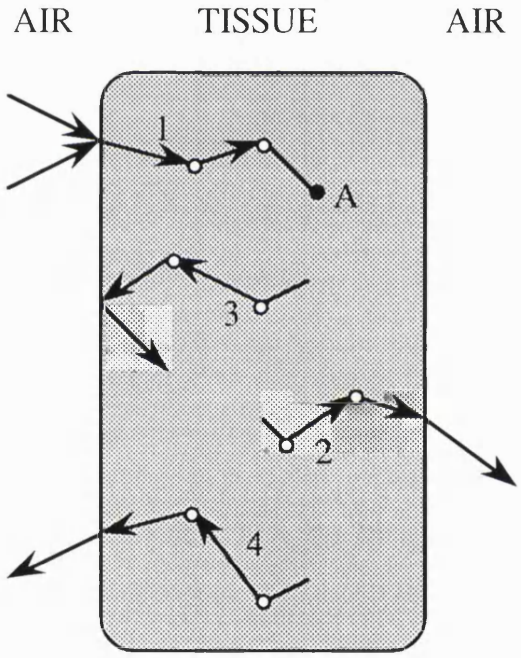


Figure 1.3
Typical light interactions with tissue.
Light enters the front tissue surface after specular reflection and refraction, Ray 1 undergoes two scattering events, and encounters an absorber (A). Ray 2 exits after refraction at the rear tissue surface. Ray 3 is back-scattered to the front surface and undergoes total internal reflection. Ray 4 is back-scattered to the front surface and exits the tissue after refraction.

A relatively small fraction of the light is lost by specular reflection at the front surface. Light scattering by microscopic inhomogeneities within the tissue leads to spreading of the beam and loss of directionality. Flux scattered in a forward hemisphere reaches the rear interface over a wide angular range. If the refractive index of the tissue exceeds that of the external medium, only those rays that reach the internal surface at less than the critical refraction angle can exit from the tissue. The other rays are internally reflected. The transmittance is the fraction of incident light that exits in all directions at the rear face, including the residual forward beam. Similarly, part of the back-scattered light reaching the front interface may be internally reflected. The fraction of incident light that exits in all directions from within the material at the front face is the diffuse reflectance. Most of the light incident on tissue is absorbed by molecular chromophores; this leads to heat generation, light emission by fluorescence, or photochemical reactions (Grossweiner, 1990).

1.3.4.(B) Lasers

In the development of PDT a wide range of light sources have been used, with the laser being preferable in many cases due to its high intensity output at a defined wavelength and, therefore, the ability to expose a specific area of tissue to light with greater selectivity than other light sources with matching of the wavelength to the sensitiser being used. The laser also has the additional advantage that its output can be focused into the thin flexible optical fibres which may be passed down an endoscope for treatment of internal lesions without the need for surgery.

Laser is an acronym for light amplification by stimulated emission of radiation. Although Einstein predicted that stimulated emission could be achieved, it was not until 1960 that the first laser was produced by T. H. Maiman using a synthetic ruby as the lasing medium (Maiman, 1960). Since then a large number of laser systems have been developed. The lasing medium is contained in the laser tube which has a

fully reflective mirror at one end and a partially reflective mirror at the other, which allows access to the laser beam. The lasing medium is excited or pumped electrically to invert the population ratio of excited to nonexcited molecules. An excited molecule will decay to the low-energy ground state with the release of a photon - a quantum of radiant energy. When an incident photon of appropriate energy strikes a molecule in the high-energy state, it stimulates it to emit an identical photon and to decay to the ground state - this is stimulated emission. The released photons are reflected back into the lasing medium from the mirrors and this optical feedback causes the 'cascade effect' with a rapid build up of light energy within the tube and this coherent light can then be released through the partially reflective mirror. The lasing medium and the mirror design determines the wavelength of the coherent light. The wavelength determines the site of absorption of the beam in the body tissues and this defines the clinical role of the laser (Carruth, 1987).

The type of laser most often used in PDT is the dye laser, and this has the advantage over other lasers in that it can be tuned over a significant part of the visible spectrum. The laser medium of the dye laser is a dye such as coumarin or rhodamine. When the dye solution is irradiated with light of a short wavelength, it may be energised to its first excited electronic state. Transitions between the bottom of this excited electronic state and the numerous vibrational and rotational levels of the ground state of the complex dye molecule radiate light within a continuous, broad band of longer wavelengths. At high pump irradiance, these radiative transitions are able to lase. The exciting energy for the dye laser often comes from an argon laser. The beam from the argon laser is passed into the dye stream of the dye laser. The gold vapour laser has also been used for PDT but the wavelength is fixed at 628 nm and cannot be tuned to activate a specific sensitiser. The metal in the gold vapour laser may be changed to copper and the copper vapour laser can then be used to excite a dye laser.

Regardless of the type of PDT laser used, light generated by a laser is usually coupled to an optical fibre permitting access to viscera otherwise inaccessible for PDT. Light distributions can be adapted to the target tissue configuration by modification of the optical fibre allowing both external and interstitial treatment of surface, intraluminal, and intracavitary lesions where contours can be quite diverse, causing difficulty for direct light irradiation to some target areas. In organs or body cavities with irregular contours and large areas to treat, calculation of the surface area and, subsequently, energy density may be difficult. The tumour topography must be considered for accurate surface area or volume calculation; inaccurate determination of the treatment area may lead to delivery of excessive power which may cause undesired thermal effects, including charring, which will interfere with light absorption. Conversely, underestimation of the true treatment area could lead to treatment failure. Light doses for optimal effect in PDT are currently somewhat empirical and vary with the tumour type.

1.3.5. Cytotoxic mechanisms of PDT

Membranes are believed to be among the primary sites of photodynamically induced cellular damage (Kohn and Kessel, 1979). Lipid peroxidation and protein crosslinking are types of membrane damage identified following treatment of cells with porphyrins and light. AlSPc has been shown to accumulate in membrane fractions and following PDT morphological changes in membrane organelles have been observed. This has led to the suggestion that plasma membrane, microsomes and mitochondria are the major sites of photodynamic action (Robinson *et al.*, 1987; Rosenthal and Ben-Hur, 1989). Therefore, PDT-induced cellular damage should be enhanced by membrane active drugs. The K^+/H^+ ionophore nigericin drastically increases killing of V79 Chinese hamster cells pretreated with unsulphonated aluminium phthalocyanine (AlPc) and exposed to light, proposed to be due to the drug perturbing the ion transport across either mitochondrial or plasma membrane (Varnes *et al.*, 1990). Photooxidative damage of nucleic acids and proteins also have

been reported but may be of less consequence because the effect of PDT appears to be equally toxic throughout all phases of the cell cycle (Manyak, 1990). When using the bacterium *Escherichia coli* (*E. Coli*) as a model for determining PDT effects of zinc phthalocyanine (ZnPc) Bertoloni *et al.* (1990) found the cytoplasmic membrane to be an important target for the photoprocess, while DNA was not involved. However, potentially cytocidal DNA-protein crosslinking can result from photooxidations catalysed by porphyrins (Dubbleman *et al.,* 1982), although this was carried out using isolated DNA not intact cells. However, different sensitisers may act on different cellular compartments and this relates to the specific patterns of intracellular localisation of each sensitiser since the generated singlet oxygen can diffuse in the order of only 0.1 μm in cells during its lifetime (Moan, 1991). Additionally, if a sensitiser is a mixture of components showing differing localisation patterns they may also have different PDT targets. Whatever the primary lethal effect of PDT the consequence is a rapid loss of cell integrity. Associated with the cell membrane damage is the release of inflammatory and immune mediators. A range of eicosanoids are released in parallel with lactate dehydrogenase from all cell types studied *in vitro* following Photofrin II photosensitisation. Histamine, another inflammatory mediator, is liberated by PDT through mast cell degranulation. All these substances are potent, fast acting and vasoactive (constrictive or dilatory) and evidence exists to implicate them in the development of PDT induced vascular damage (Henderson and Dougherty, 1992).

The evidence supporting the tumour microvasculature damage induced by PDT using HpD includes direct observation of tumour microcirculatory bleaching during PDT, microscopic arteriolar vasoconstriction and venous thrombus formation beginning during PDT, and endothelial cell necrosis in both normal and neoplastic vessels following PDT (Star *et al.*, 1985). In addition, tissue explantation immediately following PDT did not effect tumour cell clonogenicity, even when using protocols which would have led to total macroscopic tumour destruction, whereas tumour cell

death increased progressively with delay in explantation suggesting a vascular mechanism for tumour death (Henderson *et al.*, 1985). Selman *et al.* (1986) investigated the histological and blood flow changes in bladder tumours after light with pretreatment of aluminium tetrasulphonated phthalocyanine (AlS_4Pc). Tumours examined 4 and 24 hours after the completion of phototreatment showed extensive haemorrhagic necrosis. In addition, Pc and light resulted in a significant decrease in tumour blood flow within 10 minutes of completion of light exposure. These results were considered to be similar to those from using higher doses of HpD in the same tumour model. The disruption of tumour blood flow suggested that both HpD and AlSPc have a similar effect on the tumour microvasculature and that this might be the common pathway for tumour destruction from both agents. Despite accumulating evidence for a vascular mechanism for PDT, the extensive *in vitro* cytotoxicity reported by many investigators is not explained by vascular effects and direct cell kill is suggested. Hampton and Selman (1992) addressed this question by utilising a culture system where sensitisers were administered *in vivo*, tissue slices produced in minutes, placed in culture medium and exposed to light *in vitro*. Direct killing of tumour slices sensitised with HpD occurred in the absence of a functioning vascular system. Consequently, most investigators currently feel that *in vivo* tumour death is due to direct cytotoxicity in combination with disruption of tumour vasculature. Electron microscopic studies performed on tumour tissues taken from mice, sacrificed at different times after the phototreatment, showed that in the presence of aqueous administered Hp tumour necrosis was largely the consequence of vascular damage. On the other hand, in the presence of Hp administered in liposomes or complexed with low density lipoproteins (LDL), the response of the tumour to the phototreatment occurred at a faster rate and was mainly determined by direct damage of neoplastic cells (Zhou *et al.*, 1988). Therefore, the mechanism of PDT damage and location of damage will very much depend on the nature of the photosensitiser and its vehicle of administration.

1.4. Clinical PDT

After the first inconclusive treatment of skin tumours with white light following topical eosin application in 1905, the therapeutic potential of PDT remained largely dormant until the mid 1970s when clinical trials using HpD began. Since 1976 thousands of patients with various malignant lesions have been treated. These include lesions in the bladder, lung, head and neck, gastrointestinal tract, brain and skin. Most of the original patients treated by PDT had extensive cancer unmanageable by conventional treatments. The dominant theme in published work is the high incidence of success in superficial cancers, but poorer results in more advanced tumours irrespective of the site of the tumour. As PDT depends on light, which has a limited penetration in living tissues, the depth of necrosis is never more than a few millimetres. It therefore follows that for a treatment to be curative it should be applied to small and thin lesions. Despite this much work has been done on treating advanced tumours.

Advanced cases of head and neck cancer treated with either HpD, or DHE, for palliation gave disappointing results that were no more effective than standard therapeutic regimens. When tumour shrinkage could be obtained it was unpredictable and it was thought that shrinkage could be achieved more easily with conventional methods. In a number of these patients the associated skin photosensitivity actually worsened the quality of life (Gluckman, 1991). From initial studies of PDT for lesions of the bronchus and trachea it became apparent that in a large percentage of cases, malignant lesions of the bronchus and trachea do take up the photosensitising drug Photofrin I and retain it for at least 3 to 7 days. Little effect of PDT was observed beyond a tissue depth of 2 cm and the complications were significant and frequently life threatening. There was a sequence of events leading to the morbidity that was observed in all of the 17 cases studied. Shortly after exposure of the tissues to the light, there was production of significant amounts of secretions. These secretions, if not removed, form a hyaline-like cast which mechanically obstructs

airways and may be an excellent medium for infection. In general it was not felt that survival was greatly prolonged by this procedure (Vincent and Dougherty, 1984). Additionally there are reports of major haemorrhage occurring when necrosed tumour separated a few days after PDT to advanced bronchial carcinomas (Cortese and Kinsey, 1982). More recent data from a study by Monnier *et al.* (1990) in the bronchi and oesophagus gave unsatisfactory results for advanced cancers (three recurrences for eight lesions treated) which was probably due to insufficient light reaching the entire tumour. The results were more encouraging for the cancers staged *in situ* or microinvasive at endoscopy (two recurrences for 23 lesions treated). According to these experiments, PDT is efficient at destroying early squamous cell carcinomas in the pharynx, oesophagus and bronchi.

Another problem experienced when treating advanced tumours, even when there is effective destruction of tumour, is that this may lead to a transmural necrosis and subsequent perforation of the organ, which is a hazardous situation (Monnier *et al.*, 1990). However it has been found after total destruction of an experimental colonic tumour, even if the tumour has destroyed all the colonic wall, the colon can still heal safely with regeneration after PDT (Barr *et al.*, 1991). Barr *et al.* (1990c) then treated ten patients with colorectal cancers, unsuitable for operation, with endoscopic PDT. All patients were sensitised with 2.5 mg/kg HpD, 48 hours before laser treatment. Two patients were tumour free 20 and 28 months after PDT. One treatment of an advanced tumour was complicated by a haemodynamically significant secondary haemorrhage. Therefore this indicates once again that PDT may be most suitable for the treatment of small tumours, or for small areas of persistent tumour where the bulk has been removed by alternative techniques.

It appears, from the above colon studies, that some normal tissue damage during PDT may be acceptable, although ideally sensitisation and subsequent necrosis should only occur in the tumour. In treating tumours of the skin it is particularly

important, for cosmetic reasons, to carefully select a drug and light dose combination which causes selective tumour necrosis and little normal skin damage, although this selectivity is difficult to achieve. In a study by Gilson *et al.* (1988) six patients with subcutaneous or cutaneous lesions were treated with PDT using Photofrin II. The incidence of complete tumour response and skin necrosis were used to assess the therapeutic ratio of PDT. The tumour response rate was 47%. The amount of tumour and skin necrosis increased with increase in dose of photosensitiser and light used. Therefore the treatment was not selective for tumour, although damage to the skin healed completely with no scarring. However, if the normal tissue heals with no impairment of function whilst the tumour dies then the ultimate biological response is selective. It was concluded that PDT was effective in treating superficial tumours and that refinement of light delivery systems could further reduce the side effects of the treatment caused by lack of selectivity in sensitisation of normal to tumour tissue.

Another area where selectivity of PDT treatment is particularly important is in the treatment of superficial bladder cancer. This condition is usually multifocal and the value of PDT is that areas of occult dysplasia and carcinoma *in situ* do not have to be precisely defined for photodynamic therapy to be effective. Since the potential of PDT in bladder carcinoma was first demonstrated by Kelly and Snell (1976), many clinical cases have been reported which have shown promising control of resistant disease but patients receiving whole bladder PDT experienced bladder irritability and shrinkage (Dougherty, 1986). Nseyo *et al.* (1985) reported muscle fibrosis and permanently reduced bladder capacity and incontinence, symptoms which may themselves necessitate cystectomy. These side effects are probably the result of lack of selectivity of sensitiser uptake by the neoplastic tissue therefore causing unwanted photosensitiser activation and subsequent fibrosis in the deep muscle layers of the bladder wall (Pope and Bown, 1991).

The only tumour known to show real selectivity in uptake of intravenous sensitiser is the glioma. From studies in human and experimental brain tumours there is considerable selectivity of uptake of HpD compared with normal brain, with a maximum ratio of 34 to 1 at 4 hours after introduction of the sensitiser (Wharen *et al.*, 1983). This does not mean that damage to the normal brain does not occur and clinical studies have shown no significant improvement in patient survival after PDT with either primary, or recurrent, tumours (Muller and Wilson, 1990). The reason for this may relate to a lack of understanding of the mechanisms of PDT and a lack of the scientific data needed to optimise photodynamic selectivity. Unless conditions of true selectivity are met the normal brain will be damaged and without any capacity to repair normal tissue this is obviously unacceptable.

For much of the clinical PDT studies it is not known when the tumour to background tissue levels of photosensitiser are maximal, and therefore the optimum time for light treatment is not determined. Treatment parameters are generally based on animal studies. Another serious problem is underestimation of the depth of penetration of the tumour with only superficial tumour necrosis being obtained, and viable tumour being left untreated. PDT in the clinic has therefore developed on a largely empirical basis leaving questions regarding its optimum treatment parameters unanswered. This has proven disadvantageous and has led to unacceptable complications. Many problems occur due to lack of selectivity of the photodynamic treatment for tumour tissue, and therefore, treatment parameters must be carefully considered before commencing a clinical PDT programme.

1.5. Novel applications of PDT

While the primary interest for PDT has concentrated on treatment of neoplastic disease, recent studies have also been directed to the use of PDT for non-malignant disorders. These investigations have utilised the potential selectivity of this technique.

The rationale for the use of PDT in many of these treatments was based on the selective uptake and retention of photosensitisers by rapidly proliferating cells (Figge *et al.*, 1948). A number of clinical conditions stem from the rapid proliferation of cells and these include endometriosis, intimal hyperplasia, atherosclerosis and psoriasis. Endometriosis, a common gynaecological disorder in women of reproductive age, shares with neoplasia the ability to spread beyond the organ of origin. It is not malignant although the characteristics of this disorder suggested that investigation of PDT for detection and treatment of endometriosis was reasonable. The potential usefulness of Photofrin II mediated PDT for the treatment of endometriosis was studied in rabbits (Manyak *et al.*, 1989). Complete endometrial epithelial destruction was observed in 81% of animals after PDT.

Phototherapy with AlSPc has also been studied as a novel approach for the prevention and treatment of intimal hyperplasia (IH) in large blood vessels. IH occurs from proliferation of smooth muscle cells in blood vessels. A study by Ortu *et al.* (1992) has demonstrated that PDT can effectively inhibit the IH response when it is used before or during induction of cellular proliferation in an acute rat model. No adverse effects on the rats or on the structural integrity of the vessels resulted from this procedure.

The development of laser angioplasty has prompted investigations of the potential use of dyes for both the identification and PDT of atherosclerosis. Some degree of preferential accumulation of copper phthalocyanine (CuPc) in atheromatous plaques in rabbits (2:1 compared with normal arterial wall) has been reported (Eldar *et al.*,

1990). Plaques are considered to accumulate certain dyes in the same manner as hyperproliferative tissues in general (Spikes and Straight., 1990).

Psoriasis is characterised by an increased number of dividing cells per unit surface area in the basal layers of the epidermis, a shortened cycle of reproduction of germinative cells and alterations in cell differentiation. This results in the appearance of elevated, scaly red plaques on the skin as the epidermis is manufactured rapidly and abnormally. No permanent cure for psoriasis has been developed; whilst patients may have long disease-free periods, all of the therapies presently used may be followed by recurrence of the condition within an average time of nine months. Using i.v. HpD followed by illumination of psoriatic and normal areas of skin with blue-green or red light the psoriatic lesions responded well, with complete healing by 2 months. The treatment had little effect on normal skin areas suggesting that there may be selective accumulation of HpD in psoriatic lesions (McCullough *et al.*, 1987). PDT of psoriasis may prove safer than conventional psoralen-ultraviolet A treatment and is likely to need less treatments as red light penetrates much further than UV. However, skin photosensitivity is still a problem using HpD. The production of suitable topical preparations of photosensitisers should help to avoid this side effect.

The ability of PDT to cause localised blood stasis suggested potential for treatment of disorders caused by extensive neovascularisation, such as age related macular degeneration. The basis of the PDT treatment would be to completely obliterate the subretinal blood vessels and thus stop further bleeding. Assuming the existence of an appropriate time window when the dye is only localised in the neovascular vessels then selective obliteration of these vessels could be achieved without damaging the retina. The combined action of AlSPc and red light obliterated choroidal vessels in the rabbit eye (Ben-Hur *et al.*, 1989). Controlled occlusion of small blood vessels may heal other localised vascular lesions of the skin and subcutaneous tissues, such as Port wine stains (I. Rosenthal, 1991).

Using PDT it is difficult to be sure that all cells in a solid tumour are exposed to the same dose of photosensitiser and light, which is easily achieved in a single-cell suspension (Sieber and Krueger, 1989). These properties make a bone marrow graft exceptionally amenable to PDT. Furthermore when photosensitisers are used extracorporeally excess dye can be removed before reinfusion of the treated cells into the patient. This greatly reduces the risk of systemic photosensitisation or other forms of systemic toxicity. In an evaluation study of PDT as a purging procedure for bone marrow autografts it was found that clonogenic acute myeloblastic leukaemia cells were more sensitive *in vitro* to AlSPc and light than normal progenitor cells. The mechanism of discrimination between these cells was not known but was considered to be related to differences in the cell membrane lipids affecting uptake of the sensitiser molecules. An alternative explanation was thought to be due to differences in the ability of these cells to tolerate, or repair, the damage produced by PDT (Singer *et al.*, 1987 and 1988).

PDT has also been investigated for treatment of viruses. One virus investigated is the *Herpes simplex* virus (HSV). Using a suitable photosensitiser the aim was to inactivate HSV via the viral envelope, whilst avoiding damage to the DNA which may unmask the oncogenic potential of the virus (Lytle *et al.*, 1989). Whole blood spiked with vesicular stomatis virus was treated with a benzoporphyrin sensitiser and 10^7 (100%) viruses were inactivated when exposed to light (Neyndorff *et al.*, 1990). The inactivation of viruses added to whole blood was also studied with AlPc and its sulphonated derivatives (Horowitz *et al.*, 1991). Two enveloped viruses, vesicular stomatitis virus and the human immunodeficiency virus, were completely inactivated on treatment of whole blood. In addition, AlS_2Pc and AlS_4Pc, which are more hydrophilic than unsulphonated aluminium phthalocyanine, were more effective virucidal agents than the unsulphonated compound. Under the conditions examined in which viruses were selectively inactivated the red cell integrity was maintained.

These results suggest that inactivation of viruses in blood using PDT may prove beneficial in a blood bank.

PDT can be used for the killing of both prokaryotic and eukaryotic microorganisms. Gram-positive and Gram-negative bacteria, strains of fungi and yeast cells have shown susceptibility to PDT-induced damage (Krinsky, 1974). Many Gram-negative bacteria are resistant to photodynamic inactivation (Malik *et al.*, 1990). This is considered to be due to their polysaccharide cell wall which inhibits the interaction of the photosensitiser with the cell membrane (Bertoloni *et al.*, 1984). This problem may be overcome by pretreatment of Gram-negative bacteria with chemicals to disrupt their outer membrane structures (Malik *et al.*, 1990), although the need for pretreatment limits the use of PDT for killing Gram-negative bacteria clinically. In experiments designed to test PDT effects on bacteria *in vivo*, female sheep were inoculated with a culture of the Gram-positive bacterium *Staphylococcus aureus* into the cisterna magna. The sheep were then treated with Hp at a concentration of 0.25 mM and exposed to light using an optical fibre into the spinal channel. No quantitative result was given, although it was reported that a good bactericidal effect had been observed and this was considered to confirm the potential use of PDT for bacterial infections *in vivo* (Martinetto *et al.*, 1985).

In conclusion, due to the potential selectivity of PDT this technique may have much wider applications than just for the treatment of solid tumours.

1.6. Summary and thesis aims

To summarise the PDT situation at the onset of this thesis. It has been known for many years that porphyrins localise in tumours, and other tissues with rapidly proliferating cells, and that this property can aid in the detection of neoplastic tissue. More recently it has been shown that photosensitised tumours can be damaged after light and this has formed the basis of photodynamic therapy. What has become obvious is that sensitisers locate in normal tissues as well as tumours and indeed some normal tissues exhibit greater uptake than tumours. This means that after light it is not just the tumour tissue that is damaged and, therefore, PDT has not been found to be the 'selective' tumour treatment that it has historically been considered. Normal tissues have often been found to regenerate after light exposure whereas tumour tissue dies and this situation ultimately leads to a selective end point. However, it would be preferable if no normal tissue damage occurred at all, as it is this damage which has caused considerable complications in clinical treatments. Impairment of function of the treated tissue often occurs and this may even lead to mortality. To produce a truly selective PDT treatment a better understanding of the fundamental mechanisms of PDT would be preferable.

One possible means of improving biological selectivity is by utilising new photosensitisers. The conventional sensitiser Hp and its associated derivatives have not proven ideal sensitisers with only minimal normal to tumour tissue uptake and extended skin photosensitivity. One new sensitiser is AlSPc (a mixture of sulphonated components with an average of 3 sulphonate groups per molecule (McCubbin, 1985)) which shows improvements in its photochemistry, but ultimately the selectivity in uptake between normal and tumour tissues has not been enhanced. Therefore the major factor still limiting the success of PDT treatment with conventional sensitisers is the selectivity of the photosensitiser for the target tissue, generally tumour. In this study the problem of lack of tumour selectivity of photosensitisers has been addressed:

1. Initial studies were performed with aluminium sulphonated phthalocyanines as a means of increasing the dye uptake ratio between tumour and normal tissue, and determining whether different sulphonated components would exhibit differing ratios.

2. Radioprotective drugs were investigated as a means of sparing normal tissue during PDT using a protocol with AlSPc known to give little, if any tumour selectivity.

3. A new means of 5-aminolaevulinic acid induced endogenous sensitisation was exploited for use in PDT. The correlation between distribution of photosensitisation and biological effects after light were studied in normal and tumour tissues, and the benefits of i.v. against oral administration are considered.

4. The feasibility of treating the bacterial infection *Helicobacter pylori* with PDT was investigated and the potential for selective eradication discussed.

All these aspects are aimed at improving selectivity of the photodynamic processes.

CHAPTER 2 - METHODS

2.1. Basic methodology

The following experimental methods and techniques were used throughout these studies:

1. Normal and tumour tissue animal models.
2. Quantitative fluorescence microscopy using charged-coupled device camera system.
3. Phototherapy of normal and tumour tissue.

Each of these methodologies is now discussed in detail as follows.

2.1.1. Animal models

(A) Normal tissues

Normal tissue studies were performed on Wistar rats weighing 125-200 g, obtained from the Imperial Cancer Research Fund. The rats were housed in temperature controlled quarters in suspended cages with open mesh floors to prevent coprophagia.

(B) Induced tumour

Colonic tumours were chemically induced in male Wistar rats (as above) using dimethylhydrazine (DMH), (Aldrich Chemical Company) as reported by Filipe (1975). Each rat received a subcutaneous injection of 40 mg/kg body weight of DMH into the left flank at weekly intervals for five consecutive weeks. They were weighed weekly and inspected for signs of illness. Tumours tended to occur in the colon 25-30 weeks after introduction of the carcinogen. Confirmation of the presence of tumours was possible by laparotomy under general anaesthesia.

The DMH model was chosen as it was a tumour that could be treated in its site of origin and therefore paralleled the clinical situation. Due to concerns about the safety

of the DMH tumour model this method of tumour production ceased in 1989 and subsequent studies were performed on a mammary adenocarcinoma transplanted in the rat flank. This tumour model was already available in the unit.

(C) Transplanted tumour

Male Chester Beatty hooded rats (National Institute of Medical Research, Mill Hill, London) weighing 100-120 g were required for maintenance of tumour tissue. The tumour originated from a mammary carcinoma (Hosp 9AP6, Institute of Cancer Research, Royal Marsden Hospital, Sutton, Surrey). Tumour was dissected and a cube of approximately 2 mm dimensions was implanted under the skin through a small incision on the right flank under general anaesthesia with intramuscular Hypnorm (fentanyl and fluanisone, Jansen Pharmaceuticals Ltd.). The animals were inspected until the developing tumour reached a diameter of between 1-1.5 cm, this size being reached at about 2 weeks.

2.1.2. Fluorescence microscopy

Fluorescence microscopy was used to detect and localise the cellular sites of the fluorescent photosensitisers. Using computerised image processing techniques quantification of fluorescence in tissue sections, or strips of tissue, was possible. Tissues from photosensitised animals were removed immediately after sacrifice. Whole tissue strips were mounted on glass slides with the layer of interest (for example mucosa) uppermost. For the preparation of tissue sections the specimens were frozen by cooling a beaker of isopentane (BDH Ltd., UK) in liquid nitrogen and then immersing the tissue in the isopentane before storing in liquid nitrogen. Frozen sections of 10 μm thickness were cut (Cryostat E microtome, Reichert Ltd.) and stored at -70°C. Specimens were prepared and imaged with a minimum of light exposure to avoid bleaching of sensitiser.

The fluorescence microscope (Olympus IMT-2) was attached to a charged-coupled device (CCD) camera system (Wright Instruments Ltd., model 1). This photometric imaging system is highly sensitive with comparable detection efficiency to photomultipliers. Fluorescence was excited using an 8 mW helium neon laser operating at 632.8 nm, with the output directed through a liquid light-guide (via a 10 nm bandpass filter to remove extraneous light) onto a dichroic mirror in the epi-fluorescence microscope which incorporated phase-contrast attachments. Fluorescence was detected in the range 660 to 710 nm, using a combination of bandpass (Omega Optical Inc.) and longpass (Schott RG665) filters. The CCD sensor (578 x 385 pixels, model P8603, EEV Ltd.) was cryogenically cooled to minimise background thermal noise, and imaging operations and processing were controlled by an IBM AT/PC clone. Both false colour coded or black and white images were generated by the computer.

Fluorescence could be quantified digitally (software provided by Wright Instruments Ltd.) using box superimposition on the image to give an average number of counts per pixel. Specific tissue layers and/or whole images were analysed using this technique to determine the relative intensities of sensitiser fluorescence, after making small corrections for autofluorescence, luminescence from the epi-fluorescence optics, and a computer generated off-set.

For CCD imaging of normal tissue strips fluorescence excitation and emission were performed as above. Samples were illuminated with a 2 cm radius beam of uniform intensity and fluorescence was imaged onto the CCD using a 50 mm macro camera lens.

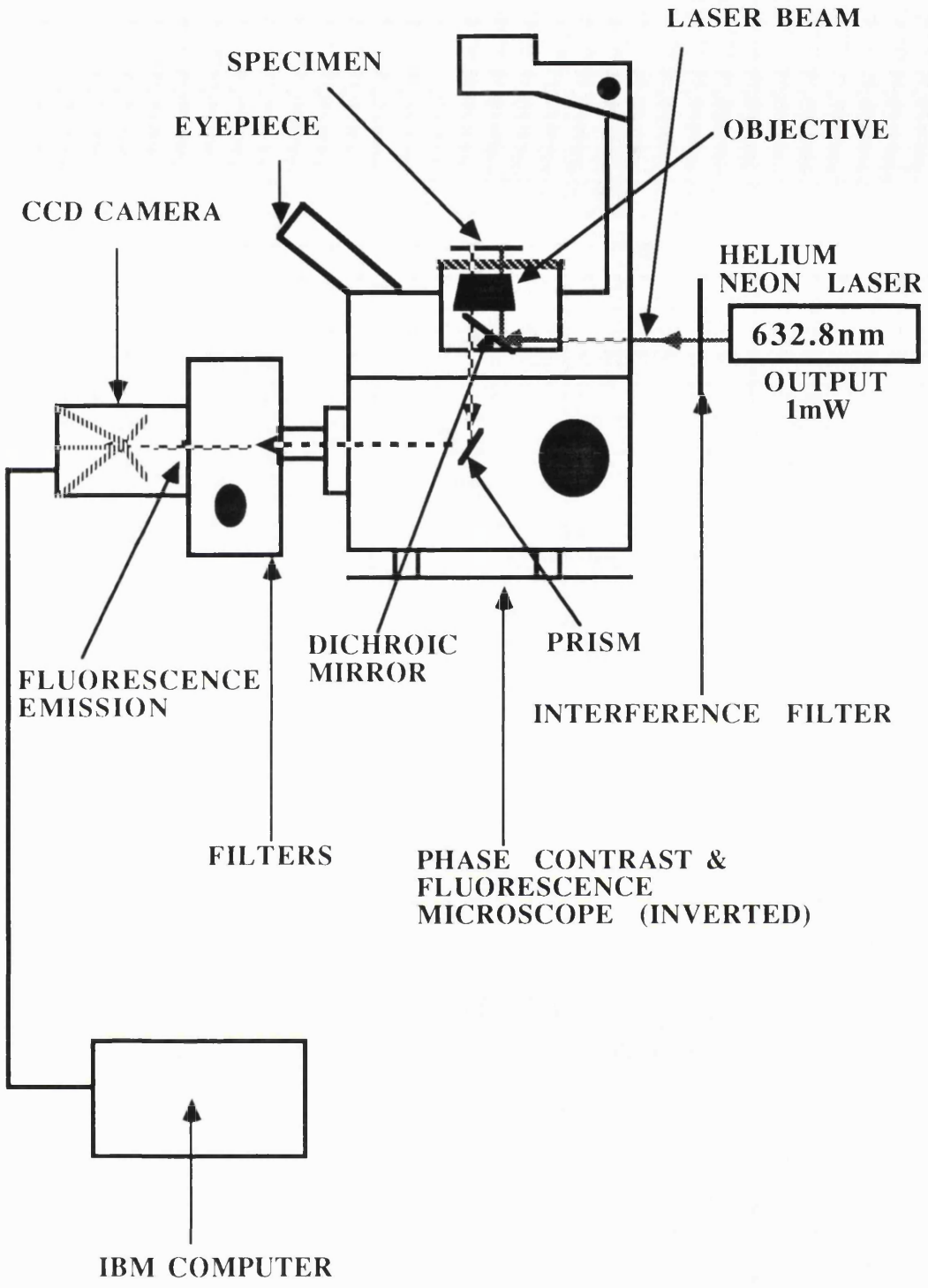


Figure 2.1
Fluorescence microscope and CCD camera system.

2.1.3. Phototherapy

The light source used was a copper vapour pumped dye laser (Oxford lasers Ltd.) which was set to deliver 50 J (100 mW for 500 seconds) from a 200 μm fibre set at a wavelength which corresponded to an absorption band of the photosensitiser. Laser treatments were performed (under general anaesthesia with intramuscular Hypnorm (fentanyl and fluanisone, Jansen Pharmaceuticals Ltd.)) either at laparotomy with the fibre just touching the normal colonic mucosa, or inserted into the apex of a tumour at a depth of 1 mm, or inserted directly into a tumour in the flank. Control animals that had not been sensitised were treated as before to quantify any thermal damage. All animals (unless otherwise specified) were killed 72 hours later when mucosal damage, when present, was at a maximum (Barr *et al.*, 1987). To assess necrosis macroscopically at any treated laser site both the greatest and the smallest diameters of damage were recorded and the mean diameter calculated. Presence of necrosis was confirmed microscopically.

CHAPTER 3 - PHARMACOKINETICS OF AlS_2Pc AND AlS_4Pc

When considering the relative merits of a new photosensitiser it is important to determine its pharmacokinetics in the tissues of interest. Such information will indicate whether there is any selectivity in uptake of the sensitiser by tumour and suggest if this is likely to lead to biological selectivity after light exposure. The time of maximal differential between concentration of sensitiser in tumour and normal tissue can be obtained and this time selected for subsequent light treatment to limit the normal tissue damage obtained in the treatment field. Therefore, we have determined the pharmacokinetics of the di- and tetrasulphonated aluminium phthalocyanine components, AlS_2Pc and AlS_4Pc respectively, in two rat tumour models and associated normal tissues.

3.1. *In vitro* and *in vivo* uptake of AlSPc and its components

To understand the basis of PDT it is important to determine which of the sensitiser species is taken up by cells and to monitor the distribution of sensitisers within individual cells. Using fluorescence microscopy it has been shown that the photosensitiser AlSPc accumulates in the cytoplasmic region of individual cells (Chan *et al.*, 1986). The importance of cell-penetrating properties of the Pc dyes in exerting biological activity has been studied by fluorescence microscopy of V-79 cells (Paquette *et al.*, 1988). Incubations with differently sulphonated AlSPc revealed that the highly photocytotoxic, disulphonated derivative gave uniform fluorescence in the cell cytoplasm, whereas cells incubated with the less photoactive and more hydrophilic tri- and tetrasulphonated derivatives showed very little dye uptake. No sensitiser uptake was observed in the cell nucleus with any of the phthalocyanine derivatives. Therefore it was concluded that differences in intracellular localisation and the degree of aggregation of the dyes contribute substantially to the differences in the efficiency of cell inactivation between lower and higher sulphonated AlPc's. However, a lower degree of dye sulphonation results in a higher tendency for the

formation of aggregates which exhibit lower photochemical activity (Brasseur *et al.*, 1987a). There are also differences in uptake and photoactivity of phthalocyanine molecules sulphonated to the same degree but with differing positions of sulphonation. Using four highly purified isomers of the disulphonated gallium phthalocyanine, it has been shown that the most hydrophilic isomer, with sulphonate groups at opposite positions of the Pc molecule, is completely devoid of photocytotoxicity. However, the most hydrophobic isomer, with sulphonate substituents at adjacent benzene rings, is extremely efficient as a photosensitiser of V-79 cell killing (Brasseur *et al.*, 1987b). In this model it was proposed that only amphiphilic isomers, with sulphonate substituents located at adjacent benzene rings of the Pc macrocycle, are capable of crossing the cell membrane and subsequently exerting phototoxicity. Disulphonated dyes with substituents on opposite positions of the Pc structure, as well as tri- and tetrasulphonated derivatives, lack the essential amphiphilic nature. This explains their poor cell penetrating properties and low photocytotoxicity.

Whereas *in vitro* photosensitisation of cell killing is mainly associated with the photochemical and cell penetrating properties of the dye, *in vivo* PDT activity appears to be governed by many more parameters. Indeed, PDT with most sensitisers involves destruction of the vascularity rather than direct cell kill and is most likely not related to cell-penetrating properties of the dye. However, tumour retention of the dye *in vivo*, like cell uptake *in vitro*, is a basic requirement for a successful PDT outcome. The photodynamic efficiency of substituted MPc will, in addition, to their photophysical properties, depend on their *in vivo* distribution pattern and stability. Furthermore, the mechanism of *in vivo* damage via a type I or II pathway will depend largely on the availability of molecular oxygen and on the intermolecular associations of the sensitiser with vital cellular constituents of the target tissue. Distribution pattern, organ specificity, and molecular interactions are strongly influenced by the nature of the Pc-substituents and the overall lipophilicity of the dyes.

3.2. Studies on the pharmacokinetics of AlS_2Pc and AlS_4Pc

Aluminium sulphonated phthalocyanine has been studied previously in this unit in a range of *in vitro* and *in vivo* PDT investigations. AlSPc as used in these studies is a mixture of isomers with a range of one to four sulphonate groups, with an average of 3 sulphonate groups per molecule (McCubbin, 1985). In a constant effort to develop a pure photosensitiser, the components of AlSPc have now become available. Preparations of mono-, di-, tri- and tetrasulphonated aluminium phthalocyanine have been synthesised, and the former three components purified using liquid chromatography (Svensen, 1990, Ambroz *et al.*, 1991). The di- and the tetrasulphonated components were the purest preparations, with the trisulphonated being of less well controlled purity and reproducibility. The monosulphonated phthalocyanine had the disadvantage of being difficult to solublise in aqueous solution. Therefore the di- and the tetra- components were used for this work. Decreasing sulphonation leads to an increase in lipid solubility and the likelihood of differences in tissue distribution. This has formed the basis for our investigation of the pharmacokinetics of AlS_2Pc and AlS_4Pc in rat tissues, including normal colon and muscle, and colonic tumour and a transplantable tumour in the flank.

3.2.1. Methods

The AlS_2Pc and AlS_4Pc were supplied by the Department of Chemistry, Imperial College, London. AlS_2Pc was initially dissolved in 0.1 M NaOH and buffered to pH 8 so that it was suitable to introduce into animals. AlS_4Pc was dissolved in 0.9 % saline. An absorption spectrum was then produced for each solution in methanol, to enable the determination of the solution concentration, using an extinction coefficient of 1.9×10^5 M^{-1} cm^{-1}. Each animal received 5.65 μmol/kg of either AlS_2Pc or AlS_4Pc (the molar equivalent of 5 mg/kg of AlSPc mixture used in previous studies in this unit (Bown *et al.*, 1986, Tralau *et al.*, 1987 and 1989, Barr *et al.*, 1987, 1990a, 1990b and 1991, Chatlani *et al.*, 1992b)). Colonic tumour animals were killed at 1, 48, 168 and 336 hours and tumour and a section of normal colon were removed.

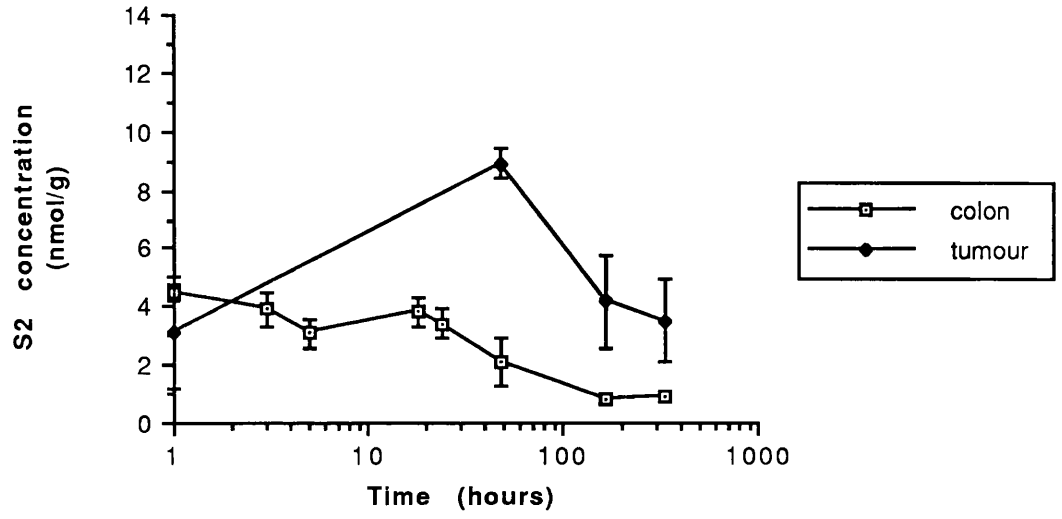
(These time points were studied as the extracted tissues were taken from animals used in other PDT experiments.) Animals with tumours transplanted in the flank were killed at 1, 3, 5, 18, 24 and 48 hours (longer time points could not be studied due to the rapid growth rate of the tumour model). At these times tumour tissue and underlying muscle, and normal colon were excised.

3.2.1.(A) Chemical extraction procedure

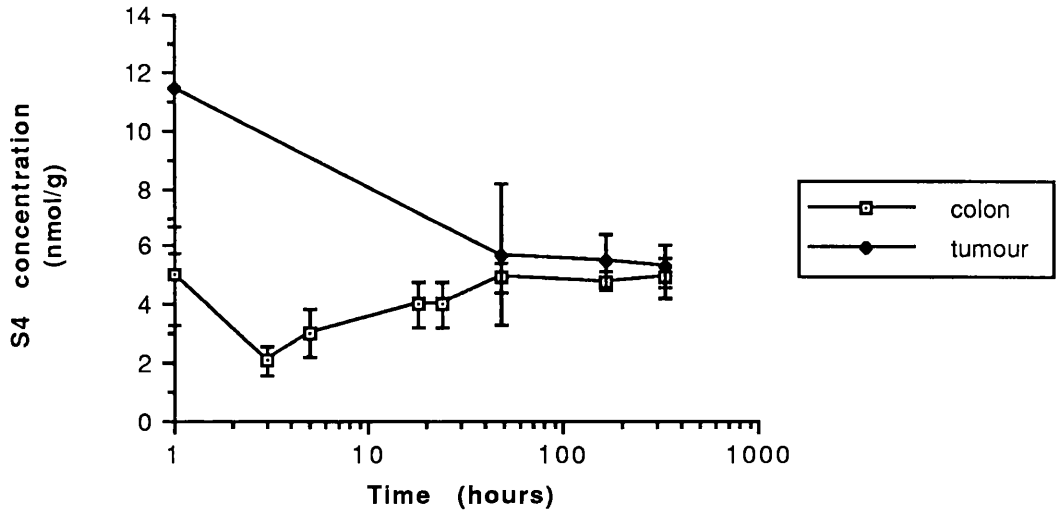
Specimens of normal and tumour tissue underwent a chemical extraction of the phthalocyanine content using a method developed by Chan *et al.* (1988). Sodium hydroxide (0.1 M) was added to thawed tissues at a ratio of 0.1 g to 10 ml NaOH. This mixture was then incubated in a shaking water bath at 50°C for 4 hours. Phthalocyanine concentrations in the resulting solutions were measured by spectrofluorimetry (LS5 Perkin Elmer Luminescence Spectrophotometer). Excitation and emission wavelengths were set to correspond to the fluorescence spectrum of each component and were 603 nm and 671 nm, and 606 nm and 674 nm, for AlS_2Pc and AlS_4Pc respectively, slit widths were always set at 10 nm. The emission was filtered with a Schott long pass (RG 645) filter in order to filter out scattered light at wavelengths below 645 nm. Standard curves were constructed using known amount of each of the phthalocyanine components added to unsensitised control tissue specimens to allow for fluorescence quenching and autofluorescence of particulate matter remaining in sample tubes. The concentration of phthalocyanine in each specimen could then be calculated.

3.2.2. Results

The results are expressed in nmol/g of tissue and are shown in Figures 3.1 (a) and (b) and 3.2 (a) and (b).

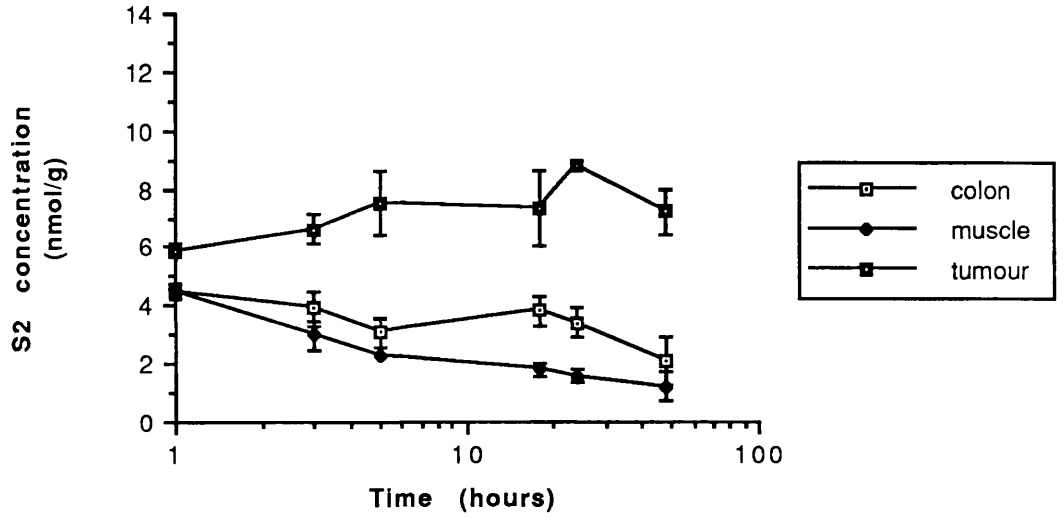


(a)

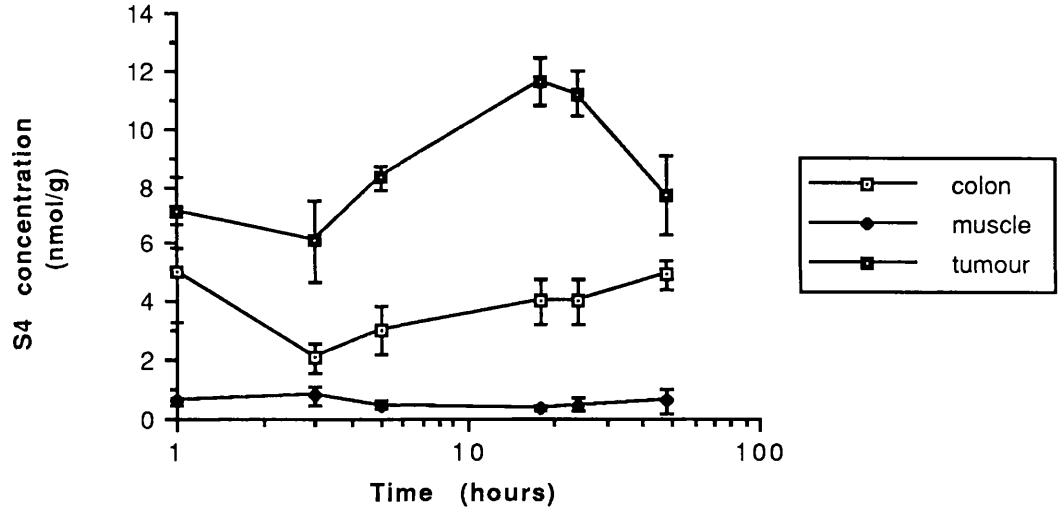


(b)

Figure 3.1
Concentration of extractable (a) AlS_2Pc, or (b) AlS_4Pc in normal colon and **colonic** tumour following an i.v. injection of 5.65 μm/kg. Each point represents the mean (± 1 s.d.) from between 2 to 5 animals.



(a)



(b)

Figure 3.2
Concentration of extractable (a) AlS_2Pc, or (b) AlS_4Pc in normal colon and muscle and **transplanted** tumour following an i.v. injection of 5.65 μm/kg. Each point represents the mean (± 1 s.d.) from between 3 to 5 animals.

From both Figure 3.1 and 3.2 it is evident that AlS_4Pc persisted in normal colon longer than the AlS_2Pc and the concentration was still high at 336 hours (shown in Figure 3.1). The two phthalocyanine components showed similar pharmacokinetic profiles for uptake and elimination of sensitiser by both tumour models although the AlS_4Pc concentration reached higher values. In Figure 3.1 (b) AlS_4Pc showed some selectivity of uptake between colonic tumour and normal colon at 1 hour, although the tumour concentration was only about twice that of the normal tissue and due to the large standard deviation in the tumour results at this point there may only be limited selectivity. At all other times tumour and normal tissue accumulation of AlS_4Pc were equivalent. The transplanted tumour to normal colon ratio (Figure 3.2 (b)) was at best 3 to 1 with the AlS_4Pc and this occurred from 3 to 24 hours after injection of the photosensitiser. This differed greatly to the tumour to muscle ratio which reached 29 to 1 at 18 hours. AlS_2Pc in colonic tumour (Figure 3.1 (a)) showed a peak at 48 hours with a tumour to normal ratio of 4 to 1. This differential was retained at the longer times. The transplanted tumour (Figure 3.2 (a)) showed a maximum ratio of AlS_2Pc uptake of 3 to 1, tumour to normal colon, at 18, 24 and 48 hours. Normal muscle took up considerably more AlS_2Pc than AlS_4Pc and the maximum tumour to normal muscle concentration was 6 to 1.

3.2.3. Discussion

The degree of sulphonation of phthalocyanines affects a number of properties of the dyes of importance for their potential use in PDT. These properties include water solubility, cell membrane uptake and penetration, normal and tumour localisation, tendency to aggregate and consequently capacity to generate singlet oxygen. From a study by Chan *et al.* (1990) uptake and retention of the mono- to tetrasulphonated aluminium sulphonated phthalocyanine derivatives by cells in tissue culture correlated inversely with the degree of sulphonation. However, it was found in the same study that Colo 26 cells growing subcutaneously in Balb/c mice accumulated photosensitiser to a greater extent when the degree of sulphonation increased, such

that AlS_4Pc > AlS_3Pc > AlS_2Pc > AlS_1Pc. This showed that the extent of *in vivo* tissue uptake may be affected profoundly by the the composition of the phthalocyanine molecule. The complex distribution and retention characteristics were considered to be a consequence of relative hydrophobicity/ hydrophilicity properties of the sulphonated species. In this study only slightly higher concentrations of AlS_4Pc were observed in tissues over time than AlS_2Pc, indeed there was greater uptake of AlS_2Pc in muscle than AlS_4Pc. This may well be due to better solublisation of AlS_2Pc in this study than that by Chan *et al.* (1990) who used PBS followed by sonication, which may not dissolve all aggregates. For this investigation AlS_2Pc was dissolved in NaOH and buffered, which should minimise aggregation in serum after an i.v. dose of sensitiser. Aggregates are removed from the circulation by the liver and spleen leaving less sensitiser available for tumour and other normal tissues. Indeed, Chan *et al.* (1989a) has shown that the very aggregated AlS_1Pc was retained selectively by the phagocytic kupffer cells of the liver, and this may be the reason for the differing results between these two AlS_2Pc studies.

The aim of this study was to determine whether greater selectivity of tumour uptake could be attained by using components of AlSPc rather than the mixture. The greatest differential of colonic tumour to normal colon was 4 to 1 with AlS_2Pc. AlS_4Pc has shown selectivity of uptake comparable to that of AlSPc mixture (2-3 to 1, tumour to normal (Tralau *et al.*, 1987)), although it does exhibit a tumour to muscle ratio of 29 to 1. This is not surprising since muscle is known to be a relatively avascular tissue with only very small amounts of sensitiser uptake (Crane *et al.*, 1989). This must be considered when determining the relative merits of a photosensitiser, a high tumour to muscle ratio is generally obtained but is only relevant for treatment of tumours growing in muscle. This is a situation which does not often occur clinically.

In a subsequent study performed in association with P. Chatlani, and reported in Chatlani *et al.* (1992a), phototherapy was applied to DMH-induced rat colonic

tumours and areas of normal colon, sensitised with AlS_2Pc and AlS_4Pc. Quantitative differences in PDT effect were obtained and related to the time interval between photosensitiser and light exposure. The differences in effect were more marked in colonic tumours than in normal colon with AlS_2Pc being more effective than AlS_4Pc, although the concentration of AlS_2Pc was generally lower than that of AlS_4Pc. At 48 hours, although the concentration of the two components were similar in tumour, PDT with AlS_2Pc produced twice as much necrosis in tumour as when AlS_4Pc was used. The difference is considered to be due to different cellular localisation patterns of the components, since the singlet oxygen yield of disulphonated and tetrasulphonated phthalocyanines do not differ (Wagner *et al.*, 1987). AlS_4Pc is apparently localised at sites where photosensitised damage is less critical for cell survival than damage caused to the sites where AlS_2Pc localises. Peng *et al.* (1991) reported that AlS_2Pc localised diffusely in the cytoplasm of a human melanoma cell line whilst AlS_4Pc localised in lysosomes. Using the DMH-induced rat colonic tumour model selective tumour necrosis could be achieved using 1.15 μmol/kg AlS_2Pc, although the diameter of tumour damage was only 2 mm and is unlikely to permit significant therapeutic advantage (Chatlani *et al.*, 1992a). This study suggests that although AlS_2Pc is a more potent photosensitiser than AlS_4Pc and of approximately equivalent potency to that of AlSPc mixture (Barr *et al.*, 1990b), it has not dramatically enhanced selectivity of uptake, or biological response after light, between tumour and normal tissue. A possible reason for the relative selectivity shown by sensitisers for tumours not leading to a selective biological response could be that photosensitisers are also distributed to structures other than tumour cells and that photosensitisation of these structures may contribute to the overall tissue response to PDT, for example, blood vessels.

In conclusion, there are differences in uptake and retention of the photosensitisers AlS_2Pc and AlS_4Pc. These differences are considered to be due to the sensitisers lipid solubility, and this affects their subcellular localisation and efficiency as

photosensitisers. It is therefore important to use pure photosensitisers. However, ultimately using components of AlSPc has not substantially increased the tumour to normal selectivity of phthalocyanines and further studies are required. One approach to overcome the lack of selectivity in biological response between normal and tumour tissues after AlSPc PDT is by using radioprotective drugs and this study is reported in chapter 4.

CHAPTER 4 - ENHANCED SELECTIVITY OF PDT USING A RADIOPROTECTIVE AGENT

When there is only minimal selectivity of uptake of sensitiser by tumour over normal tissue this leads to little, if any, biological selectivity in necrosis after light. Therefore, normal tissues can be damaged as much as tumour tissue. Radioprotective drugs have been investigated to protect normal tissues during light treatment and in this study a phosphorylated thiol drug called WR-77913 was utilised for this purpose. Using fluorescence microscopy the claim that radioprotectives enhance photobleaching of the photosensitiser as a mechanism of protection was investigated. As an introduction previous results of AlSPc phototherapy are summarised below together with observed effects in normal tissues.

4.1. Phototherapy using AlSPc

Agarwal *et al.* (1992) studied skin papillomas induced chemically and with UVB in mice to study the effects of AlSPc. These tumours develop in their own tissue matrix and they are more comparable to the clinical situation than implanted tumour model systems. When tumour bearing animals were given an i.p. injection of 5 mg/kg AlSPc a maximal tumour to normal tissue ratio of 2.4 was obtained. Tumour ablation studies were carried out at this time on animals with comparably sized tumours. This treatment resulted in greater than 80% ablation in tumour volume at 20 days post irradiation. PDT using AlSPc has also proven useful in veterinary medicine of spontaneously arising tumours in cats, dogs and snakes. PDT led to a total disappearance of the tumour with no recurrence to the date of the publication in 67% of animals. However, this result is difficult to interpret as the tumours treated were not of uniform size. The tumour responses were considered to be comparable to those seen with conventional cryotherapy, hyperthermia, or surgery (W.G. Roberts *et al.*, 1991). These studies show that tumour tissue can be killed with PDT utilising AlSPc, but what is not known is what happens to normal tissue in the light field during PDT.

Pancreatic tissues are the only tissues which have shown true biological selectivity after PDT, since tumour to normal AlSPc uptake are comparable. No histological necrosis could be observed in normal pancreas after PDT using 5 mg/kg AlSPc with light doses below 200 J. These are parameters which kill pancreatic tumour tissue. Selectivity was considered to have been achieved by some intrinsic property of the normal pancreatic tissue. The presence of radical scavengers such as glutathione was suggested but could not be proven (Chatlani *et al.*, 1992b). This suggested a therapeutic window for damage between normal pancreas and pancreatic tumour which might prove useful for the treatment of pancreatic cancer. This absence of normal tissue damage is not seen in other normal tissues investigated (Bown *et al.*, 1986; Barr *et al.*, 1987; Pope and Bown, 1991; Loh *et al.*, 1992) unless there is minimal uptake of sensitiser as in bone and tracheal cartilage (Bedwell *et al.*, 1990 and Meyer *et al.*, 1990).

Fluorescence microscopy of trachea sections revealed that negligible quantities of AlSPc were being taken up by the cartilage and this allowed for sparing of this structure during PDT. At no time did the PDT treated tracheas show any significant reduction in strength. These results indicate that normal respiratory tissue is likely to withstand the damaging effects of this treatment (Smith *et al.*, 1993). The effects of PDT using AlS_2Pc on the oral cavity of the rabbit has shown that bone is resistant to PDT. This is considered to be due to relatively low concentrations of phthalocyanine found in the bone (Meyer *et al.*, 1990), although mucosa, muscle and salivary gland do not show this resistance. All tissues healed well following PDT (Meyer *et al.*, 1991). Therefore, provided treated tumour tissue dies after PDT selectivity of PDT may be obtained due to differential healing between tumour and normal tissues.

Using a light source well directed at the tumour will allow little normal tissue to be damaged; although, to ensure that light covers the entire tumour area some exposure to light of normal tissue around the treatment site is inevitable and it is reassuring that

damaged normal tissue will heal after PDT. Ideally no normal tissue damage should be obtained during PDT. Barr *et al.* (1991) studied how normal colon would heal after total destruction of the colonic tumour when the tumour had destroyed all the colonic wall. Healing occurred safely with regeneration of the colonic mucosa, so some degree of normal tissue damage is acceptable. From a previous study it had already been shown that full thickness necrosis of normal rat colon healed without loss of mechanical strength as the submucosal collagen was undamaged and presumably acted as a template for healing by regeneration (Barr *et al.*, 1987).

The situation with brain tumours is markedly different as a concentration difference of 30 to 1 has been obtained between tumour bearing mouse brain and normal mouse brain after i.v. AlSPc. After light irradiation of these animals there was selective necrosis of glioma cells in a tumour bed, using treatment parameters that did not damage normal brain. This selectivity can only be achieved if the effects of PDT on normal brain and tumour are precisely defined (Sandeman *et al.*, 1987).

Truly selective PDT necrosis may be achieved by using low doses of AlSPc. It has proved possible to produce a superficial necrosis in the rat bladder without muscle damage. This heals by epithelial regeneration with no long term impairment of the bladder function (Pope and Bown, 1991). The sparing of muscle was considered to be due to photobleaching of the sensitiser from this layer. The absence of normal tissue damage using 0.5 mg/kg AlSPc was exploited to obtain selective tumour damage in a induced rat colonic tumour model (Barr *et al.*, 1990b). Due to the 2 to 1 tumour to normal ratio for normal tissue surrounding the tumour; the concentration of sensitiser for tumour damage was above the threshold dose but that of the normal tissue was below the threshold dose. Therefore, 2 mm of necrosis could be produced in the tumour without damaging the normal colon given the same light dose of 50 J. The requirement for accurate light dosimetry is less strict when lower drug doses are used. In order to increase tumour damage the AlSPc dose may be increased but this

will lead to some normal tissue damage. Therefore alternative means to increase biological selectivity of PDT treatment are required, and we have studied a radioprotective agent as a means of achieving this goal.

4.2. Radioprotectant studies

To avoid normal tissue damage during PDT we have studied the value of using a radioprotective drug. These agents have been developed to protect against ionising radiation and have shown promising results in experimental animals (Yuhas and Storer, 1969; Phillips *et al.*, 1973). Radioprotective agents have been found to protect normal tissues in both the skin (Dillon *et al.*, 1988) and the eye (J.E. Roberts *et al.*, 1991) against porphyrin induced phototoxicity. These agents are believed to preferentially accumulate in normal tissues due to their hydrophilic properties (Yuhas, 1980). Additionally many tumours do not contain alkaline phosphatase which is required to remove the phosphate group; as a consequence malignant tissue is not able to activate the protective thiol component (Calabro-Jones, 1985). We have investigated a phosphorylated thiol protectant WR-77913 (W7) to determine if it shows the same potential to protect normal tissue during PDT, with AlSPc in the rat colon.

Several mechanisms of protection have been proposed (Dillon *et al.*, 1988; Roberts *et al.*, 1991). One is the scavenging of singlet oxygen by the released sulphydryl of the radioprotectant. In addition, sulphydryls are excellent scavengers of free radicals (Millar and Henderson, 1986). Another possibility, which has been proposed for the photoprotection against phototoxicity induced by porphyrins, is the photobleaching of the sensitiser itself by sulphydryls.

The following experiments were undertaken to determine if the W7 could protect normal colonic tissue from PDT damage whilst allowing damage to occur to the tumour; and whether this tumour damage was of the same magnitude irrespective of

presence or absence of protectant. A further set of experiments was performed to test the hypothesis that W7 protects the normal tissue by enhancement of photosensitiser photobleaching.

4.2.1. Methods

The sensitiser AlSPc (a mixture with an average of 3 sulphonate groups per molecule (McCubbin, 1985)) was obtained from Ciba-Geigy and dissolved in normal saline at a concentration of 2 mg/ml. This solution was stored in the dark. The W7 (3-amino-2-hydroxypropyl phosphorothioate) protectant was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland. This powder was stored at -4^{0}C until required, portions were then dissolved in normal saline and used within 24 hours. Colonic tumours were chemically induced in male Wistar rats as specified in section 2.1.1.(B).

Rats found, at laparotomy, to have two colonic tumours were injected intravenously with 5 mg/kg AlSPc 24 hours before light exposure. W7, when introduced, was given intravenously at a dose of 200 mg/kg 30 minutes before laser treatment (this drug dose and time of administration before light treatment was considered to be effective for normal tissue protection as determined from studies by J.E. Roberts (personal communication)). Phototherapy was carried out as for section 2.1.3. with light delivered from a 200 μm fibre, at a wavelength of 675 nm (maximum absorption for AlSPc). Each animal underwent the following procedures. An area of normal colon and a tumour were treated with the laser at 24 hours after sensitiser administration. Immediately after this the W7 was administered. The animal was then treated at separate normal and tumour sites 30 minutes after the protectant had been injected and in this way each animal acted as its own control. The fibre was then removed and the abdomen closed, allowing recovery from the anaesthetic. The animals were killed 72 hours later and assessed for necrotic tissue (see section 2.1.3.). Further animals, all with normal colons, were treated at only one site with

identical parameters to the previous group of animals, but no protectant was introduced at any stage. This control group was studied to determine whether W7 had caused an effect on lasered sites post treatment.

To test the hypothesis that protection is conferred from the radioprotective by enhancement in the rate of photosensitiser bleaching, we imaged AlSPc fluorescence in strips of normal colon treated *in vivo*, with or without the presence of W7, using the fluorescence microscopy system (section 2.1.2.). By quantification of the fluorescence at the centre of laser irradiated zones and fluorescence at a distant untreated site it was possible to compare the extent of photobleaching of the sensitiser in the presence of W7 to that without W7. Rats with normal colons were treated once 24 hours after AlSPc administration, either 30 minutes after W7 introduction or with no W7 present, using identical drug concentrations and laser parameters as specified above. Animals were sacrificed immediately after laser treatment and their colons removed. Quantitative comparison of intensity of fluorescence was made at the treatment site with fluorescence at an untreated portion by obtaining transverse line scans of the fluorescence. These scans bisected the laser treated zone. Control strips were also examined to enable quantification and exclusion of autofluorescence from unsensitised normal colon.

4.2.2. Results

To determine whether W7 was conferring protection of normal colon during PDT, we compared the mean diameter of necrosis of normal and tumour tissue after PDT before, and after, introduction of W7. This data is shown in Table 4.1.

Mean diameter of necrosis after PDT

(mm) (Mean ±1SD (number of animals))

	Tumour Tissue	Normal Tissue
Light exposure before W7	4.4 ± 0.5 (3)	4.2 ± 1.2 (3)
Light exposure after W7	3.5 ± 0.9 (3)	0.0 (3)
AlSPc only (no W7)	___	4.1 ±1.1 (5)

Table 4.1
Each animal was sensitised with 5 mg/kg AlSPc. 24 hours later a tumour and an area of normal colon were treated with 50 J of light. Immediately after this, 200 mg/kg of W7 were administered and 30 minutes later a second tumour and second area of normal colon were also treated with 50 J of light. Animals without tumours were used as an additional control and not given W7.

In normal colon no necrosis was observed macroscopically in sites treated 30 minutes after administration of the protectant (n = 3). This observation was confirmed histologically in Figure 4.1(a) where preserved glandular architecture is apparent. Necrosis of mean diameter 4.2 mm (n = 3) was seen at sites given the protectant after light exposure (showing histologically as disruption of cellular architecture (Figure 4.1(b)) but this was not significantly different from lesions produced with the same parameters in animals not given the protectant at any time (95% confidence limits), where a mean diameter of necrosis 4.1 mm (n = 5) was obtained. Therefore a 100% reduction in necrosis in normal tissue can be observed using W7 and this difference is significant (95% confidence limits). At tumour sites the mean diameter of necrosis

was reduced by 20% (n = 3), although this difference is not significant (95% confidence limits). (All confidence limits were calculated using the Students 't' test.)

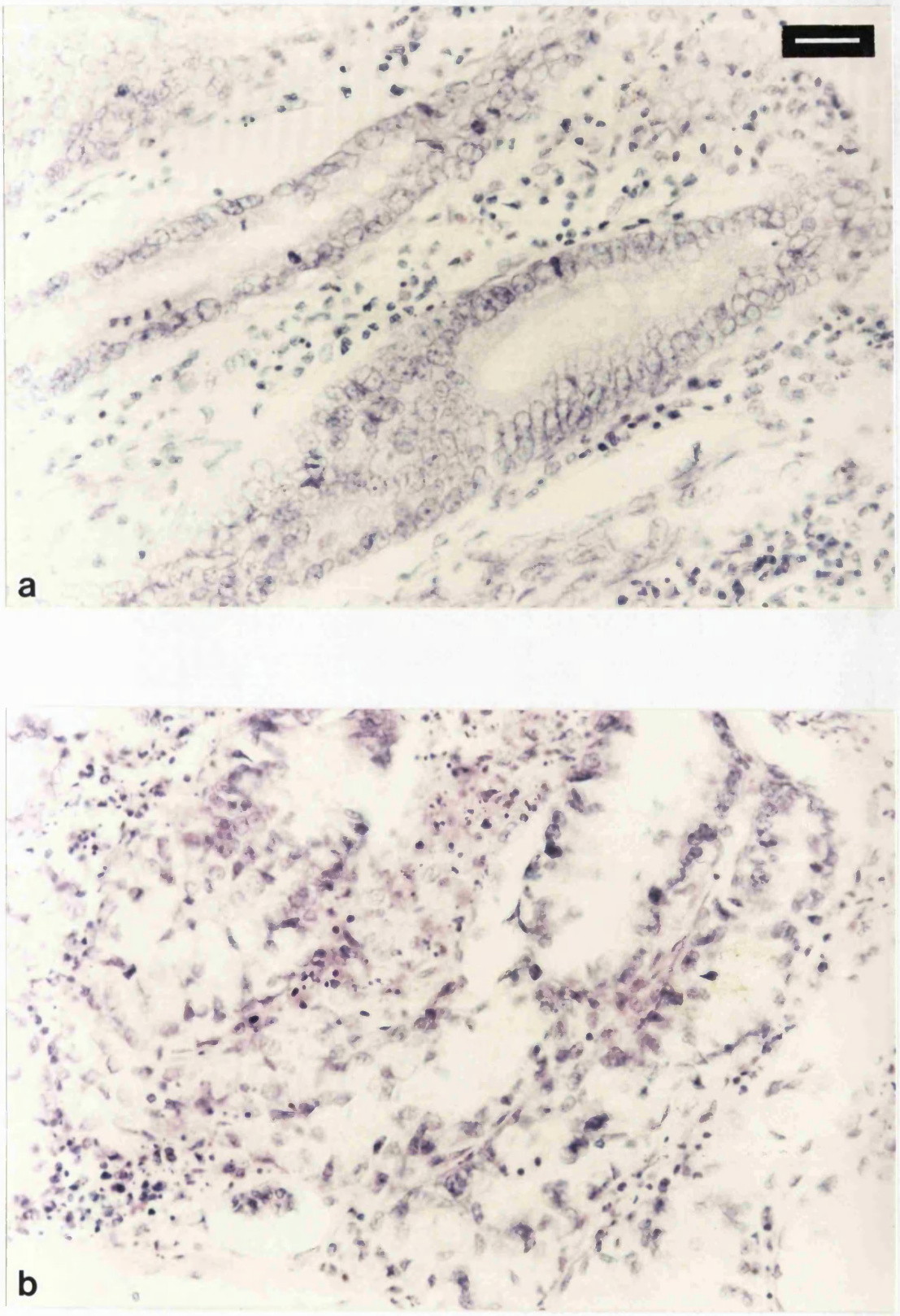


Figure 4.1
Histological sections of normal colonic mucosa sensitised with 5 mg/kg AlSPc treated 24 hours later with 50 J of light after (a), and before (b), 200 mg/kg W7. Scale: white bar represents 15 μm.

To determine if protection is conferred by enhancement in the rate of AlSPc photobleaching, we imaged AlSPc fluorescence in strips of normal colon after *in vivo* light treatment with and without W7 present, and unsensitised control strips, using the fluorescence microscopy system (section 2.1.2.). From the acquired images we obtained transverse line scans of the fluorescence which bisected the laser irradiated zone. The images and two typical scans are shown in Figure 4.2, (a) and (b) with AlSPc and W7 present and (c) and (d) with AlSPc but no W7 present. The fluorescence level is lowest in the laser treated area, and this reduction is attributed to photobleaching of the phthalocyanine (Barr *et al.*, 1990b). The line scans demonstrate that the signal level in the centre of the irradiated zone in each case is approximately 70% lower than the distal areas which received a significantly lower light dose. A series of 5 colon strips in each group were scanned for comparison and full statistical analysis was performed by A.J. MacRobert (Bedwell *et al.*, 1991). The degree of fluorescence bleaching in the centre of the irradiated zone compared to distal areas was found to be (b) 65 ± 10% with W7 and (d) 70 ± 10% without W7 present. The two results are comparable and given the errors involved no difference is discernible. The presence of W7 did not effect the AlSPc fluorescence level in non-irradiated tissue.

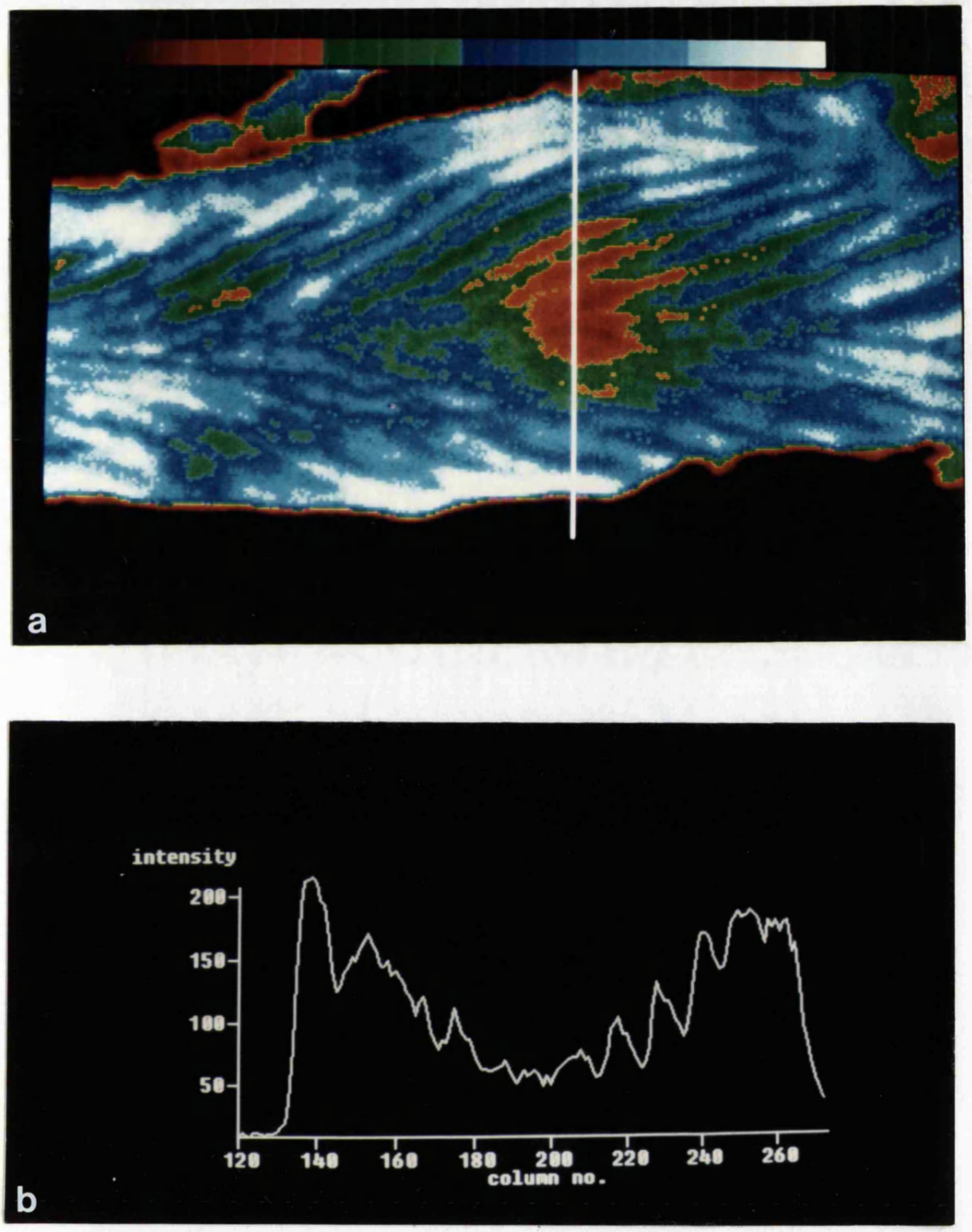
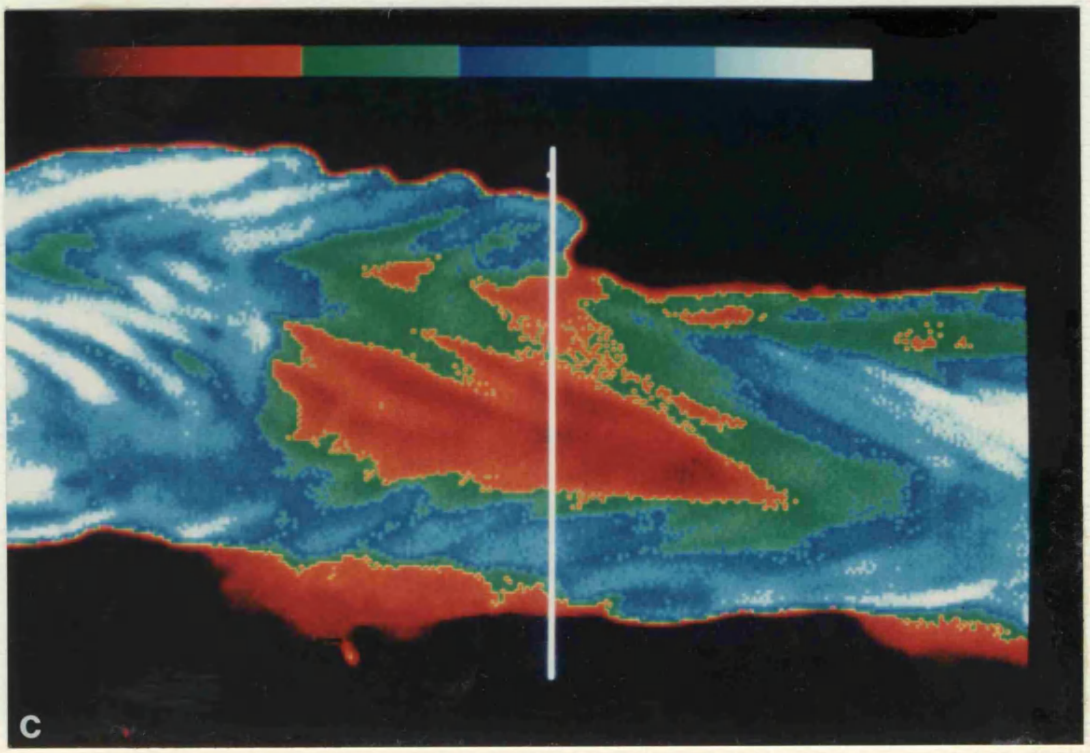


Figure 4.2

CCD images of sensitised normal colon after *in vivo* laser treatment (50 J). (a) with AlSPc (5 mg/kg) and W7 (200 mg/kg) present and (c) with AlSPc (5 mg/kg) present (no W7). Subsequent transverse line scans are shown in (b) (scan of (a)) and (d) (scan of (c)). Lines bisect laser irradiated zone. AlSPc fluorescence intensity (arbitrary units) verses pixel number; length of scan is 150 pixel corresponding to 11 mm. In each case the line scans demonstrate that the signal level in the centre of the irradiated zone is approximately 70% lower than the distal areas which received a significantly lower light dose. The fluctuating profile particularly notable in (b) results from the uneven mucosal surface.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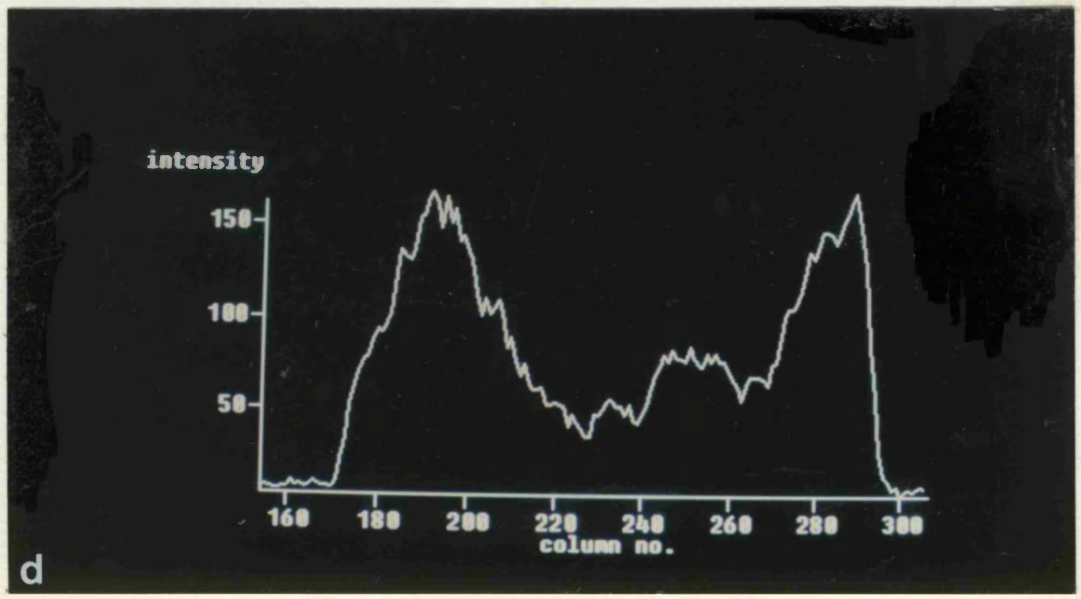
intensity
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4.2.3. Discussion

Much of the interest in PDT centres on photosensitisers being selectively retained by tumour tissue compared with normal tissue. We have shown only a limited degree of selectivity with the di- and tetrasulphonated phthalocyanine components (section 3.2.). In the DMH induced colonic tumour model, utilised in this thesis, this ratio is as low as 2:1 with AlSPc (Tralau *et al.*, 1987). Using radioprotective drugs we aimed to overcome this selectivity problem. Radioprotective agents, although developed to protect against ionising radiation, have shown potential for normal tissue sparing during PDT. Dillon *et al.* (1988) have shown reduction of porphyrin-induced phototoxic damage to mouse skin *in vivo* with protective drugs WR-2721 and WR-77913. We investigated the latter drug (referred to here as W7) to determine if we could obtain the same reduction in phototoxic damage to normal colon using AlSPc as the photosensitiser, and to establish whether the extent of tumour damage was consistent to that without protectant. In this study we have obtained complete and selective protection of normal colon by administration of W7 prior to laser treatment. No protection was observed if the W7 was given after light treatment. Tumour tissue exhibited little or no protection. These results indicate a possible role for W7 in the enhancement of selectivity of PDT in the colon, warranting a more detailed study. With manipulation of protectant dose, and time of administration prior to light exposure, it may be possible to use higher light and photosensitiser doses to allow more complete tumour eradication without significant damage to surrounding normal tissue.

Several mechanisms of protection by radioprotective drugs during PDT have been proposed, as outlined above. The one considered in this paper was enhanced photobleaching by the thiols *in vivo*. This effect has been demonstrated *in vitro* with solutions of porphyrins and eye lens protein (Dillon *et al.*, 1988). The steady state photolysis of porphyrin resulted in a 12% decrease in absorptions in the Soret region. After addition of the thiol penicillamine the photobleaching increased to give a 38%

reduction in absorption for the same light dose. Aluminium sulphonated phthalocyanine does not photobleach in aqueous solution (McCubbin and Phillips, 1986) but has been shown to bleach in solutions containing amino acids (Ferraudi *et al.*, 1988) and *in vivo* during PDT (Barr *et al.*, 1990b). In our experiments in normal rat colon, photobleaching reduced the fluorescence by 70% and this figure did not change in the presence of W7. We would therefore conclude that even if photobleaching is enhanced to a minor extent, this factor can only be of secondary importance. In this case of AlSPc photosensitisation the mechanism of protection in normal tissue may rely on scavenging and deactivation of reactive intermediates (for example, free radicals, singlet oxygen, peroxides) by the hydrolysed form of the protectant containing free sulphydryl groups, as is proposed to occur with endogenous thiols e.g. glutathione (Miller and Henderson, 1986; Thomas and Girotti, 1989; Roberts *et al*, 1991).

In summary, by using the radioprotective agent W7 we can improve the selectivity of PDT in the rat colon, and this may allow us to treat larger tumours without significant damage to the surrounding normal tissue. These improvements are unlikely to be due to enhanced photobleaching of AlSPc.

CHAPTER 5 - ENDOGENOUS PHOTOSENSITISATION FOR PDT USING 5-AMINOLAEVULINIC ACID

It has been shown in chapter 4 that we can protect normal tissue during PDT using a radioprotective drug to overcome the lack in photosensitiser selectivity for tumour. Ultimately it would still be preferable to work with a sensitiser that shows true biological selectivity for tumour damage after light exposure. A novel means of producing photosensitisation has been reported which involves the introduction of a compound called 5-aminolaevulinic acid (ALA). This is not a photosensitiser itself but participates in haem synthesis, the naturally occurring biochemical pathway responsible for producing porphyrins in the body. These porphyrins can then act as sensitisers for PDT. We have investigated ALA as a means of producing greater selectivity in photosensitisation of tumours to normal tissues with the aim of producing a selective PDT treatment. The merits of ALA have been determined in a range of normal rat tissues and two tumour models, and both an i.v. and an oral route of administration were compared. Little was known about ALA for use in PDT when these studies were performed, although considerable amounts were known on the role of ALA in haem synthesis.

5.1. ALA Introduction

The enzyme 5-aminolaevulinate synthetase catalyses the synthesis of 5-aminolaevulinic acid which in the liver, and possibly other tissues also, is the rate limiting step in haem synthesis (Marriott, 1968). This synthetic pathway is a series of irreversible reactions and a defect in one or more of these reactions can lead to a disease state with accumulation of porphyrins and their precursors (Neuberger, 1968). These diseases are the porphyrias and many symptoms are similar to those found in patients after systemic administration of photosensitisers particularly that of skin photosensitivity, as accumulated porphyrins circulate and eventually result in cutaneous photochemical reactions. Patients with porphyrias have worsening effects

after certain drugs particularly the barbiturates (Waldenstrom, 1937) and this led to the discovery that by chemically disrupting haem synthesis accumulation of porphyrins occurred (Granick, 1965). Chemical porphyria, although generally used as an experimental model for understanding the porphyria disease states, has now been suggested as a novel means of endogenous sensitisation for PDT (Divaris *et al.*, 1990). This approach involves the administration of ALA which results in endogenous photosensitisation in certain bacterial cells (Tuveson and Sammartano, 1986), cultured cells (Malik and Djaldetti, 1979) and in whole animals (Sima *et al.*, 1981).

5.1.1. Haem synthesis

Little is known about the regulation of haem biosynthesis in tissues other than the liver, and the haem synthesis pathway described here is therefore a summary of how it occurs in liver tissue (Ades, 1990). The production of ALA is the committed step in haem synthesis. ALA is produced from the condensation of glycine and succinyl CoA in the reaction catalysed by ALA synthetase. The enzymes involved in haem synthesis are distributed between several compartments in eukaryotic cells with ALA synthetase located in the mitochondrial matrix. ALA produced in the matrix moves to the cytoplasm of the cell where ALA dehydratase catalyses the formation of porphobilinogen from two molecules of ALA. Four porphobilinogen molecules are polymerised then by porphobilinogen deaminase into hydroxymethylbilane. Uroporphyrinogen III synthetase catalyses the closure of hydroxymethylbilane to uroporphyrinogen III. The decarboxylations of the acetic acid side chains, carried out by uroporphyrinogen decarboxylase, yield coproporphyrinogen III. The decarboxylations and oxidations of the proprionic acid side chains to vinyl groups in two rings then follow in the inter-membrane space of mitochondria; the steps are catalysed by coproporphyrinogen oxidase with protoporphyrinogen IX being the product. Protoporphyrinogen IX is oxidised to protoporphyrin IX by protoporphyrinogen oxidase, an oxygen requiring enzyme bound to the inner-

mitochondrial membrane. The insertion of ferrous iron into protoporphyrin IX occurs in the mitochondrial matrix and is catalysed by the inner membrane enzyme ferrochelatase. This pathway is depicted in Figure 5.1. Haem is considered to be synthesized in most tissues as it is a constituent of the cytochromes, myoglobin, and several oxidative enzymes as well as haemoglobin (Gibson *et al.*, 1961). It would seem probable that tissues other than the liver might regulate their synthesis of haem in a similar manner to that of the liver.

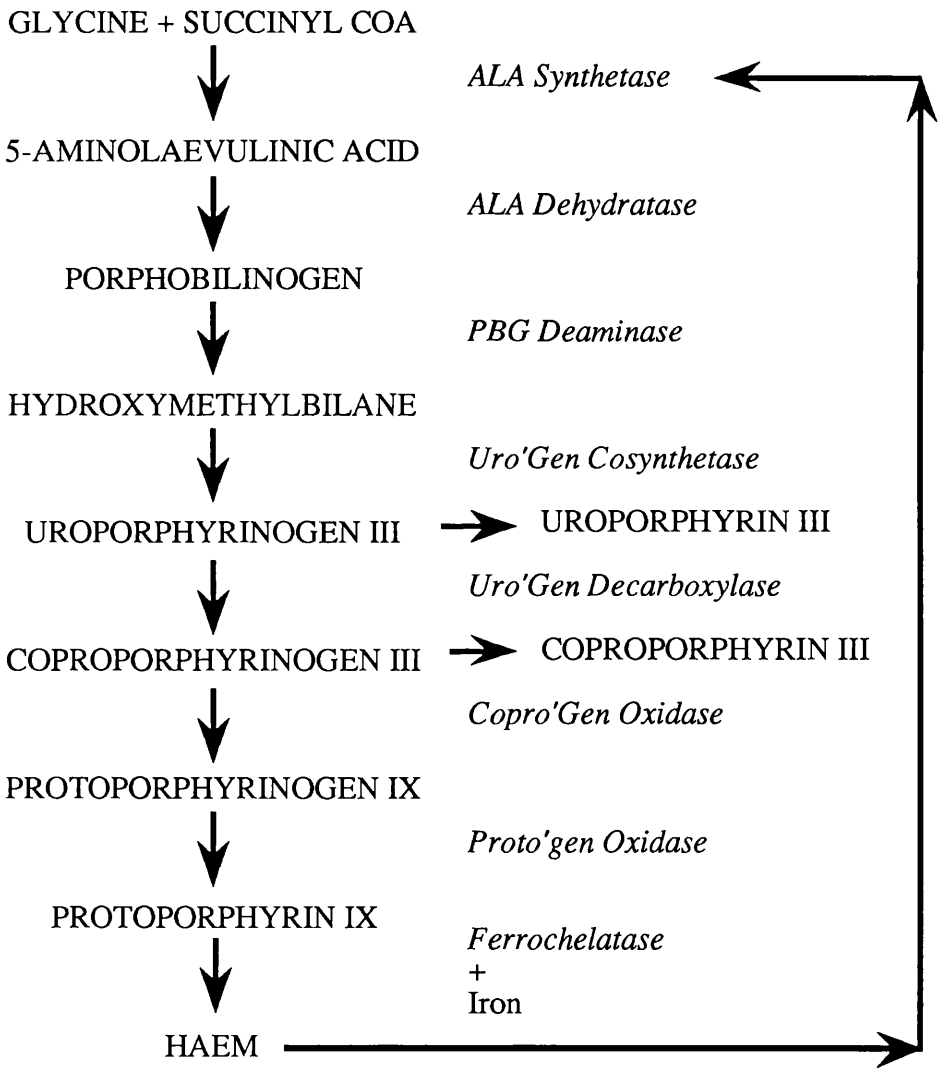


Figure 5.1
Haem biosynthesis pathway.

5.1.2. Production of ALA

Compared to the activity of the other enzymes in haem synthesis the activity of ALA synthetase is lowest. The activity of the enzyme becomes markedly elevated in humans and in animals exposed to any number of diverse drugs and chemicals. Inducers generally lead to the accumulation of several haem precursors in addition to inducing ALA synthetase activity. The effects of these agents can be quite adverse and are due primarily to the over production and accumulation of porphyrins in various tissues (Granick, 1965). Therefore a tight control of ALA synthetase activity is required because of the deleterious effects of porphyrin over production. Its activity is effectively suppressed by an elevation in intra-cellular haem concentration. An increase in haem concentration negatively affects the production of the enzyme (Hindmarsh, 1986). It is this negative feedback which is disrupted upon introduction of excess ALA. Through the introduction of an excess of ALA the regulatory feedback system is overloaded causing an accumulation of porphyrin precursors to haem, particularly protoporphyrin IX (PP IX) (Pottier *et al.*, 1986).

5.1.3. ALA *in vitro*

Porphyrin synthesis has been shown to be stimulated by exogenous ALA in uninduced Friend erythroleukemic cells. The products were then isolated and identified using thin layer chromatography. Initially the main accumulated product was found to be uroporphyrin which was then converted enzymatically to protoporphyrin. The accumulation of protoporphyrin was a consequence of the lack of ferrochelatase in the mitochondria, needed for iron insertion into the ring (Malik and Djaldetti, 1979). Malik and Lugaci (1987) have reported on the photoinactivation of these Friend erythroleukaemic cells mediated by the photodynamic effect of the accumulated endogenous porphyrins. Proerythroblasts were grown in culture medium enriched with ALA. The mitochondrion was the first organelle to be affected by light exposure in addition to an enlargement of the nuclear envelope inner space. From this it was concluded that endogenous porphyrin was initially accumulated in

mitochondria. Cells cultured for more than 3 days and exposed to light were sensitised in a variety of loci, including the chromatin and plasma membrane. This was thought to indicate that the endogenously produced protoporphyrin and possibly, to some extent other porphyrins, were gradually translocated to other cytoplasmic membranes and other sensitive sites in the cell and by photosensitisation induce the cellular damage. The interrelationship between the effect of serum on the induction of porphyrin synthesis, intercellular porphyrin accumulation and photodynamic sensitisation of human K562 after supplementation with ALA was studied (Hanania and Malik, 1992). Porphyrins were shown to accumulate in the cells, whilst addition of serum caused translocation of porphyrin from the cell to the serum. Addition of ethylenediaminetetraacetic acid (EDTA) with serum enhanced porphyrin accumulation in the cells, since EDTA reduces the concentration of available cellular ferrous iron. The photoactivation of these cells was thus increased by 3 orders of magnitude. Enhancement of porphyrin accumulation has also been shown with the potent iron complexing compound desferioxamin (Ortel and Honigsmann, 1991). The chemicals EDTA and desferioxamin, although useful for *in vitro* porphyrin accumulation have only limited, if any, application in clinical PDT due to their considerable systemic toxicity.

Tissues *ex vivo* have also shown the ability to synthesize porphyrins in the presence of ALA. Tissues from both dogs and humans were removed at autopsy and homogenised and incubated an aqueous solution of ALA for 18 hours at which time the synthesized porphyrins were extracted. The tissues formed coproporphyrin and protoporphyrin in order of decreasing amounts: liver > adrenal gland > spleen > kidney > heart > skeletal muscle > brain. Protoporphyrin was generally produced in greater quantities than coproporphyrin (Sardesai *et al.*, 1964). Preliminary studies revealed that porphyrin synthesis after ALA was significantly enhanced in explant cultures of human breast carcinoma (Navone *et al.*, 1988). From these results it was speculated that tumour cell porphyrin synthesis deviated from synthesis in normal

tissues. Porphyrin biosynthesis from ALA was then compared using tissue explant cultures, in human breast cancer and its normal tissue. The activity of ALA-dehydratase, porphobilinogenase and uroporphyrinogen decarboxylase were determined in the tumour and normal mammary tissues. Porphyrin synthesis capacity of human breast carcinoma was 20-fold enhanced, as compared with normal tissue, at least between the stages of porphobilinogen and coproporphyrinogen formation. The activity of the three enzymes examined were always lower in normal tissue than in tumour tissue and porphyrin biosynthesis was increased in breast cancer tissue (Navone *et al.*, 1990).

5.1.4. ALA *in vivo*

Pottier *et al.* (1986) have used a sensitive spectrofluorimetry system to determine the fluorescence emission spectra of photosensitised mice. After an i.p. injection of 1000 mg/kg a fluorescent species was detected that was consistent with PP IX. Therefore it was considered that it was this porphyrin that accumulated as opposed to other intermediates of haem synthesis after introduction of ALA. The intensity of the skin PP IX fluorescence increased up to 6 hours and then gradually decreased over a period of 10 to 12 hours, with background fluorescence levels reached by 24 hours. Divaris *et al.* (1990) have performed *in vivo* studies which showed that after intraperitoneal administration of ALA to mice PP IX accumulated in the skin in sufficient amounts to cause photosensitised damage on exposure to light. The key to this treatment is that only certain cells when exposed to ALA become sensitised sufficiently to elicit a biological response upon light treatment. It has been concluded that ALA-induced PP IX accumulates primarily in tissues that line surfaces (epidermis, conjunctiva, oral mucosa, respiratory mucosa, vaginal mucosa, rectal mucosa, serosal surfaces, endometrium, urothelium), or in glands with ducts that empty onto such surfaces (liver, sebaceous glands, mammary glands, salivary glands, seminal vesicles). In contrast, major tissues of mesodermal origin (muscle, connective tissue, cartilage and bone marrow and red blood cells) do not develop

significant PP IX fluorescence after a single dose of ALA *in vivo* (Kennedy and Pottier, 1992). This group has also shown that the urothelium of the bladder and the endometrium in the uterus become highly sensitised following intraperitoneal administration of ALA, whereas the underlying layers in these organs exhibited relatively little sensitisation which could enable selective destruction of superficial cancers in the urothelium and endometrium without causing perforation to the bladder or uterus. The failure of ALA to induce PP IX fluorescence in certain types of cells and tissues is proposed not to be due to a complete absence of the necessary metabolic pathway but due to a differing capacity of some cells to synthesize PP IX (Kennedy and Pottier, 1992).

It has been reported that ALA in aqueous solution passes readily through abnormal keratin, but not through normal keratin. The topical application of ALA in aqueous solution to actinic keratoses or superficial basal cell or squamous cell carcinomas induces PP IX photosensitisation that is restricted primarily to the abnormal epithelium. Subsequent exposure to photoactivating light selectively destroys some lesions. Some promising results have been obtained in a clinical trial using the topically applied ALA, which achieved a 90% complete response rate and a 7.5% partial response in the treatment of basal cell carcinomas. The cosmetic results were excellent and patient acceptance of the technique was very good (Kennedy *et al.*, 1990). It should be noted that direct administration of PP IX itself has been limited by the poor water solubility of this particular porphyrin (Berenbaum *et al.*, 1982). Warloe *et al.* (1992) have investigated the biolocalisation of ALA-induced porphyrins in human basal cell carcinoma and normal skin tissue after topical application of an ALA-emulsion. Surgical excision of the carcinoma was performed 1 to 5 hours after application of the ALA and were examined using fluorescence and light microscopy. Topical ALA-induced porphyrins selectively accumulated in the carcinoma. Considerably less porphyrin was found in the adjacent non-neoplastic epidermis and dermis, with no accumulation of porphyrins in normal skin.

Initial experiments by Sima *et al.* (1981) demonstrated that the toxicity of exogenous ALA to mice was acceptably low, and that after the systemic administration of a high dose of 1000 mg/kg of ALA there was only a transient depression of motor nerve conduction velocity. All animals recovered full neurological function by 1 week. It was not known whether this limited toxicity was due to the excess of ALA or the induced PP IX. A close to lethal dose of HpD (100 mg/kg) caused a similar depression of motor nerve conduction velocity, although this took three weeks to recover. Riopelle and Kennedy (1982) used a single sensory neurone bioassay for nerve growth factor to quantitate the toxic influences of products of the haem biosynthesis pathway. This study enables analysis of direct toxic effects rather than toxicity of metabolites. The study indicated that PP IX, uroporphyrin I and coproporphyrin I displayed dose dependent toxicity to neurones within a 24 to 30 hour time period suggesting that these porphyrins are directly toxic to neurones. Whereas uroporphyrin I and coproporphyrin I were toxic in the micromolar range, PP IX was at least two orders of magnitude more toxic on a molar basis. ALA itself was not toxic at concentrations of up to 1.5 mM. HpD was shown to have similar toxicity to uroporphyrin I and coproporphyrin I.

In summary, it has become apparent that by systemic administration of ALA photosensitisation of certain tissues occurs. This is probably due to accumulation of PP IX, although other porphyrins cannot be ruled out. The key to this treatment is that only certain cells become sensitised. Linings of hollow tissues and tumours have shown a high capacity to synthesis porphyrins, and sensitisation in the skin has been shown to return to background levels by 24 hours. ALA itself and its induced porphyrins appear to cause no greater neurotoxicity than the conventional sensitiser HpD and that any *in vivo* neurotoxicity is reversible.

5.2. Preliminary normal colon studies

At the time of these experiments there was no published data to indicate what might be a therapeutically useful dose of ALA. Therefore a very preliminary CCD fluorescence microscopy study was performed on normal rat colon sections to determine what ALA dose may induce sensitisation and at what time after i.v. administration this might occur.

5.2.1. Methods

5-ALA was obtained from Sigma Chemical Company (UK), with a purity of 98%, dissolved in phosphate buffered saline at a concentration of 80 mg/ml and used within 24 hours. ALA was administered intravenously in doses of 50, 100, 150 and 200 mg/kg via tail vein injection to rats (section 2.1.1.(A)) under general anaesthetic from intramuscular Hypnorm (Fentanyl and fluanisone, Jansen Pharmaceuticals Ltd.). Animals were killed and normal colonic tissue removed at 3, 6 or 24 hours after each of the ALA doses. Imaging and quantification of fluorescence in sections of normal tissue were achieved using fluorescence microscopy (see section 2.1.2.). Imaging of porphyrin fluorescence has usually employed excitation near 400 nm corresponding to the intense Soret band absorption (in contrast to this work where excitation was 632.8 nm). However, excitation at these shorter wavelengths gives rise to considerable background luminescence from the microscope optics and much higher tissue autofluorescence and therefore offers no clear-cut advantage to the excitation wavelength employed here which, moreover, is very close to the selected therapeutic wavelength of 630 nm. Whole images were analysed using this technique to determine the relative intensities of porphyrin fluorescence between different sections.

5.2.2. Results and discussion of preliminary normal colon studies

For these times greatest fluorescence was found at 3 hours with background levels being reached by 24 hours as shown in Figure 5.2.

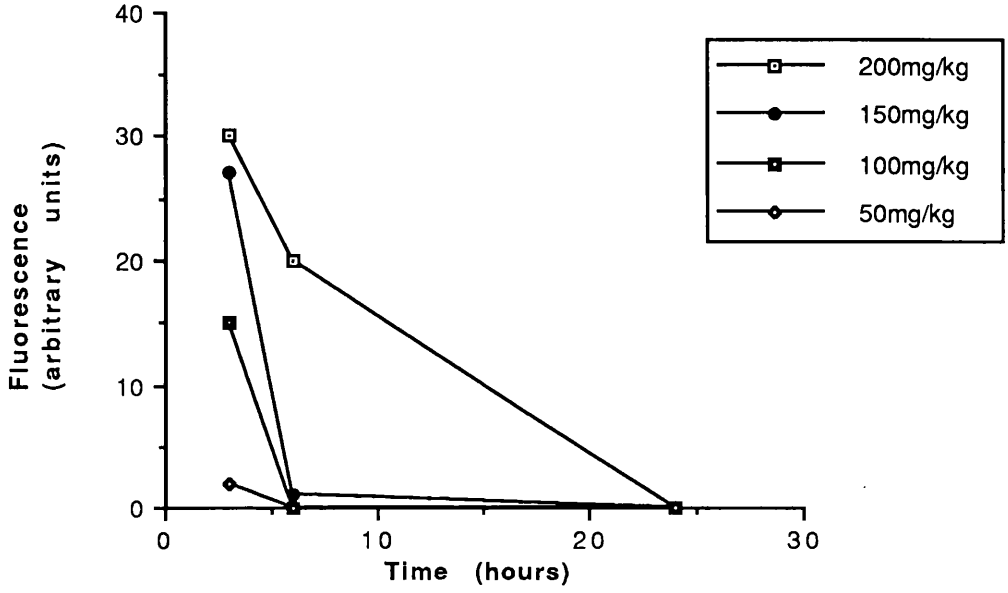


Figure 5.2
Microscopic fluorescence levels in normal colon sections as a function of time after administration of 50, 100, 150 and 200 mg/kg ALA.

After the 200 mg/kg dose there was still significant sensitisation at 6 hours and this dose was therefore considered to be the most useful therapeutically as it provided a longer range of times available for PDT treatment.

5.3. Normal tissue studies

Once a suitable ALA dose had been determined a range of normal rat tissues were studied using fluorescence imaging analysis as a means of identifying where the ALA-induced fluorescence was located.

5.3.1. Methods

After introduction of an i.v. dose of 200 mg/kg ALA (as for section 5.2.1.) into a series of normal rats (section 2.1.1.(A)) the colon, uterus, stomach and bladder were removed at 1, 3, 6 and 24 hours after ALA. Tissue sections were prepared and fluorescence imaging performed as in section 2.1.2. Specific tissue layers were analysed using this technique to determine the relative intensities of porphyrin fluorescence. A fluorescence count was performed for each tissue layer. After fluorescence imaging, the sections were fixed and stained with haematoxylin and Van Gieson's (HVG) stains or haematoxylin and eosin (H & E) and the same microscopic areas were then photographed for confirmation of histology.

5.3.2. Results and discussion of normal tissue studies

A typical fluorescence image and a corresponding stained section produced from each tissue is shown in Figures 5.3 (a) and (b) colon, 5.4 (a) and (b) uterus, 5.5 (a) and (b) stomach and 5.6 (a) and (b) bladder. All tissues gave peak levels of fluorescence at 3 hours, except uterus which gave a peak at 6 hours. The time of peak mucosal fluorescence was also the time of greatest differential between mucosa and underlying muscle layers. Background levels of fluorescence across all tissue sections were observed by 24 hours.

In normal colon fluorescence levels were maximal at 3 hours (Figure 5.3). At all times the mucosa exhibited the highest fluorescence, with a ratio of 1 to 5 between submucosa and mucosa, and 1 to 8 between muscle and mucosa at 3 hours. It is very important to maintain the integrity of the colon wall during PDT. Sensitisation of the

mucosa is not a problem as this has been shown to regenerate after PDT (Barr *et al.*, 1987), although sparing of underlying layers leaves a framework for colonic wall regeneration. Therefore, this pattern of ALA-induced sensitisation would seem ideal. PDT with ALA in the colon may offer a treatment for polyps and cancers without disturbing the strength of the colon so that perforation would be unlikely.

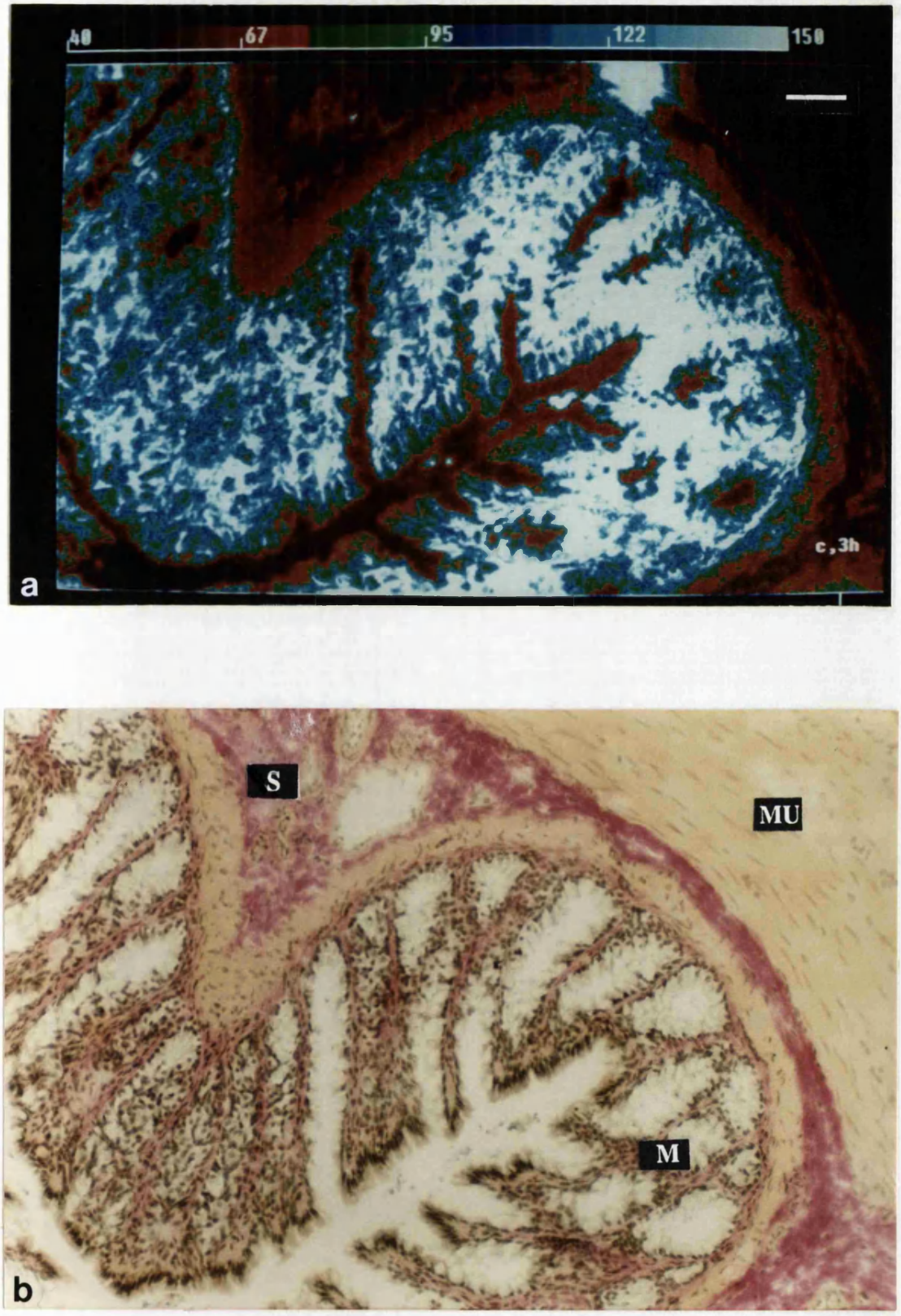


Figure 5.3
Fluorescence image of normal colon (a) after 200 mg/kg ALA together with the subsequent photographs of the same section stained with an HVG stain (b) (mucosa (M), muscle (MU) and submucosa (S)). Scale: white bar represents 60 μm.

Rat uterus gave specific sensitisation of endometrium at all times when fluorescence was above background levels (Figure 5.4). A maximal ratio of 1 to 4 between myometrium and endometrium was observed at 6 hours. Since selective endometrial ablation is a preferable option to hysterectomy for excessive menstrual bleeding, we believe PDT after treatment with ALA might be a highly selective and safe treatment for menorrhagia. In the human uterus, the thick myometrium would allow for adequate endometrial destruction with a large safety margin even if there was some damage to the underlying muscle. A preliminary phototherapy study has been undertaken in the rabbit uterus, within this unit. Reliable necrosis of the endometrium without significant myometrial damage has been obtained and the long term effects on endometrial regeneration are now being investigated (Judd *et al.*, 1992).

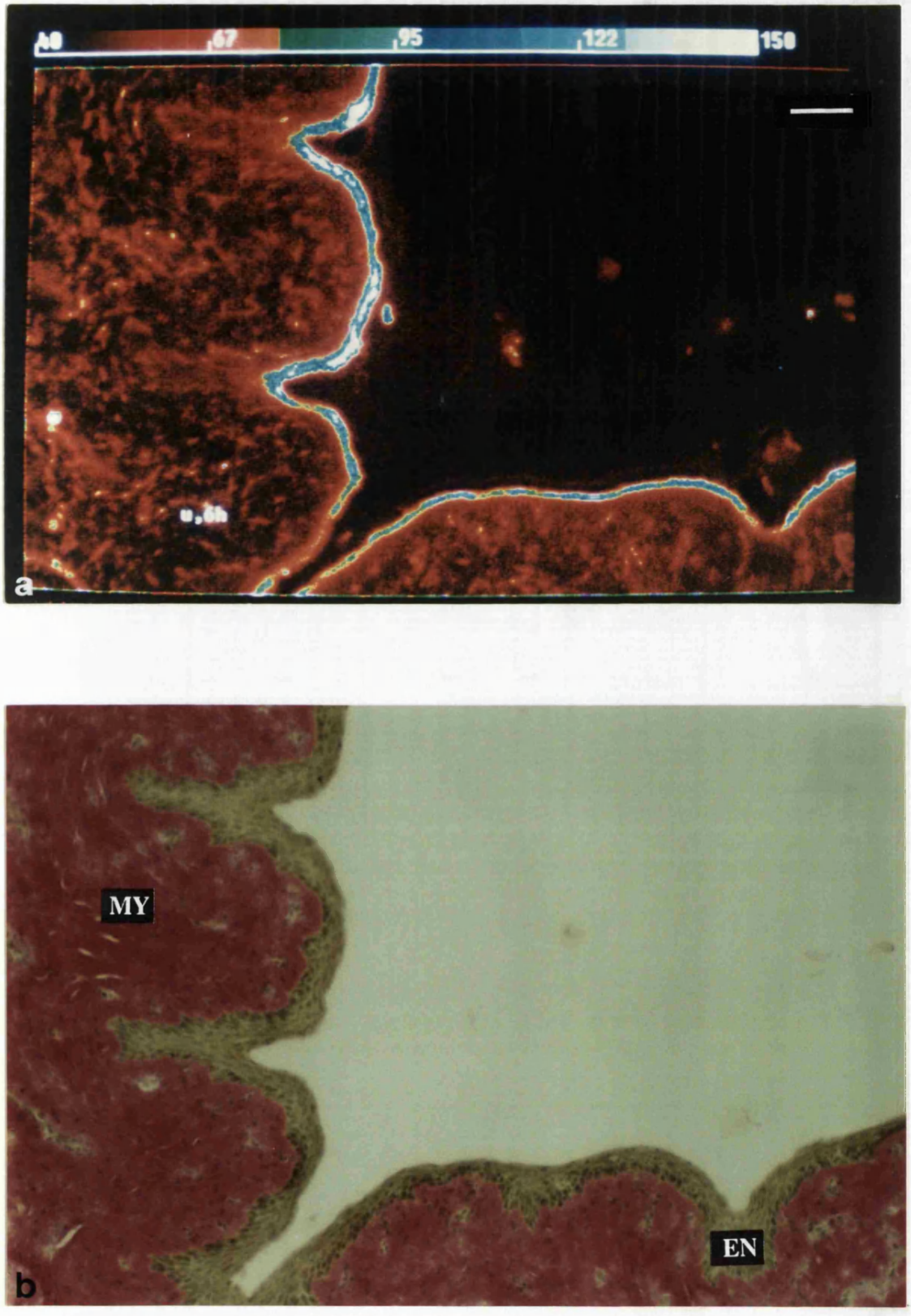


Figure 5.4
Fluorescence image of normal uterus (a) after 200 mg/kg ALA together with the subsequent photographs of the same section stained with an HVG stain (b) (myometrium (MY), endometrium (EN)). Scale: white bar represents 60 μm.

Normal rat stomach also shows selective sensitisation of the mucosa (Figure 5.5). At all times the fluorescence pattern was predominantly mucosal with very little fluorescence seen over the submucosal and muscle layers. At the time of peak mucosal fluorescence a fluorescence ratio of approximately 1 to 5 was obtained between submucosa and mucosa, and 1 to 10 between muscle and mucosa. After further investigation we have shown selective photosensitisation which correlates with PDT damage. ALA-induced sensitisation produced selective photosensitisation of the gastric mucosa for its photodynamic ablation with sparing of the underlying tissue layers with appropriate doses of ALA (Loh *et al.*, 1992). This work could prove useful for the treatment of dysplasia in the upper gastrointestinal tract since this carries a risk of invasive malignant change. Surgical excision of the affected organ is the only treatment currently available. PDT has been shown to be promising in the treatment of early and superficial tumours and may be useful for the ablation of dysplastic mucosa. This disease has a diffuse nature and treatment would necessarily involve destruction of large areas of mucosa and it is desirable to confine its effects to the mucosa in order that safe healing can take place. Another consideration is whether PDT necrosed dysplastic necrosis heals as normal or dysplastic mucosa. These studies need to be undertaken.

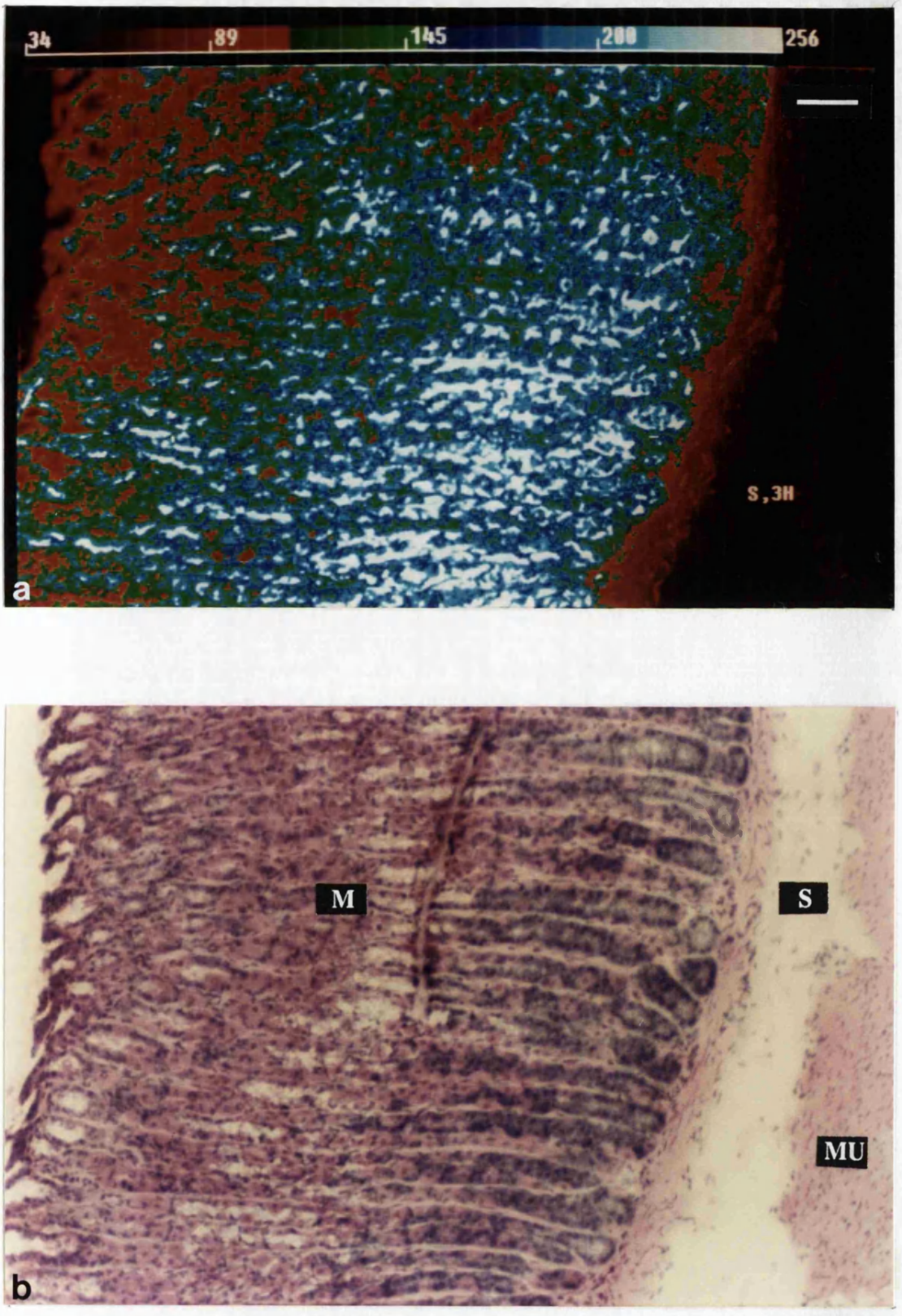


Figure 5.5
Fluorescence image of normal stomach (a) after 200 mg/kg ALA together with the subsequent photographs of the same section stained with an H & E stain (b) (mucosa (M), muscle (MU) and submucosa (S)). Scale: white bar represents 60 μm.

Normal bladder has shown specific sensitisation of the urothelium (Figure 5.6). The peak fluorescence ratio was 1 to 6 between the underlying muscle and the urothelium. The only exception was 24 hours where background levels of fluorescence were observed throughout the tissue section. Bladder seems ideally suited to PDT as it is readily accessible endoscopically and the entire mucosa can be treated simultaneously, although muscle fibrosis and permanently reduced bladder capacity, incontinence and upper tract deterioration has been reported (Nseyo *et al.*, 1985; Harty *et al.*, 1989). It has been shown in the normal rat bladder that if the wall muscle is not damaged then resulting functional impairment is less severe, and recovers more quickly and completely than after treatments where the muscle is damaged (Pope and Bown, 1991). Using AlSPc at low doses and administering light when there was considerably more sensitisation of the urothelium it was possible to produce a superficial necrosis without muscle damage across a range of light doses. This healed by epithelial regeneration with no long term functional impairment. We believe normal bladder with ALA-induced sensitisation due to its selectivity to the urothelium will heal without muscle damage, in a similar fashion, after light exposure.

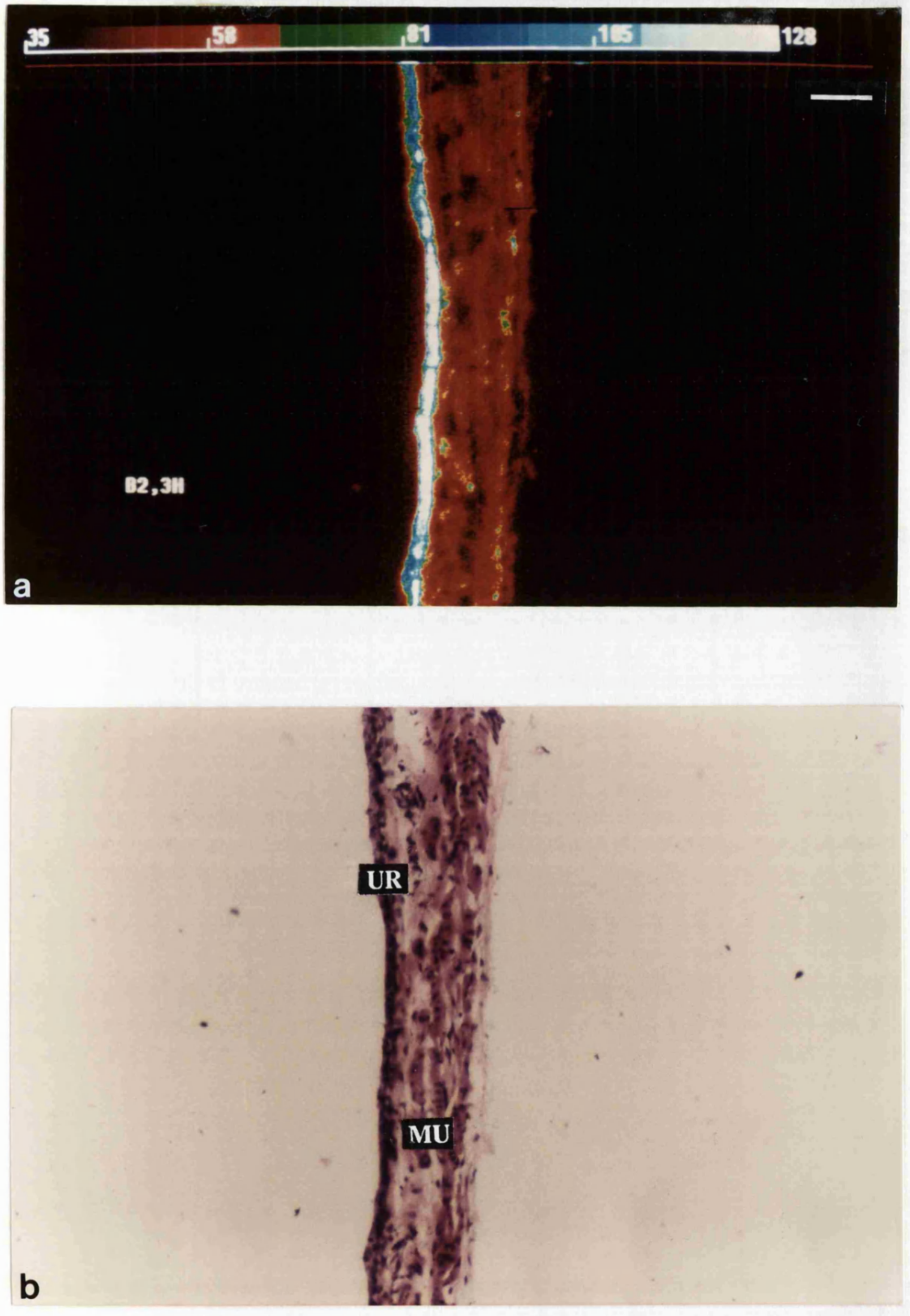


Figure 5.6
Fluorescence image of normal bladder (a) after 200 mg/kg ALA together with the subsequent photographs of the same section stained with an H & E stain (b) (urothelium (UR), muscle (MU)). Scale: white bar represents 60 μm.

The reason for such selective sensitisation of linings of these normal tissues is still speculative. Following administration of exogenous ALA accumulation of PP IX is dependent upon there being a differential rate of its synthesis versus its conversion to haem in the cells of the various tissues. The greater accumulation of PP IX seen in the mucosal cells can either be due to a differentially larger PP IX synthesising capacity or a lower conversion capacity of PP IX to haem by mucosal cells as compared to the underlying tissue layers. Haem is required in every nucleated cell. As the quantity of haem required by different cells varies, the capacity for its synthesis can be expected to vary with different tissues, or tissue layers. Since the mucosal layers of the tissues investigated in this study have a higher rate of cellular turnover they are, therefore, probably also the most metabolically active. By inference, neoplastic tissue may also prove to have a high capacity to produce porphyrins and this has been studied in section 5.4.

5.4. Normal and tumour tissue studies

We have shown specific sensitisation of linings of hollow organs including the colon in section 5.3. and in this section a more extensive study has been undertaken on the colon after intravenous administration of ALA. The aim was to investigate and quantify the porphyrin fluorescence in rat colonic tissue, both normal and tumour, and a transplanted tumour in the flank, and to determine microscopically which sites exhibited porphyrin accumulation as a function of dose and time after administration. These tissues were then treated with laser light for assessment of photosensitising activity and selectivity of damage. A comparison was made between the fluorescence distribution and the biological effect of ALA-induced photosensitisation. Additionally, a study was performed to determine whether the sensitising species underwent photodegradation.

5.4.1. Methods

The induction of colonic tumours and production of the transplantable flank tumour were performed as for section 2.1.1. (A) and (B) respectively. All the studies in this section were then carried out after an i.v. injection of 200 mg/kg ALA (as for section 5.2.1.).

5.4.1.(A) Fluorescence microscopy studies (normal and tumour tissue)

Imaging and quantification of fluorescence in sections of normal and tumour tissue were achieved using fluorescence microscopy. At 5 and 30 minutes and 1, 2, 3, 4, 5, 6, 8, 17 and 24 hours following administration of ALA animals were killed and normal colon removed, at 4, 6 and 24 hours colonic tumour was removed and at 5 and 30 minutes, 1, 2, 4, 6 and 24 hours transplantable tumours were taken and immediately frozen and prepared for fluorescence microscopy as for section 2.1.2. The fluorescence microscope set up has been described in section 2.1.2. Both false colour coded or black and white images were generated by the computer and fluorescence was quantified digitally in the different tissue layers.

5.4.1.(B) Fluorescence spectroscopy studies (normal tissue only)

Fluorescence emission spectra were observed *ex vivo* from normal rat colon strips showing ALA-induced sensitisation. Porphyrins exhibit characteristic fluorescence profiles and this provides a means of confirming the presence of (and possibly distinguishing) the porphyrin species synthesized at a given time after introduction of ALA. Normal rats were given a 200 mg/kg dose of ALA and killed at 30 minutes, 1, 2 and 6 hours and their colon immediately removed. Strips of colon were opened, cleaned and mounted flat on glass slides with the mucosal surface uppermost. The slides were placed in a fluorimeter (Perkin-Elmer LS 5B) at a 30 degree angle to the excitation beam in order to minimise scattered light which was further attenuated by a longpass filter (OG550, Schott) mounted on the emission port. The emission spectra (uncorrected) of each sample was then recorded using an excitation wavelength of

514 nm which enabled examination of the complete band of fluorescence extending from approximately 600 - 750 nm. The excitation wavelength was chosen because it has better light penetration than the more commonly used wavelength of 400 nm and since 514 nm does not correspond to the peak of any porphyrin will not bias the resultant spectrum to a particular porphyrin species. Control strips were also examined to enable quantification and exclusion of autofluorescence and scattered light from unsensitised colon.

5.4.1.(C) Fluorescence spectroscopy for photodegradation studies

Normal colons were treated 4 hours after ALA with the laser at a wavelength of 630 nm in an identical manner to that already described (section 2.1.3.) but animals were sacrificed immediately after light treatment to allow for monitoring of photodegradation. The specimens were then prepared as for the fluorescence spectroscopy (5.4.1.(B)) and a CCD scan was performed along the strip exciting at 488 nm and detecting with a 10 nm bandpass filter centred at 633 nm. A fluorescence emission spectrum was measured from the centre of the light treated region on the same specimens as described in section 5.4.1.(B).

5.4.1.(D) Phototherapy studies (normal and tumour tissue)

The effect of light on normal and tumour tissue after i.v. administration of 200 mg/kg ALA was studied. The phototherapy procedure was identical to that of section 2.1.3. although a wavelength of 630 nm was used which corresponds to a porphyrin absorption band. Normal colonic tissue was treated at 5, 20, 30 and 40 minutes and 1, 2, 3, 4, 5, 6 and 24 hours, colonic tumour was treated at 6 hours and transplanted tumour was treated at 4 hours. Control animals that had not been given ALA were treated as before to quantify any thermal damage. Macroscopic and microscopic analysis was performed on all specimens. The time interval that gave the greatest macroscopic necrosis in normal colon was 4 hours after ALA. At this time interval further animals were treated as above but killed at 1 and 3 weeks after light treatment.

Light microscopy of sections cut through the treated area stained with H & E, and HVG were used to assess the extent of PDT damage and subsequent healing.

5.4.2. Results

5.4.2.(A) Fluorescence microscopy

Sections of normal colon were examined after administration of ALA and the fluorescence levels in the mucosa, submucosa and muscle layers were determined separately. The results are shown in Figure 5.7.

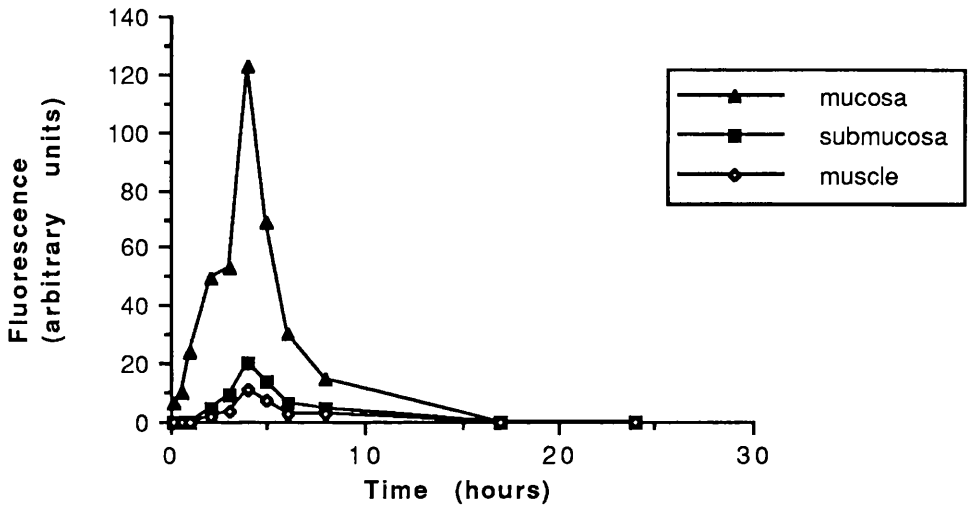


Figure 5.7
Microscopic fluorescence levels in tissue layers of the normal colon as a function of time after administration of 200 mg/kg ALA. Each point represents the mean of 2 animals.

At 30 minutes, relatively low fluorescence was observed in the mucosa, with no discernible fluorescence in submucosa and muscle. Fluorescence levels increased rapidly reaching a maximum in all layers at 4 hours, thereafter returning to background levels at 17 hours. All layers showed comparable fluorescence profiles versus time although fluorescence levels varied considerably according to the tissue site. At all times the mucosa exhibited the highest fluorescence, approximately 6 times greater than submucosal fluorescence, with muscle barely above background levels.

Figure 5.8 shows the fluorescence images of normal (a) and tumour tissue (c) 6 hours after introduction of 200 mg/kg ALA together with the subsequent photographs of the same sections stained with an HVG stain ((b) and (d)). There is specific sensitisation of tumour cells with tumour stroma exhibiting a three times lower average fluorescence level. At this time (6 hours post-administration) we estimate that the tumour glands gave on average a fluorescence ratio of approximately 6:1 to normal mucosal glands, 30:1 to normal submucosa and 60:1 to normal muscle. Confirmation of the specificity of sensitisation of tumour cells was obtained by re-imaging Figure 5.8 (c) using a higher power and is depicted in Figures 5.9 (a) and (b), which also show that sensitisation is restricted to extra-nuclear sites.

Colonic tumours imaged 4 hours after ALA gave a fluorescence count that was not greater than normal colonic mucosa sensitised at an identical time, and tumours imaged at 24 hours gave fluorescence levels barely above background. Therefore, the relative normal colon to colonic tumour levels at these times were not considered likely to lead to biological selectivity after PDT.

At all times studied, blood vessels exhibited low fluorescence levels and this is shown in Figure 5.10 (a) and (b). This figure is of images from normal colon 4 hours post-administration demonstrating that blood vessel sensitisation is comparable to that of the surrounding connective tissue, in contrast to the intense mucosal fluorescence.

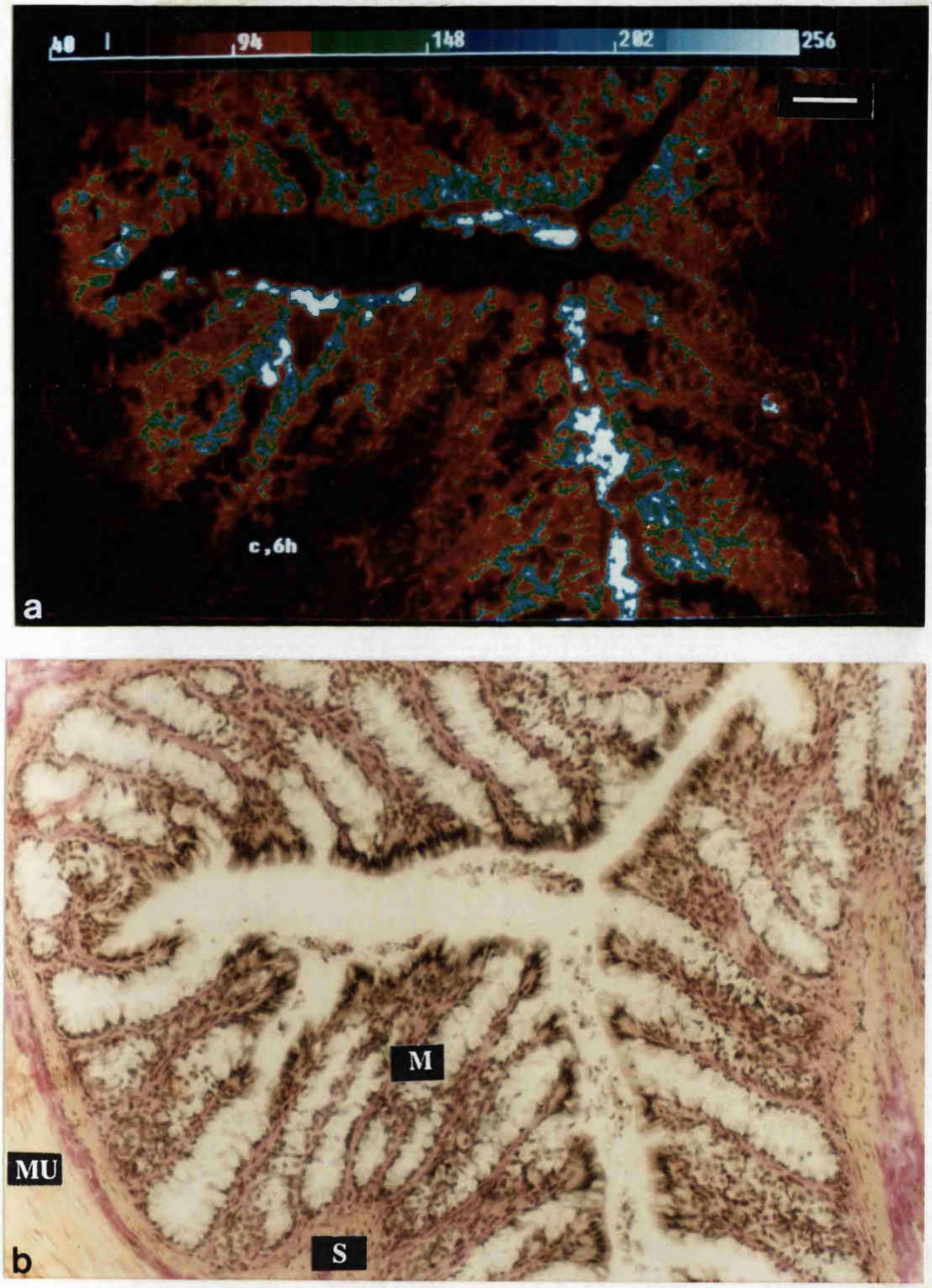
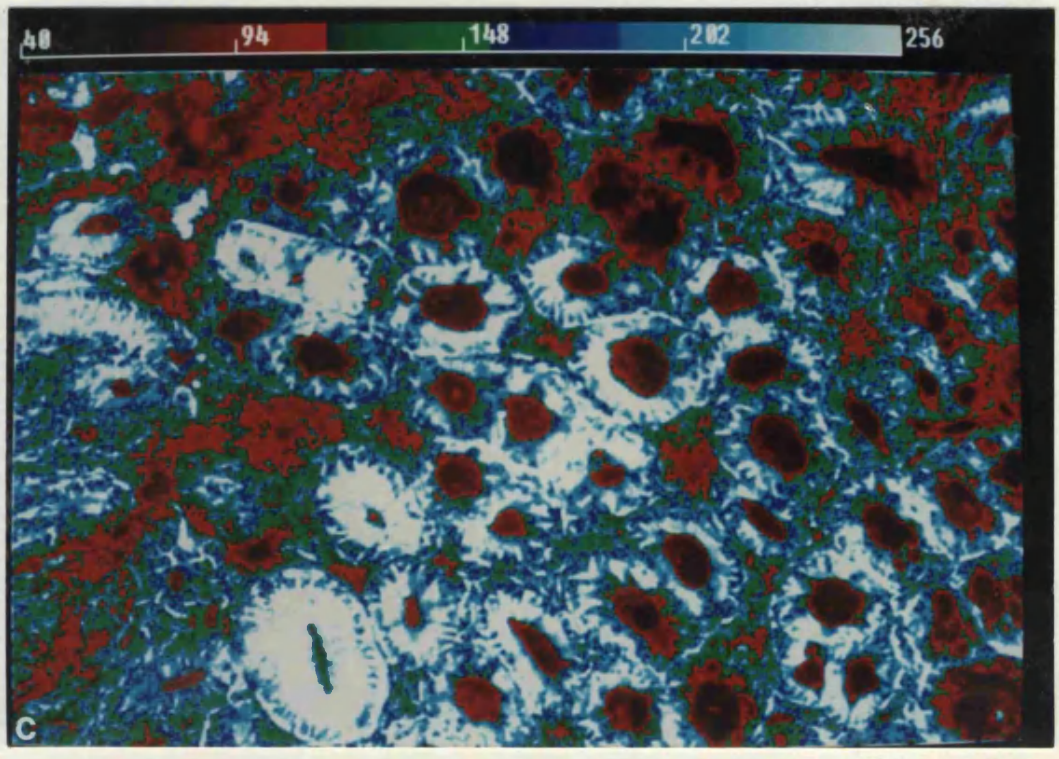


Figure 5.8
Fluorescence images of normal (a) and tumour tissue (c) 6 hours after introduction of 200 mg/kg ALA together with the subsequent photographs of the same sections stained with an HVG stain ((b) normal mucosa (M), muscle (MU) and submucosa (S) and (d) tumour stroma (TS) and tumour gland (TG)). Scale: white bar represents 60 µm. The false colour code begins at 40 counts to allow for background fluorescence.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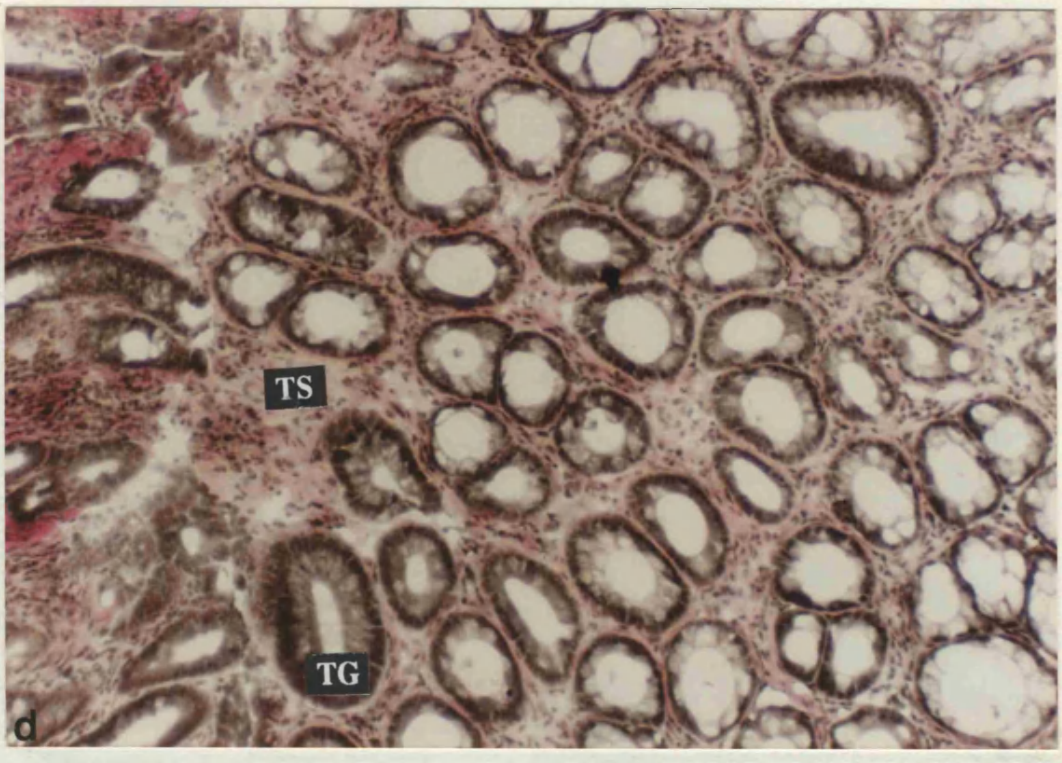
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TG
d

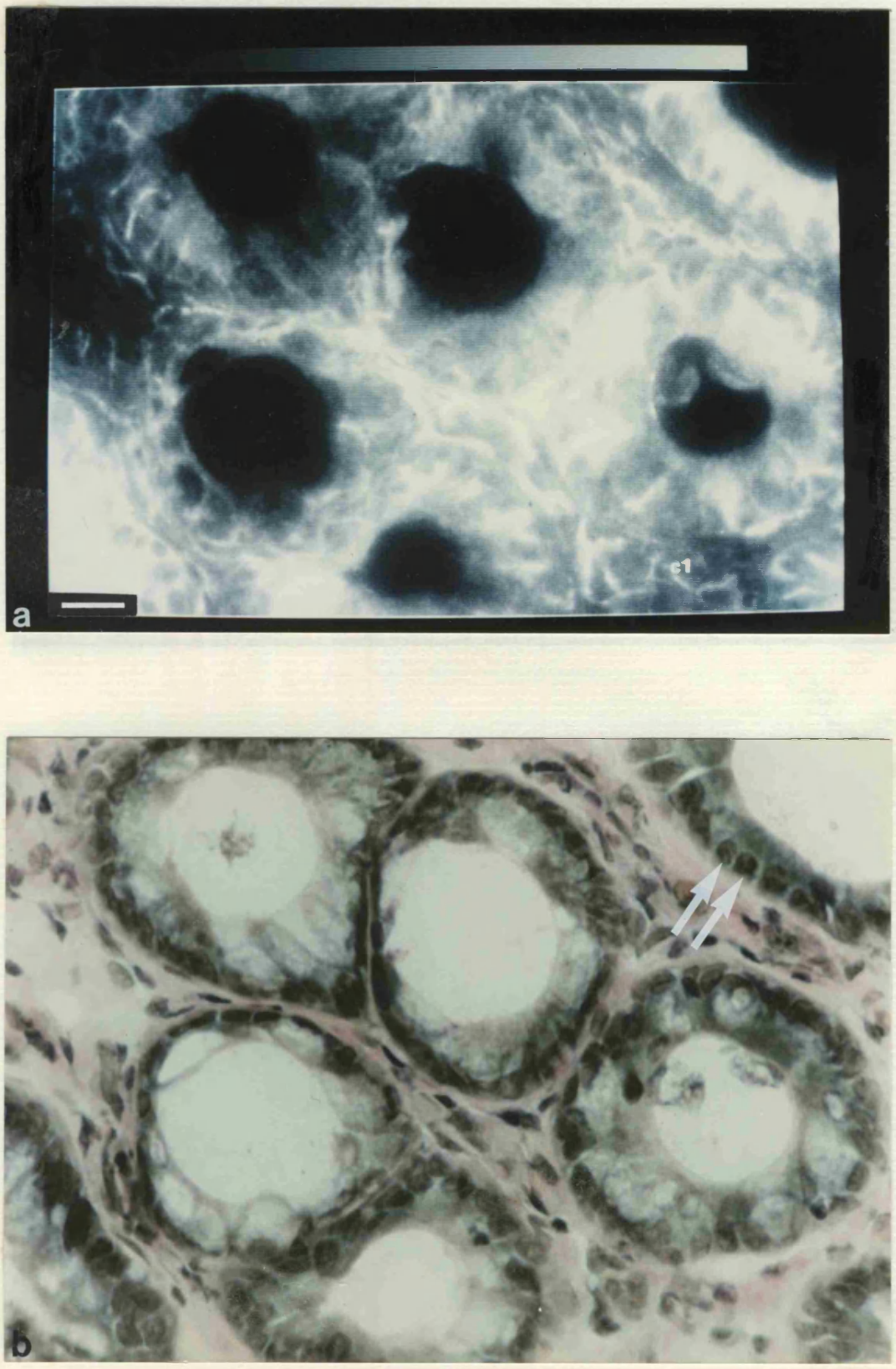


Figure 5.9
High power image of Figure 2 (c) shown in (a) with subsequent stained section (b). Arrows in (b) indicate two typical tumour nuclei depicting non-fluorescence of these structures. Scale: white bar represents 15 μm.

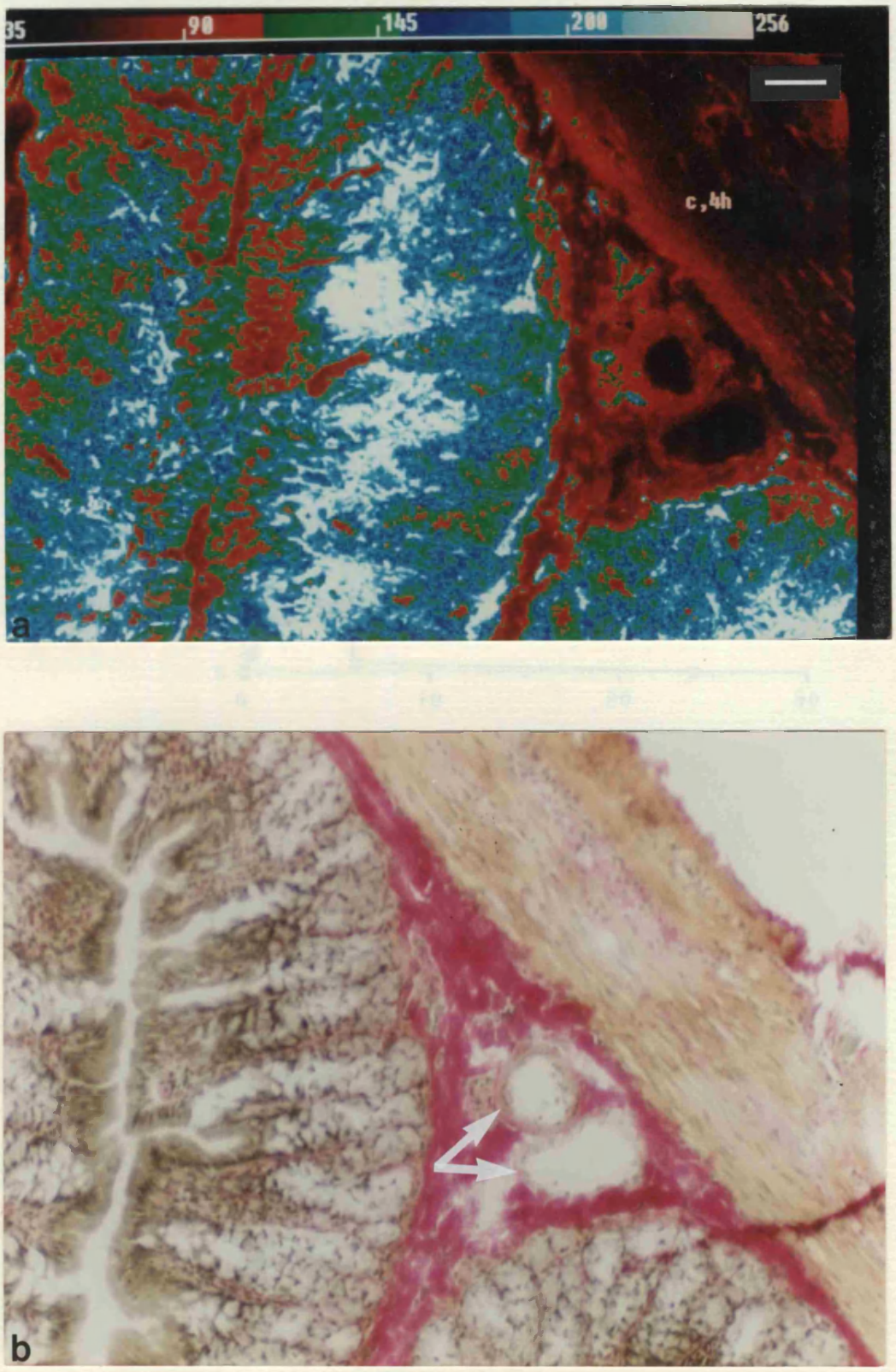


Figure 5.10
Fluorescence image of normal colon (a) 4 hours after administration of 200 mg/kg ALA together with the subsequent stained section (b). Arrows in (b) indicate blood vessels. Scale: white bar represents 60 μm.

Quantitative fluorescence imaging of the transplanted tumour (Figure 5.11) showed maximal fluorescence at 4 hours, which was gone by 24 hours, although the intensity of fluorescence was considerably lower than that of the induced tumour.

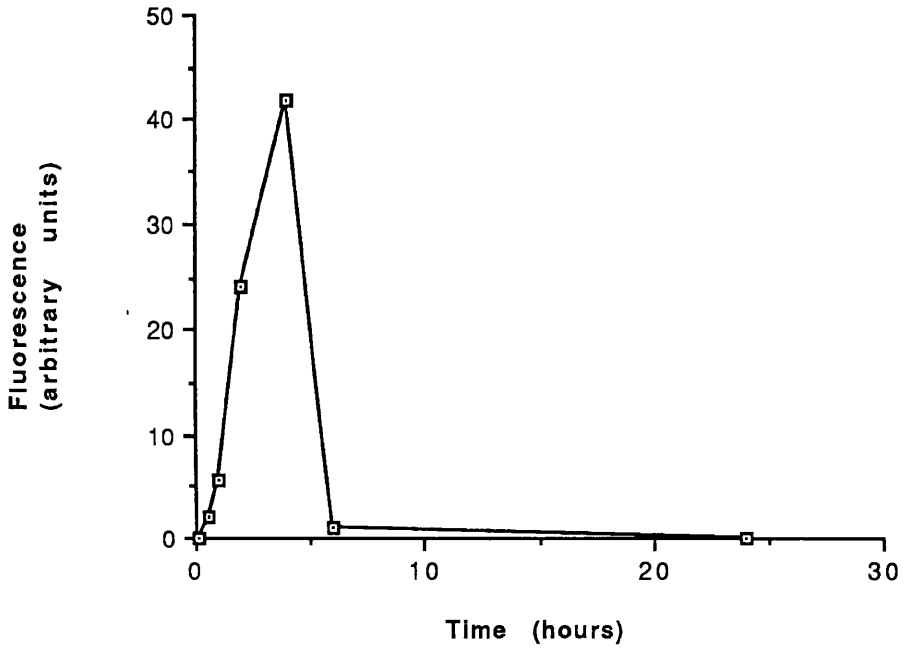


Figure 5.11
Microscopic fluorescence levels in transplanted tumour as a function of time after administration of 200 mg/kg ALA. Each point represents the mean of 2 animals.

A typical CCD image of a transplanted tumour 4 hours after sensitisation (peak fluorescence) is shown in Figure 5.12.

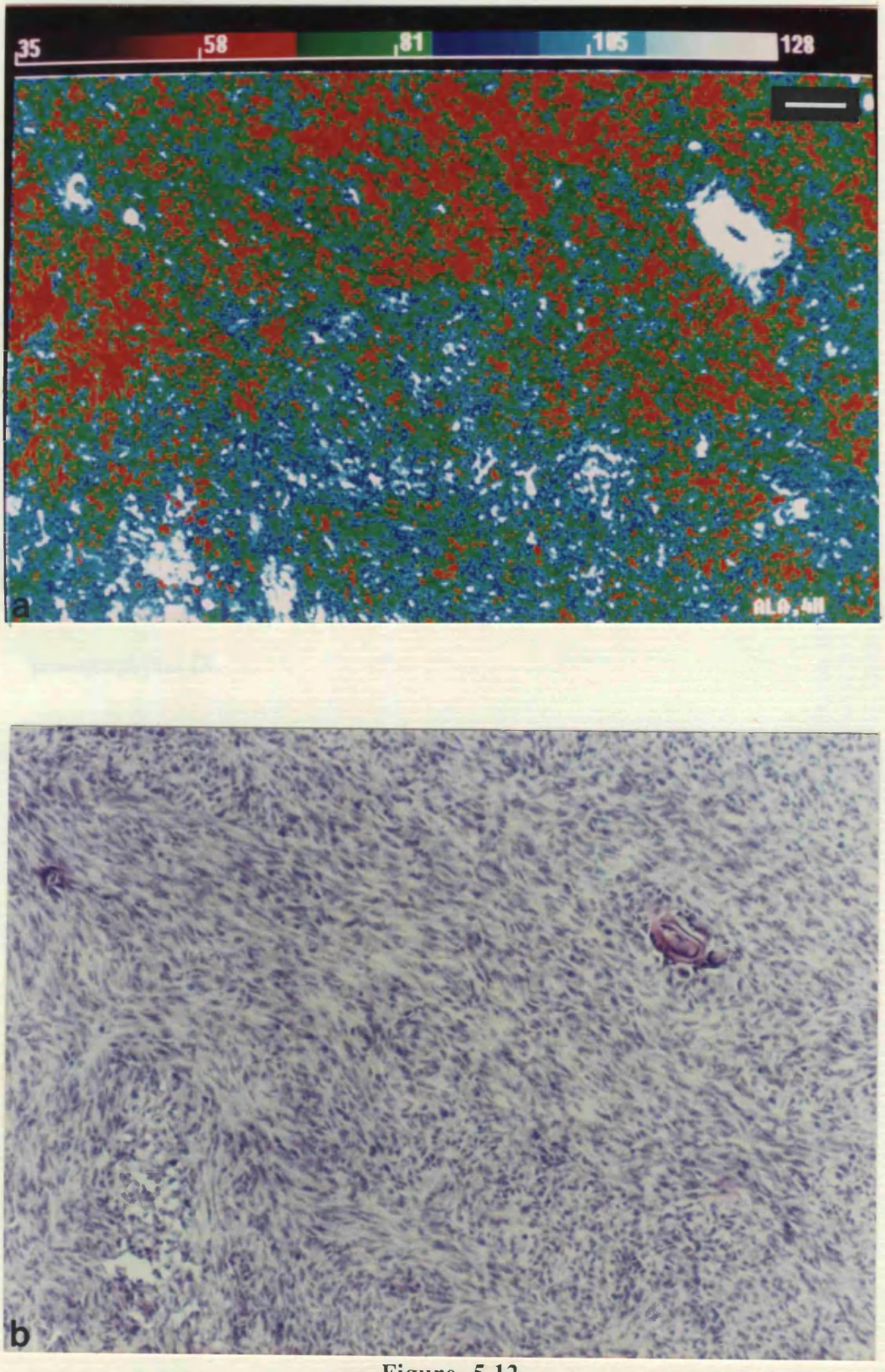


Figure 5.12
Fluorescence image of transplanted tumour (a) 4 hours after introduction of 200 mg/kg ALA together with the subsequent photograph of the same section stained with an H & E stain (b). Scale: white bar represents 60 μm. (Area of high fluorescence corresponds to artefact revealed in stained histological section.)

5.4.2.(B) Fluorescence spectroscopy

The fluorescence emission spectra from normal rat colon strips (excitation wavelength 514 nm) 30 minutes and 6 hours after ALA-induced sensitisation and unsensitised control colon are shown in Figure 5.13. The spectra from the sensitised colons differ in intensity of fluorescence but appear to exhibit the same spectral profile with maxima at approximately 636 and 704 nm; both spectra are considerably more intense than the control spectrum which apart from scattered light may also contain a contribution from endogenous porphyrin species. Further emission spectra were recorded at 1 and 2 hours which gave the same maxima. All spectra were consistent with a spectrum of PP IX in dimethylsulphoxide (DMSO) (Figure 5.14). The results are essentially identical to the *in vivo* spectra obtained by Pottier *et al.*, (1986) from skins of mice injected i.p. with ALA, who assigned the spectrum solely to protoporphyrin IX.

Figure 5.13
Fluorescence emission spectra (excitation wavelength 514 nm) from normal colon strips 6 hours (1) and 30 minutes (2) after 200 mg/kg ALA, and unsensitised control tissue (3).

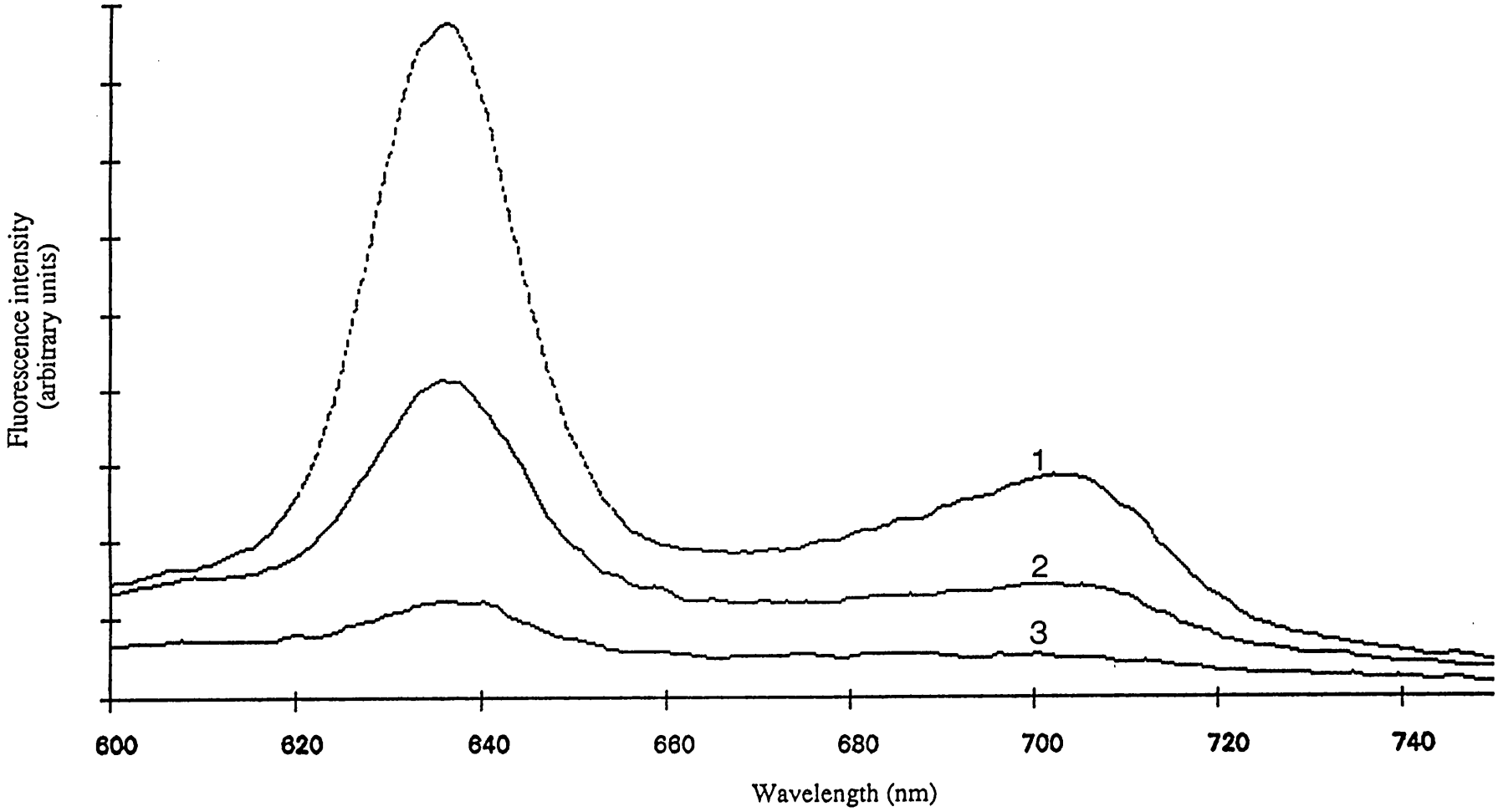
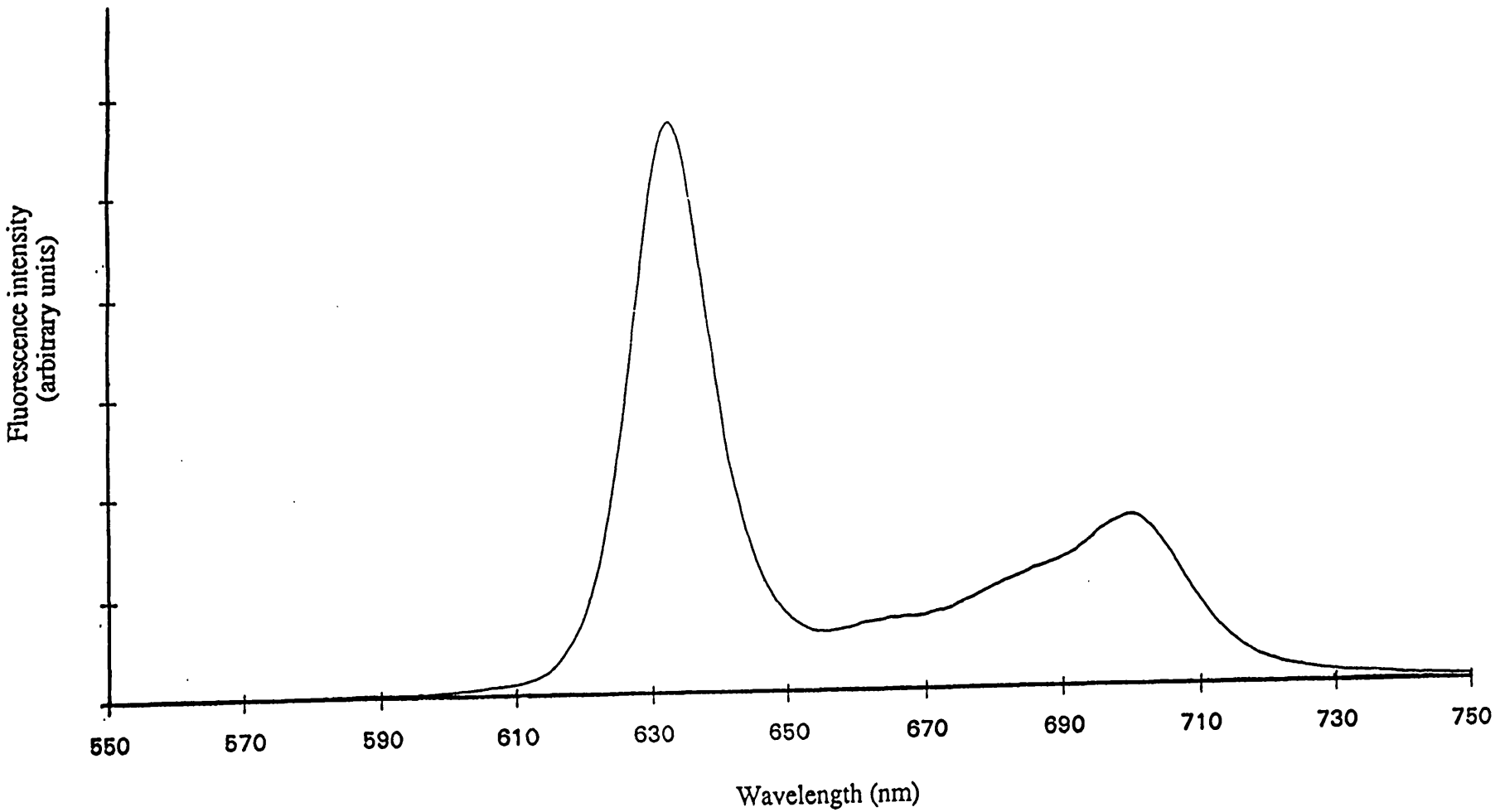


Figure 5.14
Fluorescence emission spectrum (excitation wavelength 514 nm) from PP IX in DMSO.



5.4.2.(C) Fluorescence spectroscopy for photodegradation studies

CCD monitoring of an *ex vivo* normal colon strip 4 hours after administration of ALA after 50 J of light given immediately prior to sacrifice gave a fluorescence reading reduced by 3 in the centre of the treated zone and bleaching was evident over an extent of 1 cm (Figure 5.15). After imaging, the treated section was mounted in a fluorimeter and a fluorescence emission spectrum produced. The laser treated region showed the appearance of a different band at 675 nm in its fluorescence emission spectrum (Figure 5.16) which was not present in untreated specimens. This corresponds to the band produced from *in vitro* studies of PP IX in DMSO treated for 30 minutes with a tungsten-halogen lamp, filtered between 350 and 450 nm, with an incident power of 40 mW, as shown in Figure 5.17. We propose that this new band corresponds to a photoprotoporphyrin product. The dimethyl ester form of this compound has previously been shown to sensitise the production of singlet oxygen (Keene *et al.*, 1986).

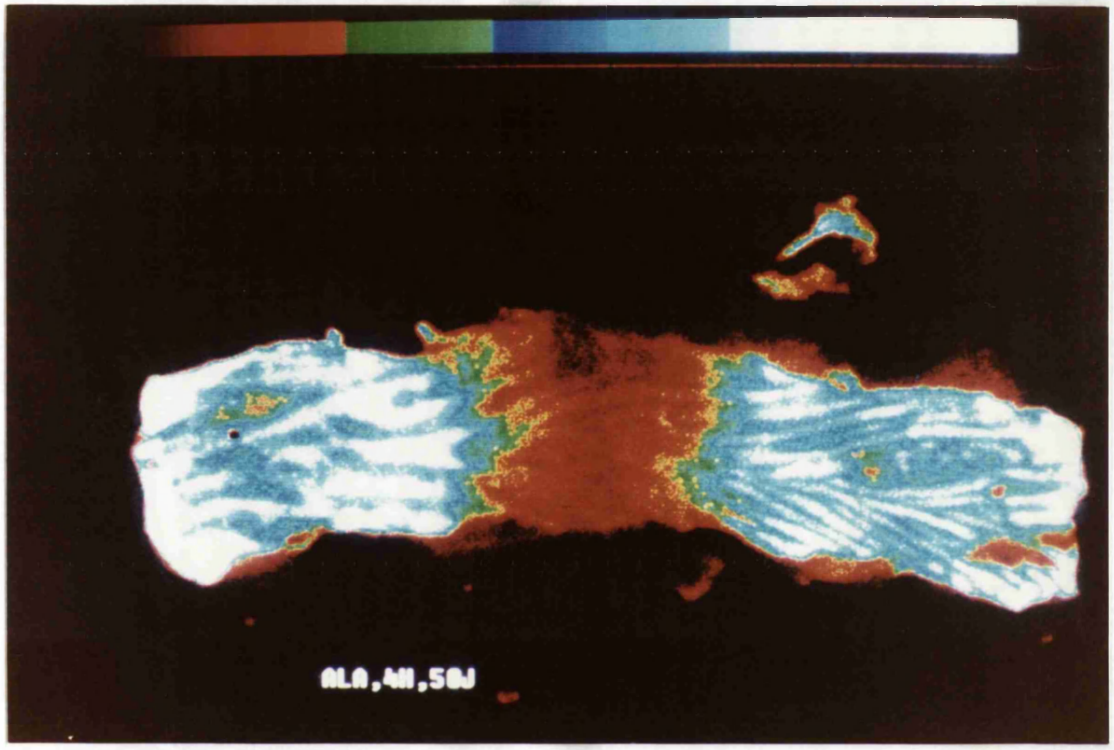


Figure 5.15
CCD image of ALA-induced PP IX sensitised normal colon strip after *in vivo* laser treatment (50 J).

Figure 5.16
Fluorescence emission spectrum (excitation wavelength 514 nm) from a ALA-induced PP IX sensitised normal colon strip after *in vivo* laser treatment (50 J). The spectrum was recorded from centre of laser treated area.

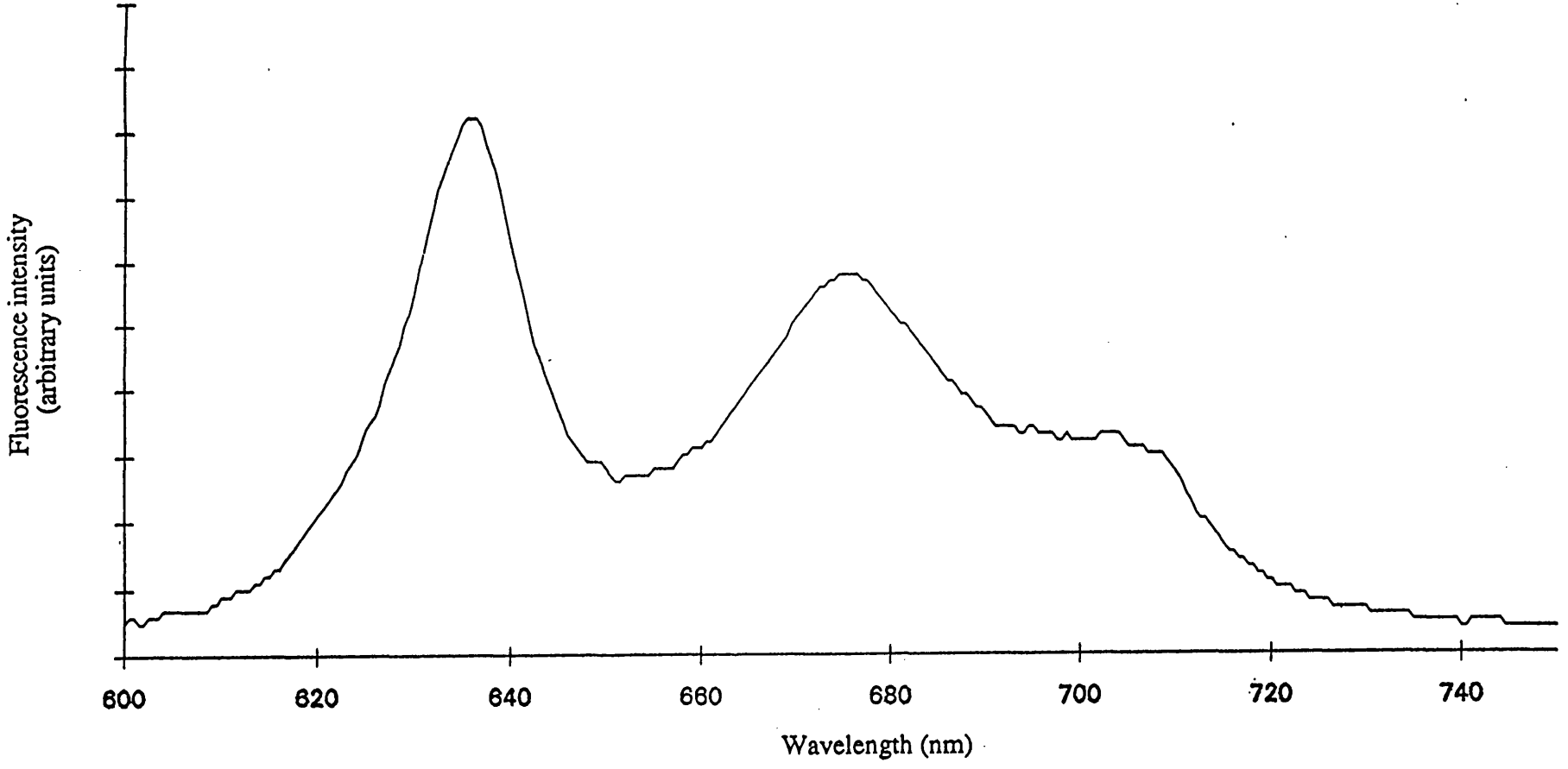
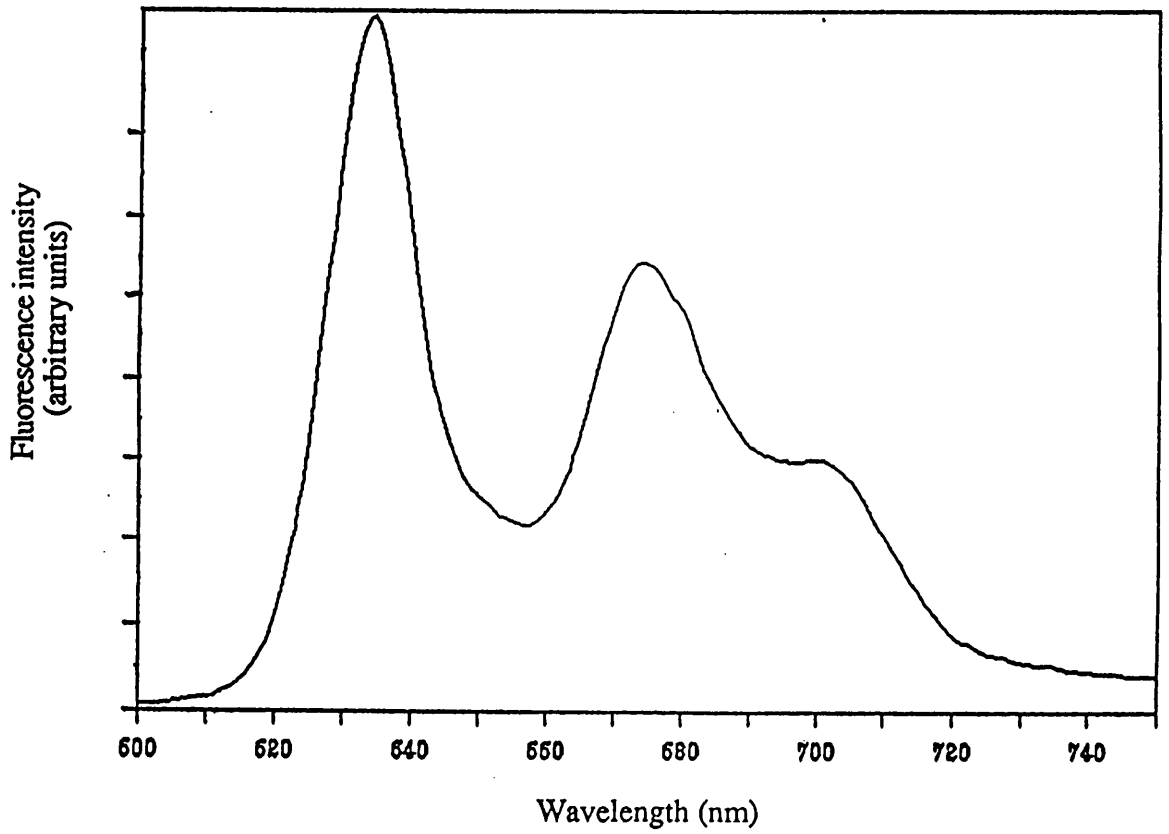


Figure 5.17
Fluorescence emission spectrum (excitation wavelength 514 nm) from PP IX in DMSO, treated with 40 mW of light from a tungsten-halogen lamp.



5.4.2.(D) Phototherapy studies

The mean diameter of necrosis in normal colonic mucosa was measured as a function of time after administration of ALA and results are shown in Figure 5.18.

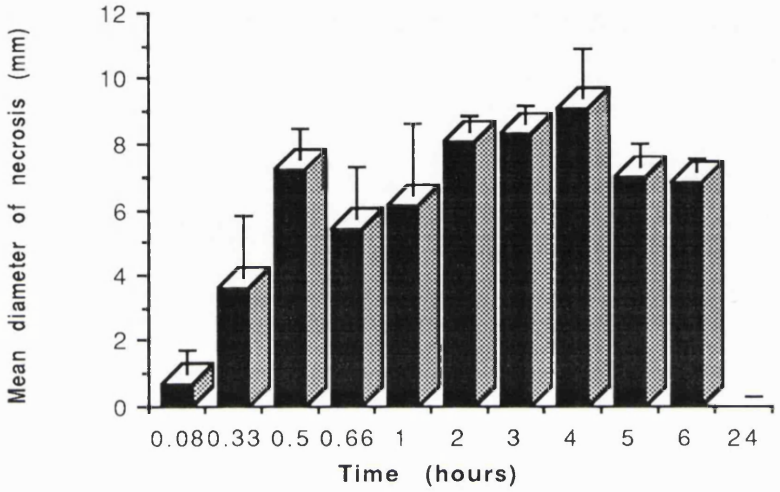


Figure 5.18
Diameter of necrosis in normal colonic mucosa measured as a function of time after administration of ALA. Each point represents the mean (± 1SD) of 5 animals.

These results show broad correlation with the corresponding mucosal fluorescence measurements from the CCD imaging studies with greatest damage also found at 4 hours (mean diameter 9.1± 1.5 mm) with no visible necrosis at 24 hours. The notable exception was at 30 minutes where considerable necrosis was seen (mean diameter 7.2 ± 1.0 mm) even though mucosal fluorescence was more than an order of magnitude lower than at 4 hours.

Histological examination was undertaken on all specimens showing macroscopic necrosis 72 hours after laser treatment. At all treatment times after introduction of ALA, an acute inflammatory infiltrate, mainly comprising of monocytes and polymorphonuclear neutrocytes, was seen throughout all tissue layers of the colon but there was little evidence of muscular layer necrosis in the specimens examined. This relative sparing of the muscle layer of normal colon is shown in Figure 5.19 (a) treated at 6 hours, with the corresponding high power image (b). Specimens treated at 4 hours after ALA (showing the greatest area of necrosis at 72 hours) still showed extensive ulceration by 1 week with interstitial haemorrhage and inflammatory infiltrate throughout the tissue layers with new collagen fibres being deposited. By 3 weeks muscle and glands were regenerated with normal looking collagen.

High power histological images were produced of submucosal blood vessels both from a laser treated area (2 hours after introduction of ALA) (Figure 5.20 (a)) and an untreated area of the same colon specimen (Figure 5.20 (b)). After treatment damage to the wall of the vein can be seen, with the artery showing loss of endothelial lining to the vessel and necrosis of smooth muscle cells of the media with evidence of oedema. These changes were not seen in the untreated vessels. The longer term effects of ALA-induced PDT on these blood vessels has not been determined in this study, although this is something that could be addressed in the future, but ultimately it has been shown that even when there is little fluorescence in blood vessels after administration of ALA this can still lead to vessel damage.

Induced colonic tumour treated at 6 hours shows obvious necrosis (Figure 5.21). Two different areas of a laser treated transplanted tumour (4 hours after administration of 200 mg/kg ALA) are shown in Figure 5.22 (a) and (b). A high power image of a viable area of tumour from the opposite side of the tumour to light treatment is shown in (a). This shows large pleomorphic nuclei with well stained chromatin and cytoplasm. The cells are arranged in highly structured sheets. These

observations are characteristics of an active viable tumour. In (b), a light treated area, the tissue is dead with loss of cellular architecture. There is breakdown of cell membranes and release of nuclei which are scattered throughout the section.

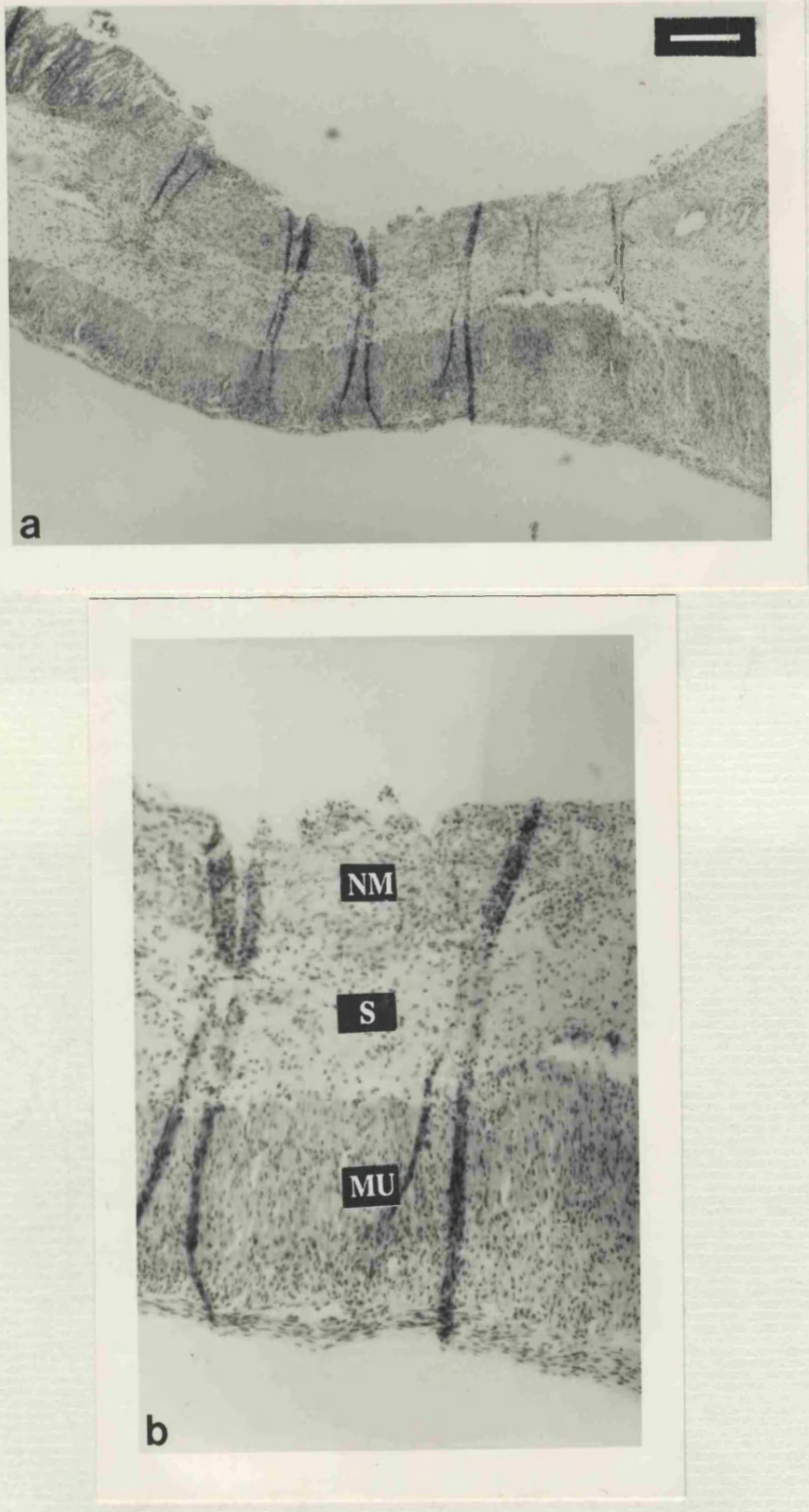


Figure 5.19
Histological section of normal colon treated 6 hours after 200 mg/kg ALA showing necrotic mucosa (NM), inflammatory infiltrate in the submucosa (S) and relative sparing of the muscle layer (MU). Scale: white bar represents 200 μm in (a) and 80 μm in (b).

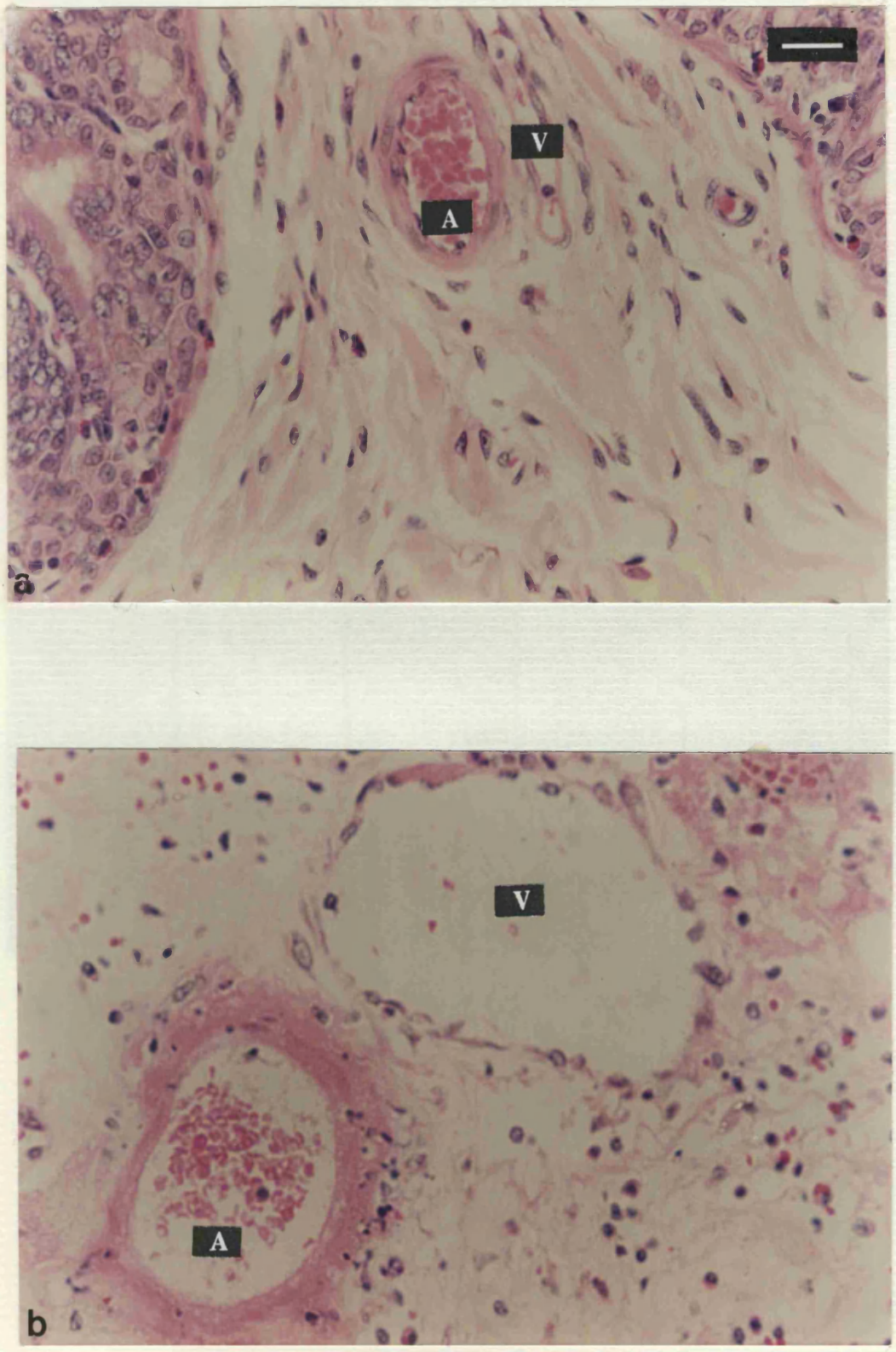


Figure 5.20
Histological sections of blood vessels from a normal colon treated 2 hours after ALA. (a) area not exposed to light and (b) light treated area (artery (A), vein (V)). Scale: white bar represents 15 μm.

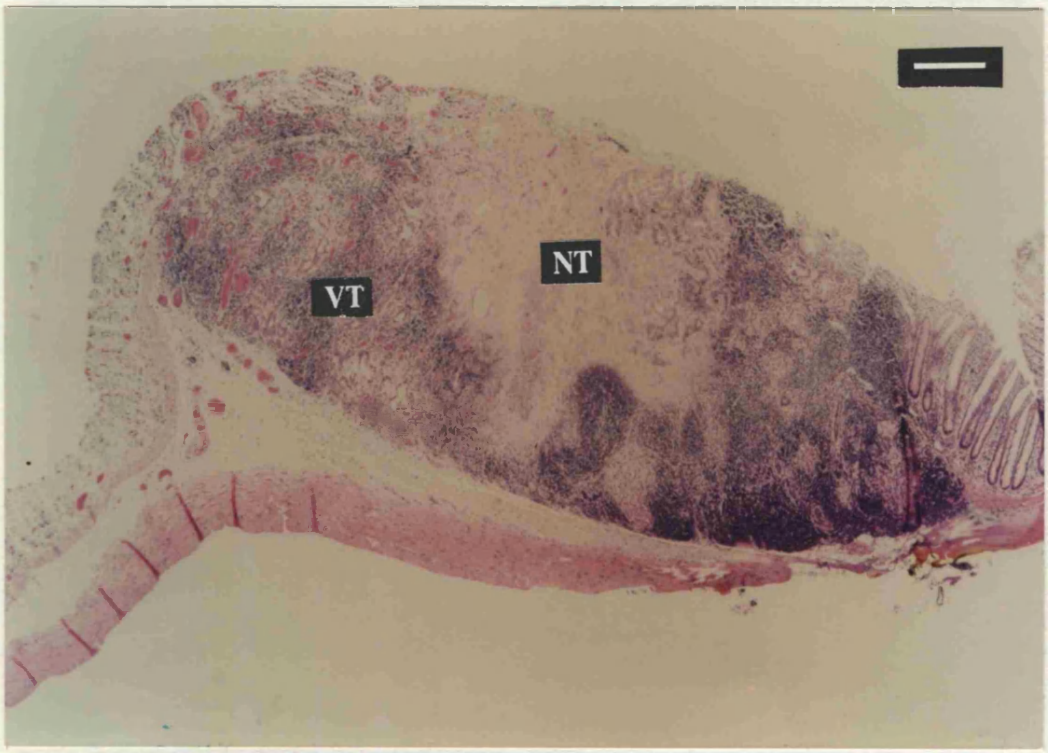


Figure 5.21
Histological section of colonic tumour treated 6 hours after 200 mg/kg ALA showing necrotic tumour (NT) and viable tumour (VT) beyond the treatment site. Scale: white bar represents 150 μm.

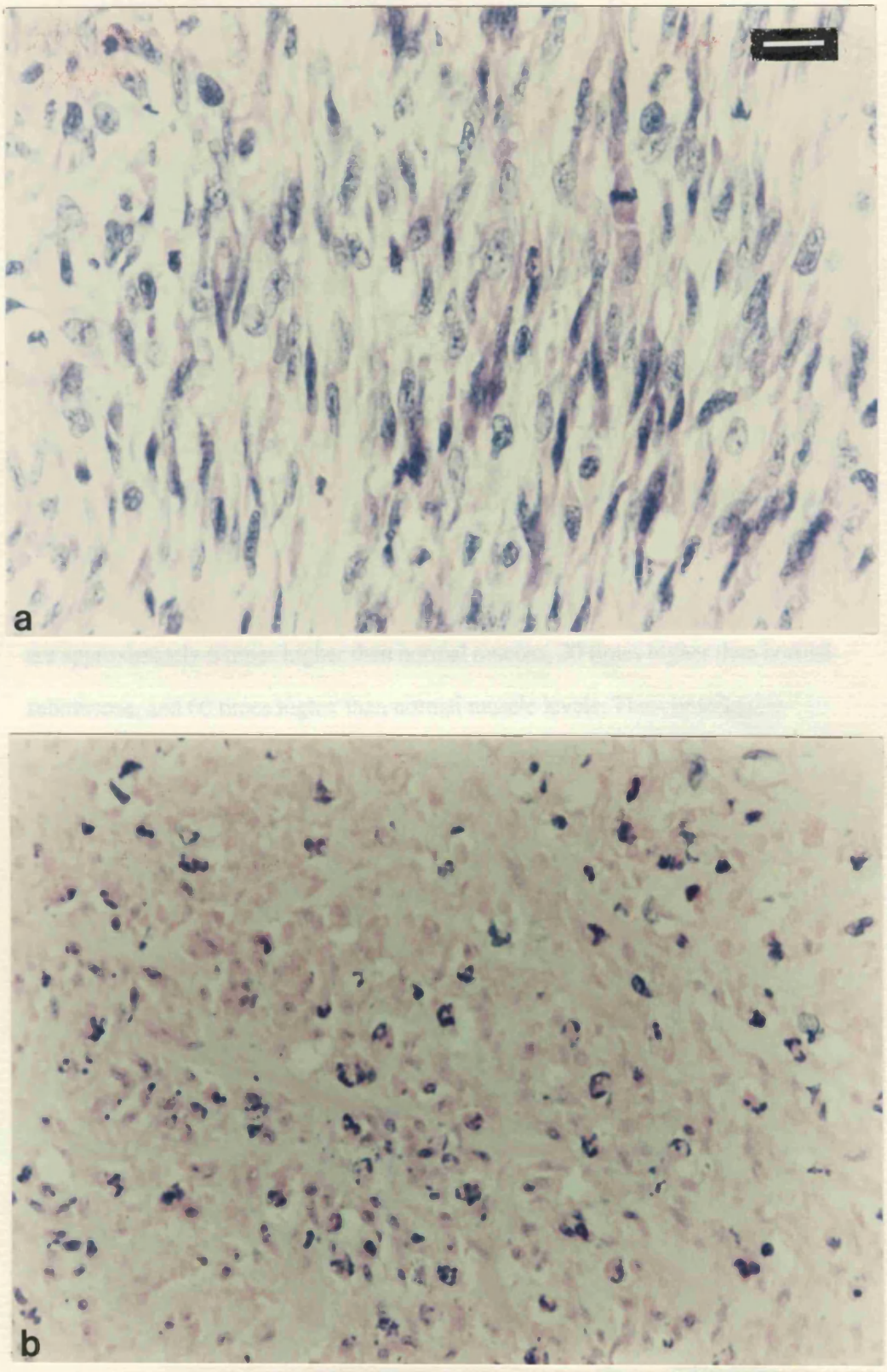


Figure 5.22
Histological sections of transplanted tumour treated 4 hours after 200 mg/kg ALA showing (a) viable area of tumour not exposed to light and (b) necrotic tumour from area exposed to light. Scale: white bar represents 15 μm.

5.4.3. Discussion

A new means of sensitisation for PDT has been studied which involves the introduction of ALA to induce endogenous porphyrin sensitisation. We have investigated the microscopic distribution and spectroscopy of ALA-induced porphyrin fluorescence at various time intervals after the introduction of ALA, in both normal and tumour tissue in the rat colon. It has been shown that the fluorescence spectrum in normal colon is consistent with that of protoporphyrin IX and using quantitative fluorescence microscopy that the fluorescence is mainly localised in the mucosa, with approximately 6 times less fluorescence in submucosal tissue and with muscle barely exceeding background fluorescence levels. We have found that at 6 hours there is a considerable differential between porphyrin fluorescence in tumour and normal colon. At this time colon tumour cells exhibit specific fluorescence with average levels that are approximately 6 times higher than normal mucosa, 30 times higher than normal submucosa, and 60 times higher than normal muscle levels. This considerable accumulation of porphyrins over normal tissue could be due to low ferrochelatase activity in cancerous cells. This has been suggested as a mechanism whereby tumour cells accumulate more exogenous porphyrin than normal cells (Dailey and Smith, 1984). Our fluorescence spectroscopic and pharmacokinetic data are consistent with previous work by Pottier *et al.* (1986) who have likewise shown that sensitisation by PP IX occurs rapidly reaching a maximum near 3 hours in the skin and declining to background levels by 24 hours.

The assumption that localisation and intensity of microscopic fluorescence correlates with PDT induced damage has generally been confirmed with our phototherapy studies. The mean diameter of necrosis in normal colon (allowing for the geometry of the light delivery) correlated with the relative porphyrin fluorescence levels between 5 minutes and 24 hours, except at 30 minutes (discussed below). Additionally, necrosis in all specimens has been restricted to the colonic mucosa with muscle remaining viable. Divaris *et al.* (1990) have shown previously that after systemic administration

of ALA to mice the intensity of the ALA-induced PP IX fluorescence in skin also correlated with the amount of phototoxic damage. With phototherapy it has proved possible to kill tumour tissue with ALA-induced sensitisation. Laser irradiation at 630 nm of DMH-induced colonic tumour caused tumour cell kill at 6 hours post-administration when necrotic damage to normal colon was limited to mucosa (which is capable of healing through regeneration - Barr *et al.*, 1987). It is reassuring that necrosis did not extend into the muscle layer, although there is evidence of an inflammatory infiltrate. The observation that any damaged layer of the normal colon regenerates after PDT even when the highest dose of PP IX was present (4 hours after a 200 mg/kg dose of ALA) is encouraging as it suggests that the full strength of the colon will be preserved after PDT. This can be compared to the maintenance of the bursting pressure seen in normal colon treated using AlSPc, even after full thickness necrosis of this tissue, probably due to a lack of any deleterious effect on the submucosal collagen (Barr *et al.*, 1987); however experiments on bladder show that even if there is histological evidence of muscle healing by regeneration, the muscle function may be permanently damaged (Pope and Bown 1991) and so clearly it is better that there is no muscle damage. Colonic induced tumours were only treated 6 hours after administration of ALA, as results from fluorescence microscopy taken at this time showed considerable potential for selective tumour treatment. Transplanted tumour was investigated as the fluorescence level observed was considerably lower than that of the induced tumour. This may have been due to a less developed blood supply in the rapidly growing transplanted tumour, although after light treatment tumour damage was still observed. We have also demonstrated a significant advantage of ALA-induced porphyrin over conventional sensitisers in that the level of sensitisation returns to background values in both normal and tumour tissue by 24 hours. This suggests that continued tissue sensitisation post-treatment will not be a problem. Consideration must be made for the possible contribution of photoactive degradation products to the biological effect of PDT using ALA-induced PP IX. Furthermore, monitoring of phototherapy using fluorescence photodegradation must

take account of the formation of the new fluorescence band produced during light treatment.

An unexpected result was found when treating normal tissue 30 minutes after introduction of ALA. A relatively low fluorescence reading was obtained in the mucosa at this time with no discernible fluorescence in the submucosa and muscle layers, so we would have predicted little, if any, damage after light treatment. However, significant photodamage was found with a diameter of necrosis of 7.2 ± 1.0 mm, which could be observed histologically as comprising necrotic mucosa and a particularly heavy inflammatory infiltrate in the submucosa and muscle layers. The mechanism of photodamage in Friend erythroleukaemic cells from the use of ALA has been shown to be a combination of the cellular location and the chemical nature of the photosensitiser at a particular time (Malik and Lugaci, 1987). They have reported that endogenous porphyrins initially accumulated in the mitochondria and then translocated to other photosensitive sites within these cells. A possible explanation of our anomalous *in vivo* results would be that 30 minutes after ALA administration the induced porphyrin (in this case PP IX) is located at particularly photosensitive sites, such as the mitochondria, and that thereafter the porphyrin is located in other less photosensitive sites in the cell. Another explanation is that other photoactive porphyrins (e.g uroporphyrin) were present at the shorter times but given the consistency of the fluorescence spectra recorded from 30 minutes to 6 hours we believe this to be unlikely, although we can offer no explanation for the specific production of PP IX *in vivo* which is contrary to the *in vitro* results. Sensitive chromatographic analysis of the porphyrin content of colon specimens at different times after ALA has been carried out and at all times PP IX has been identified as the main porphyrin present (Loh *et al.*, 1993a).

Interesting comparisons can be made with our recent studies of AlSPc sensitisation of normal rat colon (Chatlani *et al.*, 1991), which showed that AlSPc fluorescence was

particularly high in and adjacent to blood vessel walls, in complete contrast to the results presented here. This observation was confirmed by Star *et al.* (1992) using fluorescence microscopy they were unable to visualise blood vessels at all times after administration of i.v. ALA. Even with this apparent very low magnitude of sensitisation of the blood vessels histological damage of submucosal blood vessels was observed and was as severe as after treatment using AlSPc (Barr *et al.*, 1987). Although the histological damage appears the same, we have no evidence that necrosis is caused by the same mechanism with both photosensitising agents. This could mean that blood vessels are more sensitive to PDT after PP IX sensitisation than other normal tissues. Peng *et al.* (1992) used light and electron microscopy and showed that the mitochondria of the tumour cells and the basal lamina of vascular walls beneath the endothelium in the tumour tissue were initially extensively destroyed after PDT with ALA-induced porphyrins. Thereafter, diffuse degeneration followed by local and/or diffuse severe necrosis of the tumour cells was found. This may be due mainly to the initial damage to mitochondria in the endothelial cells and also to the destruction of the vascular wall in the tumour tissue. The mechanism of photodamage may still differ between these two methods of sensitisation in extent of vascular effect versus direct cell kill. ALA - induced sensitisation would appear to also rely on direct cell kill whereas the mechanism with AlSPc relies primarily on vascular effects. This conclusion may have the most far reaching implications for ALA. With the current photosensitisers which localise mainly to the vascular stroma of tumours and normal areas, it is only possible to destroy tumour cells infiltrating normal areas by also necrosing the normal tissue (Barr *et al.*, 1991). In contrast, the combination of tumour to normal selectivity and synthesis of PP IX in individual malignant cells may make it possible to kill infiltrating cells without so much damage to normal structures. Disruption to supporting tissue and vascular structure feeding the mucosa is less likely and may lead to better healing with less scarring. Naturally, this is only applicable to tumours that can synthesize PP IX from ALA, but could be extremely valuable for sterilising the resection margins after tumour surgery.

In summary, this new method of sensitisation has several advantages over conventional exogenous photosensitiser administration. Sensitisation is rapid with near maximal tissue levels of PP IX being reached at 3-4 hours. Photosensitisation of tissues including skin (Pottier *et al.*, 1986) is likely to be comparable in intensity to that after HpD, but would only be expected to last 24 hours instead of several weeks. The absorption peak of PP IX in the red part of the spectrum is low (comparable to that for HpD, and much lower than that for AlSPc), but this just means that more light is required and since sensitisation has gone by 24 hours the phototherapy may be repeated a number of times to obtain further tumour kill. The real bonus is the direct sensitisation of individual malignant cells, and if this can be exploited, the potential for the technique is very considerable.

5.5 Oral ALA studies

PDT with intravenously administered ALA has shown considerable potential for tumour treatment (section 5.4). Certain problems become apparent when considering producing a drug for i.v. administration into patients such as sterility of preparation; also there is a need to produce a solution of neutral pH. This is required since intravenous injection of 24 mg of unbuffered solutions of ALA caused significant bradycardia and hypotension in rats. These effects were not present when a solution of neutral pH was injected (Edwards *et al.*, 1984). We have experienced problems when producing a neutral solution of ALA, since with the concentration of solution we require for laboratory animals (60-100 mg/kg) as neutrality is approached the clear solution rapidly degrades to form a yellow/orange solution which is no longer active (when given to animals does not induce enhanced porphyrin synthesis). Therefore, oral administration of the ALA at acidic pH would be preferable if the ALA is absorbed without too much degradation and that selectivity of PP IX localisation produced after i.v. injection of ALA could be maintained. Additionally, oral administration of a drug is considerably more acceptable to a patient. A study of porphyrin metabolism in man revealed photosensitisation of the skin after ingested doses (0.07-0.21 mmol/kg the equivalent of 11.7-35.2 mg/kg) of the hydrochloride of ALA dissolved in water (Berlin *et al.*, 1956a and 1956b), and therefore it was considered that oral ALA-induced PP IX may cause photosensitisation of other tissues in addition to the skin. Additionally, oral administration of ALA to rats results in progressive accumulation of porphyrin in a colonic carcinoma transplanted into the liver, with less porphyrin accumulation in the surrounding normal liver (maximal tumour to normal ratio of 4:1) (van Hillegersberg *et al.*, 1992). This indicates that oral ALA should cause PP IX sensitisation of tumour tissue. Using the normal colon rat model we have investigated the porphyrin fluorescence after an oral dose of ALA and compared this with data obtained after an i.v. dose of ALA (section 5.4.2.(A)).

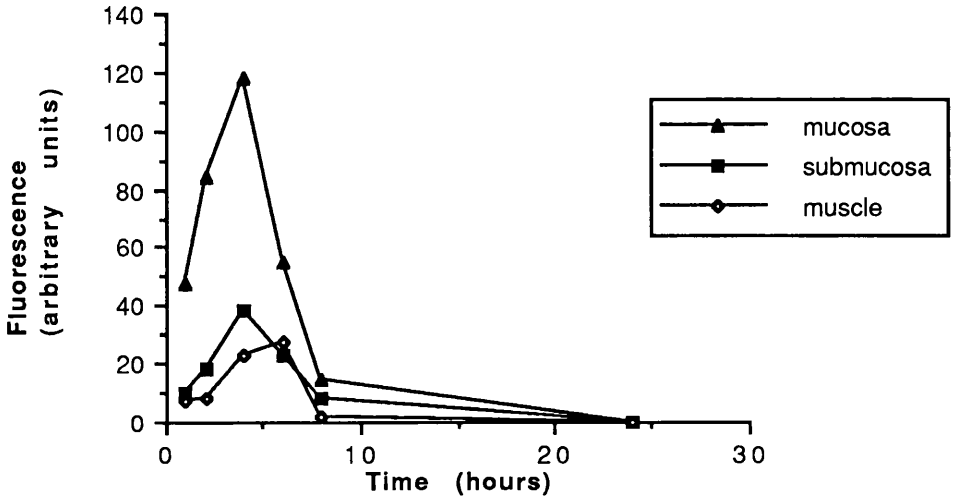
5.5.1. Methods

All methods were identical to those given in section 5.3.1., except each animal received 400 mg/kg of ALA dissolved in 1 ml of phosphate buffered saline. This was administered using a feeding needle introduced orally down the oesophagus into the stomach. Rats are incapable of vomiting or regurgitation and therefore all animals ingested the full delivered dose. A higher dose was administered orally than used previously for the i.v. studies (sections 5.2., 5.3. and 5.4.) because of likely first-pass metabolism. Animals were then sacrificed at 1, 2, 4, 6, 8 and 24 hours after ALA administration and colon specimens were removed for CCD fluorescence microscopy analysis as in section 2.1.2.

5.5.2. Results

Measurements of fluorescence after oral ALA were made from muscle, mucosa and submucosa against time and are shown in Figure 5.23(a) and can be compared with those after i.v. ALA shown in (b) (data as for Figure 5.7). In (a) and (b) fluorescence in the mucosa reached a peak at 4 hours with approximately a 50% reduction in fluorescence by 6 hours and background levels by 24 hours. The submucosa and muscle show an approximate doubling in their peak fluorescence after oral ALA. The fluorescence profiles in (a) were similar to those after i.v. administration of ALA (b) both in shape and similar intensity of fluorescence, with fluorescence differentials between layers being generally retained.

(a)



(b)

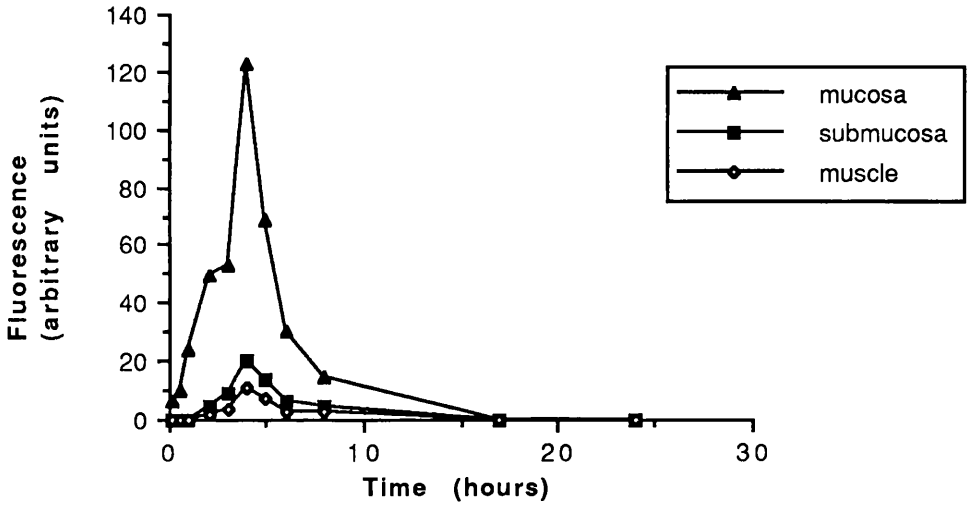


Figure 5.23
Microscopic fluorescence levels in tissue layers of the normal colon as a function of time after (a) oral administration of 400 mg/kg ALA and (b) i.v. administration of 200 mg/kg ALA (data from Figure 5.7). Each point represents the mean of 2 animals.

5.5.3. Discussion of oral ALA studies

We have undertaken considerable studies using ALA as a means of producing endogenous porphyrin for PDT although they have all involved i.v. administration of this drug (sections 5.2., 5.3. and 5.4.). This study addresses the possibility of the oral route as a means of ALA administration to avoid the inherent disadvantages of giving a preparation intravenously. Oral administration is not appropriate with the conventional photosensitisers HpD and AlSPc.

ALA is analogous to amino acids for which there are active transport mechanisms for uptake in the gut. Therefore, it is reasonable to expect that a degree of local uptake of ALA into the bowel lumen will result after oral administration. From the results obtained, ratios of fluorescence in the individual tissue layers are comparable with those obtained after i.v. administration of ALA. These findings suggest a vascular route of administration to cells. It is thus likely that following oral administration the ALA will be absorbed and so enter the blood stream leading rapidly to a high serum level of drug which will be available to tissues in the same manner as an i.v. administered dose of ALA. We believe this indicates that there is rapid absorption from the gut in the upper alimentary tract. All significant sensitisation of the colon is probably obtained via the blood vessels. It is thus possible to assume that the selectivity of production of porphyrins in tumour against normal colon (section 5.4.) will be the same after oral administration of ALA. The only drawback is that twice the concentration of ALA was required for oral administration to obtain the same porphyrin tissue levels, which is considered to be due to the first-pass effect. That is after oral administration a drug will reach the systemic circulation only if it is absorbed from the gastrointestinal tract and if it escapes metabolism in the gastrointestinal tract, liver and lungs;with the liver being the most important site of first-pass metabolism. A high extent of hepatic first-pass metabolism of a drug will greatly reduce its systemic bioavailability. We believe that ALA can be administered i.v. at considerably lower doses than 200 mg/kg to obtain appropriate

photosensitisation for PDT. Loh *et al.* (1992) have shown considerable necrosis of the gastric mucosa even with an i.v. dose of 20 mg/kg. Therefore, the requirement for a higher dose of ALA for oral administration may not be a problem. This study indicates that oral administration of ALA may prove to be as useful as i.v. administration of this drug for PDT, and indeed the preferable option of administration in the clinic.

CHAPTER 6 - *IN VITRO* KILLING OF *HELICOBACTER PYLORI* WITH PDT

Bacteria have generally been used as a convenient model for determining the cellular mechanisms of photodynamic damage, although more recently PDT is being considered as a modality for killing bacterial infections. We have therefore utilised PDT as a means of killing the bacterium *Helicobacter pylori* (*H. pylori*) *in vitro* as the first stage in testing the hypothesis that PDT might be of value in treating *H. pylori in vivo*. This organism is considered to play a role in the development of gastritis, duodenitis, duodenal and gastric ulceration and more recently gastric cancer.

6.1. Inactivation of bacteria with PDT

Many studies using PDT to inactivate bacteria *in vitro* have been reported. Uptake of HpD and its sensitisation of the skin bacteria *Propionibacterium acnes* (*P. acnes*) to light was investigated (Kjeldstad *et al.*, 1986). *P. acnes* is light sensitive due to endogenously produced porphyrins, although photosensitivity is considerably increased by the addition of exogenous HpD. The amount of cell bound HpD and the light sensitivity increased non-linearly with increasing incubation concentration of HpD. This was thought to be due to a saturation of binding sites on the cell. It is claimed that Gram-negative bacteria are insensitive to PDT with HpD (Nitzan *et al.*, 1983; Bertoloni *et al.*, 1984, 1985) and phthalocyanines (Bertoloni *et al.*, 1990). The uptake of HpD in several bacterial strains, both Gram-positive and Gram-negative, was compared (Kjeldstad *et al.*, 1986). It was found not to be strictly dependent on the Gram staining. Different strains of *Propionibacterium* (Gram-positive) and strains of *Haemophilus* (Gram-negative) bacteria were found to take up more HpD than other types of bacteria in studies including both Gram-positive and Gram-negative bacteria. Although, *H. influenzae* (Gram-negative) had high HpD uptake (Kjeldstad *et al.*, 1986) but no photosensitivity (Malik *et al.*, 1982) whereas *Streptococcus faecalis* (Gram-positive) took up less HpD (Kjeldstad *et al.*, 1986) but is reported to

be photosensitive (Bertoloni *et al.*, 1984). This indicates that it is not the total amount of porphyrin taken up which is the essential parameter for photosensitising different cells, but rather the localisation of the porphyrin, that is, in close proximity to photosensitive structures. The lack of accordance between uptake and sensitisation is reported to be due to differences in cell wall construction. All Gram-negative bacteria contain an outer membrane in their cell wall, outside the peptidoglycan layer. This structure plays important physiological roles by both making Gram-negative bacteria resistant to host defence factors and by acting as a strong permeability barrier to many antibiotics that are effective against Gram-positive bacteria. The outer membrane could trap the dye thus preventing the interaction of the photogenerated reactive species with sensitive cell constituents. Malik *et al.* (1990) have suggested that the cell wall of Gram-negative bacteria may act as a sponge for the adsorption and absorption of porphyrins, therefore demonstrating a false positive reaction of porphyrin uptake. Increasing the light dose may lead to better photodynamic kill of Gram-negative bacteria. *E. coli* could not be killed at light doses less than 31 J/cm^2 in combination with the photosensitiser methylene blue (2 μg/ml) (Menezes *et al.*, 1990). Martinetto *et al.* (1985) have shown kill of *E. coli* with either high concentrations of Hp (1 mM) or high powers of light (6 W for 30 mins). There was no report of light only controls so kill at high powers may be due to thermal effects; although, kill at lower light doses and higher concentrations of Hp must be a PDT effect as the higher concentrations of Hp were not toxic to the bacteria. In another study *E. coli* were reported to be insensitive to the photosensitising action of both the lipid soluble ZnPc and the water-soluble mixture of mono- and di- sulphonated phthalocyanine (ZnSPc) (Bertoloni *et al.*, 1990). Not all Gram-negative bacteria are resistant to PDT and this could be due to either the specific chemical composition of the photosensitiser or the particular structure of the outer membrane of a strain of Gram-negative bacteria. Rose bengal is an anionic compound and is found to slowly penetrate the outer membrane of the Gram-negative bacterium *Salmonella typhiurium* wild type strain. It has been proposed that the barrier presented to anionic compounds

provided by the Gram-negative outer membrane is not complete, therefore it can be crossed slowly allowing the accumulation of photosensitiser with time (Dahl *et al.*, 1988). Photodynamic action can be improved by making cells permeable, thereby enabling a greater amount of dye to penetrate into them. Previously insensitive bacteria were made sensitive to PDT by alteration of the outer membrane (Bertoloni *et al.*, 1990). This was achieved by treatment of the *E. coli* with a solution of calcium chloride, or Tris-EDTA. The sensitisers ZnPc and ZnSPc could then bind at the level of the cytoplasmic membrane. After biochemical analysis it was suggested that the cytoplasmic membrane was important as a target of the photoprocess, while DNA was not involved. This information on the ability to kill Gram-negative and Gram-positive bacteria with different sensitisers and with, or without, pretreatment is summarised in Table 6.1.

Therefore, it would seem that the much quoted statement that Gram-negative bacteria are not killed by PDT without pretreatment is not correct and very much depends upon the type of bacterium and the exact experimental conditions. Variables such as the nature of the Gram-negative outer membrane structure, the chemical structure of the photosensitiser, and the experimental parameters such as the length of time the bacteria are incubated with sensitiser, the fluence rate of light exposure (varied by time or power of exposure) and the physiological state of the cells may all contribute to the efficacy of a treatment.

Bacterium	Gram stain	Photosensitiser	Pretreatment[1]	Kill[2]	Authors
Propionibacterium acnes	+	HpD	X	√	Kjeldstad *et al.*, 1986
Haemophilus influenzae	-	HpD	X	X	Malik *et al.*, 1982
Streptococcus faecalis	+	HpD	X	√	Bertoloni *et al.*, 1984
Escherichia coli	-	methylene blue	X	√	Menezes *et al.*, 1990
		Hp	X	√	Martinetto *et al.*, 1985
		ZnPc	X	X	Bertoloni *et al.*, 1990
			√	√	Bertoloni *et al.*, 1990
		ZnSPc	X	X	Bertoloni *et al.*, 1990
			√	√	Bertoloni *et al.*, 1990
Salmonella typhiurium	-	rose bengal	X	√	Dahl *et al.*, 1988

Table 6.1
Photodynamic killing of bacterial cells
[1] X indicates no pretreatment, √ indicates pretreatment
[2] X indicates no kill, √ indicates some kill

6.2. *Helicobacter pylori*

Helicobacter pylori (formerly *Campylobacter pylori* (Goodwin *et al.*, 1989)) is a Gram-negative spiral organism which colonises the superficial layers of the stomach and duodenum. It is a localised infection found nowhere else in the body. *H. pylori* almost certainly plays a pathogenic role in the aetiology of type B gastritis, duodenitis and duodenal ulceration. Additionally, the organism is associated with gastric ulceration and more recently has been linked with gastric cancer.

It is generally considered that *H. pylori* is the major aetiological agent of chronic active (type B) gastritis, and that it may further predispose to peptic ulceration. Conformational studies in piglets (Krakowka *et al.*, 1987) and in two human volunteers (Marshall *et al.*, 1985a; Morris and Nicholson, 1987) have reproduced the typical features of gastritis. Attempted clearance of the organism with amoxycillin (Glupczynski *et al.*, 1988), furazolidone (Morgan *et al.*, 1988), or bismuth salts (McNulty *et al.*, 1986) led to improvement or even total resolution of gastritis, although recolonization generally occurred within a few weeks of cessation of drug treatment. However, one particular study has greatly stimulated interest for drug treatment of *H. pylori* infection by showing that patients with a healed duodenal ulcer who were considered to be rid of *H. pylori* relapsed much less frequently than those not considered to be cleared of the organism (Marshall *et al.*, 1988). On the other hand, the potential benefit of anti-*H. pylori* treatment in gastric ulceration remains presently unknown. However, when patients with known causes of gastric ulcers were excluded, thus yielding a subgroup of cases with idiopathic gastric ulcer disease, *H. pylori* was found to have a frequency of 96% (Borsch, 1989) equivalent to the frequency of *H. pylori* in duodenal ulcer disease (Rauws *et al.*, 1988). A link has been suggested between *H. pylori* and tumour-like lesions (Frommer *et al.*, 1988). *H. pylori* is now thought to be involved in causing as many as 50 % of all gastric cancers. Forman *et al.* (1991) carried out a study investigating blood samples of 29 middle-aged men who developed stomach cancer. 20 (69 %) showed increased

levels of antibodies to the *H. pylori* indicating that they had at some stage been infected by it. This prevalence of infection with *H. pylori* in gastric cancer patients compared to control groups has been confirmed in other studies (Parsonnet *et al.*, 1991; Nomura *et al.*, 1991). *H. pylori* also induces tissue monocytes to produce reactive oxygen intermediates, which can act as potent genotoxic carcinogens (Ames and Gold, 1990). A major problem when determining links between *H. pylori* and different clinical conditions is that the bacterium is very difficult to totally eradicate with conventional treatments. By eliminating the bacterium there will be a much clearer picture as to which conditions are caused by *H. pylori*, and therefore those which are likely to be cleared with eradication of the organism, and those which are just associated with *H. pylori*.

6.2.1. Conventional drug therapy for *H. pylori* infection.

The search for the optimal treatment of *H. pylori* has not been easy. *In vitro*, *H. pylori* is sensitive to most antibiotics (Glupczynski and Burette, 1990). However, the clinical experience with this type of drug has been disappointing, especially when they are administered as a monotherapy. Amoxycillin clears *H. pylori* in 70-90% of patients, but the relapse rate shortly after treatment is high, and long term eradication has not been achieved in more than 20% of individuals (Rauws *et al.*, 1988). In one study, furazolidone produced encouraging results, with an initial clearance rate of 93% and a six week eradication rate of approximately 40% (Morgan *et al.*, 1988). The demonstration that bismuth has an antibacterial effect on *H. pylori* (Marshall *et al.*, 1985b) has encouraged many studies using bismuth preparations. However, there is still no optimum agent or treatment regime for *H. pylori*, although the combination of antibiotics with bismuth salts proves superior *in vivo* to either of the agents administered alone (Glupczynski and Burette, 1990). There are many different factors that may be responsible for the lack of clinical efficacy in the treatment of *H. pylori* infection. These include insufficient drug concentration in gastric mucus and crypts due to poor penetration, decreased antibacterial activity of some antibiotics at

low pH, the physicochemical properties of the molecule will have crucial importance in the activity of the drug at the site of infection. The respective importance of the local and systemic effects of antibiotics is still poorly understood. The optimal drug formulation is still unknown, with differing formulations potentially resulting in variable systemic or local concentrations. It is possible that patchy distribution of a locally active antimicrobial over the gastric mucosa may cause wide variation in concentrations in the oesophagus, stomach or duodenum, and hence, subsequently lead to relapse or to the development of antibiotic resistance. Additionally many patients are unable or unwilling to take a prolonged course of several antibiotics which is required for the best chance of eradication of *H. pylori*. PDT killing of these bacteria may offer a new way of eradicating *H. pylori* in such patients, and in patients with persistent infection despite optimal drug treatment. *H. pylori* only colonises endoscopically accessible parts of the upper gastrointestinal tract, and so might be amenable to local endoscopic therapy.

6.3. Studies on *in vitro* killing of *Helicobacter pylori* with PDT

We have studied the killing of *H. pylori in vitro* using PDT to test the hypothesis that PDT might be of value in treating *H. pylori in vivo*. From a clinical point of view it is important to confirm a possible difference in sensitivity between microorganisms and underlying tissue. This selectivity may be obtained by use of a suitable photosensitiser or by some form of local application of drug or light to areas rich in bacteria. The treatment parameters used are compared with those known to affect normal gastrointestinal tissues to assess the feasibility of destroying *H. pylori* without unacceptable damage to normal mucosa. In view of the importance of bismuth salts as antibacterial agents against *H. pylori*, PDT studies were also carried out in the presence of subtherapeutic concentrations of bismuth subcitrate to determine if there was any synergistic bacterial kill effect of using PDT combined with bismuth.

6.3.1. Methods

H. pylori was isolated from biopsies taken from the antrum of six patients with active gastritis and stored in glycerol broth at -70°C. Prior to use, strains were subcultured on to brain heart infusion agar (containing 10 μg/ml amphotericin, to prevent overgrowth by fungi) and incubated in micro-aerobic conditions. Preliminary studies showed that all six strains had similar sensitivity to PDT, so just one was used for subsequent experiments.

The photosensitiser used was aluminium sulphonated phthalocyanine (a mixture with an average of 3 sulphonate groups per molecule (McCubbin, 1985)), supplied by Ciba-Geigy and used as received. For phototherapy studies, a three day agar culture of *H. pylori* was harvested into 0.9% saline and resuspended in aqueous AlSPc solution (concentration 2.5 mM to 2.5×10^{-2} μM) for four hours. 50 μl were then plated on to an agar plate and exposed to red light at 675 nm (the main absorption peak of AlSPc) from a copper vapour pumped dye laser (Oxford Lasers). The exposure times used were chosen to give total light doses of 1.5 and 5.5 J/cm^2. These plates were incubated for five days and growth compared with that on control plates (AlSPc alone, light alone or neither) by counting colonies. Colony counting and all other microbiological techniques were performed by J. Holton.

The minimum inhibitory concentration (MIC) of bismuth subcitrate was determined for all six strains of *H. pylori* initially isolated by standard plate assay techniques. Again there was no difference between strains, so further studies were limited to the strain used for the main experiments. Bismuth subcitrate at a concentration of 25% of the MIC was added to *H. pylori* exposed to 2.5 μM AlSPc for four hours and then exposed to light doses of 0.3, 0.6, or 0.9 J/cm^2 (energies sufficient to give partial but not complete kill if the bismuth subcitrate had not been added).

Independent confirmation of uptake of AlSPc by bacteria was achieved by direct visualisation using fluorescence microscopy with the CCD camera system, as described in section 2.1.2., on *H. pylori* cultures exposed to AlSPc for four hours at 25 μM. The bacteria were treated as for the phototherapy studies except the cells were washed three times in 0.9% saline to remove any AlSPc not attached to or taken up by the bacteria before being smeared onto glass microscope slides. The slides were then stored frozen until examined.

6.3.2. Results

Fluorescence microscopy of bacterial smears revealed discrete areas of fluorescence considered to correspond to AlSPc stained bacteria (Figure 6.1).

A typical control bacterial plate is shown in Figure 6.2 (sensitised with 25 μM AlSPc, but not exposed to light). Light microscopy of similarly treated bacteria stained with a Gram stain are shown in Figure 6.3.

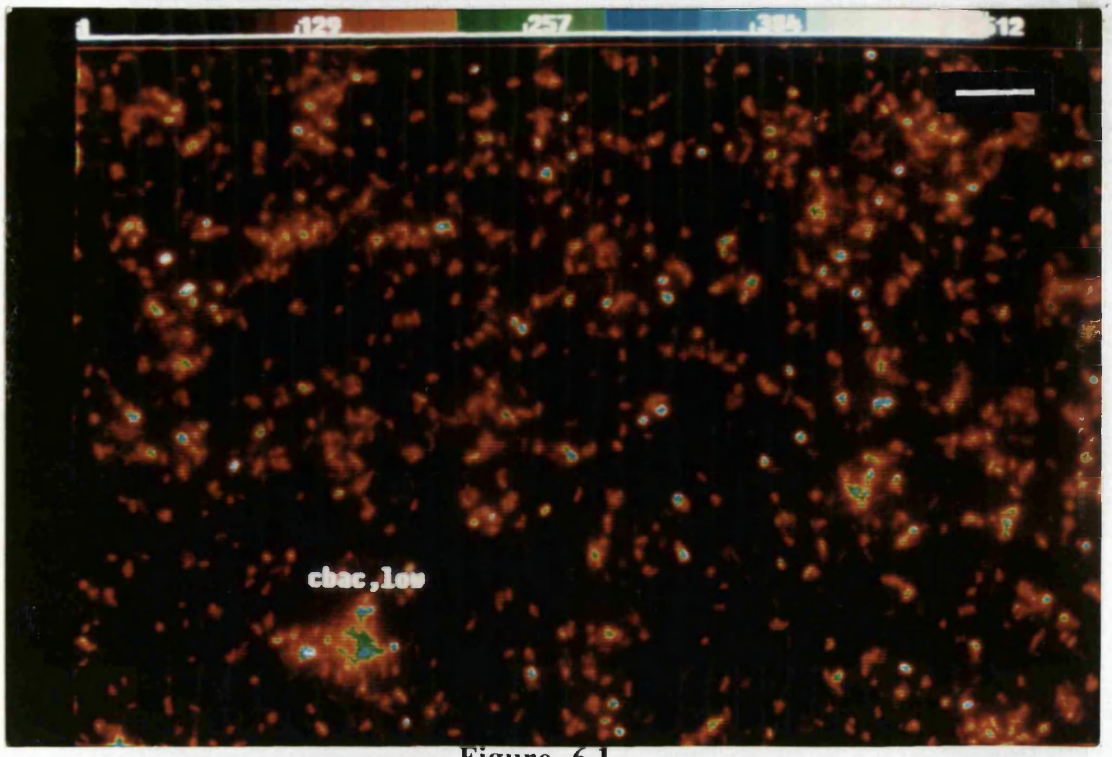


Figure 6.1
CCD image of *H. pylori* smear exposed to 25 μM AlSPc.
Scale: white bar represents 15 μm.

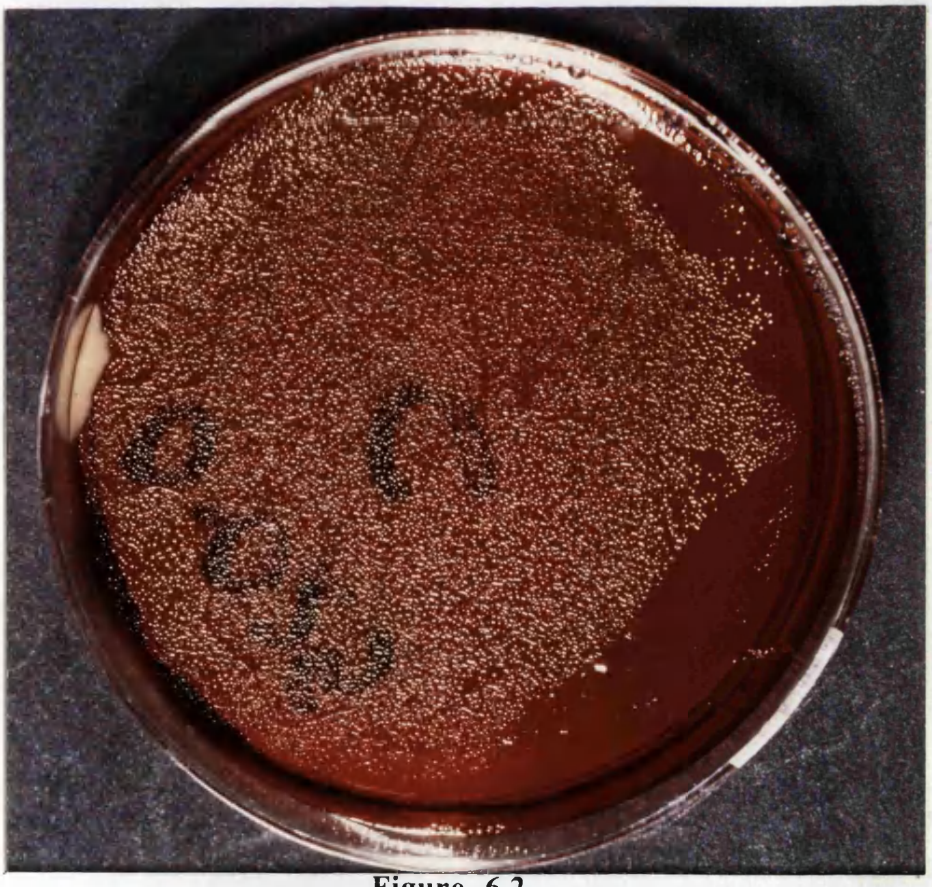


Figure 6.2
Typical control bacterial plate (sensitised but not lasered).

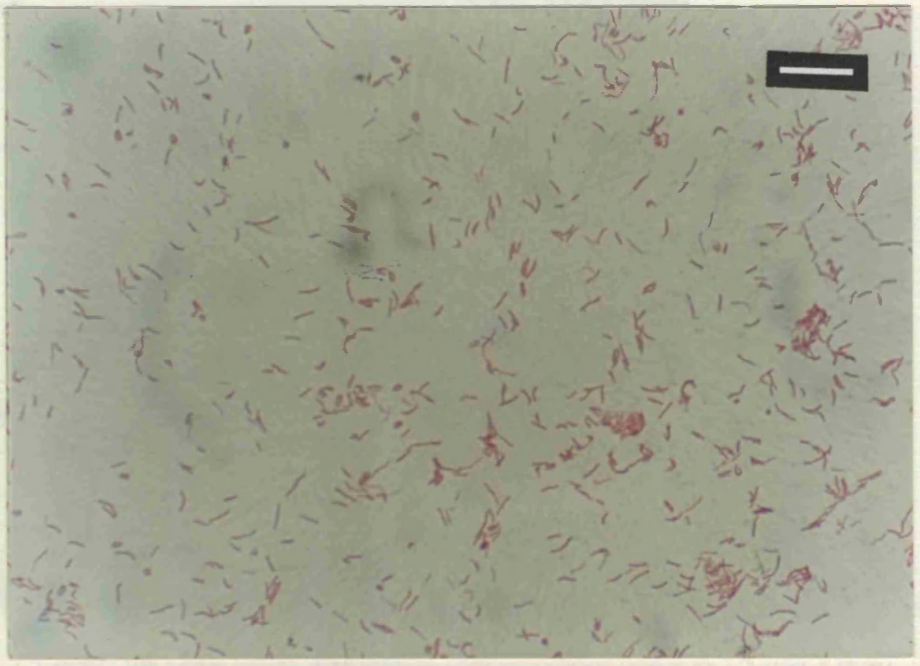


Figure 6.3
Gram stained *H. pylori*. Scale: white bar represents 10 μm.

The percentage of bacterial kill after PDT for the two energy doses applied is shown in Figure 6.4 as a function of the concentration of AlSPc solution used.

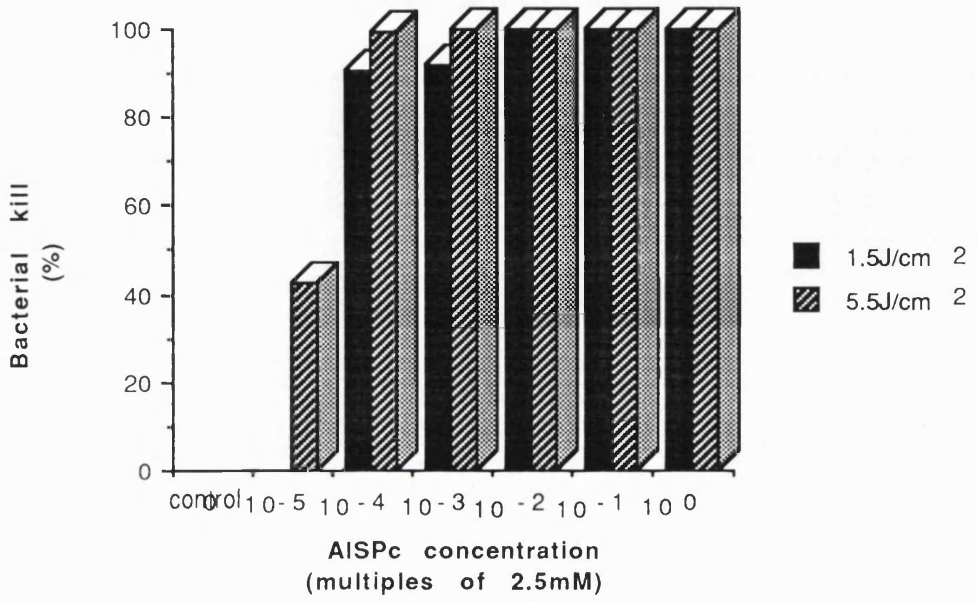


Figure 6.4
Percentage bacterial kill at two laser energies with varying AlSPc concentration.

1.5 J/cm^2 caused 100% *H. pylori* kill at concentrations of AlSPc down to 25 μM and with 5.5 J/cm^2 the same effect was seen at concentrations down to 2.5 μM. On all control plates (light only, sensitiser only, or no treatment) no bacterial cell kill was observed.

The MIC of bismuth subcitrate for the isolates of *H. pylori* was between 16-32 mg/l. Thus sensitised *H. pylori* was treated with bismuth subcitrate concentration of 4 mg/l (25% of MIC) prior to light exposure. In this pilot experiment, this concentration abolished the PDT effect, permitting the same growth of *H. pylori* colonies as was seen in control plates.

6.3.3. Discussion

These experiments have shown that *H. pylori* take up AlSPc *in vitro*. Uptake, that is absorption or adsorption, of sensitiser by the bacteria does not necessarily mean the sensitiser is located in a site to inflict lethal damage after light. Although photosensitised bacteria are subsequently killed by exposure to red light. The inhibitory action of bismuth on the photodynamic effect needs further investigation, but may be due to free radical scavenging by the bismuth. The inhibition of *H. pylori* kill could probably be avoided by treating with bismuth after rather than before PDT but this study has yet to be performed.

We have previously shown that after an intravenous dose of 5 mg/kg of AlSPc (mixture) in normal rats the peak concentration reached in colon is approximately 5 μg/g of tissue (Barr *et al.*, 1987) and we have subsequently shown that similar peak concentrations are obtained with AlS_2Pc in the stomach of rats (unpublished data). In AlS_2Pc sensitised normal rats, a light dose of 50 J delivered from a fibre just touching the gastric mucosa, produced local, full thickness necrosis which healed safely by regeneration of normal tissue (Loh *et al.*, 1992). Additionally, it has been shown that light doses as low as those used in the present experiments had essentially no effect on AlSPc sensitised normal colon (Barr *et al.*, 1987), 1 J (100 mW for 10 seconds) given with the fibre in contact with the tissue gave a lesion no different from control unsensitised animals. We were particularly interested in the mucosal layer as the *H. pylori* infection in man is on the surface of the mucosal cells in the stomach. Fluorescence microscopy studies on rat colons show that a estimated maximum mucosal concentration in the order of 50 μM was reached after administration of the 5 mg/kg AlSPc dose (personal communication - A.J. MacRobert). We were able to obtain 100% *H. pylori* kill at half this concentration even with the lower light dose of 1.5 J/cm^2. From the above data it is likely that gastric mucosa will remain intact during PDT kill of *H. pylori*. In the rat studies the photosensitiser was given i.v., and it is unlikely that this route will deliver AlSPc to the *H. pylori*. Oral

administration of the photosensitiser is likely to be a better option. In preliminary fluorescence microscopy studies in normal rats we have seen only background levels of fluorescence in the gastric wall at 30 minutes, 1, 2, 4 and 6 hours after an oral dose of 5 mg/kg AlS_2Pc. This lack of photosensitisation of the stomach may have been due to precipitation out of solution of AlS_2Pc, known to occur at acidic pH's. Even when omeprazole (a drug which inhibits gastric acid secretion) was given before administration of the sensitiser no greater tissue fluorescence was observed. Therefore, we believe due to the hydrophilicity and large size of AlS_2Pc molecule it is not able to penetrate the gastric mucosal tissue. If under these treatment conditions the sensitiser could access photosensitive sites on the *H. pylori* whilst not sensitising the stomach, aluminium sulphonated phthalocyanine sensitisers could prove ideal for this application. However, much work is still required in order to investigate this possibility.

The key to clinical application of this technique will be to ensure that all areas likely to harbour *H. pylori* are exposed to both the sensitiser and light. For this, the sensitiser must be prepared in a form that will reach all the appropriate mucosal surfaces in the stomach and duodenum. It is not necessary to deliver the same light dose to every point, but just that the dose at every point should be above the threshold for a PDT effect. Pope *et al.* (1991) has investigated the use of PDT for treating carcinoma in situ in the rat bladder. At an appropriate tissue concentration, the photosensitiser can be photobleached out of the muscle layer in such a way that no biological effect is produced however high the light dose, while at the same time one can achieve safe uniform necrosis of the mucosa as is required to treat carcinoma in situ. This means that it should be possible to achieve the desired clinical effect without the need for uniform illumination of the bladder mucosa. The only requirement is that the light dose at every point should be above the threshold level, but there is no upper limit for avoiding unacceptable damage to normal areas. In the case of *H. pylori* infection, the ideal would be that the threshold for a PDT effect is achieved in the bacteria but not in

the mucosa, whatever the light dose. Even if this cannot be achieved and the threshold is exceeded in the mucosa, it should heal safely so this would be clinically acceptable.

Therefore, this is a new technique which raises the possibility of using PDT either alone, or as an adjuvant to antimicrobial drugs, for the eradication of *H. pylori*. The potential of this new approach for the treatment of other localised infections is very considerable, for example, in the mouth. The results presented here are very preliminary, and the efficacy of the technique must be tested in an *in vivo* model before it will be justified to start clinical trials. An animal model suggested for this study is the ferret (Otto *et al.*, 1990). A gastric *H. pylori*-like organism (*Helicobacter mustelae*) very similar in morphology has been isolated from the ferret. The ferret bacterium is closely related to *H. pylori* and shares many characteristics. These Gram-negative, microaerophilic, urease positive, spiral bacilli occupy the same ecological niche beneath gastric mucus attached to the gastric epithelium, although, their association with gastritis is inconsistent (Marshall, 1989). Mature ferrets have a high incidence of infection (Tompkins, 1989). This raises the possibility of the ferret being a useful model for the study of *H. pylori*-related diseases. In our own study out of 8 ferrets biopsied 6 were positive for *H. mustelae* infection. Upon histopathological analysis of an infected ferret stomach post-mortem there was no evidence of inflammatory response or ulceration and all layers of the stomach were normal. Initial studies with *H. mustelae* have shown a similar susceptibility to PDT with AlSPc. Therefore, this is a suitable model for our investigation and these studies are now being continued.

CHAPTER 7 - DISCUSSION

Historically photodynamic therapy has been considered as "the selective tumour treatment". This assumption has greatly hindered the development of the technique and has caused serious, somewhat unexpected, side effects in the clinic, some with potentially fatal consequences. An example of a particularly unfortunate side effect has been reported when treating carcinoma in situ in the bladder. The condition was successfully eradicated but due to a marked reduction in bladder capacity, with incontinence, cystectomy was still required (Nseyo *et al.*, 1985). This was thought to occur because of a lack of selectivity of sensitiser for the tumour compared with the underlying normal tissues, and after light muscle fibrosis occurs (Pope and Bown, 1991). It is therefore obvious that clinical PDT will not be widely accepted until selectivity of tumour damage can be significantly enhanced, or it can be used in situations in which selectivity is not essential. Research must not be just totally based on trying to obtain significant tumour damage, but also on retaining the integrity and normal function of any normal tissues which may fall within the field of the treatment light. During the time the work for this thesis has been performed it has become apparent that the idea of only limited selectivity of tumour sensitisation (if indeed there is any at all with normal tissues at the tumour margin (Barr *et al.*, 1991)) has been more widely accepted and considerable work is being performed to enhance this selectivity of treatment with some promising developments. Enhancing selectivity of PDT can be achieved in more ways than just by obtaining a better tumour to normal tissue ratio; indeed, if a particular sensitiser acts primarily on tumour vasculature, as is the case with HpD and the phthalocyanines, enhancing this ratio may have only a limited effect. Therefore, in addition to preferential localisation of photosensitisers in tumour tissue, selectivity can also be obtained by applying a treatment to which tumour cells are more sensitive than normal cells and tissues; or by bringing upon the tissues a type of damage from which normal tissue is only partially damaged and subsequently heals and the tumour tissue dies; and by confining the light treatment to the tumour region. New strategies have been examined to enhance PDT selectivity.

Using a low dose of the AlSPc mixture Barr *et al.* (1990b) have indeed shown selective tumour damage by utilising the ability of this sensitiser to photobleach. It was postulated that at a low dosage (0.5 mg/kg), selective necrosis could occur because the photosensitiser was photodegraded in the normal colon before a threshold photodynamic dose was reached, whereas in tumour containing approximately twice as much sensitiser as the normal tissue, a photodynamic threshold dose could be achieved and necrosis produced. Unfortunately, this technique is limited by the extent of tumour necrosis, which under these circumstances is only 1-2 mm for realistic treatment times and is therefore unlikely to be of clinical significance.

Therefore, new photosensitisers have been investigated to attain better tumour to normal ratios. Enhanced ratios have tended to be observed only between transplanted tumour models and underlying flank muscle. As we have shown in section 3.2. this is only relevant when treating tumours situated in this normal tissue, and the greater tumour selectivity is likely to be due to a relatively poor uptake of sensitiser in muscle. Even the limited selectivity generally seen between tumour and the surrounding normal tissue is actually between the vascular stroma of normal and tumour tissue, and not between individual normal and malignant cells. Thus it is difficult to achieve truly selective eradication of malignant tumours with PDT as in the most crucial region where tumour cells are infiltrating normal areas, tumour and normal cells will contain similar quantities of photosensitiser (Barr *et al.*, 1991). We have studied the di- and tetrasulphonated aluminium phthalocyanines with the intent of attempting to increase normal to tumour ratios, and then exploiting the photodegradation properties of AlSPc discussed above to increase this limited tumour necrosis. We have not been able to show any appreciable enhanced selectivity of tumour uptake with these components of AlSPc. Additionally, no increase in selectivity of biological effect over that obtained with a mixture of AlSPc has been observed (Chatlani *et al.*, 1992a). However, the benefit of using a component of

AlSPc for clinical PDT is that photosensitisers with greater purity and reproducibility are always preferable for patient administration.

To avoid normal tissue damage during PDT in the rat colonic tumour model, when there is known to be only a limited tumour selectivity of 2 to 1 for the mixture of AlSPc (Tralau *et al.*, 1987), a radioprotective drug has been investigated in combination with a conventional PDT protocol. We have studied the phosphorylated thiol protectant W7 and shown complete protection of normal tissue whilst still retaining the magnitude of tumour necrosis. In this way biological selectivity of PDT was obtained, however, high doses of the protectant were required and this may have inherent disadvantages in the clinic due to intolerable levels of systemic toxicity.

The other major aspect of this work has been to explore a totally new approach to photosensitisation. This involved the introduction of 5-aminolaevulinic acid, as described in chapter 5. This is not itself a sensitiser, but is naturally occurring in cells, and in the presence of excess ALA causes accumulation of porphyrin sensitisers, via the haem synthesis pathway. This endogenous sensitisation has been exploited for PDT. Clinical data has been produced using topical ALA with promising results. Kennedy *et al.* (1990) have shown a 90% complete response rate and a 7.5% partial response in the treatment of basal cell carcinomas. The cosmetic results were excellent and patient acceptance of the technique was very good; but PDT following intravenous ALA was largely an unknown field of study. This route of sensitisation not only gave considerable selectivity but tumour cells were specifically sensitised, and all sensitisation was eliminated by 24 hours. Extended skin photosensitivity should not be a problem to patients treated after ALA-induced sensitisation. After light treatment, damage correlated well with the pattern of sensitisation leaving the colonic muscle intact and, therefore, there was also selectivity in the biological effect. An additional finding was that the pattern of sensitisation was the same after both i.v. and oral administration in the normal colon,

and we therefore believe tumour selectivity may be of the same magnitude as after i.v. ALA. PDT after oral ALA could form an important protocol for use in patients and indeed we have begun clinical trials of PDT after oral ALA in a variety of tumours (Loh *et al.*, 1993b). These tumours have been shown to synthesize porphyrins and show areas of necrosis after light exposure (Grant *et al.*, 1993), although considerable optimisation studies of this treatment are still required. PDT with ALA may also prove an important technique not just in the treatment of solid tumours but in the situation of 'field change' disease where the lining of an organ is abnormal and liable to malignant degeneration over an extended area as seen in the stomach, bowel and vagina. It is difficult to treat without removal of the entire organ, therefore if the diseased layer can be destroyed without serious damage to deeper layers then it could prove a significant advance. It has been shown in the preliminary studies in section 5.3 that in a range of normal tissues the underlying muscle is only minimally sensitised and if biological selectivity is consistent with colon studies the muscle will remain intact after this mode of treatment. Therefore as long as the tissue heals by regeneration of normal mucosa and not by regeneration of the same premalignant mucosa this could prove a very useful clinical technique.

PDT has also been shown to be efficacious in killing bacteria. We have studied killing a specific bacterium called *Helicobacter pylori*, a organism linked with duodenal and gastric ulceration and more recently gastric cancer. Selectivity of treatment is of particular importance when treating *H. pylori* as ulceration is not life threatening, therefore severe necrosis of the gastrointestinal mucosa is not desirable. Considerable work is still required before any attempt is made to treat patients, although preliminary results show we can kill 100 % *H. pylori in vitro* at a laser energy known not to cause necrosis of normal rat colonic mucosa. There are many questions still to answer, not least how to optimally sensitise the bacteria and how to treat the entire gastric and duodenal mucosa with light. Even if these problems

eventually prove insurmountable for this particular application, we believe PDT will still prove a powerful tool in treatment of other localised infection.

The findings from the studies in this thesis and their relevance to clinical selectivity are summarised in Table 7.1.

Method of achieving selectivity (chapter)	Mode of selectivity	Selectivity achieved *in vivo*	Relevance to clinical selectivity
AlSPc components (3)	Greater uptake in tumour	+	Biological selectivity did not differ greatly from that achieved with the AlSPc mixture, although it is always preferable to use photosensitisers of greater purity in the clinic.
Radioprotective drug (4)	Protection of normal tissues	+++	Complete biological selectivity was achieved, although high doses of the protective drug were required, this magnitude of dose may prove intolerably toxic for patient use.
ALA (5)	Greater sensitisation of tumour	++	Considerable biological selectivity seen between tumour and underlying normal tissues. Unlikely to cause photosensitivity much beyond 24 hours. Drug may be given orally. Potential for clinical applications other than treatment of solid tumours.
H. pylori eradication (6)	Bacterial kill with underlying tissue left intact	Still to be investigated	Observations from other *in vivo* studies suggest potential for selective eradication of bacteria whilst retaining integrity of underlying normal tissues. Considerable potential for treating other localised infections.

Table 7.1
Methods investigated for achieving selectivity in PDT
+ indicates limited selectivity
++ indicates considerable selectivity
+++ indicates complete selectivity

In summary, clinical PDT has evolved on a largely empirical basis whilst there are still fundamental questions regarding its mechanism of action and optimum treatment parameters unanswered. These include the choice of photosensitiser and the optimum drug dose, the time delay between sensitisation and light treatment to allow for the most advantageous distribution between tumour and normal tissue, and the wavelength and energy of the activating light. Photosensitisers tend not to be selectively retained by malignant cells to any great extent so it becomes essential to obtain photosensitisers with enhanced tumour selectivity or, at least, to understand the damaging action that PDT has on normal tissues surrounding tumours. The treatment should be developed to produce the desired effect in the tumour but with minimal unwanted normal tissue damage to avoid the complications seen in clinical PDT.

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APPENDIX 1 - Data for figures and tables

Data for Figure 3.2

Concentration of extractable (a) AlS_2Pc, or (b) AlS_4Pc in normal colon and colonic tumour following an i.v. injection of 5.65 μm/kg.

(a)

AlS_2Pc in colonic tumour.

Time (hours)	Concentration (nmol/g of tissue)					Mean	Standard deviation
1	1.9	5.8	1.8	2.7		3.1	1.9
48	9.2	8.5				8.9	0.5
168	3.1	5.3				4.2	1.6
336	2.7	4.4	5.5	2.0	3.1	3.5	1.4

AlS_2Pc in normal colon.

Time (hours)	Concentration (nmol/g of tissue)					Mean	Standard deviation
1	4.9	4.1	4.4	4.3	4.8	4.5	0.3
3	3.3	4.4	4.1			3.9	0.6
5	2.5	2.8	3.1	3.6	3.6	3.1	0.5
18	4.2	3.2	3.9			3.8	0.5
24	2.9	3.9	3.3			3.4	0.5
48	1.2	2.3	2.1	1.4	3.3	2.1	0.8
168	0.8	0.8	0.6	0.8		0.8	0.1
336	0.8	0.8	1.2			0.9	0.2

(b)

AlS_4Pc in colonic tumour.

Time (hours)	Concentration (nmol/g of tissue)					Mean	Standard deviation
1	6.2	10.7	17.2			11.4	5.6
48	3.7	3.9	6.2	8.9		5.7	2.4
168	6.1	4.7	6.5	4.8		5.5	0.9
336	5.4	4.7	4.8	6.3		5.3	0.7

AlS_4Pc in normal colon.

Time (hours)	Concentration (nmol/g of tissue)					Mean	Standard deviation
1	4.2	4.7	7.3	5.8	2.9	5.0	1.7
3	2.3	1.5	2.5			2.1	0.5
5	3.8	3.8	2.8	2.7	2.0	3.0	0.8
18	4.3	4.5	3.1			4.0	0.8
24	4.5	4.5	3.1			4.0	0.8
48	4.7	5.7	4.7	4.9	4.3	4.9	0.5
168	4.9	4.5	4.3	4.7	5.2	4.8	0.3
336	4.6	5.3	4.9	5.7	3.8	4.9	0.7

Data for Figure 3.3

Concentration of extractable (a) AlS_2Pc, or (b) AlS_4Pc in normal colon and muscle and transplanted tumour following an i.v. injection of 5.65 μm/kg.

(a)

AlS_2Pc in transplanted tumour.

Time (hours)	Concentration (nmol/g of tissue)			Mean	Standard deviation
1	5.7	5.9	6.0	5.9	0.2
3	7.2	6.2	6.4	6.6	0.5
5	8.3	6.3	8.0	7.5	1.1
18	8.8	6.5	6.7	7.3	1.3
24	8.7	8.8	8.9	8.8	0.1
48	6.6	8.1	6.9	7.2	0.8

AlS_2Pc in normal colon.

Time (hours)	Concentration (nmol/g of tissue)					Mean	Standard deviation
1	4.9	4.1	4.4	4.3	4.8	4.5	0.3
3	3.3	4.4	4.1			3.9	0.6
5	2.5	2.8	3.1	3.6	3.6	3.1	0.5
18	4.2	3.2	3.9			3.8	0.5
24	2.9	3.9	3.3			3.4	0.5
48	1.2	2.3	2.1	1.4	3.3	2.1	0.8

AlS_2Pc in normal muscle.

Time (hours)	Concentration (nmol/g of tissue)	Mean	Standard deviation
1	4.2 4.6 4.8	4.5	0.3
3	3.4 3.1 2.5	3.0	0.5
5	2.3 2.3 2.3	2.3	0.0
18	1.6 1.9 2.0	1.8	0.2
24	1.7 1.4 1.7	1.6	0.2
48	0.9 0.9 1.7	1.2	0.5

(b)

AlS_4Pc in transplanted tumour.

Time (hours)	Concentration (nmol/g of tissue)	Mean	Standard deviation
1	7.9 7.7 5.7	7.1	1.2
3	4.5 7.1 6.8	6.1	1.4
5	8.7 7.9 8.4	8.3	0.4
18	10.9 12.4 11.4	11.6	0.8
24	11.3 11.9 10.3	11.2	0.8
48	9.3 6.5 7.4	7.7	1.4

AlS_4Pc in normal colon.

Time (hours)	Concentration (nmol/g of tissue)	Mean	Standard deviation
1	4.2 4.7 7.3 5.8 2.9	5.0	1.7
3	2.3 1.5 2.5	2.1	0.5
5	3.8 3.8 2.8 2.7 2.0	3.0	0.8
18	4.3 4.5 3.1	4.0	0.8
24	4.5 4.5 3.1	4.0	0.8
48	4.7 5.7 4.7 4.9 4.3	4.9	0.5

AlS_4Pc in normal muscle.

Time (hours)	Concentration (nmol/g of tissue)	Mean	Standard deviation
1	0.6 0.5 0.6	0.6	0.1
3	0.9 0.4 1.0	0.8	0.3
5	0.4 0.5 0.5	0.5	0.1
18	0.4 0.3 0.4	0.4	0.1
24	0.5 0.7 0.4	0.5	0.2
48	1.0 0.3 0.5	0.6	0.4

Data for Table 4.1

Diameter of necrosis in normal colonic mucosa and colonic tumour after PDT with and without W7.

	Tumour tissue			Normal tissue		
	S.D.	Mean	S.D.		Mean	
Light exposure before W7	4.5 3.8 4.8	4.4	0.5	5.5 3.5 3.5	4.2	1.2
Light exposure after W7	2.5 3.6 4.3	3.5	0.9	0.0 0.0 0.0	0.0	0.0
AlSPc only (no W7)				5.0 2.5 4.5 3.5 5.0	4.1	1.1

Data for Figure 5.2

Microscopic fluorescence levels (arbitrary units) in normal colon sections as a function of time after administration of 50, 100, 150 and 200 mg/kg ALA.

	ALA dose (mg/kg)			
Time (hours)	50	100	150	200
3	2	15	27	30
6	0	0	1	20
24	0	0	0	0

Data for Figure 5.7

Microscopic fluorescence levels in tissue layers of the normal colon as a function of time after administration of 200 mg/kg ALA.

	Fluorescence level in tissue layer (arbitrary units)								
	Muscle			Submucosa			Mucosa		
Time (hours)			Mean			Mean			Mean
0.08	0	0	0	0	0	0	5	6	6
0.05	0	0	0	0	0	0	10	9	10
1	0	0	0	0	0	0	22	26	24
2	2	1	2	5	5	5	48	50	49
3	4	3	4	11	7	9	52	54	53
4	10	12	11	17	23	20	111	135	123
5	6	8	7	16	12	14	64	73	69
6	3	3	3	5	6	6	27	33	30
8	5	1	3	2	8	5	15	14	15
17	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0

Data for Figure 5.11

Microscopic fluorescence levels (arbitrary units) in transplanted tumour sections as a function of time after administration of 200 mg/kg ALA.

	Fluorescence level in tissue layer (arbitrary units)		
Time			Mean
0.08	0	0	0
0.5	3	1	2
1	7	4	6
2	20	28	24
4	44	40	42
6	1	1	1
24	0	0	0

Data for Figure 5.18

Diameter of necrosis in normal colonic mucosa measured as a function of time after administration of 200 mg/kg ALA.

Time (hours)	Diameter of necrosis (mm)					mean	Standard deviation
0.08	1.3	1.3	1.0	0.0	0.0	0.7	0.7
0.33	1.0	6.0	5.0	3.0	3.0	3.6	2.0
0.5	7.5	6.5	7.5	8.5	6.0	7.2	1.0
0.66	4.5	8.0	4.5	6.0	4.0	5.4	1.6
1	7.5	4.0	7.0	3.5	8.5	6.1	2.2
2	7.5	8.5	8.0	8.5	8.0	8.1	0.4
3	8.0	8.5	8.5	7.5	9.0	8.3	0.6
4	7.5	11.0	10.0	7.5	9.5	9.1	1.6
5	7.5	6.5	7.5	7.5	6.0	7.0	0.7
6	6.5	6.5	7.5	7.0	6.5	6.8	0.4
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Data for Figure 5.23

Microscopic fluorescence levels in tissue layers of the normal colon as a function of time after oral administration of 400 mg/kg ALA.

	Fluorescence level in tissue layer (arbitrary units)								
	Muscle			Submucosa			Mucosa		
Time (hours)			Mean			Mean			Mean
1	6	8	7	4	16	10	23	72	48
2	9	8	9	23	13	18	82	87	85
4	19	27	23	31	45	38	129	106	118
6	34	20	27	18	28	23	42	68	55
8	15	14	15	8	10	9	1	2	2
24	0	0	0	0	0	0	0	0	0

APPENDIX 2 - Publications and selected reprints

J. Bedwell, J. Holton, D. Vaira, A.J. MacRobert, S.G. Bown. *In vitro* killing of *Helicobacter pylori* with photodynamic therapy. The Lancet, 335, 1287, 1990.

W-S. Chan, J.F. Marshall, R. Svensen, J. Bedwell, I.R. Hart. Effect of sulphonation on the cell and tissue distribution of the photosensitiser aluminium phthalocyanine. Cancer Research, 50, 4533-4538, 1990.

J. Bedwell, P.T. Chatlani, A.J. MacRobert, J.E. Roberts, J. Dillon, S.G. Bown. Enhanced tumour selectivity of photodynamic therapy in the rat colon using a radioprotective agent. Photochemistry and Photobiology, 53, 6, 753-756, 1991.

P.T. Chatlani, J. Bedwell, A.J. MacRobert, H. Barr, P.B. Boulos, N. Krasner, D. Phillips, S.G. Bown. Comparison of distribution and photodynamic effects of di- and tetra- sulphonated aluminium sulphonated phthalocyanines in normal rat colon. Photochemistry and photobiology, 53, 6, 745-751, 1991.

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P.T. Chatlani, P.J.O. Nuutinen, N. Toda, H. Barr, A.J. MacRobert, J. Bedwell, S.G. Bown. Selective necrosis in BOP-induced hamster pancreatic tumours using photodynamic therapy with phthalocyanine photosensitisation. British Journal of Surgery, 79, 786-790, 1992.

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C.S. Loh, J. Bedwell, A.J. MacRobert, N. Krasner, D. Phillips, S.G. Bown. The kinetics of ALA photosensitisation in the rat stomach. In: Photodynamic therapy and biomedical lasers. Excerpta Medica International Congress Series, P. Spinelli, M. Dal Fante, R. Marchesini (eds), 286-291, 1992.

P.T. Chatlani, J. Bedwell, A.J. MacRobert and S.G. Bown. Distribution and photodynamic effects of di- and tetra- sulphonated aluminium phthalocyanines (AlS_2Pc and AlS_4Pc) in normal and neoplastic rat colon. In: Photodynamic therapy and biomedical lasers. Excerpta Medica International Congress Series, P. Spinelli, M. Dal Fante, R. Marchesini (eds), 539-544, 1992.

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C.S. Loh, D. Vernon, A.J. MacRobert, J. Bedwell, S.G. Bown and S.B. Brown Endogenous porphyrin distributions induced by 5-aminolaevulinic acid in the tissue layers of the gastrointestinal tract of the normal rat. Journal of Photochemistry and Photobiology (B:Biology), *in press.*

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Selective necrosis in hamster pancreatic tumours using photodynamic therapy with phthalocyanine photosensitization

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Photodynamic therapy (PDT) is often thought to be able to effect selective tumour necrosis. This therapeutic selectivity, based on transient differences in tumour:normal tissue photosensitizer concentration ratios, is rarely useful clinically in extracranial tumours, although PDT itself may be of value by virtue of the nature of the damage produced and healing of normal tissue by regeneration. This report describes the effects of PDT on normal pancreas and chemically induced pancreatic cancers in the hamster, where a different mechanism of selective necrosis may be seen. Photosensitizer distribution in normal and neoplastic pancreas was studied by chemical extraction and fluorescence microscopy. Correlation of distribution studies with necrosis produced by PDT shows that the photodynamic dose (product of tissue concentration of sensitizer and light dose) threshold for damage is seven times as high for normal pancreas as for pancreatic cancer. Tumour necrosis extended to the point where tumour was invading normal areas without damaging the normal tissue. In rat colonic cancer, photodynamic dose thresholds in tumour and normal tissue are similar and so such marked selectivity of necrosis is not possible. The reason for this selectivity in the pancreas is not clear, but recent evidence has suggested a difference in response to PDT between normal and neoplastic pancreatic cell lines and the presence of a singlet oxygen scavenger in normal pancreas is postulated. Furthermore, the present fluorescence microscopy studies suggest that tumour stroma contains the highest level of photosensitizer and thus receives the highest photodynamic dose during PDT. These results suggest a possible role for PDT in treating small pancreatic tumours or as an adjuvant to other techniques, such as surgery, that reduce the main bulk of tumours localized to the pancreas.

Photodynamic therapy (PDT) has attracted much interest over the past 10 years as the concept of selective retention of photosensitizers in malignant tumours has raised the possibility of their selective destruction. In general, this dream has not been realized. For all solid extracranial tumours so far studied, the ratio of sensitizer concentration between tumour and normal tissue does not exceed 3:1. Few quantitative studies of necrosis have been reported but, in most that have been carried out and well documented, there is just as much damage to normal tissue as to malignant areas when both are exposed to the same light dose. Clinically, this may be acceptable if damage to normal areas heals mainly by regeneration of normal tissue. However, there are circumstances under which truly selective necrosis can be achieved. The most notable is in the brain, where the photosensitizer ratio between tumour and normal tissue can be as high as 28:1; necrosis of transplanted gliomas has been shown in mice and rats without damage to adjacent normal brain tissue[1,2].

Truly selective necrosis has been shown in chemically induced colonic cancers in rats, based on transient differences in pharmacokinetics of the photosensitizer. However, under the circumstances in which this could be achieved, it was possible to obtain only 2 mm of necrosis in the tumour and this did not include the region where the cancer invaded adjacent normal tissue[3].

Two thresholds must be exceeded for a PDT effect to be seen. The first is the absolute concentration of sensitizer in the tissue. If this is not reached, the sensitizer is inactivated by photobleaching, presumably before enough singlet oxygen is formed to produce a biological effect[4]. The second is the product of the absolute concentration of the sensitizer and the light dose at each point in the target tissue. If the concentration is just below the first threshold in normal areas but just above in the tumour, selective damage is possible, as was shown in the colon experiments[3]. However, the sensitizer doses in these studies were well below those normally used. This inevitably resulted in a very small extent of necrosis being produced, although if the target pathology is <2 mm thick this may be acceptable. For example, *in situ* bladder carcinoma may be amenable to treatment using these dosimetry principles[5].

The situation with the pancreas may be different. Recent reports[6] have suggested that pancreatic carcinomas induced by 2,2'-dioxodi-*n*-propylnitrosamine (BOP) in Syrian golden hamsters may be more susceptible to PDT than normal pancreatic tissue after sensitization with dihaematoporphyrin ether (DHE). In the present study, the microscopic distribution of aluminium sulphonated phthalocyanine (AlSPc) was examined in normal and neoplastic pancreas using fluorescence microscopy with a charge-coupled device (CCD) camera; these results were correlated with the distributions measured by

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chemical extraction and with the response to light. The light dose was varied to determine the photodynamic threshold for damage to the normal pancreas and pancreatic tumour.

Materials and methods

Experiments were carried out on female Syrian golden hamsters. For studies on normal pancreas, animals aged 6–10 weeks and weighing 100 g were used. Pancreatic tumours were induced by ten subcutaneous injections of 10 mg/kg BOP at 1 week intervals[7]. Animals treated with carcinogen were used between 16 and 24 weeks after the first injection. The photosensitizer used was AlSPc. This was obtained from Ciba-Geigy (Basel, Switzerland), and made up in 1 mol/l NaCl to a concentration of 2 mg/ml. Sensitization was by intravenous injection of 5 mg/kg into the inferior vena cava at laparotomy, at which time the presence or absence of tumour was confirmed.

Distribution studies from chemical extractions

Animals injected with 5 mg/kg AlSPc were killed 4, 24 or 48 h after sensitization. Pancreatic tissue was removed at autopsy and stored at −4°C before assay. AlSPc was extracted using 0·1 mol/l NaOH as previously described[8]. Briefly, the assay required dissolution of a weighed amount of tissue in NaOH by incubation of the mixture in a water bath at 50°C for 4 h. The resulting mixture was spun down and the AlSPc content of the supernatant determined using fluorescence measurement by comparison with standard curves prepared using 0·1 mol/l NaOH on unsensitized pancreas or pancreatic tumour to which known amounts of photosensitizer had been added.

Fluorescence microscopy and photometry

Animals sensitized with 5 mg/kg AlSPc were killed 48 h after injection. Pancreatic tissue (tumour or normal) was removed at autopsy and immediately frozen using isopentane and liquid nitrogen. Frozen sections (10 μm) were used for fluorescence microscopy as previously described[9,10]. Using a combination of phase-contrast and light microscopy of haematoxylin and eosin stained sections cut immediately adjacent to those used for fluorescence microscopy, it was possible to identify areas of interest. The technique of fluorescence microscopy has been described recently[11], but is summarized here. Frozen tissue sections on glass slides were placed on the stage of an inverted microscope (IMT-2; Olympus, Tokyo, Japan) with epifluorescence and phase-contrast attachments, and an 8-mW helium–neon laser (632·8 nm) used as the excitation source. The laser output was passed through a 10-nm bandpass filter, centred at 633 nm, to remove extraneous light and then directed on to the section using a liquid light guide and dichroic mirror. Fluorescence was detected between 665 and 700 nm, using a combination of bandpass (Omega Optical, Vermont, USA) and longpass (RG665; Schott, Mainz, Germany) filters, and imaged using the cryogenically cooled CCD camera (Model 1; Wright Instruments, London, UK, resolution 400 × 600 pixels) fitted to the microscope. Image processing and camera operation were carried out using a personal computer. This slow-scan CCD system has sensitivity comparable to that of photomultiplier photon counting detection and is significantly superior to conventional video camera imaging[12]. The black-and-white digital fluorescence images were falsely colour-coded by image processing software and photographed for comparison with the corresponding images from adjacent stained sections. Computer software was used to calculate values for mean fluorescence intensity within a rectangle drawn over areas of interest on the monitor[11]. With tumour-bearing pancreata, values were obtained from tumour epithelium and stroma, together with values for normal acinar tissue and ducts within the same pancreas.

Photodynamic therapy

Normal pancreas and pancreatic tumours (≥5 mm) were treated at laparotomy 48 h after sensitization. Red light (675 nm) from a copper vapour pumped-dye laser (Oxford Lasers, Oxford, UK) was delivered using a 200-μm fibre just touching the tissue to be irradiated. Energy doses studied were 50, 100 and 200 J for normal pancreas and 50, 25 and 12·5 J for pancreatic tumour, using a laser power of 25 mW for 50 J or lower or 50 mW for levels above 50 J. Control experiments were carried out on unsensitized animals using 50, 100 or 200 J for normal pancreas and 50 J for pancreatic tumour using the same power levels. Necrosis was assessed at autopsy 72 h after light exposure, both macroscopically and microscopically, using haematoxylin and eosin stained sections of the treated areas.

Results

Distribution studies from chemical extractions

As shown in *Figure 1*, AlSPc concentration was maximal in normal pancreas at 4 h (the shortest time interval studied) and decreased with time after injection. In tumours, peak concentration was noted at 24 h. The tumour:normal tissue ratios at 4, 24 and 48 h were 0·74:1, 3·0:1 and 2·3:1 respectively.

Fluorescence microscopy and photometry

Fluorescence in normal pancreas was greatest in ducts and blood vessel walls (*Table 1*). The cellular areas of acini were poorly fluorescent although there was slightly more fluorescence at the peripheries of acini (*Figure 2*).

Pancreata in tumour-bearing animals selected for fluorescence microscopy contained areas of normal pancreas (*Figures 3* and *4*). Fluorescence was greatest in stromal areas (*Table 1*). Fluorescence from tumour epithelium was less than from stromal areas, but was slightly greater than from normal acinar tissue.

Photodynamic therapy

Although the maximum tumour:normal tissue ratio of AlSPc concentration occurred at 24 h, the standard deviations of these concentrations were smallest at 48 h, so this was the time chosen for PDT. These results are shown in *Table 2*. There was little difference in macroscopic and microscopic appearances between sensitized (*Figure 5*) and unsensitized normal pancreas

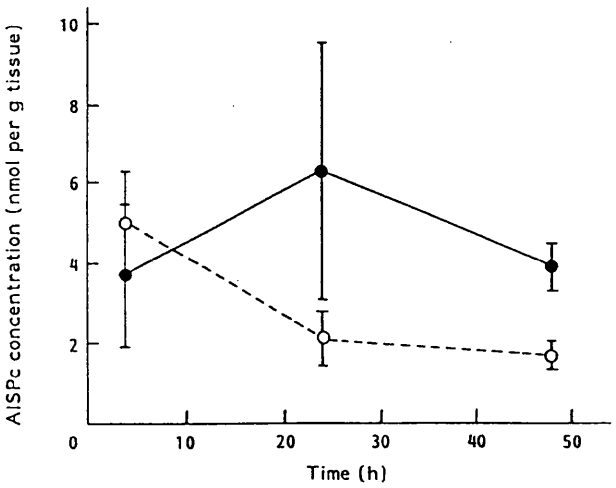

Figure 1 *Mean(s.d.) aluminium sulphonated phthalocyanine (AlSPc) concentration in (○) normal pancreas and (●) pancreatic tumour* versus *time from photosensitizer administration (n ≥ 3 for each value). Although the tumour:normal tissue ratio was highest at 24 h, 48 h was chosen for fluorescence studies and photodynamic therapy as the variation of AlSPc concentration was least at this time*

Table 1 *Fluorescence photometry of normal pancreas and pancreatic tumour 48 h after photosensitizer administration*

	Fluorescence intensity (counts/pixel)
Normal tissue	
Acinar areas	53(14·7)
Ducts	96(11·3)
Tumour	
Epithelium	68(6·9)
Stroma	155(30)

Each value is the mean(s.d.) of readings from at least five different specimens. Background fluorescence values using unsensitized pancreatic sections on plane glass slides have been subtracted

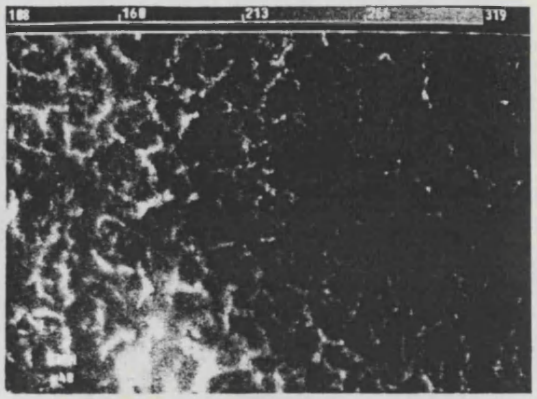

Figure 2 *Fluorescence micrograph of normal pancreas 48 h after photosensitizer administration. Fluorescence is localized to cell membranes and at the peripheries of acini. The grey fluorescence intensity scale is at the top of the image. (Actual size 200 × 120 μm)*

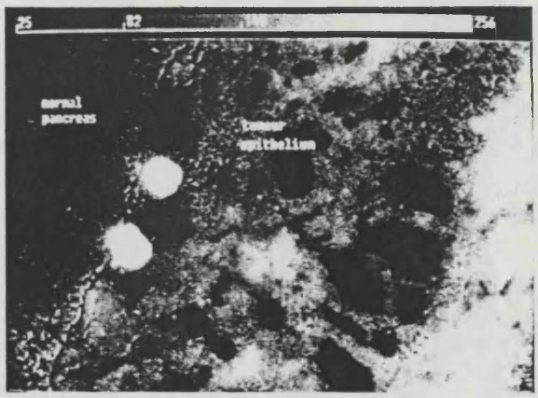

Figure 3 *Fluorescence micrograph of frozen section of pancreatic tumour 48 h after photosensitizer administration. Maximum fluorescence is noted in tumour stroma (bottom right). Tumour epithelium fluorescence is higher than that of normal acinar tissue. (Actual size 800 × 550 μm)*

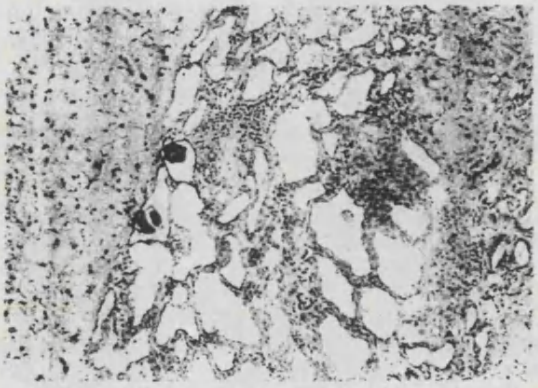

Figure 4 *Micrograph of frozen section used in* Figure 3 *after haematoxylin and eosin staining. This permits visual comparison of the fluorescence image. (Actual size 800 × 550 μm)*

treated with up to 100 J, suggesting no PDT damage but slight thermal damage, with oedema and vascular stasis, at the site of irradiation (<2 mm in diameter). With 200 J (*Figure 6*), there was evidence of haemorrhagic coagulative necrosis on light microscopy in sensitized animals, indicative of photodynamic damage. This was different from thermal controls in which, although necrosis was present, there was no evidence of haemorrhage and the inflammatory cell infiltrate was less marked. Adjacent viable pancreas (*Figure 6*) exhibited some damage with a moderate inflammatory cell infiltrate, although this had not progressed to coagulative necrosis. With light doses of 50 J and above, three of nine sensitized animals demonstrated gastric ulcers in areas adjacent to treated pancreas. All sensitized tumours, even those treated with only 12·5 J, demonstrated macroscopic evidence of coagulative haemorrhagic necrosis after PDT, which was confirmed on microscopy (*Figure 7*), although no damage was evident in adjacent normal pancreas. Tumour necrosis was noted to be <5 mm in diameter with 12·5 J, and a viable rim of surviving tumour in areas furthest from the site of light application was seen. When 25 or 50 J light doses were used, damage appeared to extend to the edges of tumour nodules 5 mm in diameter ($n = 2$), and up to 8 mm in larger tumours. No damage was seen in adjacent normal pancreas (*Figure 7*) or stomach in any of these tumour-bearing animals at the light doses used (maximum 50 J).

Table 2 *Results of photodynamic therapy of hamster pancreas at 48 h after photosensitizer administration*

Light dose (J)	Normal	Tumour
12·5	—	Haemorrhagic coagulative necrosis
25	—	Haemorrhagic coagulative necrosis
50	No effect	Haemorrhagic coagulative necrosis
100	No effect	—
200	Damage	—

Necrosis was assessed histologically from tissues taken at necropsy 72 h after light administration. Normal pancreas demonstrated damage only after light doses of 200 J, whereas tumour necrosis was seen after even as little as 12·5 J

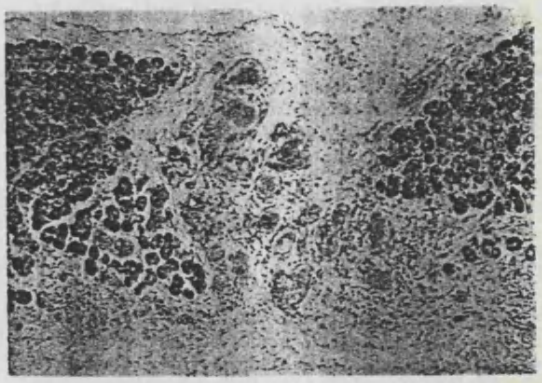

Figure 5 *Micrograph of normal pancreas 72 h after photodynamic therapy using a light dose of 100 J. There is oedema and vascular stasis suggestive of thermal damage. (Actual size 800 × 550 μm)*

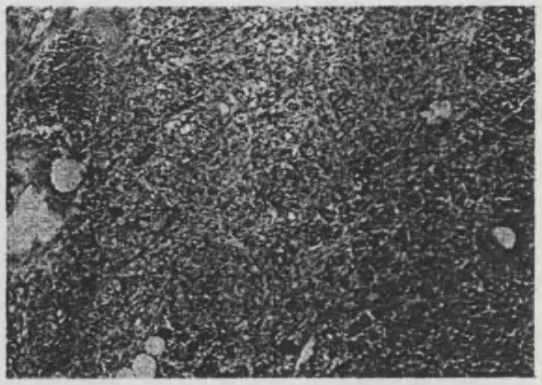

Figure 6 *Micrograph of normal pancreas 72 h after photodynamic therapy using a light dose of 200 J. There is a relatively well defined area of haemorrhage and coagulative necrosis suggestive of photodynamic damage (upper left). Adjacent pancreas exhibits some damage with a moderate inflammatory cell infiltrate, but damage has not progressed to coagulative necrosis. (Actual size 800 × 550 μm)*

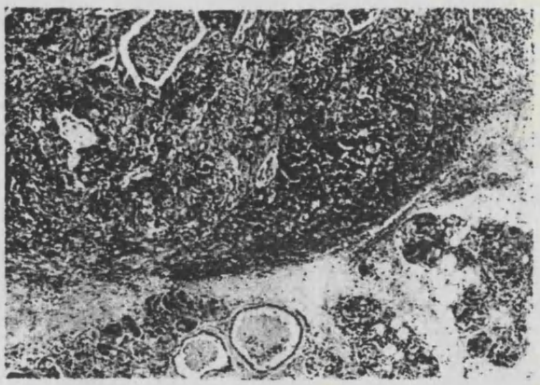

Figure 7 *Micrograph of edge of tumour nodule 72 h after photodynamic therapy using a light dose of 25 J. Coagulative necrosis and haemorrhage is seen in the tumour, whereas adjacent normal pancreas (bottom right) is unaffected. (Actual size 800 × 550 μm)*

On the basis of the above, the minimum light dose required to produce damage to normal pancreas at 48 h was 200 J. The threshold photodynamic dose required to produce damage was thus 200 × 1·7 (light dose × mean concentration of AlSPc at 48 h), that is 340 arbitrary units. By a similar method, for pancreatic tumour, the threshold dose was approximately 12·5 × 3·9, that is 49 arbitrary units. Thus the threshold photodynamic dose for damage to normal pancreas was about seven times as high as for damage to pancreatic tumour.

Discussion

Selective destruction of tumours within their organ of origin without damage to the normal tissue in which the tumour arose is the aim of all local treatment of cancer. The selectivity shown in transplanted brain tumours in mice[2] and dimethylhydrazine-induced colonic tumours in the rat[3] relies on transient differences in photosensitizer concentration, related to preferential retention of photosensitizer in tumour tissue. In brain, the tumour:normal tissue ratio of photosensitizer concentration (as high as 28:1) is probably caused by an inability of the photosensitizer to cross the intact blood–brain barrier in normal areas of brain, whereas this barrier is breached in tumour areas. With the colon, the maximum tumour:normal tissue ratio of 2:1 can be exploited to give selective necrosis only by keeping the tissue concentrations of photosensitizer very close to the photobleaching threshold. In these experiments, Barr *et al.*[3] demonstrated that the threshold photodynamic doses were similar for normal and neoplastic colon, and the large difference in the present report between normal and neoplastic pancreas was not seen. This is good evidence that a different mechanism of selectivity is occurring in the pancreas. True selectivity is confirmed by the histological studies showing haemorrhagic necrosis in tumour areas extending to the junction of normal and neoplastic pancreas but with no damage to immediately adjacent normal pancreas (*Figure 7*).

Schroder *et al.*[6] studied PDT of BOP-induced pancreatic cancers in Syrian golden hamsters. They used the porphyrin DHE for photosensitization, and light application was carried out 3 h after photosensitizer administration, when the tumour DHE concentration was highest. At this time, all adjacent organs also contained high levels of DHE and these were also exposed to the light. PDT was carried out on seven hamsters, four of which died during the first 4 days, three with intestinal perforations. Histology of the pancreatic tumours revealed carbon debris, indicating hyperthermia, and areas suggestive of photodynamic damage with haemorrhage and necrosis. Animals that survived 1 week demonstrated damage localized to pancreatic tumour tissue with unaffected areas of normal pancreas. Although in the present study gastric lesions were seen using higher light doses ($\geqslant 50$ J) in sensitized normal animals, no perforation occurred. The absence of gastric lesions after PDT of pancreatic tumours probably relates to selectivity of damage by application of light principally to the tumour surface and the smaller light doses ($\leqslant 50$ J) used for tumour irradiation. The light dose delivered to the stomach during irradiation is higher when normal pancreas is treated than during tumour light exposure, as tumour bulk shields the stomach, with light flux falling off towards the deep surface of the tumour.

There are several possible explanations for the enhanced selectivity of PDT seen in the pancreas. Mang *et al.*[13] studied photobleaching kinetics of DHE in normal and neoplastic pancreas. They found that photobleaching in tumours was three times higher than in normal pancreas. Singlet oxygen has been shown to be an active intermediary in the necrosis produced by PDT[14]. Mang and colleagues postulated that there may be a singlet oxygen scavenger in normal pancreas that prevented the singlet oxygen produced during PDT from attacking the photosensitizer molecule. If singlet oxygen is involved in photobleaching, the same singlet oxygen scavenging mechanism that protects the photosensitizer from degradation may also protect normal pancreas from damage by PDT. Possible agents for this scavenging role could be glutathione or other intracellular thiols[15,16] although, in a small preliminary study, we were unable to demonstrate a fall in acid-soluble sulphydryl content[17] of normal pancreas after PDT (data not presented here). This does not, however, exclude intracellular thiols as protection, because replenishment may occur.

Matthews and Cui[18] have suggested that biochemical differences exist between normal pancreatic acinar cells and an exocrine pancreatic carcinoma cell line (AR4-2J) in responses to PDT using AlSPc photosensitization. Photodynamic action inhibited amylase secretion by AR4-2J cells, which contrasted with stimulation of amylase release by normal acinar cells. They suggested that membrane proteins or lipids are inactivated by PDT, leading to inhibition of amylase secretion. Another study[19] (which partly overlapped with the aforementioned one) demonstrated that, although amylase release from isolated pancreatic acini was increased, this was not caused by structural damage, as no changes could be demonstrated on transmission electron microscopy. It was postulated[8] that the negatively charged AlSPc (with a mean of 3·2 sulphonate groups per phthalocyanine molecule) localized to the plasma membrane of intact acini, and little was taken up intracellularly, presumably effectively reducing the photodynamic dose delivered to cells in intact acini.

The present fluorescence microscopy studies on normal pancreas confirmed the localization of AlSPc around the edges of acini and on cell membranes but, in addition, there was localization in duct walls. In pancreatic tumours the photosensitizer was localized mainly to stroma, which accords with work carried out on other tumour models using autoradiography[20] and fluorescence microscopy[9]. Microscopic localizations of AlSPc in normal and neoplastic tissues are related, as the polar photosensitizer molecules tend to accumulate in interstitial spaces, which in tumour tissue are particularly associated with stroma[21,22], whereas in normal pancreas transfer across cell and basement membranes is restricted by polarity. The net result of PDT with such photosensitizer molecule distribution is delivery of high photodynamic doses to tumour stroma with light exposure.

Although pharmacokinetic differences exist between tumours and the normal tissues in which they arise, leading to some degree of selective retention of photosensitizers in tumour, it is generally difficult to translate this differential retention into selective necrosis. However, this study has demonstrated greater selectivity of necrosis in BOP-induced pancreatic tumours compared with normal pancreatic tissue than is possible by photosensitizer retention alone. PDT may thus have a role in the destruction of small or strategically placed cancers or as adjuvant therapy when the bulk of tumour has been removed

by other means such as surgery. However, work is required on the effects of PDT on organs closely related to the pancreas, particularly the bile duct, stomach and duodenum, before PDT can be deemed safe to be used in the management of pancreatic carcinoma.

Acknowledgements

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Distribution and photodynamic effects of di- and tetra-sulphonated aluminium phthalocyanines (AlS_2Pc and AlS_4Pc) in normal and neoplastic rat colon

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Introduction

There have been few studies looking at photodynamic therapy (PDT) of tumours and normal tissues from which they arise. Chemically induced tumour systems offer the potential of synchronous comparison of pharmacokinetics and treatment effects in normal and tumour tissue if the model chosen is appropriate.

AlSPc is a mixture of compounds with 1-4 sulphonate groups. Recently individual components have become available [1]. This study compares the distribution and photodynamic effects of AlS_2Pc and AlS_4Pc on normal rat colon and colonic tumours in dimethylhydrazine (DMH) treated rats.

Materials and methods

Colon tumour model

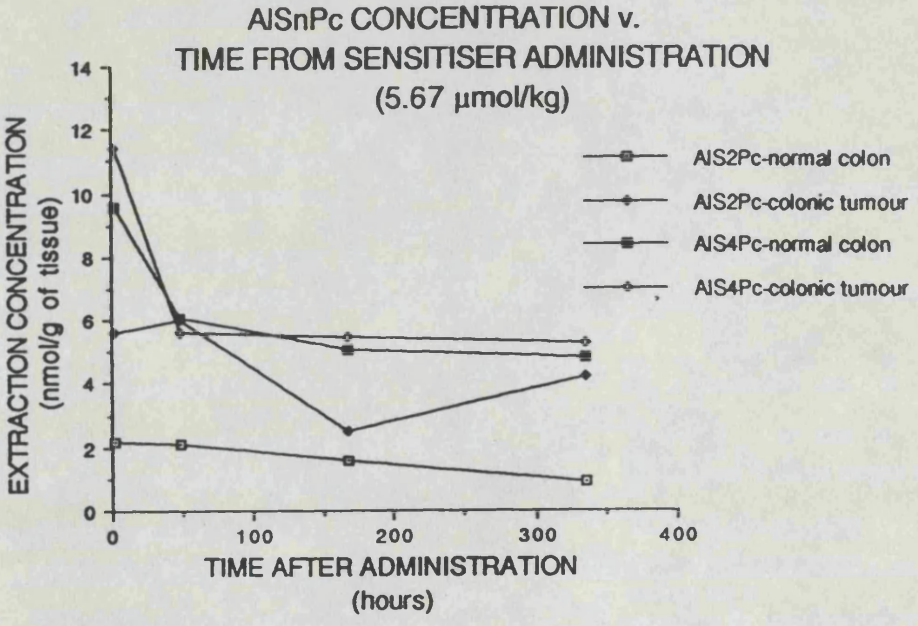
DMH was used to induce colonic tumours in weanling Wistar rats[2]. Tumours developed at least 6 months after a course of 5 weekly subcutaneous injections of $5mg.kg^{-1}$ of DMH. Colonic tumours were diagnosed by examination of faeces for blood or mucus, monitoring for abdominal masses by palpation and regular weighing and confirmed by laparotomy.

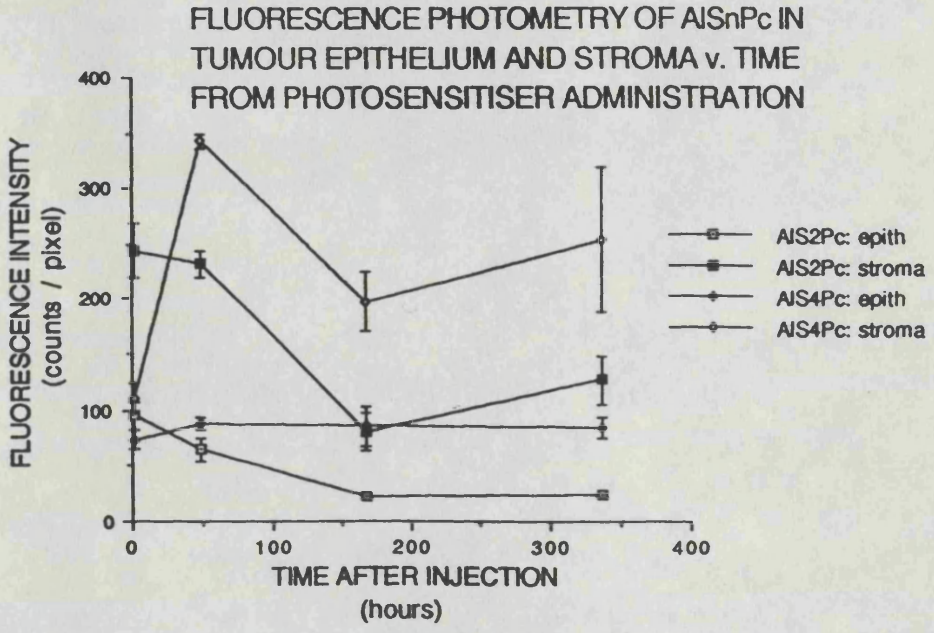
Distribution of photosensitisers in normal colon and colonic tumours by extraction

Tumour bearing animals were used to study the distribution of AlS_2Pc and AlS_4Pc in tumour and normal colon in the same animal. Animals were injected (tail vein) with $5.65\ \mu mol.kg^{-1}$ and specimens obtained at necropsy at 1h, 48h, 168h or 336h after photosensitiser administration, by tail vein injection. Tumours were bisected and half kept for fluorescence microscopy and half for extraction studies. Segments of adjacent normal colon were also kept. Tissues for extraction studies were kept at -4°C prior to chemical extraction with 0.1M NaOH [3].

Distribution of photosensitisers in colonic tumours by fluorescence microscopy

Tumour tissues for fluorescence microscopy obtained as described above were trimmed to obtain a suitable "face", and frozen in iso-pentane using


AlSnPc CONCENTRATION v.
TIME FROM SENSITISER ADMINISTRATION
(5.67 µmol/kg)
EXTRACTION CONCENTRATION
(nmol/g of tissue)
TIME AFTER ADMINISTRATION
(hours)
AlS2Pc-normal colon
AlS2Pc-colonic tumour
AlS4Pc-normal colon
AlS4Pc-colonic tumour
0
2
4
6
8
10
12
14
0
100
200
300
400


FLUORESCENCE PHOTOMETRY OF AlSnPc IN
TUMOUR EPITHELIUM AND STROMA v. TIME
FROM PHOTOSENSITISER ADMINISTRATION
FLUORESCENCE INTENSITY
(counts / pixel)
TIME AFTER INJECTION
(hours)
AlS2Pc: epith
AlS2Pc: stroma
AlS4Pc: epith
AlS4Pc: stroma
0
100
200
300
400

liquid nitrogen. These specimens were stored in liquid nitrozen until 10μm frozen sections were cut for fluorescence microscopy.

Fluorescence microscopy [4,5] was carried out using HeNe excitation of the frozen section on the stage of an inverted microscope and a charge-coupled device camera used to capture the fluorescence image. Camera operation and image processing were carried out by computer. The combination of light microscopy of stained sections obtained adjacent to the frozen sections together with phase contrast microscopy of the frozen sections at the time of fluorescence microscopy enabled tumour stroma and epithelium to be identified on the fluorescence image and fluorescence intensity of these structures measured at 1h, 48h, 168h and 336h after administration of 5.65 $\mu mol.kg^{-1}$ AlS_2Pc or AlS_4Pc.

Photodynamic therapy of normal and neoplastic colon

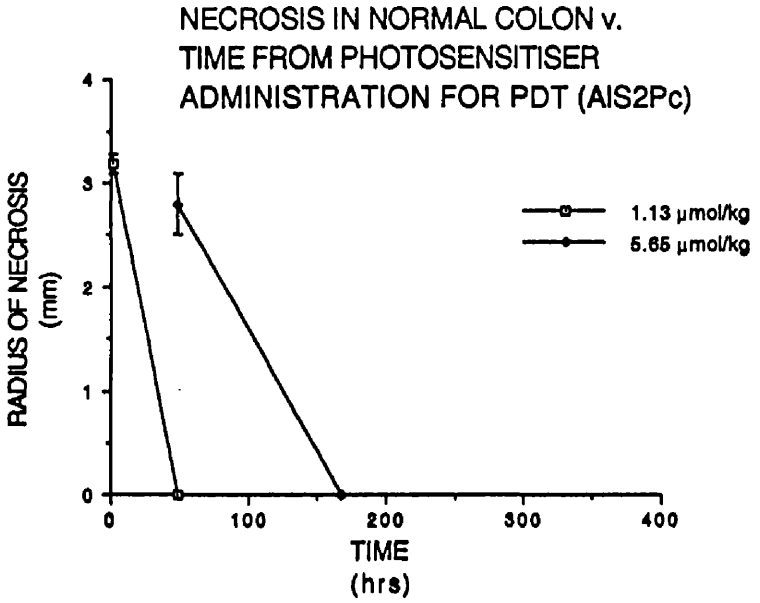
Normal colon and colonic tumour were irradiated at laparotomy in each animal at 1h, 48h, 1 week or 2 weeks after administration of 1.13 or 5.65 μmol.kg-1 of of AlS_2Pc or AlS_4Pc. Irradiation was carried out using 675nm light from a Cu vapour pumped dye laser producing 50mW at the tip of a 200μm fibre. The light dose at each treatment site was 50J. For normal colon, the fibre was threaded into the colon and positioned so that the tip just touched the mucosa on the antimesenteric side, not less than 4cm away from the tumour. After irradiation, the entry site of the fibre into the colonic lumen was closed by a marker suture. For colonic tumour irradiation, the fibre tip was just inserted into the apex of the tumour at colotomy. Tumour dimensions were measured prior to irradiation and the colotomy closed with interrupted sutures.

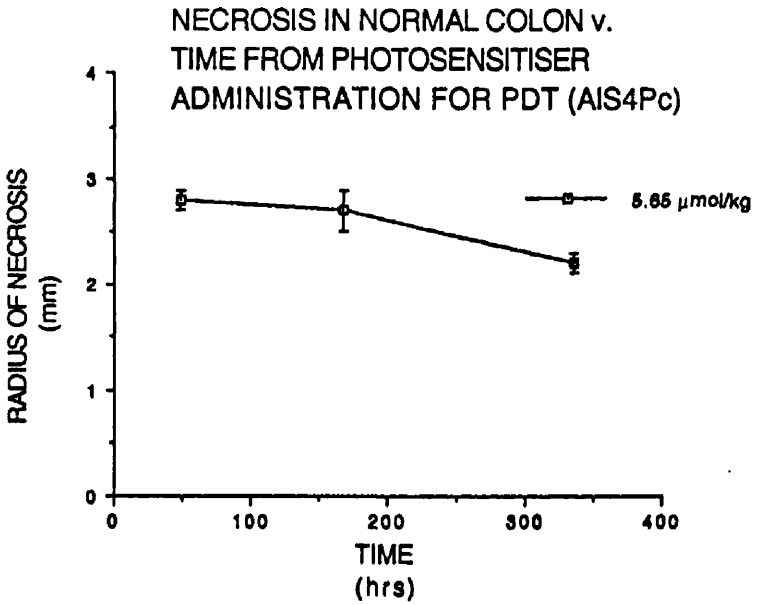
The radius of necrosis, if present, in normal colon was taken as half of the longest axis of the well-defined sloughly lesion noted at post mortem 72h after light exposure. Tumour damage was represented by a well defined cap of necrosis which occasionally had separated to leave an ulcer. The extent of tumour necrosis was taken as the change in the tumour dimensions along the longest light path [6].

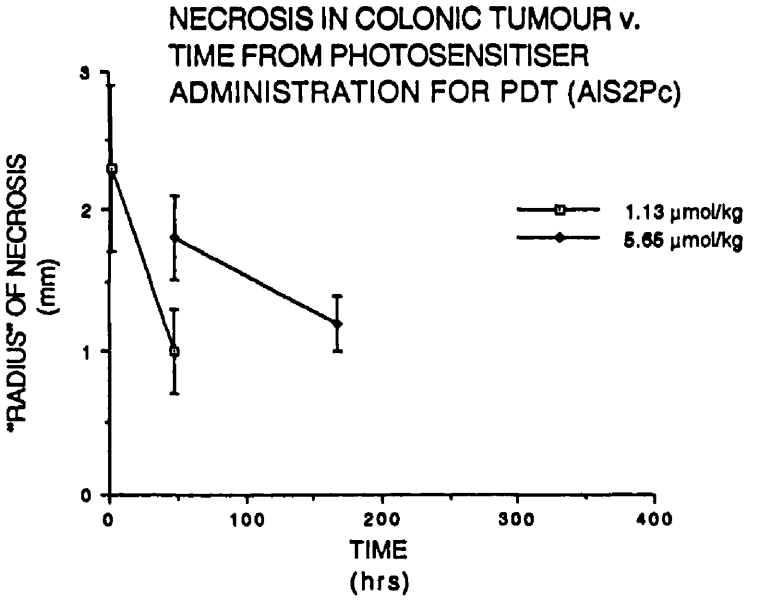
Results

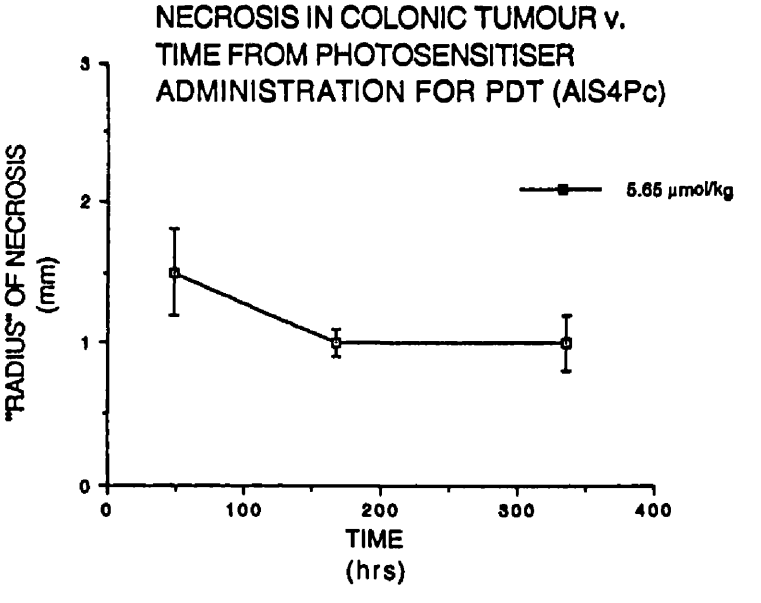
Distribution of photosensitisers in normal colon and colonic tumours by extraction

After photosensitiser administration, normal colon and tumour concentrations of AlS_4Pc were similar. In contrast, tumours tended to retain AlS_2Pc to a greater extent than normal colon. The extraction results are shown in the figure below.


NECROSIS IN NORMAL COLON v.
TIME FROM PHOTOSENSITISER
ADMINISTRATION FOR PDT (AIS2Pc)
RADIUS OF NECROSIS
(mm)
1.13 µmol/kg
5.65 µmol/kg
TIME
(hrs)


NECROSIS IN NORMAL COLON v.
TIME FROM PHOTOSENSITISER
ADMINISTRATION FOR PDT (AIS4Pc)
RADIUS OF NECROSIS
(mm)
5.65 µmol/kg
TIME
(hrs)


NECROSIS IN COLONIC TUMOUR v.
TIME FROM PHOTOSENSITISER
ADMINISTRATION FOR PDT (AIS2Pc)
"RADIUS" OF NECROSIS
(mm)
1.13 µmol/kg
5.65 µmol/kg
TIME
(hrs)


NECROSIS IN COLONIC TUMOUR v.
TIME FROM PHOTOSENSITISER
ADMINISTRATION FOR PDT (AIS4Pc)
"RADIUS" OF NECROSIS
(mm)
5.65 µmol/kg
TIME
(hrs)

Distribution of photosensitisers in colonic tumours by fluorescence microscopy

Fluorescence kinetics in colonic tumours following photosensitiser administration are shown below. For each tumour, 2 to 5 matching values were obtained for fluorescence intensity of stroma and epithelium within rectangles drawn over these structures on the fluorescence image. Background values of unsensitised tumour sections were subtracted and the mean fluorescence intensity (±s.e.) of these structures after photosensitiser administration calculated using at least three tumours at each time.

AlS_2Pc exhibited greater fluorescence in stroma than epithelium and fluorescence intensity in both these structures was greatest 1h after sensitiser administration and fell thereafter. With AlS_4Pc, fluorescence was least at 1h after injection, maximum at 48h and decreased slowly thereafter, but nevertheless remaining relatively high in tumour stroma during the time period studied.

Photodynamic therapy of normal and neoplastic colon

Necrosis produced by photodynamic therapy is shown below. The light dose was the same at all these sites and it is important to note that each value for necrosis in normal colon has a paired value for necrosis in tumour, although these are not directly comparable as irradiation geometry and measurement of necrosis are different for these two types of tissue. With 1.13 $\mu mol.kg^{-1}$ of AlS_4Pc, no PDT necrosis could be produced either in normal colon or in tumour. With 1.13$\mu mol.kg^{-1}$ of AlS_2Pc, necrosis was produced in tumour following light exposure at 1h and 48h, whereas normal colon damage was only damaged with light exposure at 1h. A similar pattern was noted following 5.65 $\mu mol.kg^{-1}$ of AlS_2Pc, with which necrosis was produced with light exposure in tumour at 48h and 1 week, but normal colon damage only with light exposure at 48h after photosensitiser administration. With AlS_4Pc, necrosis was produced with light exposure at 48h, 1 week and 2 weeks after photosensitiser administrationin both normal colon and tumour. Tumour necrosis was greater using AlS_2Pc than AlS_4Pc.

Discussion and conclusions

AlS_2Pc and AlS_4Pc exhibit different pharmacokinetics in colonic tumour and normal colon in rats bearing DMH induced tumours. Differences are noted in fluorescence kinetics of these photosensitisers in tumours, with a tendency for AlS_4Pc (and to a lesser extent AlS_2Pc) to be retained in tumour stroma. There are quantitative differences in necrosis following PDT suggesting AlS_2Pc is the more potent photosensitiser, when similar light doses are used.

Following AlS_4Pc administration, tumour and normal colon photosensitiser concentrations are similar and at no time could selective necrosis be produced. AlS_4Pc pharmacokinetics in tumour and normal colon probably reflect retention in tumour stroma paralleling retention in normal colon submucosa[5].

With AlS_2Pc photosensitisation of normal colon persists for a shorter time than in colonic tumours and for a much shorter time than when AlS_4Pc is used. This permits selective tumour necrosis with PDT as a tumour:normal photosensitiser concentration exists when AlS_2Pc is used as a photosensitiser. The magnitude of selective tumour necrosis obtained under these circumstances is small and likely to permit significant therapeutic advantage only in exceptional circumstances.

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THE KINETICS OF 5-AMINOLAEVULINIC ACID PHOTOSENSITISATION IN THE RAT STOMACH.

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INTRODUCTION

Epithelial dysplasia, a premalignant condition, is confined only to the mucosa and photodynamic therapy (PDT) may be a useful treatment modality for this condition. Due to its diffuse nature, treatment would necessarily involve destruction of large areas of mucosa. It is thus essential to limit the photodynamic effect to the mucosa to minimise complication and ensure prompt and safe healing. Photodynamic effect only occurs when the product of the local light dose and photosensitiser concentration exceeds a threshold [1]. In addition, because of photodegradation of photosensitiser by the activating light, concentration of photosensitiser has to exceed a particular level irrespective of light dose before photodynamic action can take place [2]. When a sufficient differential photosensitiser distribution exist between mucosa and underlying structure, it is possible to produce a predominant photodynamic effect in the mucosa by keeping the concentration of photosensitiser in the mucosa above this threshold while that in the underlying tissue below it. This approach has been adopted experimentally in the bladder using aluminium sulphonated phthalocyanine, and the resultant photodynamic effect caused minimal disruption of both the anatomical and functional integrity of the bladder while still achieving the desired objective of complete mucosal ablation [3].

ALA is a natural porphyrin precursor and exogenous administration can lead to accumulation of photoactive porphyrin intermediates, particularly protoporphyrin IX (PPIX), in sufficient quantity for photodynamic effect [4-5]. Although in theory all cells capable of haem synthesis are liable to become photosensitised, Divaris et al. has found that following administration of exogenous ALA to mice, there was a marked difference in the levels of PPIX accumulation in the various tissue structures in the skin as studied by fluorescence microscopy. The epidermal cells and cells of the pilosebaceous apparatus were markedly fluorescent as compared to the dermis [5]. They have also reported that in the bladder and uterus, ALA administration resulted in preferential photosensitisation of the mucosa and endometrium over the other underlying structures of the respective organs. These findings prompted us to investigate ALA as a possible photosensitiser for photodynamic ablation of gastric mucosa.

MATERIALS

ALA was obtained as a hydrochloride (formula weight = 167.6) in 98% pure powder form from Sigma Chemical Company Limited (Poole, UK). It was dissolved in phosphate buffered saline to the desired concentration for intravenous injection via the tail vein. All studies were performed on 4 to 8 weeks old female Wistar rats ranging from 100 g to 200 g in weight. Injections of photosensitisers were carried out under intramuscular Hypnorm (fentanyl and fluanisone) anaesthesia. Photodynamic therapy was carried out during laparotomy under intramuscular Hypnorm and diazepam anaesthesia.

METHODS

Fluorescence distribution of PPIX

This was studied by means of fluorescence microscopy and photometry. Animals were killed at a range of times up to 8 hours after administration of ALA. A small disc of stomach wall was excised from the glandular part and immediately frozen. 10 μm frozen sections were prepared from the specimens for fluorescence microscopy. An inverted microscope (Olympus IMT-2) with epifluorescence and phase-contrast attachments was used as described previously [6]. Fluorescence excitation came from an 8 mW HeNe laser (632.8 nm). The beam was directed through a 10 nm band-pass filter centred at 633 nm to remove extraneous light onto the dichroic mirror (Omega Optical Inc.) for epifluorescence study. Fluorescence was detected between 665 and 700 nm using a combination of band-pass (Omega Optical Inc.) and long-pass (Schott RG665) filters. Fluorescence signal was detected by a highly sensitive cryogenically cooled CCD (charge-coupled device) camera (Wright Instruments, model 1, resolution 400 x 600 pixels) fitted to the microscope and processed by a personal computer into a falsely colour-coded microscopic image. Fluorescence, measured arbitrarily as counts per pixel, was corrected for tissue autofluorescence. After fluorescence microscopy, specimens were fixed in formalin and stained with H & E for comparative light microscopy. As the conversion of ALA to the photoactive PPIX is dose dependent, a range of doses (20 mg/kg , 100 mg/kg and 200 mg/kg) were investigated.

Photodynamic therapy

Light source came from a pulsed (12 kHz) copper vapour pumped dye laser (Oxford Lasers) tuned to 630 nm and delivered via a 200 μm fibre threaded through the forestomach and held just touching the mucosa of the glandular stomach. The fibre was maintained at approximately 90° to the mucosal surface. The rest of the abdominal viscera were shielded from forward light scatter. Only one point was treated in each animal. Each animal was irradiated at 50 mW for 1000 s. Three sub-groups of animals were treated. In the first, all animals were given 200 mg/kg of ALA and exposed to laser light at a range of time from 30 mins to 8 hours. In the second, animals were sensitised with different doses of ALA (1 mg/kg, 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 100 mg/kg, 200 mg/kg, and 400 mg/kg) and then exposed to laser light at the time of peak photosensitisation for the respective doses as determined from fluorescence photometry. Control unsensitised animals were irradiated

using similar parameters to exclude thermal effects. Treated areas were marked with sutures placed 1 cm proximal and distal to the point of contact. Animals were then allowed to recover and kept in standard laboratory conditions until sacrificed at 72 hours. On killing the animal, the stomach was immediately excised and opened along the lesser curve for macroscopic inspection. The specimens were laid out on a piece of card and the size of the PDT induced lesions were determined by taking the mean of the longest diameter and the broadest diameter of the lesion [7]. The specimen was then fixed in formalin , sectioned and stained with H & E and haematoxylin van Gieson stains for conventional light microscopy study. In the last subgroup, a range of doses were employed (20 mg/kg, 100 mg/kg and 200 mg/kg) but animals were only sacrificed at 2 weeks, 3 weeks, 4 weeks and 6 weeks after PDT to study healing.

RESULTS

Fluorescence photometry

With 200 mg/kg of ALA, the fluorescence signal in the mucosa layer rose rapidly to a peak at 3 hours while signal over the other layers rose much less (Figure 1). Peak fluorescence appeared to be achieved earlier with lower doses of ALA (Figure 2). The levels of maximum fluorescence achieved increased non linearly with increasing dose of ALA

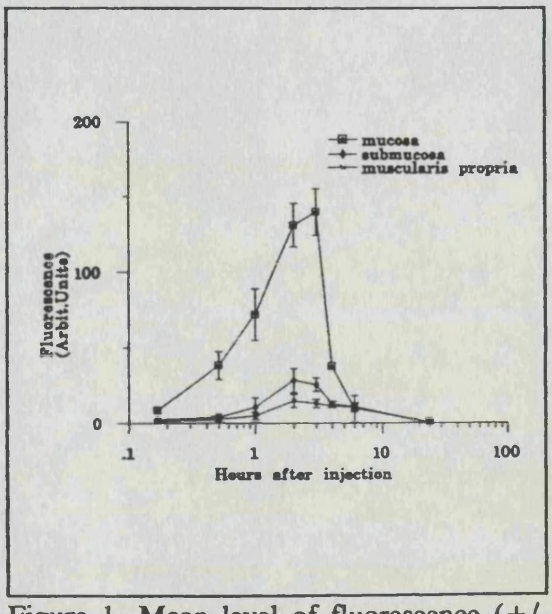

Figure 1. Mean level of fluorescence (+/- S.D.) of the stomach after i.v. ALA (200 mg/kg).

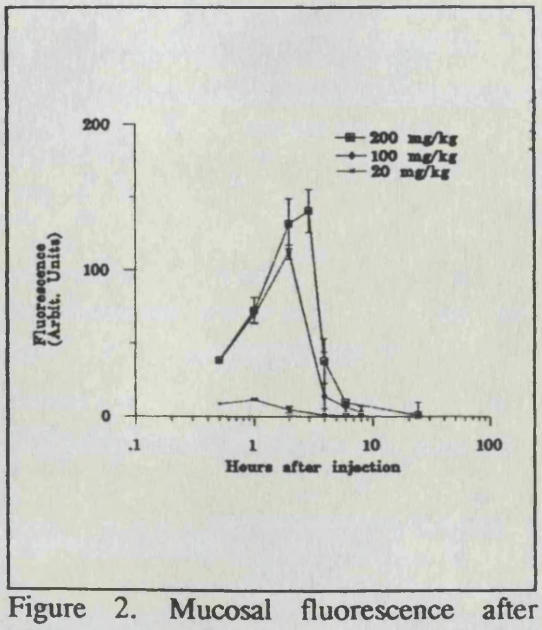

Figure 2. Mucosal fluorescence after different doses of ALA as a function of time.

administered. Fluorescence declined very rapidly and almost reached background level by 6 to 8 hours. The resultant PPIX fluorescence was predominantly over the mucosa with very

little fluorescence seen over the submucosa and muscularis propria. The ratio of fluorescence level between mucosa and muscle varied with time (Figure 3). With all doses, this ratio rose to a peak in excess of 14 one hour after administration. However, with 20 mg/kg of ALA, this ratio fell back rapidly to almost unity at 2 hours. When higher doses were given, the high fluorescence ratio was sustained over a longer duration so that with the dose of 200 mg/kg, it did not reach unity until 8 hours after administration. ALA induced PPIX fluorescence appears intracellular in location and was mainly perinuclear in distribution.

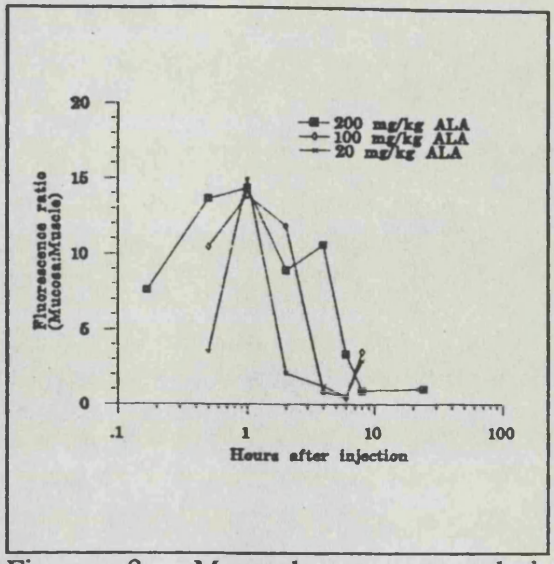

Figure 3. Mucosal vs. muscularis fluorescence ratio as a function of time after different doses of ALA.

Photodynamic therapy

No macroscopic lesion was seen in the control groups. In the first subgroup of animals, apart from a lesser extent of damage produced when light exposure occurred at ½ hour after administration, the extent of damage appeared to plateau off subsequently when light exposure occurred between 1 and 6 hours after administration (Figure 4). In the second subgroup, no PDT damage was evident when less than 15 mg/kg of ALA was used. The size of the lesion produced was larger with 20 mg/kg but remained relatively constant despite a 20 times increase in the dose of ALA given from 20 mg/kg to 400 mg/kg (Figure 5).

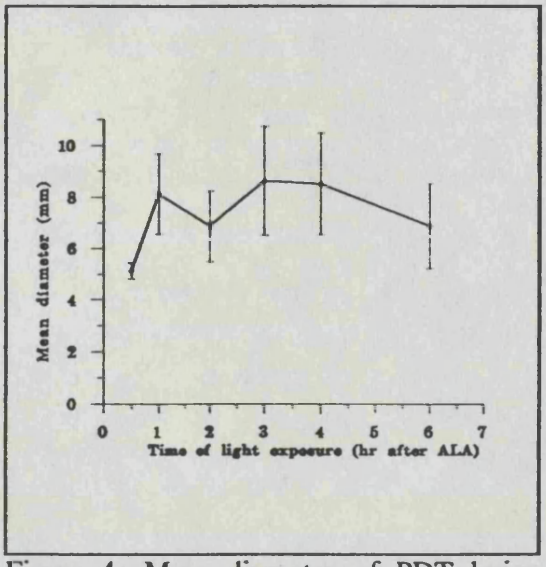

Figure 4. Mean diameter of PDT lesion produced as a function of time after 200 mg/kg ALA.

Histologically, necrosis of all layers were seen after 100 and 200 mg/kg of ALA following light exposure at times of peak fluorescence. A degree of muscularis preservation was seen if light exposure occurred at 30 mins after ALA. With the dose of 20 mg/kg however, necrosis was consistently confined only to the mucosa. Following panmural necrosis, healing was marked by submucosal and serosal fibrosis. When necrosis was only confined to the mucosa, healing was quicker and involved no fibrosis. In all cases, the regenerated mucosa initially bears only mucus secreting cells but after about 4 weeks, acid secreting oxyntic cells return.

DISCUSSION

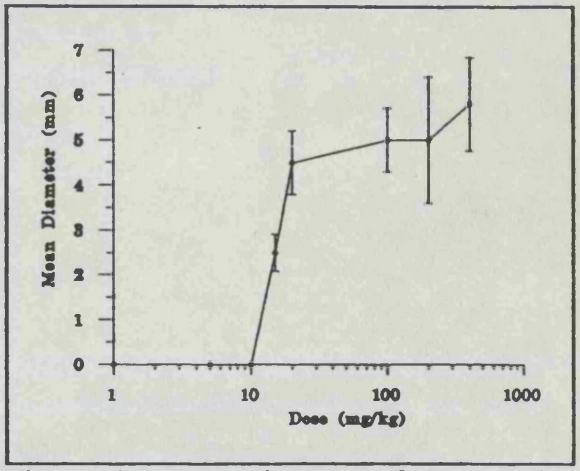

Figure 5. Mean diameter of PDT lesion produced with different doses of ALA.

Dysplasia in the alimentary tract is a difficult clinical problem. Although its malignant potential is well known, many clinicians are reluctant to advice excisional surgery in the absence of invasive malignant change. PDT may be an effective but less invasive treatment for this condition. The ideal goal would be the selective destruction of only the dysplastic mucosa. Barr *et al.* [7] had shown that truly selective tumour necrosis (i.e. necrosis of tumour tissue but not the adjacent normal tissue from which the tumour arise) could be produced by judicious manipulation of treatment parameters in such a way that the photosensitiser concentration in the normal tissue fell below the photodynamic threshold while that in the tumour tissue remained above it. The volume of tumour necrosis produced was however very small. This was due to the small therapeutic ratio of conventional photosensitiser (2:1) between tumour and adjacent normal tissue [8]. In addition, this true selectivity could only be applied to part of the tumours treated, and did not apply to the region where the tumour was invading normal tissue [9]. Although some workers had shown qualitiatively that haematoporphyrin derivative can localised in benign tumours [10-11], carcinoma in situ [12] and severe dysplasia [13], quantitatively this differential is not likely to exceed that seen between established tumour and its normal tissue. Clearly, without a substantial therapeutic ratio between dysplastic and normal tissue, true selective ablation of dysplastic mucosa would be impractical clinically. Alternatively, selectivity can be between mucosa and muscle rather than between dysplastic mucosa and normal mucosa, as long as safe healing of large areas of mucosal defect with healthy epithelium can be ensured. ALA appears to provide a substantial therapeutic ratio between mucosa and muscularis which we are able to exploit for producing selective mucosal necrosis. However, as this ratio changes with time and dose of ALA used, accurate determination of treatment parameters is important. It would seem likely that the concentration of PPIX achieved in the muscularis after 200 mg/kg of ALA is below photodynamic threshold in the first 30 minutes but exceeds it thereafter while with 20 mg/kg, PPIX concentration in the muscularis never rise above photodynamic threshold. We have demonstrated that healing after selective mucosal ablation involves little or no fibrosis. Although we only produce focal lesions, larger areas of mucosal ablation should be feasible with appropriate light delivery.

This study shows that the mucosa has the largest capacity for porphyrin synthesis following exogenous ALA. Although the precise mechanism for this remains unclear, it is notable that the mucosa has the highest rate of turnover and is probably most metabolically active. By inference, neoplastic tissue may proved to have an even higher capacity in this respect. Indeed, a higher porphyrin biosynthetic capacity has been reported in human breast tumour tissue [14]. Our group has also shown that significantly more PPIX is synthesised in the neoplastic colonic mucosa than in normal mucosa of rats treated with dimethylhydrazine

[15]. It remains to be seen if ALA offers an adequate therapeutic ratio between dysplastic and normal mucosa to be exploited for truly selective ablation of dysplastic mucosa.

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Comparison of the distribution of phthalocyanine and ALA-induced porphyrin sensitisers within the rabbit uterus.

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INTRODUCTION

Excessive menstrual bleeding is found in approximately 20% of women during their reproductive years [1] and hysterectomy still remains the commonest surgical procedure for women whose menorrhagia has failed to respond to medical treatment. Recently techniques for local endometrial destruction have become more widely used and include the use of the resectoscope [2], laser ablation [3] and radiofrequency induced ablation [4]. These techniques are successful but are not without their problems [5] and include fluid overload, haemorrhage, uterine perforation and damage to extra-uterine organs.

Photodynamic therapy (PDT) involves the pre-treatment of target tissues with photosensitising agents which enable light within the visible or infrared region up to about 1000nm to produce a severe cytotoxic effect. It has been observed that certain tissues, particularly tumours, selectively retain the photosensitiser while adjacent tissue areas contain very little, although this selectivity has often been over emphasised [6]. Light at a specific wavelength corresponding to an absorption peak of the photosensitiser is then used to activate this drug to produce local necrosis in the required area and leave adjacent tissue undamaged.

The use of newer photosensitisers has several advantages over the haematoporphyrin derivatives on which most PDT work has been based over the last ten years, in terms of chemical purity and stability, and some like the phthalocyanines have their major absorption peak in the red part of the spectrum where there is better tissue penetration.

We have studied the distribution of two photosensitisers within the endometrium to explore the possibility that PDT might be a potentially highly selective and safe treatment for menorrhagia. In rat bladder highly selective mucosal destruction can be achieved without damage to the underlying musculature or loss of bladder function by manipulating the concentration of the photosensitiser and the timing of treatment after sensitisation. [7]. In many cases, like the bladder, there seems to be more

selectivity between different normal tissue layers than between tumour and the normal tissue from which the tumour arose.

In the human uterus, the thick myometrium would allow for adequate endometrial destruction with a large safety margin even if there was some damage to muscle. Adequate tissue penetration is required for complete endometrial destruction and the newer sensitisers have their major absorption bands at longer wavelengths (in the red and near infrared part of the spectrum) which can produce a maximum depth of necrosis of approximately 5mm.

METHOD

The distributions of aluminium disulphonated phthalocyanine (AlS_2Pc) and aminolaevulinic acid (ALA) were studied in the rabbit uterus. AlS_2Pc is a photosensitiser, whereas ALA is a natural precursor of haem and in the presence of excess ALA there is an accumulation of protoporphyrin IX (PpIX), a natural photosensitiser and the last intermediate product before haem. Normal rabbits were given either an intravenous dose of ALA 200mg per kg or AlS_2Pc 1mg per kg and then killed at intervals between 30 minutes and one week. The uterus was removed at these times and the distribution of PpIX and AlS_2Pc studied using quantitative fluorescence microscopy with a CCD (charge-coupled device) imaging system. This technique produces high quality colour images which can be digitally analysed to determine relative photosensitiser fluorescence intensities in various parts of the tissue sections [8,9]. The fluorescence levels in the endometrium, stroma and myometrium were individually assessed at each time interval.

RESULTS

Table 1
Fluorescence intensity ratios in each component of the uterine wall when using AlS_2Pc

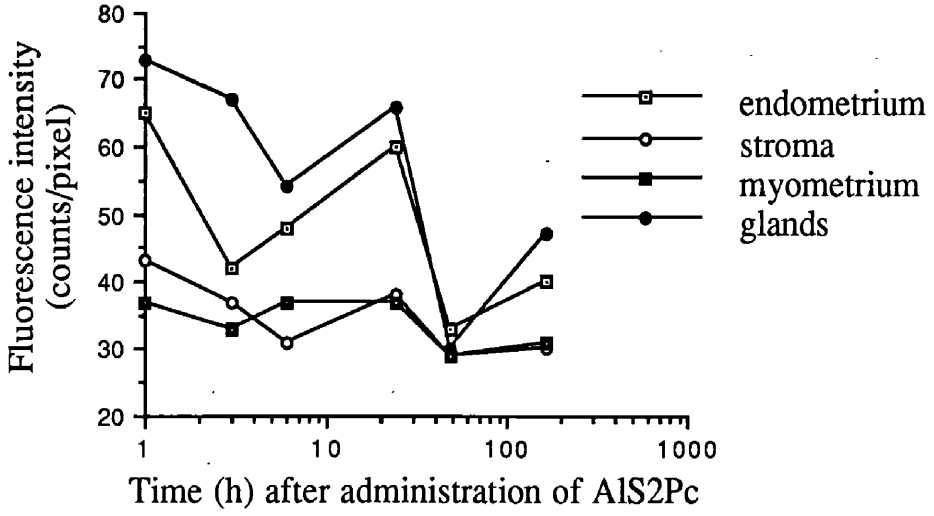
Time (h)	Endometrium	Stroma	Myometrium	Glands
1	5	2	1	6
3	4	2	1	12
6	2.5	1	1	3
24	4	1	1	1

Changes in fluorescence intensity in the uterine wall are shown in Figures 1 and 2 and Tables 1 and 2. At one hour after administration of the AlS_2Pc, the endometrium and glands showed fluorescence levels 5-6 times higher than the myometrium and approximately 3 times greater than the stroma. At 3 hours after administration of the ALA, the endometrium and glands showed fluorescence levels 9 times greater than the myometrium and stroma. By 24 hours for ALA and 48 hours for AlS_2Pc, all uterine layers had only background levels of fluorescence.

Table 2
Fluorescence intensity ratios in each component of the uterine wall when using ALA

Time (h)	Endometrium	Stroma	Myometrium	Glands
2	9	1	2	9
3	9	1	1	9
4	7	1	2	8

Figure 1. Fluorescence kinetics of the microscopic distribution of AlS_2Pc in the normal uterine wall. Each point represents the mean of separate measurements. Logarithmic scale for time.



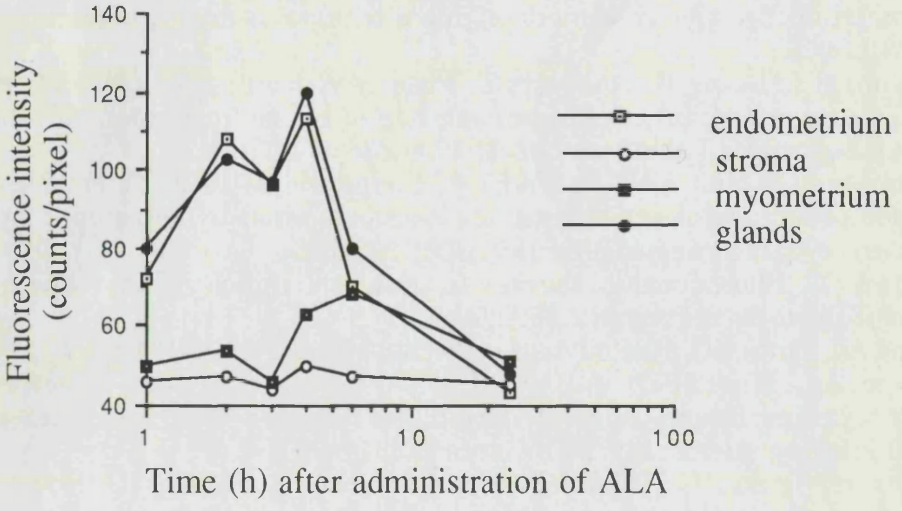
We have since applied these parameters to an animal model and produced reliable necrosis of the endometrium without significant myometrial damage and are presently studying the long term effects on endometrial regeneration. Should this be an effective means of preventing

endometrial regrowth, this technique might prove to be a highly selective treatment for women suffering from menorrhagia.

CONCLUSION

For women whose menorrhagia remains resistant to medical treatment, a surgical procedure is often the only option. Methods of local endometrial destruction are now widely used and allow women to be treated and home usually within 24 hours. However these techniques are not without their own risk and the aim is to develop techniques with minimal morbidity which could potentially be easily repeatable and performed on an outpatient basis.

Figure 2. Fluorescence kinetics of the microscopic distribution of ALA in the normal uterine wall. Each point represents the mean of separate measurements. Logarithmic scale for time.



To understand the tissue effects produced by photodynamic therapy, it is essential to know the distribution of the photosensitiser at the microscopic level. Fluorescence microscopy has high sensitivity and has made possible quantitative measurements of photosensitiser distribution.

The use of new photosensitisers for PDT has the potential to produce extremely accurate endometrial and glandular destruction without the prolonged cutaneous photosensitivity of the haematoporphyrin derivatives. This is because they are either cleared more rapidly from the skin (like

ALA), or have a localised absorption peak at a longer wavelength than the porphyrins, away from the main wavelengths in sunlight and thus produce less of a skin reaction (like the phthalocyanines) [10].

Adenomyosis is not improved by local endometrial techniques and it is exciting to consider that by manipulating photosensitiser concentrations and pretreatment intervals that glandular destruction could be maximised. From our early studies on the distribution of sensitisers, the myometrium appears to have negligible uptake while glands even when islolated from the main endometrial components show high fluorescence levels and thus a high uptake of photosensitiser. However, much careful research will be required before it is clear what role PDT may have to play in treating diseases of the uterus.

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Effect of Sulfonation on the Cell and Tissue Distribution of the Photosensitizer Aluminum Phthalocyanine

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ABSTRACT

Aluminum sulfonated phthalocyanine has potential as a suitable photosensitizer for use in the photodynamic therapy of cancer. In the present study, cellular uptake and retention of the individual mono-, di-, tri-, and tetrasulfonated derivatives ($AlS_{1-4}Pc$) were examined in tissue culture and in normal and neoplastic tissue of tumor-bearing mice. Uptake and retention of the various derivatives by cells in tissue culture correlated inversely with the degree of sulfonation. Accordingly, Colo 26 cells in monolayer culture, 24 h after addition of 10 μM of appropriate photosensitizer, had accumulated approximately 25-fold more AlS_1Pc than AlS_3Pc and retained this species longer than more sulfonated derivatives. In contrast to these *in vitro* results, it was found that Colo 26 growing s.c. in BALB/c mice accumulated photosensitizer to a greater extent when the degree of sulfonation increased, such that $AlS_4Pc > AlS_3Pc > AlS_2Pc > AlS_1Pc$. By 24–48 h after the i.v. injection of 0.1 ml 2.27 mM solution of individual photosensitizer, the relative ratios of tumor:adjacent tissue varied from >10:1 to <2:1, showing that selective tumor uptake may be affected profoundly by the composition of the phthalocyanine compound. The livers and spleens of both normal and tumor-bearing mice, unlike other normal tissue, took up the sulfonated derivatives in an order that provided a mirror image of that observed in neoplastic tissue. These complex *in vivo* distribution and retention characteristics appear to be a consequence of relative hydrophilicity/hydrophobicity properties of the sulfonated species and indicate the extent to which these characteristics may influence photosensitizer distribution and accumulation.

INTRODUCTION

Metallophthalocyanines are porphyrin-like, photoactivatable dyes with strong red light absorption characteristics, which have been considered as second generation photosensitizers for the PDT[2] of cancer (for review, see Refs. 1–4). Because most metallo-Pc are poorly soluble in water, they are not suitable for clinical administration. Problems of insolubility can be overcome by the use of specific carrier vehicles, such as entrapment of monomeric form Pc in unilamellar vesicles (5), or by the creation of water-soluble derivatives. This latter change can be achieved by the addition of substituents like amino, carboxylic acid, and sulfonate groups to the outer ring of the isoindole units. To date, the sulfonated derivatives have been the most commonly used, since their synthesis is relatively easy, although subsequent purification may be difficult. One such sulfonated derivative, AlSPc, that we have been investigating is capable of photoinactivating cells in tissue culture (6), exhibits good tumor-localizing capacity (7, 8), and can be used to achieve marked reduction in tumor burden of various murine tumors of different histological origins (7). This dye, therefore, seems to have considerable potential as a photosensitizer for use in PDT.

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[2] The abbreviations used are: PDT, photodynamic therapy; AlS_nPc, chloroaluminum sulfonated phthalocyanine with *n* sulfonate groups; AlSPc, a mixed sulfonate preparation of chloroaluminum sulfonated phthalocyanine; ex/em, excitation/emission; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; Pc, phthalocyanine(s).

However, the AlSPc used in our studies, and in studies from other groups which also have demonstrated the possible utility of this agent (9–11), consisted of a mixture of mono-, di-, tri-, and tetrasulfonated derivatives. It would seem to be important to know which derivative(s) is (are) responsible for the observed biological activities. Some of the *in vitro* characteristics of the individual species have been investigated (12–14), although to date there is little information on the *in vivo* behavior of these various agents (15) and nothing on their distribution patterns. In this paper, we report not only the uptake of individual derivatives of AlSPc by cells *in vitro* but also their *in vivo* uptake and retention by normal and neoplastic tissue in tumor-bearing mice.

MATERIALS AND METHODS

Photosensitizer Preparation. AlSPc was obtained from Ciba-Geigy Dyestuffs and Chemicals (Basel, Switzerland). This material was a complex mixture of mono- to tetrasulfonated derivatives; according to the supplier, it had an average of three sulfonate groups. Individual chloroaluminum mono-, di-, and trisulfonated derivatives (AlS_1Pc, AlS_2Pc, and AlS_3Pc, respectively) were purchased from Porphyrin Products (Logan, UT). Chloroaluminum tetrasulfonated Pc (AlS_4Pc) was prepared by condensation of aluminum trichloride with four equivalents of sulfophthalic acid (monosodium salt) using the modified technique of Weber and Busch (16). Stock solutions of all of the dyes were prepared at 2–4 mg/ml in PBS, except the water-insoluble AlS_1Pc, which was dissolved to the same concentration in either 40% (v/v) ethanol:PBS (for *in vivo* studies) or in dimethylformamide (for *in vitro* studies). Solutions were sonicated for 3–5 min, heated at 100°C for 5–10 min, and then stored at 4°C in darkness. Stock solutions were measured spectrophotometrically (diode array spectrophotometer, model 8452A; Hewlett-Packard, Palo Alto, CA) after 10 μl of stock solution had been diluted in 9.990 ml 0.1 M NaOH [in the case of AlS_1Pc, the diluent was the same volume of a 3:1 (v/v) mixture of absolute ethanol:0.1 M NaOH]. Molarity of the AlS_2Pc solution was calculated from absorbance at 666 nm and a molar extinction coefficient (17):

$$E = 1.5 \times 10^5\ M^{-1}\ cm^{-1}$$

In order to determine the molarity of the solutions of the other derivatives, the area under the curve obtained by measuring absorbance between 500 and 750 nm was compared to that obtained with AlS_2Pc.

HPLC Analysis. Analysis of the individual Pc species was by reverse-phase HPLC essentially as described elsewhere (18). Briefly, the various sulfonated Pc were dissolved in 10 mM NaOH, buffered with 10 mM phosphate to pH 5, filtered (Waters HV 0.45 microfilters SJHVD43N3), and then injected onto a reverse-phase column (Waters NOVA-PAK C-18 radial compressed unit). Samples were resolved by a gradient mode elution (0–80% methanol in 10 mM phosphate buffer) over an 18-min run period. Eluted Pc were detected by absorption measurements at 365 nm.

Tumor Cells. Colo 26 cells from a murine colorectal carcinoma, syngeneic to BALB/c mice, were passaged routinely in E4 growth medium containing 10% fetal calf serum exactly as detailed previously (7).

Animals. Specific pathogen-free BALB/c mice were obtained from the Imperial Cancer Research Fund breeding unit. For individual

experiments, animals were sex matched and used at 8–14 weeks old, at which time they weighed ~20–25 g.

In Vitro Pc Uptake and Clearance Experiments. Colo 26 cells were plated (10^5–10^6 cells) into 55-mm Petri dishes containing 5 ml growth medium and allowed to attach overnight. For uptake experiments, cultures were then refed with fresh medium containing 10 μM of the specific Pc under test. At varying times thereafter, cells were washed three times with PBS. The viable, adherent cells were then detached by trypsinization, and total cell yields were determined by Coulter counting an aliquot. The remaining cells were pelleted and digested in 0.1 M NaOH at 1–2 × 10^5 cells/ml. To determine the cellular uptake of AlS_1Pc, the pellet was digested initially with 0.1 M NaOH at one-fourth the final required volume. Twenty-four h later, absolute ethanol was added at a ratio of 3 parts ethanol:1 part cell extract to bring the solution to the desired volume. All cell extracts were stored at −20°C.

Quantitation of Pc in the various extracts was by fluorospectrophotometry (Perkin-Elmer LS-3 spectrofluorometer) at the following ex/em wavelengths: AlS_1Pc, 605 nm/675 nm; AlS_2Pc, 605 nm/672 nm; AlS_3Pc, 605 nm/674 nm; AlS_4Pc, 607 nm/675 nm; AlSPc, 605 nm/674 nm. A slit width of 10 nm was used for both excitation and emission, and the emission was filtered with a Schott 645 filter (50% transmission at 645 nm) to eliminate scattered light. Experimental results were referenced to standard curves and are expressed as nmol Pc/10^6 cells. Data points represent means of three to four independent experiments, with two to three duplicated per time point.

In order to determine dye retention, cells were plated and loaded with the individual Pc species exactly as described above. Cultures were refed with fresh growth medium without dye, and this medium was replaced every 24 h; the cells were terminated and extracted at intervals thereafter. Pc concentrations were normalized to 100%, obtained at time zero after dye removal, to facilitate comparison of clearance kinetics of the individual sulfonated species.

Tissue Distribution of Pc Derivatives. Progressively growing tumors were produced in BALB/c mice by s.c. injections of 1 × 10^6 Colo 26 cells into the flank region. When the tumors reached a size of between 8 and 13 mm in diameter, the mice received a single i.v. injection, via the lateral tail vein, of 0.1 ml 2.27 mM Pc in PBS (40% ethanol-PBS for AlS_1Pc as described). Two to three animals per group were killed at various times after dye injection and weighed, and samples of tissues, including tumor, adjacent normal skin, thigh muscle, liver, spleen, and kidney, were dissected out exactly as described previously (7). Plasma and urine samples were taken from the same animals. Tissue and fluid samples were digested with 0.1 M NaOH (10 ml/0.1 g wet weight tissue, or 100 μl fluid) for 4 h at 50°C. However, samples obtained from animals which had received AlS_1Pc were digested with 2.5 ml 0.1 M NaOH/0.1 g wet tissue, as described above, before the addition of 7.5 ml absolute ethanol. Fluorescence of the various extracts was measured at the following ex/em wavelengths:- AlS_1Pc, 606 nm/677 nm, AlS_2Pc, 605 nm/673 nm; AlS_3Pc, 605 nm/675 nm; AlS_4Pc, 607 nm/677 nm; AlSPc, 605 nm/675 nm. The wavelengths which gave maximum fluorescent intensity were slightly different from those which gave maximum intensity on cell extracts. Effects of tissue absorption and temperature at each of these settings were allowed for by reference to standard curves, and the data points were expressed as nmol/g wet tissue (or per ml fluid). Values presented are the means of four or five independent experiments.

In a small series of additional experiments, normal or tumor-bearing mice received either an i.v. or an i.p. injection of the specific Pc. Twenty-four or 48 h later, tissue samples were taken after peritoneal exudate cells had been harvested as described (19), and the content of phthalocyanine in these samples was assayed as outlined above. Data presented were derived from two independent experiments with a total of four mice per data point.

RESULTS

HPLC Analysis of AlS_nPc Species. The individual sulfonated species used in this study, with the exception of AlS_4Pc, were purchased from commercial sources, and their purity was verified by spectroscopy and HPLC analysis. The absorption spectra of the sulfonated Pc are presented in Fig. 1. Although peak shape is consistent with the presence of monomeric Pc (17), the asymmetry of some of the peaks (660–680 nm) indicated the possible presence of more than one component. HPLC analysis (Fig. 2) suggested that the derivatives were relatively pure as regards their degree of sulfonation and that this asym-

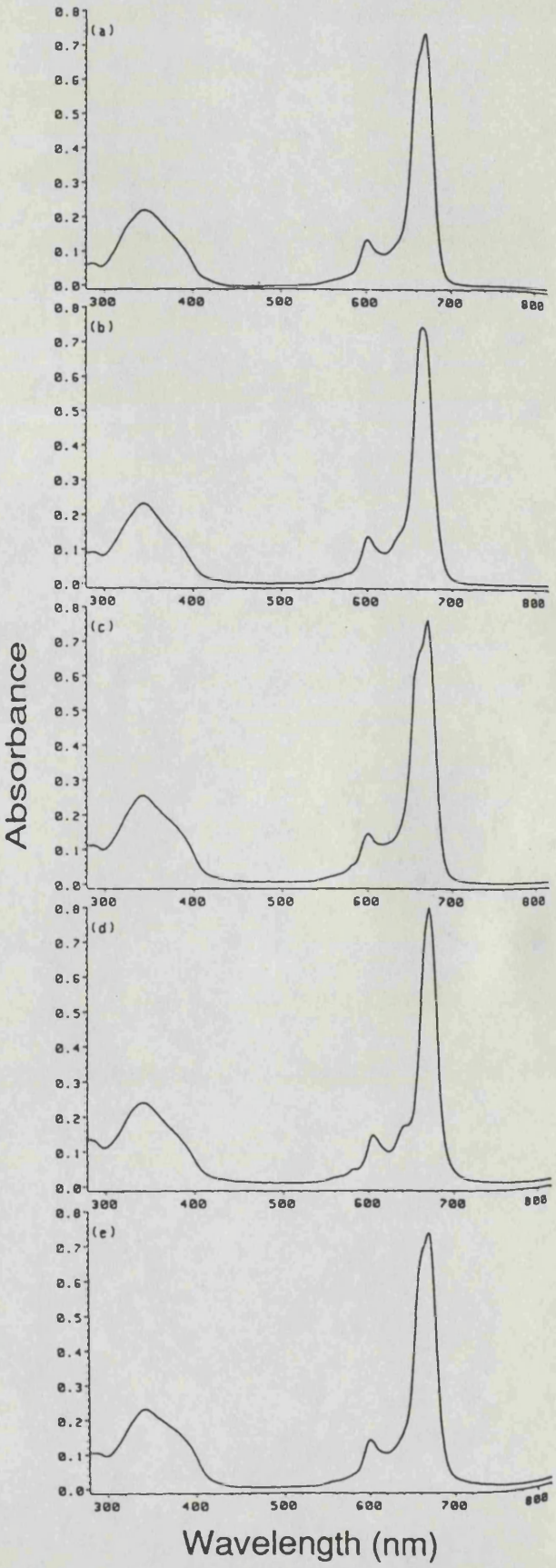

Fig. 1. Absorbance spectra of (*a*) AlS_1Pc, (*b*) AlS_2Pc, (*c*) AlS_3Pc, (*d*) AlS_4Pc, and (*e*) AlSPc; 10 mM in 0.1 M NaOH (1 part 0.1 M NaOH and 3 parts ethanol for AlS_1Pc).

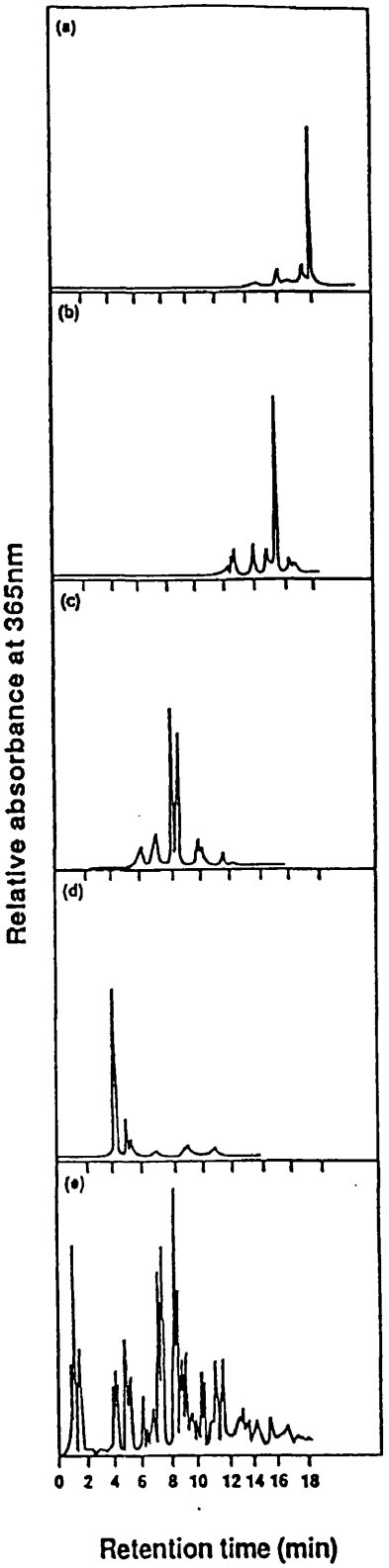

Fig. 2. HPLC traces of (*a*) AlS_1Pc, (*b*) AlS_2Pc, (*c*) AlS_3Pc, (*d*) AlS_4Pc, and (*e*) AlSPc. Detailed techniques are described in the text.

metry might be attributable to different isomers or the asymmetric substitution of a single Pc isomer. There are four nonequivalent potential sites for sulfonation in each of the four rings of the Pc molecule which could give rise to a large number of isomers. No attempt was made to determine either the actual number or arrangement of these isomers. The HPLC trace of the AlSPc used in our earlier work (6–8) revealed it to be a complex mixture containing a larger number of activity peaks than would be obtained by the simple admixing of the AlS_1Pc-AlS_4Pc species used in the present study (Fig. 2*e*).

Cellular Uptake of Pc Derivatives *in Vitro*. The kinetics of uptake of the various sulfonated species of AlS_nPc, by Colo 26 cells growing in tissue culture, are presented in Fig. 3. Although the shape of the uptake curves is similar for all of the derivatives examined, the relative amount of each individual species taken up by the cells varies considerably, with $AlS_1Pc > AlS_2Pc > AlSPc > AlS_4Pc > AlS_3Pc$, with a ratio at 24 h of approximately 25:4.7:3.7:1.5:1.

Cellular Retention of Pc Derivatives *in Vitro*. Fig. 4 illustrates the clearance of individual Pc species from Colo 26 cells which had been "loaded" with dye for 24 h. Although the differences in clearance rates were not great, it was apparent, over a series of experiments, that those sulfonated Pc derivatives taken up to the greatest extent (see Fig. 3) were retained longer than the less well taken up AlS_3Pc and AlS_4Pc (Fig. 4).

Retention of Pc Derivatives by Tumor Tissue. The retention patterns of the various Pc derivatives by the Colo 26 tumor, growing in a s.c. site, relative to adjacent normal skin and muscle tissue are illustrated in Fig. 5. The first time point examined was 3 h after dye injection. With the dye species used, the tumor showed greater selectivity of retention, with ratios of >10:1 for tumor:muscle and 2–3.5:1 for tumor:skin consistently being achieved 24–48 h after Pc administration. The exception was AlS_1Pc, for which tumor:muscle and tu-

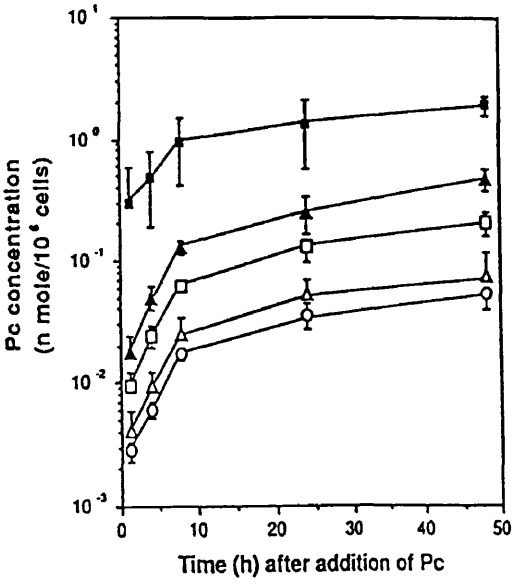

Fig. 3. Kinetics of cellular uptake *in vitro* of sulfonated Pc by Colo 26 cells. Media contained 10 μM of individual sulfonated Pc. ■, AlS_1Pc; ▲, AlS_2Pc; ○, AlS_3Pc; △, AlS_4Pc; □, AlSPc. *Bars*, SD. Earliest time point, 1 h after addition of Pc.

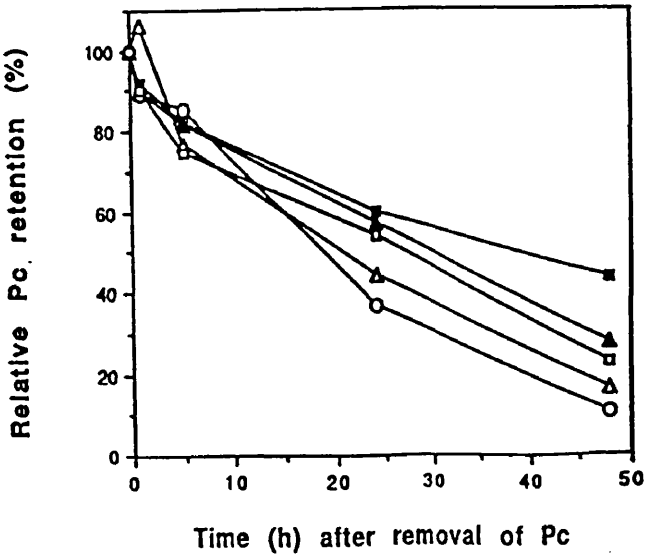

Fig. 4. Kinetics of sulfonated Pc retention in Colo 26 cells *in vitro*. Cells were exposed to dyes for 24 h before examination for retention. ■, AlS_1PC; ▲, AlS_2Pc; ○, AlS_2Pc; △, AlS_4Pc; □, AlSPc. SD < 10%. Pc concentrations were normalized to 100% obtained at time zero after dye removal.

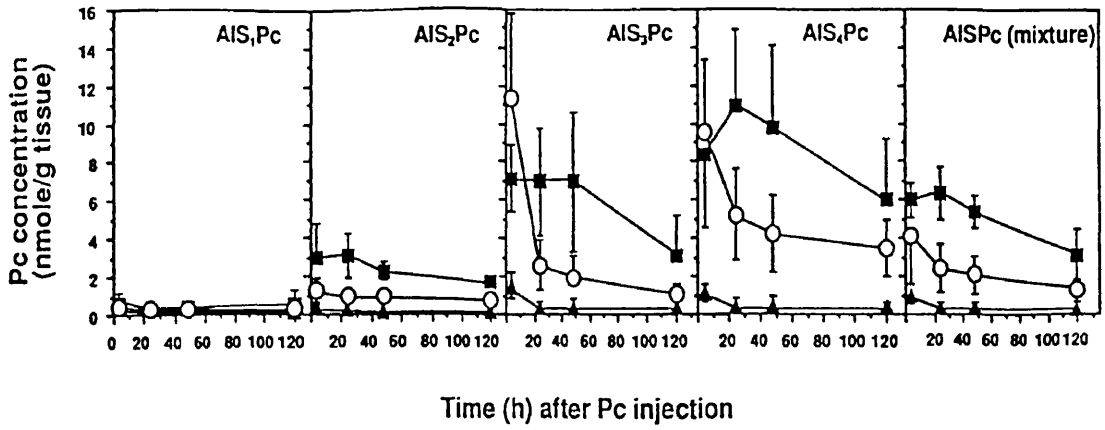

Fig. 5. Kinetics of sulfonated Pc uptake and retention by Colo 26 tumors growing in s.c. site of syngeneic mice compared with adjacent skin and thigh muscle. Mice received 0.1 ml 2.27 mM Pc solution i.v. by injection into the lateral tail vein. ■, Colo 26 tumor; O, skin adjacent to tumor tissue; ▲, thigh muscle. Earliest time point, 3 h after injection of dye.

mor:skin ratios were less than <2:1, although this is unclear because of the scale used in Fig. 5*a*. However, the absolute amounts of the individual sulfonated species found in the tissues varied substantially. Accordingly, tissue samples took up $AlS_4Pc > AlS_3Pc \geq AlSPc > AlS_2Pc > AlS_1Pc$ in a pattern which was almost a mirror image of that found in cellular uptake *in vitro*. Interestingly, dye accumulation to maximum levels generally occurred more rapidly in normal tissues (<3 h) than it did in the tumor (peak levels attained 24–48 h after Pc injection).

Pc Derivative Distribution in Normal Tissues. The major site of uptake for AlSPc (7, 8) and many porphyrin compounds (20, 21) has been found to be the reticuloendothelial system of the livers and spleens of injected animals. Results presented in Fig. 6 show that the amount of Pc uptake by liver and spleen closely resembled that of tumor cells in tissue culture (Fig. 3) and thus was very different from that seen in tumor, skin, and muscle tissues (Fig. 5), with $AlS_1Pc > AlS_2Pc > AlSPc > AlS_4Pc > AlS_3Pc$ (Fig. 6). Thus, liver:tumor ratios at 24 h after Pc administration varied from approximately 600:1 for AlS_1Pc to 1:1.5 for AlS_3Pc. The kidney, which took up less dye than liver and spleen, shares a pattern of uptake which parallels that seen in tumor tissue (Fig. 6). Whereas high levels of AlS_3Pc and AlS_4Pc were rapidly evident in plasma and urine subsequent to i.v. injection, much lower levels of AlS_1Pc and AlS_3Pc were found in these fluids (Fig. 6).

Comparison of i.v. and i.p. Route of Dye Delivery on Tissue and Cell Uptake. The relative uptake of the various sulfonated Pc by liver, spleen, or peritoneal exudate cells after i.p. or i.v. injection of equal amounts of dye is illustrated in Fig. 7. It can be seen that the i.p. route of administration led to the uptake of large amounts of all of the Pc by peritoneal exudate cells, whereas, with the exception of AlS_3Pc and AlS_4Pc, the same cells took up relatively little material after i.v. injection (Fig. 7*c*). In contrast, the i.p. delivery of AlS_1Pc and AlS_2Pc resulted in accumulation of less dye in the liver and spleen than was achieved after i.v. injection. Interestingly, whether AlS_3Pc and AlS_4Pc were delivered by either the i.p. or the i.v. route had relatively little effect on the degree of uptake achieved by the liver and spleen (Fig. 7, *a* and *b*). AlSPc, the mixture of sulfonated derivatives, presented a pattern which lay between that obtained with AlS_1Pc/AlS_2Pc on the one hand and AlS_3Pc/AlS_4Pc on the other.

DISCUSSION

It has been shown that the degree of sulfonation is correlated inversely to the lipid/water partition coefficient of Pc (13), so that a reduction in the number of sulfonate groups increases Pc lipophilicity. Generally, the greater the partition coefficient, the higher the concentration of the drug in the membrane and the faster its rate of diffusion into the cell (22). The *in vitro* results presented in this paper tend to support this concept, with the less sulfonated Pc being taken up at a faster rate, and to a greater extent, than the more sulfonated Pc (Fig. 3). AlS_4Pc was taken up by the cells used in this study to a slightly higher extent than was AlS_3Pc, although the difference between the two was not marked. Berg *et al.* (13) have shown that the difference in partition coefficients between these two species is not great. Comparison of HPLC traces of the commercially supplied Pc with the traces of similar materials prepared by both the condensation method and by the treatment of chlo-

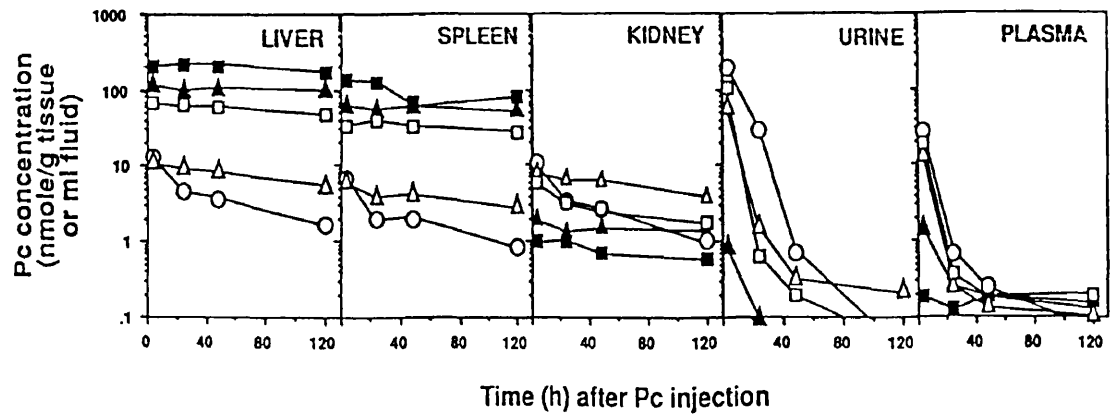

Fig. 6. Kinetics of sulfonated Pc uptake and retention in normal tissues and fluids obtained from mice bearing s.c. located Colo 26 tumors. Amounts of Pc below 0.1 nmol were not shown. SD varied from 10 to 50%. ■, AlS_1Pc; ▲, AlS_2Pc; O, AlS_3Pc; △, AlS_4Pc; □, AlSPc. Earliest time point, 3 h after injection of dye.

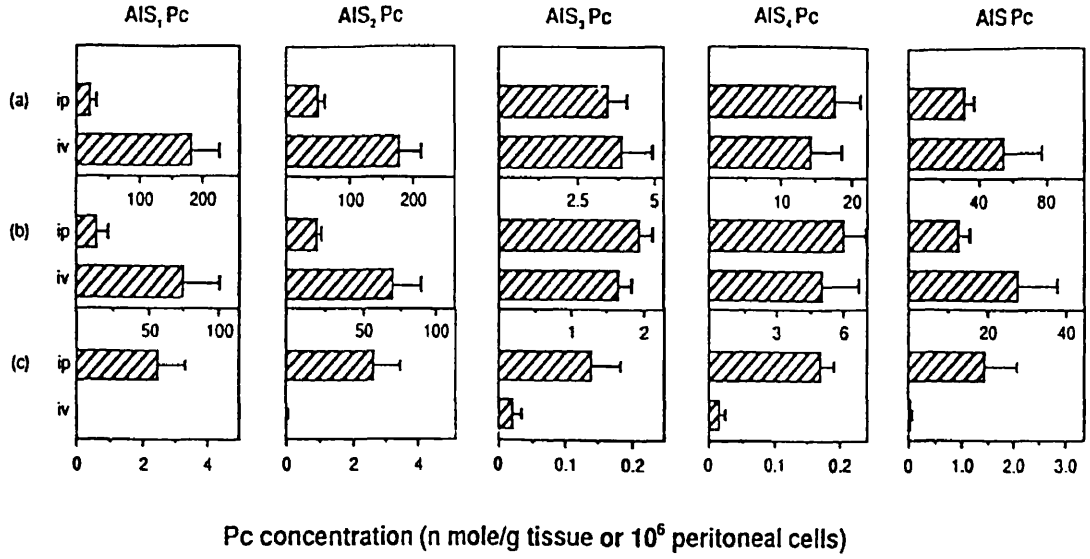

Fig. 7. Comparison of sulfonated Pc uptake and retention in tissue and cells 24 h after i.v. or i.p. injection of 0.1 ml 2.27 mM Pc solution. *a*, liver; *b*, spleen; *c*, peritoneal exudate cells. Quantities of Pc in samples are individually scaled.

roaluminum Pc with oleum (data as seen in Ref. 18) suggested that the commercial materials have been prepared by treatment with oleum (18). These different preparative techniques have been shown to result in different populations of isomers (23). This could account for the increased uptake of AlS_4Pc in cells as compared to AlS_3Pc even though these compounds have been shown to have minor differences in their partition coefficients.

Clearance of the different Pc from the cells occurred at rates which were in reverse order to that observed for uptake (Fig. 3), although the magnitude of difference between the various Pc species was much less than that observed in kinetics of uptake.

These relatively simple correlations between cellular uptake/clearance and the degree of sulfonation are not readily apparent when the *in vivo* data are analyzed. Retention of Pc derivatives by solid tumor was greatest when the degree of sulfonation was highest, *i.e.*, opposite to the order observed in tissue culture experiments utilizing pure populations of tumor cells. This result is unlikely to derive from the rapid clearance of hydrophobic Pc from the tumor, since *in vitro* data show that the clearance rates for hydrophobic Pc were lower than those of hydrophilic Pc (Fig. 4). However, although we have shown previously that neoplastic cells within a tumor mass are capable of taking up AlSPc (7), it is not certain that this cell population is the major contributor to the selectivity of dye uptake manifested by tumor masses. By using flow cytometry, it was shown that infiltrating cells of the monocyte-macrophage lineage incorporated the greatest amounts of dye (7). Probably, the considerable uptake and retention of Pc derivatives by liver and spleen, which are greatest for the mono- and disulfonated species (Fig. 6), are attributable directly to the capacity of cells of the monocytic lineage to accumulate Pc dyes. Indeed, by charge-coupled device-camera imaging, we have shown that AlS_1Pc is retained selectively by the phagocytic Kupffer cells of the liver (19). Moreover, the rapid accumulation of AlS_1Pc and AlS_2Pc by these reticuloendothelial organs may rapidly deplete plasma levels (Fig. 6) and thus profoundly affect the quantity of dye available for subsequent uptake by tumor and other normal tissues. Such a mechanism might account for the noted differences in distribution between i.v. and i.p. modes of dye delivery. Here, the more lipophilic dyes, when injected i.p., were retained to a greater extent within the abdominal cavity, and relatively little was apparent in the liver and spleen (Fig. 7). These differences in pharmacokinetics between the various sulfonated species may be compounded further by the degree of dye aggregation occurring in the serum which could limit tissue penetration. Recent investigations have utilized incorporation of lipophilic Pc into liposomes (5) or oil emulsions (24) to overcome this phenomenon of aggregation. Another possibility which might account for the varying retention of dyes of differing hydrophilicity is that dyes bind to extracellular matrix and other stromal components rather than to neoplastic cells. Such a phenomenon has been observed when the more hydrophilic sulfonated derivatives of tetraphenylporphine have been utilized (25).

In summary, then, our results have shown that, in the relatively simple environment of tissue culture, cellular uptake correlates well with the lipophilicity as determined by the amount of sulfonation of the various Pc derivatives. The demonstration that there is comparatively little uptake of more lipophilic species of other Pc (*e.g.*, tetraneopentoxyphthalocyanine) in alternative cell systems (26) suggests that this need not be a universal phenomenon. Analysis of the *in vivo* distribution of the various sulfonated species of chloroaluminum phthalocyanine revealed the complexity of tissue uptake of these dyes. This suggests that simple biophysical characteristics relating to plasma membrane transport are not the sole determinants of organ distribution for these photosensitizers. Consideration of these aspects will be a necessary part of choosing the correct Pc, and the correct route of Pc administration, for PDT in the clinic. Although we have shown previously that AlSPc has good tumor-localizing ability, probably attributable to the tri- and tetrasulfonated components, we remain uncertain as to which constituent mediates the tumor-destructive effect, subsequent to light irradiation, exhibited by this material (7, 8). This question currently is under investigation in our laboratory.

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COMPARISON OF DISTRIBUTION AND PHOTODYNAMIC EFFECTS OF DI- AND TETRA-SULPHONATED ALUMINIUM PHTHALOCYANINES IN NORMAL RAT COLON

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Abstract—We have previously reported photodynamic therapy of normal rat colon using aluminium sulphonated phthalocyanine (AlSPc). In that study, the AlSPc used was a mixture of phthalocyanines of different degrees of sulphonation. Phthalocyanines of defined degrees of sulphonation have recently become available and we compared the distribution of the di- and tetra-sulphonates (AlS_2Pc and AlS_4Pc) in rat colon and colon wall structures employing both chemical extraction and fluorescence photometry using a charge coupled device imaging system. Also, the photodynamic effects produced by these components in rat colon were compared at various times after photosensitization. After intravenous photosensitizer administration using equimolar doses, the concentration of AlS_2Pc in colon fell off more rapidly with time than AlS_4Pc. Differences were noted in the microscopic distribution of these compounds, with the di-sulphonate exhibiting peak fluorescence in colon wall structures by 1 h after photosensitization, while mucosal fluorescence with the tetra-sulphonate peaked at 5 h. Fluorescence was also lost from the colon wall much more slowly with the tetra-sulphonate, which tended to be retained in the submucosa. Maximum photosensitizing capability was seen at 1 h with AlS_2Pc and no lesions could be produced with photodynamic therapy at 1 week, with up to 5.65 μmol/kg. With AlS_4Pc (5.65 μmol/kg), while no lesions could be produced with light treatment at 1 h, photodynamic therapy at 1 week produced lesions only slightly smaller than those produced with treatment at 48 h (the time of maximum effect), and significant photosensitization was present at 2 weeks. It is proposed that differences between AlS_2Pc and AlS_4Pc with respect to pharmacokinetics, microscopic distribution and qualitative (related to the timing) and quantitative differences in photodynamic effects in normal rat colon are related to physico-chemical characteristics of the phthalocyanines used, particularly lipid solubility. Similar studies are now required on tumour bearing animals.

INTRODUCTION

Aluminium sulphonated phthalocyanine (AlSPc)† has been studied as a sensitizer for PDT in a range of tissues and selective tumour destruction demonstrated in colon (Barr *et al.*, 1990) and brain (Sandeman *et al.*, 1987). AlSPc as used in these studies is a mixture of compounds with a range of one to four sulphonate groups (AlS_nPc, where n = 1,2,3 or 4, average = 3.2 (Tralau *et al.*, 1987)). Preparations of disulphonated aluminium phthalocyanine (AlS_2Pc) and tetrasulphonated aluminium phthalocyanine (AlS_4Pc) have now become available (Svensen, 1990, Ambroz *et al.*, 1991) and this provided the stimulus to study these components. Decreasing sulphonation leads to increased lipid solubility and the likelihood of differences in tissue distribution and PDT effects.

In the past, the tissue distribution of photosensitizers has usually been determined by chemical extraction techniques. However, it is now possible, using a highly sensitive charge coupled device (CCD) camera, to detect the microscopic localization of photosensitizers in tissue sections using fluorescence imaging (Chan *et al.*, 1989; Barr *et al.*, 1988). In this paper, we present studies on the distribution of AlS_2Pc and AlS_4Pc in rat colon using both techniques and correlated the results with the photodynamic effects produced by standard light doses at various times after photosensitizer administration.

MATERIALS AND METHODS

All experiments were carried out using female Wistar rats 4–8 weeks old and weighing 125–200 g. Full details concerning the preparation and analysis of the AlS_2Pc and AlS_4Pc used in this study are the subject of a paper by Ambroz *et al.* (1991). AlS_2Pc was the most lipophilic fraction separated by reverse phase medium pressure liquid chromatography (H_2O and methanol elution) from an AlSPc mixture prepared by oleum (30%) sulphonation of chloroaluminium phthalocyanine. It is thought that this

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†*Abbreviations*: AlSPc, aluminium sulphonated phthalocyanine (a mixture, with moieties of different degrees of sulphonation); AlS_2Pc, disulphonated aluminium phthalocyanine; AlS_4Pc, tetrasulphonated aluminium phthalocyanine; CCD, charge-coupled device; PDT, photodynamic therapy.

fraction contains AlS_2Pc with two adjacent sulphonate groups. This was dissolved in 0.1 *M* sodium hydroxide and the resulting solution buffered to pH 8 using NaH_2PO_4. The AlS_4Pc, produced by condensation of 4-sulphophthalic acid with aluminum chloride, was made up in N-saline as it was readily water soluble. The molarity of each of these solutions was measured using optical density. For distribution studies, equimolar doses (5.65 μmol/kg) of AlS_2Pc or AlS_4Pc were used. This dose was chosen as it was equivalent to 5 mg/kg of AlSPc as used in our previously published reports (Barr *et al.*, 1987). Animals were killed 1 h, 5 h, 48 h or 7 days after tail vein injection of photosensitizer. The colon was removed and immediately frozen in liquid nitrogen. A 2 cm segment was kept frozen in liquid nitrogen until transverse frozen sections were cut and used for fluorescence microscopy. The remainder of the colon was stored at −4°C prior to chemical extraction.

Chemical extraction. NaOH, 0.1 *M*, was used to extract the phthalocyanine from thawed tissue using a ratio of 0.1 g tissue to 10 mL NaOH. This mixture was incubated in a water bath at 50°C for 4 h. Phthalocyanine concentrations in the resulting solution were measured by spectrofluorimetry (603 nm excitation and 673 nm emission with 10 nm slit widths), using standard curves prepared by addition of known amounts of photosensitizer to control colon specimens treated similarly (Chan *et al.*, 1988).

Fluorescence microscopy and photometry. Frozen tissue sections (10 μm) on glass slides were placed on the stage of an inverted microscope (Olympus IMT-2) with epifluorescence and phase contrast attachments, and an 8mW helium neon laser (632.8 nm) was used as the excitation source. The laser output was passed through a 10 nm band-pass filter, centered at 633 nm, to remove extraneous light and then directed onto the section using a liquid light guide and dichroic mirror. Fluorescence was detected between 665 and 700 nm, using a combination of band-pass (Omega Optical Inc.) and long-pass (Schott RG665) filters, and imaged using the cryogenically cooled CCD camera (Wright Instruments, Model 1, resolution 400 × 600 pixels) fitted to the microscope. Image processing and camera operation were carried out using an IBM personal computer. This slow scan CCD system has comparable sensitivity to photomultiplier photon counting detection and is significantly superior to conventional video camera imaging (Aikens *et al.*, 1989). The digital fluorescence images were falsely colour-coded by image processing software and were photographed. The combination of phase contrast microscopy of the frozen sections and standard light microscopy of adjacent stained tissue sections enabled individual colonic wall structures—mucosa, submucosa, blood vessel wall, muscularis mucosa, muscularis propria—to be identified in the fluorescence image and the fluorescence intensity of these structures to be measured. These fluorescence measurements represented the average number of counts per pixel within a rectangle superimposed on the area of interest on the stored image. The minimum area used was 100 pixels, for example, to measure blood vessel wall fluorescence intensity. When the sampling rectangle was enlarged to cover the full thickness of the colon wall, whole image fluorescence intensity could be measured and compared with phthalocyanine concentration, determined using chemical extraction on segments of colon adjacent to those used for the fluorescence microscopy. Values obtained for unsensitized colon sections on glass slides were not different from background values using glass slides alone, indicating minimal tissue autofluorescence between 665 and 700 nm. The sections used for fluorescence microscopy were subsequently fixed and stained, and the areas used for fluorescence microscopic studies photographed for visual comparison.

Photodynamic therapy. PDT of the colon was carried out using standard light doses of 50 J (100 mW for 500 s) at a wavelength of 675 nm from a pulsed (12 kHz) copper-vapour pumped dye laser (Oxford Lasers). The energy was delivered *via* a plane tip 200 μm fibre threaded into the colon at laparotomy and positioned so that it just touched the colonic mucosa at a distance not less than 15 mm from the point of entry of the fibre into the colonic lumen. PDT was carried out at 1 h, 48 h or 168 h after photosensitization with either 1.13 or 5.65 μmol/kg of AlS_2Pc or AlS_4Pc, and, in addition, 336 h after photosensitization with AlS_4Pc. At 72 h after treatment, necrosis, if produced, was represented at post mortem by a well demarcated sloughy area which was easily measured in longitudinal and transverse axes. The mean of these two measurements was taken as the mean diameter of necrosis (Barr *et al.*, 1987).

RESULTS

Chemical extraction

The results are expressed in nmol/g of tissue and are shown in Fig. 1 (mean of at least two extractions for each point). Concentrations of both AlS_2Pc and AlS_4Pc decreased with time after injection although the rate of decrease was less for AlS_4Pc.

Fluorescence microscopy and photometry

In all quantitative estimations, the fluorescence of a control unsensitized colon section on a glass

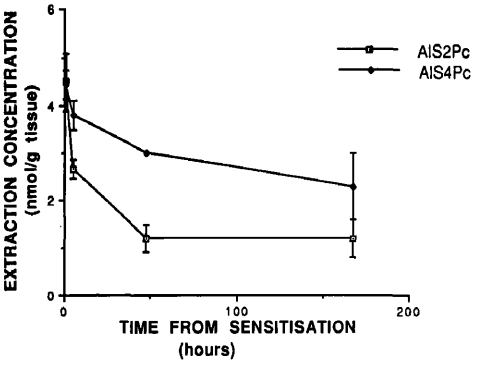

Figure 1. Concentration of photosensitizer (± 1 SD) as measured by chemical extraction *vs* time from sensitization with 5.65 μmol/kg of AlS_2Pc or AlS_4Pc.

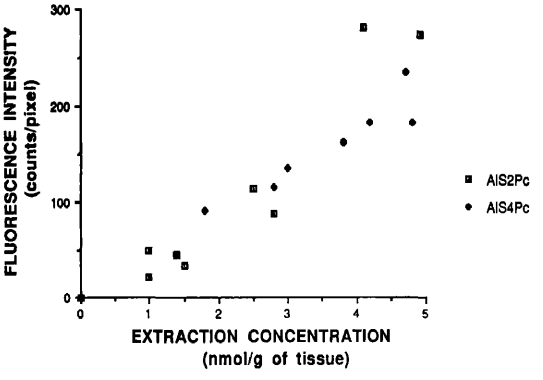

Figure 2. Whole image fluorescence intensity *vs* photosensitizer concentration as measured by chemical extraction.

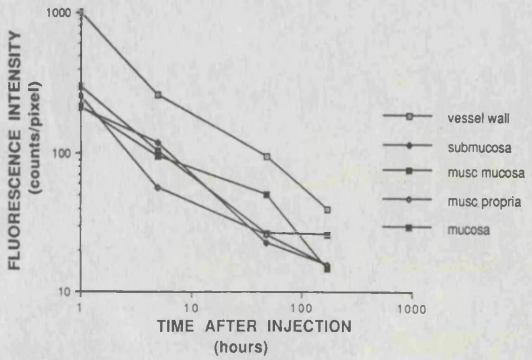

Figure 3. Fluorescence photometry of AlS_2Pc: fluorescence in colon wall structures at different times after sensitization.

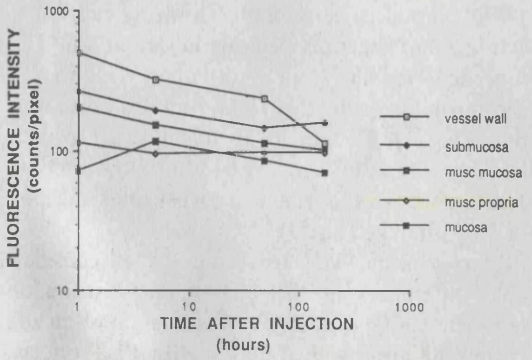

Figure 4. Fluorescence photometry of AlS_4Pc: fluorescence in colon wall structures at different times after sensitization.

slide was subtracted from values of fluorescence intensity of sensitized tissues. Figure 2 compares the level of phthalocyanine measured by chemical extraction with that from fluorescence intensity of the whole image. Good correlation was demonstrated between the two for both AlS_2Pc and AlS_4Pc (r^2 = 0.90 and 0.95, respectively).

Changes in fluorescence of colon wall structures with time are shown in Figs. 3 and 4 (mean of readings from sections from at least two animals). All structures studied exhibited maximal fluorescence intensity at 1 h except mucosa after AlS_4Pc injection (maximal at 5 h). With AlS_4Pc, fluorescence was noted principally in blood vessel walls and submucosa throughout the time period studied (1 week). With AlS_2Pc, this pattern was different with early spread of fluorescence throughout all colon wall structures, including mucosa, by 1 h. Contrasts in the patterns of fluorescence using these two compounds are seen in Figs. 5,6,7 and 8.

Photodynamic therapy

The diameter of the zone of necrosis produced by PDT with the two sensitizers and each time interval studied are shown in Table 1 and Fig. 9

Table 1. Mean diameter of necrosis (mm) produced by PDT (50J) with low doses of AlS_2Pc or AlS_4Pc

Time from sensitization for PDT	Necrosis produced by AlS_2Pc (1.13 μmol/kg) ± 1 SD	Necrosis produced by AlS_4Pc (1.13 μmol/kg)
1 h	8.9 ± 0.9	0.0
48 h	0.0	0.0
168 h	0.0	0.0

(mean of at least 6 lesions at each point). For AlS_2Pc at a dose of 1.13 μmol/kg, necrosis could only be produced with PDT at 1 h. With 5.65 μmol/kg, while larger lesions were produced with PDT at 1 h, and smaller lesions at 48 h, there was no effect at 1 week.

For AlS_4Pc, photosensitization with either 1.13 or 5.65 μmol/kg failed to produce lesions with light treatment at 1 h. 1.13 μmol/kg also failed to produce lesions with PDT at 48 h or 168 h. Using 5.67 μmol/kg, lesions were produced at 48 h, 168 h and 336 h but were maximal at 48 h.

DISCUSSION

Cell uptake and inactivation studies demonstrate increased uptake (and rate of uptake) and efficiency of inactivation on exposure to light with increasing liphophilicity, both with phthalocyanines (Berg *et al.*, 1989, a,b) and certain porphyrins (e.g. sulphonated derivatives of tetraphenylporphyrin, Kessel *et al.*, 1987). However, this increase in efficiency is limited by a tendency to aggregate as lipophilicity increases. Phthalocyanine aggregates exhibit negligible fluorescence and photoactivity (Barr *et al.*, 1988; Berg *et al.*, 1989b). Therefore, fluorescence intensities will not necessarily correlate with chemical extraction concentrations. Nevertheless, in these experiments, good correlations were noted between phthalocyanine concentration, measured by chemical extraction, and fluorescence intensity of the whole image (Fig. 2), suggesting that factors influencing fluorescence/concentration relationships, such as aggregation, fluorescence quenching and self-absorption combine to produce a linear fluorescence/concentration relationship, at the concentrations studied. However, formal analysis would require determination of fluorescence/concentration relationships at each of various specified times after administration of the photosensitizer. It is noted that linear correlation has been previously demonstrated between *in vivo* fluorescence and chemical extraction assay using "dihaematoporphyrin ether" in mouse mammary carcinoma (Mang *et al.*, 1987). The ampiphilic AlS_2Pc used in this study may be expected to bind to different plasma components than the hydrophilic AlS_4Pc leading to different plasma pharmacokinetics (Jori, 1989). However, the

rapid accumulation of AlS_2Pc in reticuloendothelial organs, such as liver and spleen tends to overshadow these plasma binding differences and may thus profoundly reduce the amount of AlS_2Pc available for uptake by other organs (Chan *et al.*, 1990).

This study demonstrates differences between the microscopic distributions of AlS_2Pc and AlS_4Pc with time (Figs. 3–8). With AlS_2Pc, fluorescence is noted to be maximum at 1 h, greatest in submucosal blood vessel walls and, over the course of 1 week, decreases in all structures. With AlS_4Pc, mucosal fluorescence is maximal at 5 h and by 1 week the submucosal layer exhibits maximum fluorescence when compared to other layers of the colon wall. Submucosa is loose connective tissue. It comprises a collagen and eleastic fibre network, dispersed within which are fluid and macromolecular constituents (glycosaminoglycans and glycoproteins) which form a hydrophilic gel phase (Granger, 1981). The pharmacokinetics of molecules within this structure, therefore, reflect those in an extracellular matrix and depend on the physical and chemical characteristics of the compounds studied (Nugent and Jain, 1984). AlS_4Pc, with four negative charges at pH 7.4, is hydrophilic and would be expected to be retained in interstitium. We feel that this has been demonstrated in submucosa, which we would suggest can be used as an interstitial tissue model. This retention in submucosa/interstitium, taken together with delayed cell uptake, demonstrated by other workers (Berg *et al.*, 1989a) serves to explain the lack of necrosis produced by PDT at 1 h. Furthermore, the tendency to be retained in submucosa is likely to be responsible for the slow clearance of AlS_4Pc from rat colon with time, leading to protracted photosensitizing capability (Fig. 9). In contrast, our fluorescence studies demonstrate that the more lipid soluble AlS_2Pc rapidly spreads throughout colon wall structures and this property, together with rapid cell uptake (Paquette *et al.*, 1988; Berg *et al.*, 1989a), produces early photosensitizing capability, with maximal necrosis being produced at 1 h. The superior "mobility" of AlS_2Pc in colon wall accompanies equally rapid loss from this tissue with time (Fig. 1), leading to an inability to produce lesions with PDT at 48 h after injection of 1.13 μmol/kg (Table 1) and at 1 week after injection of 5.65 μmol/kg (Fig. 9).

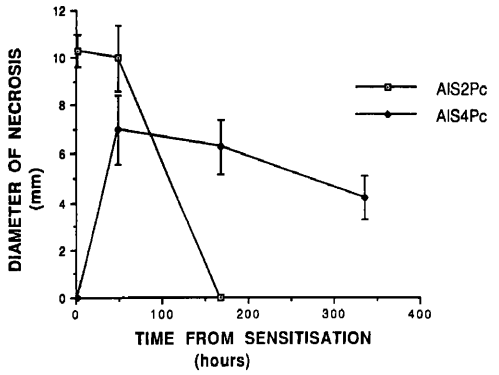

Figure 9. Diameter of necrosis (± 1 SD) produced by light exposure of 50J *vs* time from sensitization for light exposure using 5.65 μmol/kg of AlS_2Pc or AlS_4Pc.

In conclusion, we have demonstrated considerable differences in the microscopic distribution between AlS_2Pc and AlS_4Pc in normal rat colon and correlated these with differences in PDT effects. These effects are due to differences in lipid solubility and emphasize the importance of using pure photosensitizers. The next step must be to compare the two sensitizers in tumour bearing animals.

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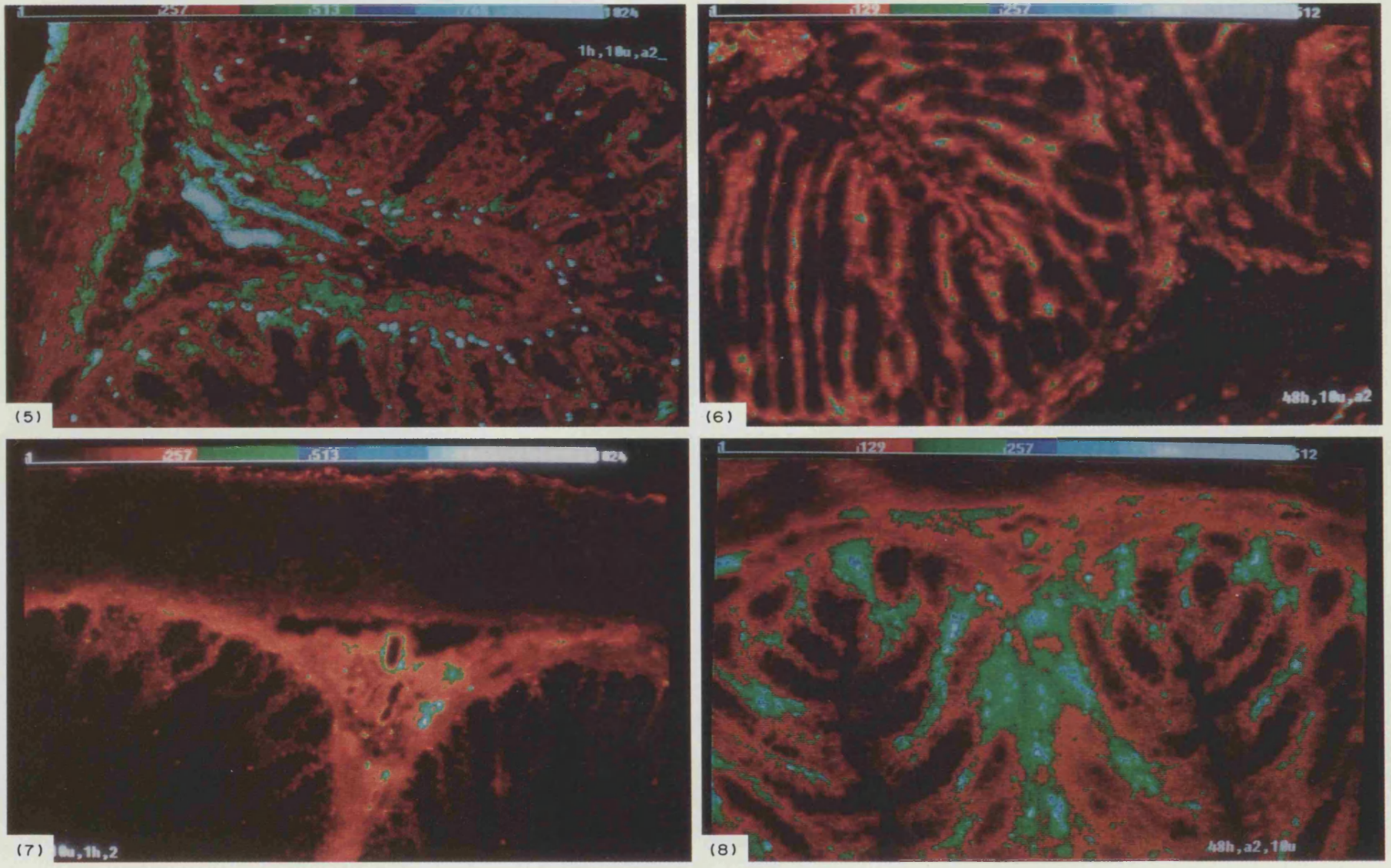
(Figs. 5–8 opposite)

Figure 5. Fluorescence micrograph of transverse frozen section of colon wall 1 h after sensitization with 5.65 μmol/kg of AlS_2Pc. The colour scale is shown at the top of the micrograph. Maximum fluorescence (1024 counts/pixel) is represented white. All structures exhibit fluorescence (greatest in submucosal vessel walls, shown blue and white). (Magnification ×300, X10 objective.)

Figure 6. Fluorescence micrograph of transverse frozen section of colon wall 48 h after sensitization with 5.65 μmol/kg of AlS_2Pc. Fluorescence has markedly decreased in all structures. (Full scale is 512 counts/pixel). (Magnification ×300, X10 objective.)

Figure 7. Fluorescence micrograph of transverse frozen section of colon wall 1 h after sensitization with 5.65 μmol/kg of AlS_4Pc. Mucosa is poorly fluorescent and maximum fluorescence is seen in submucosal vessel walls (blue and white). (Full scale 1024 counts/pixel). (Magnification ×300, X10 objective.)

Figure 8. Fluorescence micrograph of transverse frozen section of colon wall 48 h after sensitization with 5.65 μmol/kg of AlS_4Pc. Fluorescence principally remains within submucosa (green), and of all structures, submucosal blood vessels exhibit greatest fluorescence (blue). This micrograph presents a striking contrast to Fig. 6 which demonstrates AlS_2Pc in colon wall at 48 h after sensitization. (Full scale 512 counts/pixel). (Magnification ×300, X10 objective.)



Figs. 5–8 (*legends opposite*)

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ENHANCED TUMOUR SELECTIVITY OF PHOTODYNAMIC THERAPY IN THE RAT COLON USING A RADIOPROTECTIVE AGENT

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Abstract—Radioprotective agents have been found to protect normal tissues during photodynamic therapy (PDT). We have investigated a phosphorylated thiol protectant WR-77913 (W7) with the photosensitizer aluminium sulphonated phthalocyanine (AlSPc). We compared the effects of PDT on normal and tumour tissue in the rat colon, with and without this protectant. In normal colon no necrosis was seen in sites treated after administration of the W7. Necrosis of mean diameter 4.2 mm was seen in those given the protectant after light exposure. At tumour sites the area of necrosis was similar after light exposure before and after the administration of the protective agent. These results suggest a possible role for W7 in enhancement of selectivity of PDT action. Several mechanisms of protection against porphyrin phototoxicity by these drugs have been proposed, including acceleration of photobleaching. We used fluorescence to detect AlSPc in strips of rat colon before and after laser treatment, with and without W7. However, a primary role for the photobleaching of AlSPc as the mechanism for the protection shown is not supported by these observations.

INTRODUCTION

Photodynamic therapy (PDT)† involves the interaction of light with an administered photosensitizing drug to produce cellular damage. Photodynamic therapy has attracted considerable interest as the sensitizing drugs are retained with some degree of selectivity in malignant tumours and it was hoped that this could be exploited for selective destruction of tumours. However, a major disadvantage with this technique using the currently available sensitizers is poor selectivity of uptake, which does not exceed a ratio of 1:3 normal tissue:tumour in any tissue other than the brain (Tralau *et al.*, 1987). Many tumours invade normal tissue; therefore, to enable complete eradication of malignant cells a considerable amount of damage to the surrounding normal tissue is likely to occur. True selectivity of PDT can be obtained at very low doses of AlSPc (0.5 mg/kg) but to achieve this, only 2 mm of necrosis is possible in the tumour with no damage to the normal colon (Barr *et al.*, 1990). In order to increase tumour damage the AlSPc dose may be increased but this will lead to some normal tissue damage. To avoid normal tissue damage we have studied the value of using a radioprotective drug. These agents have been developed to protect against ionizing radiation and have shown promising results in experimental animals (Yuhas and Storer, 1969; Phillips *et al.*, 1973).

Radioprotective agents have been found to protect normal tissues in both the skin (Dillon *et al.*, 1988) and the eye (Roberts *et al.*, 1990) against porphyrin induced phototoxicity. These agents are believed to preferentially accumulate in normal tissues due to their hydrophilic properties (Yuhas, 1980). Additionally many tumours do not contain alkaline phosphatase which is required to remove the phosphate group; as a consequence malignant tissue is not able to activate the protective thiol component (Calabro-Jones, 1985). We have investigated a phosphorylated thiol protectant WR-77913 (W7) to determine if it shows the same potential to protect normal tissue during PDT, with the photosensitizer aluminium sulphonated phthalocyanine (AlSPc), in the rat colon.

Several mechanisms of protection have been proposed (Dillon *et al.*, 1988; Roberts *et al.*, 1990). One is scavenging of singlet oxygen by the released sulphydryl of the radioprotectant. Sulphydryls scavenge singlet oxygen in the order of 10^6 mol/s and hydrated electrons at diffusion controlled rates. In addition, sulphydryls are excellent scavengers of free radicals (Millar and Henderson, 1986). Another possibility, which has been proposed for the photoprotection against phototoxicity induced by porphyrins, is the photobleaching of the sensitizer itself by sulphydryls.

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†*Abbreviations*: AlSPc, aluminium sulphonated phthalocyanine; CCD, charged-coupled device; DMH, dimethylhydrazine; HpD, hematoporphyrin derivative; PDT, photodynamic therapy; WR-77913, 3-amino-2-hydroxypropyl phosphorothioate.

The following experiments were undertaken to determine if the W7 could protect normal colonic tissue from PDT damage whilst allowing damage to occur to the tumour, and whether this tumour damage was of the same magnitude irrespective of presence or absence of protectant. A further set of experiments was performed to test the hypothesis that W7 protects the normal tissue by enhancement of photosensitizer photobleaching.

MATERIALS AND METHODS

The sensitizer AlSPc was obtained from Ciba-Geigy (Basel, Switzerland) and dissolved in normal saline at a concentration of 2 mg/mL. This solution was stored in the dark. The W7 (3-amino-2-hydroxypropyl phosphorothioate) protectant was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. This powder was stored at −4°C until required; portions were then dissolved in normal saline and used within 24 h. Colonic tumours were chemically induced in male Wistar rats using dimethylhydrazine (DMH) (Aldrich Chemical Co., Dorset, UK) as reported by Filipe (1975).

Rats found, at laparotomy, to have two colonic tumours were injected intravenously with 5 mg/kg AlSPc 24 h before light exposure. W7, when introduced, was given intravenously at a dose of 200 mg/kg 30 min before laser treatment. The light source used was a copper vapour pumped dye laser (Oxford lasers) which was at all times set to deliver 100 mW for 500 s (50 J), from a 200 μm fibre, at a wavelength of 675 nm (maximum absorption for AlSPc). All injections and subsequent procedures were carried out under general anesthetic from intramuscular Hypnorm (fentanyl and fluanisone) (Janssen Pharmaceutical Ltd., Oxford, UK). Laser treatments were performed at laparotomy with the fibre just touching the normal colonic mucosa, or inserted into the apex of the tumour at a depth of 1 mm.

Each animal underwent the following procedures. An area of normal colon and a tumour were treated with the laser at 24 h after sensitizer administration. Immediately after this the W7 was administered. The animal was then treated at separate normal and tumour sites 30 min after the protectant had been injected and in this way each animal acted as its own control. The fibre was then removed and the abdomen closed, allowing recovery from the anaesthetic. The animals were killed 72 h later when mucosal damage, when present, is at its maximum (Barr *et al.*, 1987). The method of assessment of necrotic tissue has been defined in detail in Barr *et al.* (1987). In brief, necrosis, when produced, was represented by a sharply defined area of coagulative necrosis, which, particularly in normal colon, tended to slough off to leave an ulcer. To quantify the extent of necrosis at any treated laser site both the greatest and the smallest diameter of damage were recorded and the mean diameter calculated. Further animals, all with normal colons, were treated at only one site with identical parameters to the previous group of animals, but no protectant was introduced at any stage. This control group was studied to determine whether W7 had caused an effect on lasered sites post treatment.

For fluorescence imaging studies of normal colon treated *in vivo* a charged-coupled device (CCD) imaging system was used. This highly sensitive system has been described elsewhere (Barr *et al.*, 1988) and enables quantitative fluorescent imaging of AlSPc in tissues. Fluorescence was excited using an 8 mW helium neon laser operating at 632.8 nm and fluorescence was detected in the range 660 to 700 nm. Samples were illuminated with a 2 cm radius beam of uniform intensity and fluorescence was imaged onto the CCD using a 50 mm macro camera lens. Rats with normal colons were treated once 24 h after AlSPc administration, either 30 min after W7 introduction or with no W7 present, using identical drug concentrations and laser parameters as specified above. Animals were sacrificed immediately after laser treatment and their colons removed. Strips of treated colon and an adjacent untreated area were opened and cleaned. They were then mounted on glass slides with the colonic mucosa uppermost. This enabled quantitative comparison of intensity of fluorescence at the treatment site with fluorescence at an untreated portion. Control strips were also examined to enable quantification and exclusion of autofluorescence from unsensitized normal colon.

Table 1. Mean diameter of necrosis after PDT (mm) (Mean ± 1 SD (number of animals))

	Tumour tissue	Normal tissue
Light exposure before W7	4.4 ± 0.5 (3)	4.2 ± 1.2 (3)
Light exposure after W7	3.5 ± 0.9 (3)	0.0 (3)
AlSPc only (no W7)	—	4.1 ± 1.1 (5)

Each animal was sensitized with 5 mg/kg AlSPc; 24 h later a tumour and an area of normal colon were treated with 50 J of light. Immediately after this, 200 mg/kg of W7 were administered and 30 min later a second tumour and second area of normal colon were also treated with 50 J of light. Animals without tumours were used as an additional control and not given W7.

RESULTS

To determine whether W7 was allowing protection of normal colon during PDT, we compared the mean diameter of necrosis of normal and tumour tissue after PDT, before, and after, introduction of W7. These data are shown in Table 1. In normal colon no necrosis was observed macroscopically in sites treated 30 min after administration of the protectant (n=3). (This observation was confirmed histologically.) Necrosis of mean diameter 4.2 mm (n=3) was seen at sites given the protectant after light exposure, and this was not significantly different from lesions produced with the same parameters in animals not given the protectant at any time (95% confidence limits), where a mean diameter of necrosis 4.1 mm (n=5) was obtained. Therefore a 100% reduction in necrosis in normal tissue can be observed using W7 and this difference is significant (95% confidence limits). At tumour sites the mean diameter of necrosis was reduced by 20% (n=3), although this difference is not significant (95% confidence limits).

To test the hypothesis that protection is conferred by enhancement in the rate of bleaching, we examined the AlSPc fluorescence in strips of normal colon treated *in vivo* with 50 J at 675 nm. From the acquired images we obtained transverse line scans of the fluorescence which bisected the laser irradiated

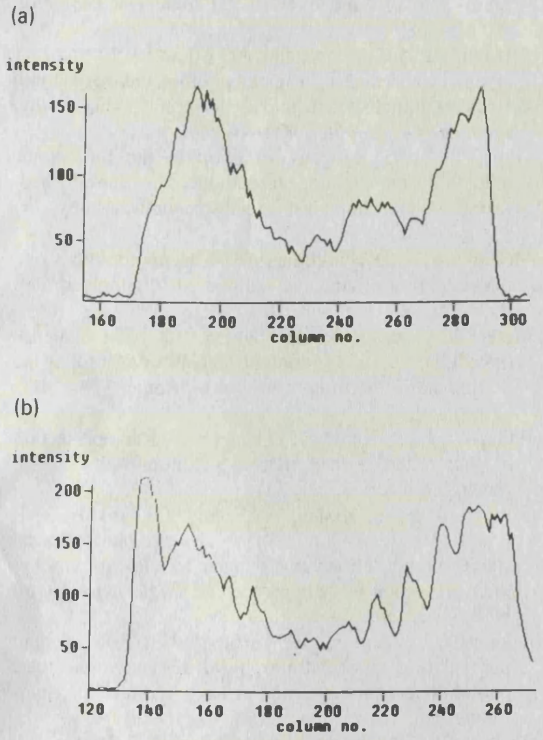

Figure 1. Transverse line scans of CCD images of sensitised normal colon after *in vivo* laser treatment (50 J). Lines bisect irradiated zone. AlSPc fluorescence intensity (arbitrary units) vs pixel number; length of scan is 150 pixel corresponding to 11 mm. (a) scan with AlSPc (5 mg/kg) present (no W7); (b) scan with AlSPc (5 mg/kg) and W7 (200 mg/kg) present. In each case the line scans demonstrate that the signal level in the centre of the irradiated zone is approx. 70% lower than the distal areas which received a significantly lower light dose. The fluctuating profile particularly notable in (b) results from the uneven mucosal surface.

zone. Two typical scans are shown in Fig. 1 (a) with AlSPc but no W7 present, and (b) with AlSPc and W7 present. The fluorescence level is lowest in the laser treated area, and this reduction is attributed to photobleaching of the phthalocyanine (Barr *et al.*, 1990). The line scans demonstrate that the signal level in the centre of the irradiated zone in each case is approximately 70% lower than the distal areas which received a significantly lower light dose. A series of five colon strips in each group were scanned for comparison. The degree of fluorescence bleaching in the centre of the irradiated zone compared to distal areas was found to be (a) 70 ± 10% without W7 and (b) 65 ± 10% with W7 present. The two results are comparable and given the errors involved no difference is discernible. The presence of W7 did not affect the AlSPc fluorescence level in non-irradiated tissue.

DISCUSSION

Much of the interest in PDT centres on photosensitizers being selectively retained by tumour tissue compared with normal tissue. This assumption is only of major importance in brain where a 28:1 tumour:normal ratio has been reported using AlSPc. In the DMH induced colonic tumour model, utilized in this study, this ratio is as low as 2:1 (Tralau *et al.*, 1987). Using radioprotective drugs we aimed to overcome this selectivity problem. Radioprotective agents, although developed to protect against ionizing radiation, have shown potential for normal tissue sparing during PDT. Dillon *et al.* (1988) have shown reduction of porphyrin-induced phototoxic damage to mouse skin *in vivo* with protective drugs WR-2721 and WR-77913. We investigated the latter drug (referred to here as W7) to determine if we could obtain the same reduction in phototoxic damage to normal colon using AlSPc as the photosensitizer, and to establish whether the extent of tumour damage was consistent to that without protectant. In this study we have obtained complete and selective protection of normal colon by administration of W7 prior to laser treatment. No protection was observed if the W7 was given after light treatment. Tumour tissue exhibited little or no protection. These results indicate a possible role for W7 in the enhancement of selectivity of PDT in the colon, warranting a more detailed study. With manipulation of protectant dose, and time of administration prior to light exposure, it may be possible to use higher light and photosensitizer doses to allow more complete tumour eradication without significant damage to surrounding normal tissue.

Several mechanisms of protection by radioprotective drugs during PDT have been proposed, as outlined above. The one considered in this paper was enhanced photobleaching by the thiols *in vivo*. This effect has been demonstrated *in vitro* with solutions of porphyrins and eye lens protein (Dillon *et al.*, 1988). The steady state photolysis of porphyrin resulted in a 12% decrease in absorptions in the Soret region. After addition of the thiol penicillamine the photobleaching increased to give a 38% reduction in absorption for the same light dose. Aluminium sulphonated phthalocyanine does not photobleach in aqueous solution (McCubbin and Phillips, 1986) but has been shown to bleach in solutions containing amino acids (Ferraudi *et al.*, 1988) and *in vivo* during PDT (Barr *et al.*, 1990). In our experiments in normal rat colon, photobleaching reduced the fluorescence by 70% and this figure did not change in the presence of W7. We would therefore conclude that even if photobleaching is enhanced to a minor extent, this factor can only be of secondary importance. In this case of AlSPc photosensitization the mechanism of protection in normal tissue may rely on scavenging and deactivation of reactive intermediates (e.g. free radicals, singlet oxygen, peroxides) by the hydrolysed form of the protectant containing free sulphydryl groups, as is proposed to occur with endogenous thiols e.g. glutathione (Miller and Henderson, 1986;

Thomas and Girotti, 1989; Roberts *et al.*, 1990).

In summary, by using the radioprotective agent W7 we can improve the selectivity of PDT in the rat colon, and this may allow us to treat larger tumours without significant damage to the surrounding normal tissue. These improvements are unlikely to be due to enhanced photobleaching of AlSPc.

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Distribution and photodynamic effect of disulphonated aluminium phthalocyanine in the pancreas and adjacent tissues in the Syrian golden hamster

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Summary Necrosis of small volumes of tumour tissue with photodynamic therapy (PDT) can be achieved relatively easily. For this to be clinically relevant, it is essential to know what the same treatment parameters do to adjacent normal tissues into which the tumour has spread. For pancreatic cancers, local spread to vital structures is common. We have studied chemical extraction, microscopic fluorescence kinetics and photodynamic effects of disulphonated aluminium phthalocyanine (AlS_2Pc) in normal pancreas and adjacent tissues in hamsters. Chemical extraction exhibited a peak duodenal concentration of AlS_2Pc 48 h after sensitisation, with levels much higher than in stomach and pancreas. With microscopic fluorescence photometry highest levels were seen in duodenal submucosa and bile duct walls 48 h after photosensitisation. Pancreatic ducts, duodenal mucosa and gastric mucosa and submucosa exhibited intermediate fluorescence with relatively weak fluorescence in pancreatic acinar tissue and the muscle layer of the stomach. As expected, on the basis of fluorescence intensity and chemical extraction studies, the duodenal and bile duct wall were the most vulnerable tissues to photodynamic therapy. When the dose of 5 μmol kg^{-1} of sensitiser was used, duodenal perforations, gastric ulcers and transudation of bile from the bile duct occurred. However, the lesions in the stomach and bile duct healed without perforation or obstruction, so only the duodenum was at risk of serious, irreversible damage. Using a lower dose of photosensitiser markedly reduced damage.

It has been shown in many publications that it is relatively easy to destroy small volumes of a wide variety of tumours with PDT (Li *et al.*, 1990; Barr *et al.*, 1991). However, what matters to a patient is whether the entire tumour volume can be destroyed without unacceptable damage to the adjacent normal tissues. This means that it is essential to understand what happens in the region where the tumour is invading normal areas. Surprisingly little work has been done on this aspect (Bown, 1990). Although much of the interest in PDT has centred around the possibility of selective destruction of tumours, this aspect is almost always over emphasised, and getting true and complete selective destruction of cancers is close to impossible (Barr *et al.*, 1990 and 1991). Thus the challenge is to understand what PDT does to normal tissues using treatment parameters that will destroy tumour invading that region. A previous report (Schroder *et al.*, 1988) showed that PDT will produce necrosis in a chemically induced pancreatic cancer in hamsters using dihaematoporphyrin ether (DHE) but at the price of duodenal perforation. The aim of the present study is to look at the effect of PDT on the normal pancreas and adjacent tissues using treatment parameters similar to those known to given pancreatic tumour necrosis to identify which normal tissues are most vulnerable to PDT damage and to find ways to minimise this damage.

Haematoporphyrin derivative (HPD), and purified fractions thereof, are the only photosensitisers currently available for clinical PDT but unfortunately are far from ideal. The properties of more suitable photosensitisers have been identified and sulphonated metallophthalocyanines have been extensively studied in this regard (Bown *et al.*, 1986; Brasseur *et al.*, 1985 and 1987; Tralau *et al.*, 1987; Paquette *et al.*, 1988; Peng *et al.*, 1990), and advantages demonstrated over HPD, including studies involving direct comparison with HPD. The main advantages (Ben-Hur *et al.*, 1987) of phthalocyanines include their strong absorption above 650 nm, where the light penetration of tissue is good, photochemical and thermal stability in solution, relatively well defined chemistry and lower skin photosensitivity to sunlight than HPD (Roberts *et al.*, 1989; Tralau *et al.*, 1989). Both the porphyrins and phthalocyanines may be photodegraded *in vivo* (Potter *et al.*, 1987; Barr *et al.*, 1990). Most of the PDT studies using aluminium sulphonated phthalocyanine (AlSPc) as a photosensitiser, have been carried out using a mixture of compounds with different degrees of sulphonation (range of one to four sulphonated groups, AlSnPc, where n is 1, 2, 3 or 4; average being 3,2 (Barr *et al.*, 1990; Tralau *et al.*, 1987)). From recent studies (Paquette *et al.*, 1987; Berg *et al.*, 1989; Chan *et al.*, 1990; Chatlani *et al.*, 1991*a*) disulphonated aluminium phthalocyanine is a more potent photosensitiser than the tetrasulphonated derivative both for *in vitro* and *in vivo* studies. For these reasons we have selected the disulphonated fraction, AlS_2Pc, for this study.

Chatlani *et al.* (1991*b*) have shown that necrosis can be produced by PDT in the same hamster pancreatic cancer model as used by Schroder using AlSPc as the photosensitiser. As a prelude for studying the effect of PDT in pancreatic neoplasms, we carried out studies in normal hamsters on the pancreas and its adjacent tissues (duodenum, stomach, bile ducts, portal vein and the main arteries) using treatment parameters similar to those shown by Chatlani (1991*b*) to produce tumour necrosis. In this paper we studied the distribution of AlS_2Pc by both chemical extraction and fluorescence microscopy (Barr *et al.*, 1988; Chan *et al.*, 1989). It has been shown by others that there is good correlation of concentrations measured by fluorescence intensity with those determined by chemical extraction, both with HPD and selectively sulphonated phthalocyanines (Mang *et al.*, 1987; Chatlani *et al.*, 1991*a*). We also studied the necrosis produced by PDT using high and low sensitising doses of AlS_2Pc to establish when damage to normal tissue might be unacceptable, and how this might be avoided.

Materials and methods

Female Syrian golden hamsters weighing 80 to 120 g were used in all experiments. AlS_2Pc was separated from an AlSPc mixture, prepared by the oleum sulphonation of aluminium phthalocyanine chloride, using reverse phase liquid chroma-

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tography (Ambroz *et al.*, 1991). This fraction as analysed by high performance liquid chromatography (HPLC) contains a range of disulphonated components dominated by the most hydrophobic component comprising 60 ± 5% of the integrated HPLC chromatograph. This particular component has been studied by Ambroz *et al.* for laser spectroscopic investigations, but is difficult to prepare in useful quantities without other components being present. The photosensitiser was administered in isotonic saline by an intracaval injection at laparotomy and the animals were killed 1, 3, 48 and 168 h after the injection. For chemical extractions and fluorescence microscopy studies, the dose of photosensitiser – 5 μmol kg^{-1} – was chosen on the basis of previous studies (Tralau *et al.*, 1987; Chatlani *et al.*, 1991*a*). Tissue samples consisting of pancreas, the free edge of lesser omentum, duodenum and middle-distal parts of stomach plus aorta and vena cava were removed at postmortem and immediately frozen using isopentane in a vessel in liquid nitrogen for subsequent fluorescence studies on 10 μ frozen sections. Adjacent tissue samples of pancreas, stomach and duodenum were removed and stored at −4°C prior to chemical extraction. The excretion of AlS_2Pc into bile was studied by cannulating the common bile duct under general anaesthesia and collecting the bile. This was done 1/2 hourly over two 4 h periods (0–4 and 4–8 h) and at 24, 48 and 168 h after sensitisation.

Extraction of AlS_2Pc

Phthalocyanine was extracted from thawed tissues using 0.1 M NaOH (ratio 0.1 g wet tissue 10 ml^{-1} 0.1 M NaOH) for 4 h in a 50°C water bath. The total tissue phthalocyanine concentration was measured in the supernatant using spectrofluorimetry with calibration against standard curves of known AlS_2Pc concentration (Chan *et al.*, 1988).

Fluorescence microscopy and photometry

An inverted microscope (Olympus IMT-2) with epifluorescence and phase-contrast attachments was used, as described previously (Chan *et al.*, 1989). Fluorescence excitation was carried out with an 8 mW helium-neon laser (632.8 nm), with the beam directed through a liquid light guide (via a 10 nm band-pass filter, centred at 633 nm, to remove extraneous light) onto the dichroic mirror (Omega Optical Inc.) for epifluorescence studies. The phthalocyanine fluorescence was detected between 665 and 700 nm using a combination of band-pass (Omega Optical Inc.) and long-pass (Schott RG665) filters. The imaging detector was a highly sensitive cryogenically cooled CCD (charge-coupled device) camera (Wright Instruments, model 1, resolution 400 × 600 pixels) fitted to the microscope. Image processing and camera operation were carried out by computer. The values for mean fluorescence intensities were calculated by image processing software (Wright Instruments) within rectangular areas of variable size (e.g. 50 × 75 pixels) corresponding to sites of interest. The sections used for fluorescence microscopy were subsequently stained with haematoxylin and eosin for later visual comparison using light microscopy and photography.

The combination of phase contrast microscopy of the frozen sections and light microscopy of haematoxylin-eosin stained adjacent tissue sections enabled different structures of the tissues (serosa, muscle layer, submucosa, mucosa, vessel wall, pancreatic and bile duct wall and acinar pancreas) to be identified in the fluorescence image and the fluorescence intensity of these structures to be measured (Chatlani *et al.*, 1991*a*).

Photodynamic therapy

Light of wavelength 675 nm (peak absorption for AlS_2Pc) from a pulsed (12 kHz) copper-vapour pumped dye laser (Oxford Lasers) was delivered via a 200 μm fibre just touching the surface of the tissue to be irradiated. Control experiments were carried out by looking at the effects of 50 and 100 mW powers at the fibre tip (50J, in either case) on unsensitised hamsters. No thermal effect was detected on stomach or duodenum at 50 mW, although minor changes (oedema and necrosis 0.5 × 1.0 mm) were seen in the pancreas. In contrast 100 mW produced thermal effects, with oedema and necrosis up to 3–4 mm in extent in the pancreas, duodenum and stomach, noted on necropsy specimens, taken at sacrifice 72 h after light exposure. Therefore, 50 mW (X 1000s = 50J energy delivered) was the power chosen for PDT with the fibre tip located on the pancreas (adjacent to stomach or duodenum), the free edge of lesser omentum or the vena cava and aorta, 48 h after sensitisation.

Since with a dose of 5 μmol kg^{-1} AlS_2Pc, PDT produced duodenal perforations and bile duct necrosis, we also used a smaller dose of sensitiser (1 μmol kg^{-1} AlS_2Pc) with the same laser power setting and exposure time (50 mW, 1000 s). The animals were killed 72 h after light exposure and changes in treated tissue were studied by macroscopic examination and subsequent light microscopy of haematoxylin and eosin stained sections. Four further animals were treated with 5 μmol kg^{-1} AlS_2Pc and 50J light, but the duodenum was shielded from the light by gentle mobilisation and insertion of a piece of opaque paper between the duodenum and the fibre tip. These animals were killed 2 weeks after treatment and the treated areas removed for histological examination.

Results

Chemical extractions

The results are shown in Figures 1 and 2. The concentration of AlS_2Pc was greatest in duodenal wall 48 h after sensitisation (Figure 1). Stomach and pancreas showed relatively low concentrations at all time points studied. The results are expressed in nmol g^{-1} of tissue (3–5 animals for each point).

The excretion of bile in these animals was fairly constant at approximately 0.1 ml h. The concentration of AlS_2Pc in bile with time is shown in Figure 2. The peak value was seen 3 h after an intravenous injection of 5 μmol kg^{-1} AlS_2Pc and only fell slightly over the next 4 h, but fell to 50% over 24 h. Thereafter, the excretion declined slowly during the following 6 days.

Fluorescence microscopy and photometry

Changes in fluorescence intensity levels in pancreas and adjacent tissues with time are shown in Figure 3, 4 and Table I.

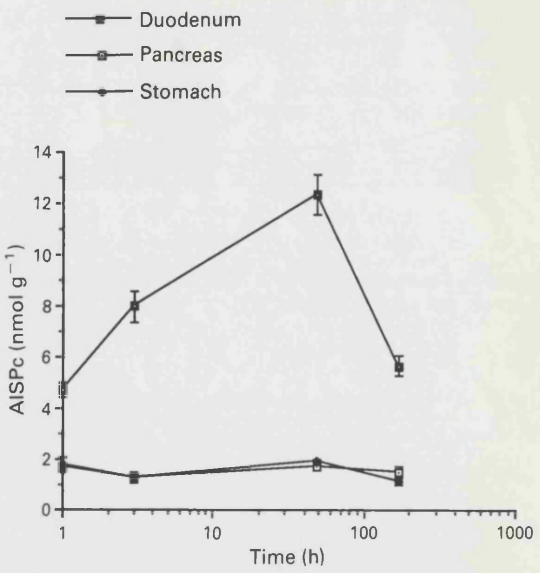

Figure 1 Concentration of photosensitiser (±s.d.) measured by chemical extraction *vs* time from sensitisation with AlS_2Pc 5 μmol kg^{-1}. All layers of the duodenal and gastric wall were included.

A wide range of values was found. Highest fluorescence was present in duodenal submucosa and in bile duct wall 48 h after photosensitisation and had only decreased slightly by 168 h. Artery wall and serosa exhibited high fluorescence intensity at the first timepoint (1 h) but showed decreasing values at all subsequent timepoints. Pancreatic ducts and duodenal mucosa showed intermediate levels with peak values at 3 h. Weaker fluorescence was noted in pancreatic acinar tissue and in the gastric muscle (Figures 6–10).

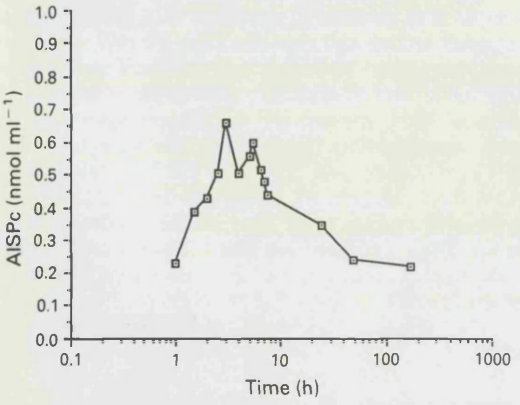

Figure 2 Concentration of AlS_2Pc in bile. Logarithmic scale for time (two animals studied at each time point).

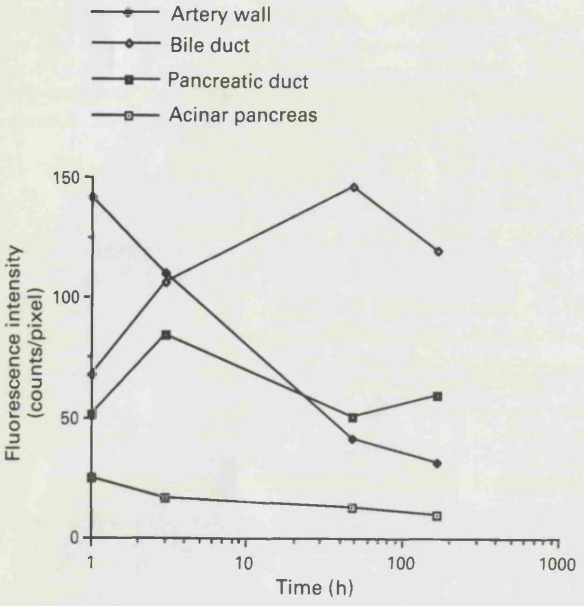

Figure 3 Fluorescence kinetics of AlS_2Pc in different parts of the pancreas and adjacent tissues.

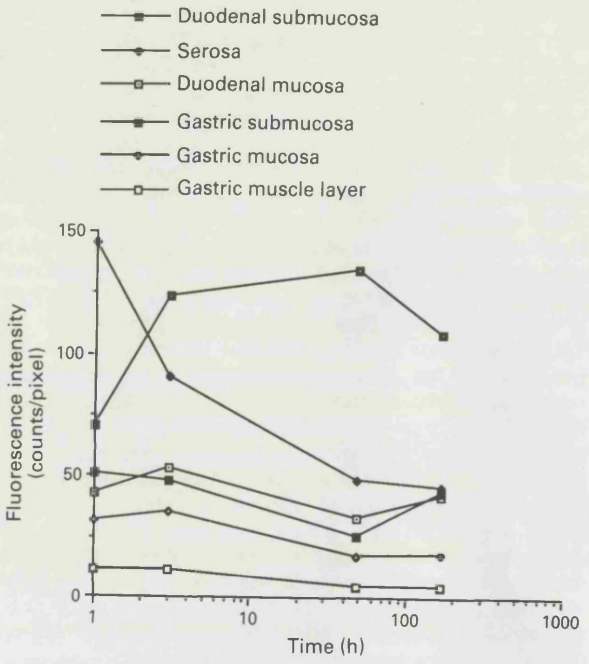

Figure 4 Fluorescence kinetics of AlS_2Pc in different structures of gastric and duodenal wall.

Photodynamic effects

To avoid thermal effects, a laser power of 50 mW was used throughout the study. For studies on the pancreas either the duodenal or gastric lobe of the pancreas (only one point in each animal) was treated. With the higher dose of AlS_2Pc, extensive necrosis of the duodenal wall with perforations was noted 3 days after light exposure (Table II). Small areas of necrosis were seen in the pancreas. There were no gastric perforations, but deep necrotic ulcers with full thickness necrosis were seen in the gastric wall, which were sealed by omental fat or the edge of the liver. When the fibre tip was sited on the common bile duct in the free edge of the lesser omentum, necrosis was seen in the common bile duct and gallbladder wall causing bile to diffuse through the wall with yellow staining of adjacent tissues. No perforation of bile ducts or gallbladder wall was discovered. The liver parenchyma close to the position of the fibre tip also showed necrosis. Because of the severe photodynamic effects with the higher dose of sensitiser, 5 μmol kg^{-1}, we carried out further experiments reducing the dose to 1 μmol kg^{-1}. With the same light dose (50J) there was no photodynamic effect seen in the pancreas, and only mild damage with erosions or small necrotic ulcers in the stomach. However, a concealed duodenal perforation was discovered in one of the two animals treated with the fibre placed on the pancreas next to the duodenum. In the animals treated with duodenal shielding and killed after 2 weeks, there was no evidence of obstruction or perforation of the bile duct, duodenum, stomach or blood vessels.

Table I Fluorescence photometry (counts/pixel) (mean ± s.d.) with time from photosensitiser injection

Time	*Pancreas*	*Duodenum*		*Stomach*			*Bile*	*Pancreatic*	*Artery*	
(h)	*acinar*	*Mucosa*	*Submucosa*	*Mucosa*	*Submucosa*	*Muscle*	*duct*	*duct*	*wall*	*Serosa*
1	25±2	43±8	71±9	32±9	51±10	12±5	68±9	52±4	142±16	146±19
3	16±2	53±4	124±15	35±12	48±12	11±1	106±0	85±9	110±12	94±9
48	13±7	33±7	135±8	17±6	26±7	5±3	146±29	50±12	42±9	48±16
168	10±3	42±14	109±12	18±5	44±12	5±2	120±13	59±16	31±7	46±9

Fluorescence values are presented as 'arbitrary units' counts/pixel (±s.d.) corrected for autofluorescence. Measurements of the bile duct included all structures of the bile duct wall as well as the main arteries around the pancreas. Measurements of the arteries included all layers of the arterial wall. The fluorescence intensity of the serosa was measured from the serosa located between the pancreas and stomach.

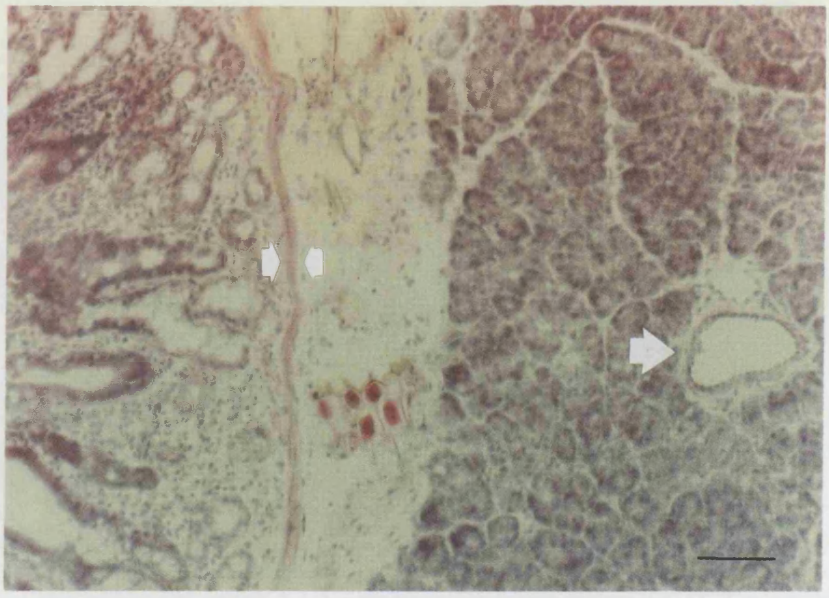

Figure 5 Microscopic picture of duodenal wall (left) and pancreas (right) with H-E staining showing the extremely thin muscular layer of the duodenum (small arrows), the connective tissue with vessels between pancreas and duodenum and a cross-section of the main pancreatic duct (large arrow). Scale: the bar (14 mm) represents 80 μm.

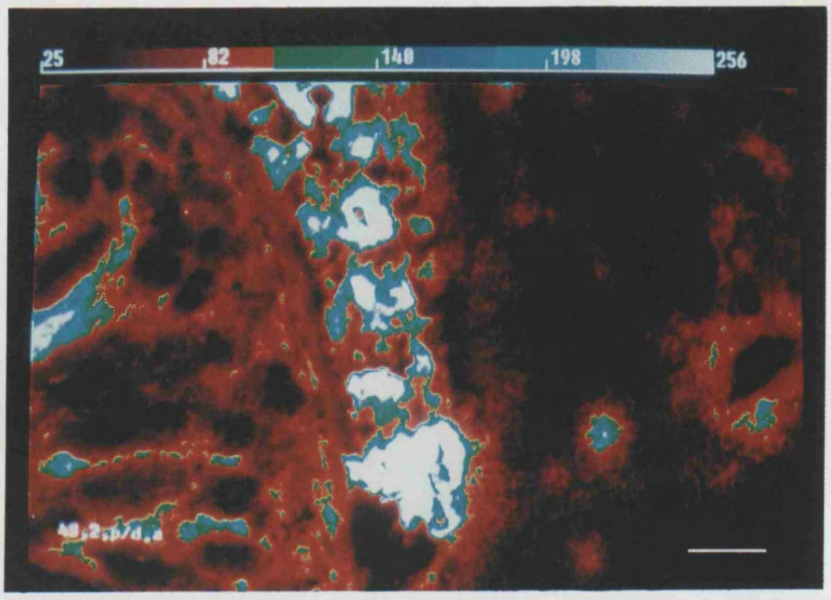

Figure 6 Fluorescence micrograph of adjacent section to that shown in Figure 5. The colour scale is shown at the top of the micrograph. Maximum fluorescence (1024 counts/pixel) is represented by white. Fluorescence is seen in all structures, but is highest in duodenal submucosa (white and blue) and connective tissue between pancreas and duodenum 48 h after sensitisation. The pancreatic duct exhibits intermediate fluorescence (blue, green and light brown) and the acinar pancreas shows low fluorescence (dark brown). Same magnification as in Figure 5 (the bar represents 80μm).

Discussion

To understand the tissue effects produced by PDT, it is essential to know the distribution of the photosensitiser at the microscopic level. Fluorescence microscopy has high sensitivity and has made possible quantitative measurements of photosensitiser distribution. Good correlation between chemical extraction and fluorescence intensity values has been reported previously in normal rat colon sensitised with sulphonated phthalocyanines (Chatlani *et al.*, 1991*a*). On the microscopic level, the distribution and behaviour of AlSPc depends on the tissues studied and the degree of sulphonation of the dye (Chan *et al.*, 1990; Chatlani *et al.*, 1991*a*). Reduction in the number of sulphonated groups in AlSPc increases lipophilicity (Berg *et al.*, 1989), which favours the rapid transport of the dye through cell membranes. Furthermore, the less sulphonated fraction (S/Pc ratio 2.0 = AlS_2Pc) was shown to be 25 times more efficient in photoinactivation of hamster lung fibroblasts than the more sulphonated fraction (S/Pc ratio 3.6) (Paquette *et al.*, 1988).

In view of its potent qualities as a photosensitiser, AlS_2Pc was chosen for our studies. It is clear from these results that

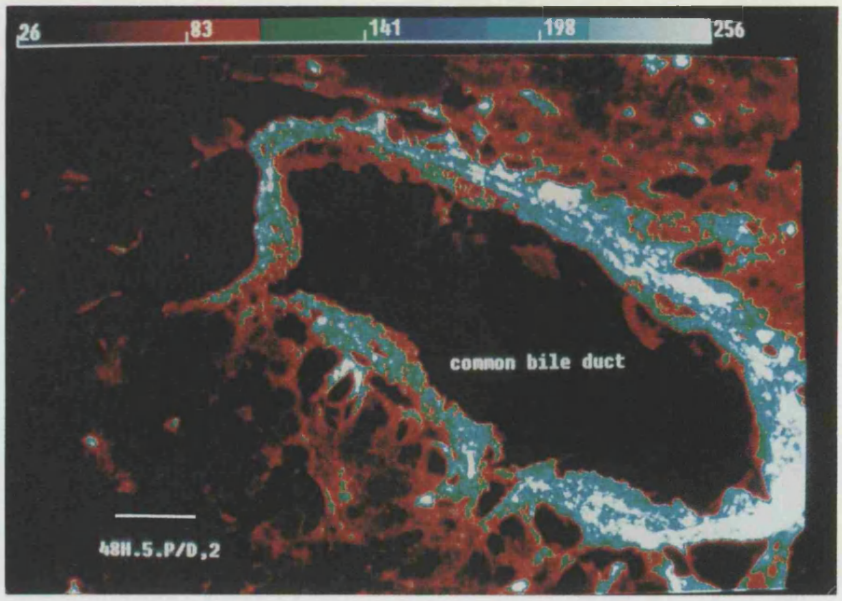

Figure 7 Fluorescence micrograph of common bile duct. High fluorescence in the bile duct wall 48 h after sensitisation compared with adjacent pancreatic tissue as shown in Figure 10. The colour scale and magnification same as in Figure 6.

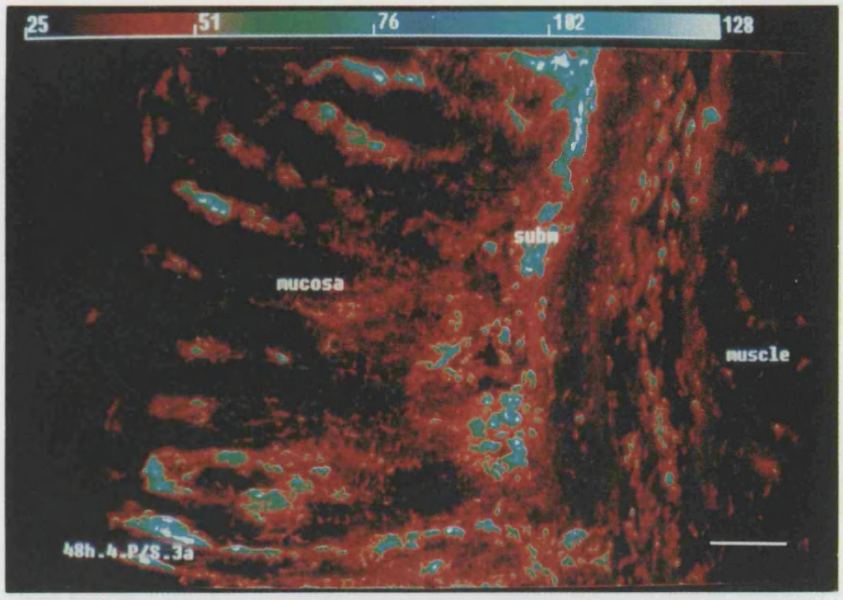

Figure 8 Fluorescence micrograph of stomach wall 48 h after sensitisation. Note that the maximum fluorescence is half of that in Figures 6, 7, 9 and 10. Muscle layers exhibit low fluorescence (right) and intermediate fluorescence is seen in the submucosa (subm). Same magnification as in Figure 6.

some of the normal tissues in the vicinity of the pancreas are vulnerable to serious PDT damage, although others are not. With the higher dose of sensitiser, extensive duodenal wall necrosis with perforations and deep gastric ulcers were seen. Our finding is in accordance with that shown in a previous study (Schroder *et al.*, 1988), which also showed vulnerability of hamster duodenal wall to PDT. They used DHE as the sensitiser and exposed the pancreas to laser light 3 h after sensitisation. We also showed damage to the bile duct when the fibre was positioned on the common bile duct in the free edge of lesser omentum. The portal vein and the main arteries adjacent to the pancreas were not damaged. Pancreatic necrosis was detected when the fibre tip was positioned on the surface of the gland. Maximum PDT damage was expected to the duodenal wall and common bile duct on the basis of the fluorescence intensity (Figures 3 and 4) and chemical extraction studies (Figure 1) and indeed this was seen. We tried reducing the dose of AlS_2Pc to 1 μmol kg^{-1}, but even at this level, a concealed duodenal perforation was discovered. There seem to be two possible explanations for the undesirable duodenal damage. Firstly, the concentration of sensitiser is particularly high at the time chosen for light exposure (48 h after sensitisation). Secondly, the muscle layer of the hamster duodenal wall is extremely thin, only 60–300 μm thick, although earlier work has shown that the main mechanical strength of colon after PDT damage comes from

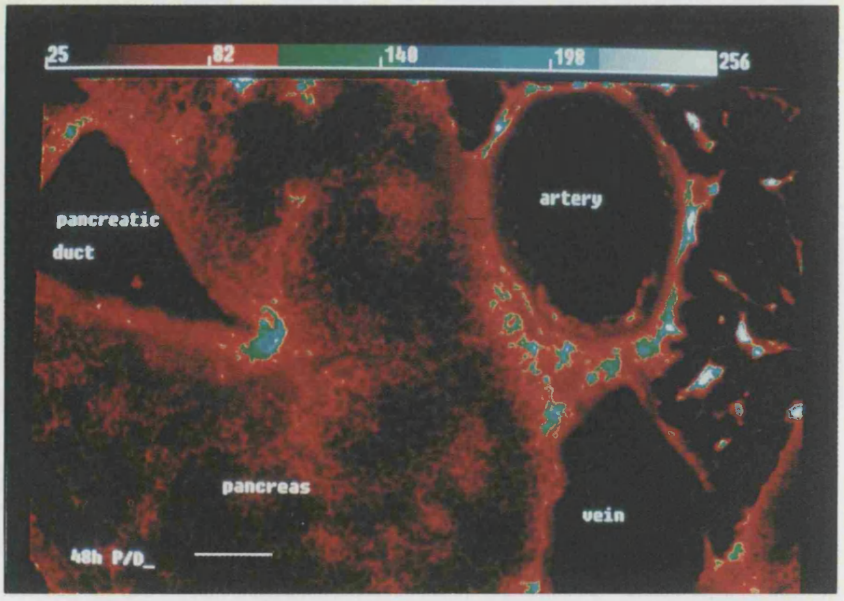

Figure 9 Fluorescence micrograph of pancreas including the main pancreatic duct and large vessels around the pancreas 48 h after sensitisation. Greatest fluorescence is shown in the connective tissue around the vessels. Same magnification as in Figure 6.

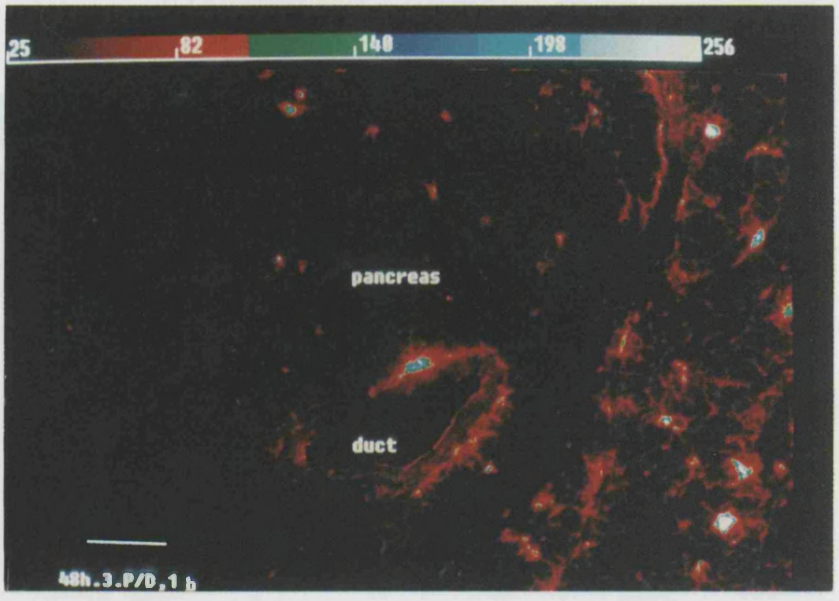

Figure 10 Relatively low florescence (dark brown) is seen in pancreatic parenchyma 48 h after sensitisation. Magnification as in Figure 6.

the collagen in the submucosa (Barr *et al.*, 1987) and so one would not expect necrosis in the muscle layer to lead to perforation. Our results suggest that both in the duodenum and the stomach the muscle wall plays some part in maintaining the integrity of the organ after PDT. There may be other reasons for the vulnerability of the duodenum. It was unexpected that the concentration of the photosensitiser should be so much higher in the submucosa of the duodenum than in the submucosa of the adjacent stomach (antrum and body). However, for measurements in duodenal submucosa, as there is no muscularis mucosae, we included the part of the submucosa that extends into the villus which has a dense capillary network suppported by collagen tissue (Wheater *et al.*, 1979) where high fluorescence was detected. In contrast, in the stomach where there is muscularis mucosae, the submucosa does not extend up into the villi and this may explain the differences found. The other organ at risk is the bile duct, high fluorescence (Figures 3 and 7) correlating with severe PDT damage although the duct did not perforate in any animal so the damage may be reversible. High levels of sensitiser in the bile duct wall could be due to high concentrations in bile although the relatively low excretion of AlS_2Pc into bile compared with AlS_2Pc values in plasma (unpublished data) did not support this possibility. Also, duodenal submucosa was highly fluorescent in contrast to the epithelium of the duodenal mucosa despite the latter being in closer contact with bile.

We conclude from these studies that the normal pancreas and the major blood vessels are relatively immune from PDT damage, and that the damage to stomach and bile duct is reversible and probably acceptable. The major problem seems to be the duodenum. Great care will be required if this

Table II Photodynamic effects 3 days after treatment

Sensitiser dose	*Site of laser fibre*	*Result*
5 μmol kg^{-1}	Pancreas adjacent to stomach ($n = 2$)	Full thickness necrosis, deep ulcers, no perforation ($n = 2$). Pancreatic necrosis.
5 μmol kg^{-1}	Pancreas adjacent to duodenum ($n = 2$)	Duodenal perforations ($n = 2$) with diffuse peritonitis, 1 dead at 48 h. Pancreatic necrosis.
5 μmol kg^{-1}	Free edge of lesser omentum ($n = 2$)	Transudation of bile. No macroscopic perforation of bile ducts ($n = 2$), but full thickness damage. No damage to portal vein or hepatic artery.
1 μgmol kg^{-1}	Pancreas adjacent to stomach ($n = 2$)	Partial thickness necrosis with deep ulcer ($n = 1$) and with superficial ulcer ($n = 1$). No pancreatic damage ($n = 2$).
1 μmol kg^{-1}	Pancreas adjacent to duodenum ($n = 2$)	Full thickness necrosis with sealed perforation ($n = 1$), partial thickness necrosis with ulcers ($n = 1$). No pancreatic damage ($n = 2$).
1 μmol kg^{-1}	Free edge of lesser omentum ($n = 2$)	No transudation of bile, minor necrotic areas in bile duct wall ($n = 2$). No damage to portal vein or hepatic artery. ($n = 2$).

PDT effects in animals killed at 72 h after exposure to 50J. All animals were treated 48 h after intravenous sensitisation with AlS_2Pc.

is treated in patients, although an organ as thick as the human duodenum may be much safer than the thin walled hamster duodenum. However, in the human situation, the scale of everything is so much larger that it will be much easier to limit the area exposed to laser light and avoid the duodenum. It is unfortunate that the duodenum is so vulnerable as there are some conditions that might be suitable for PDT in this region, such as small ampullary carcinomas in patients unsuitable for surgery. It is possible that experiments in larger animals may identify treatment conditions that are safe to use in the duodenum, but with the currently available information, it would be wise to exercise caution in this region.

In contrast, these results suggest that it may be possible to treat tumours of the bile ducts, as bile duct damage appears to heal safely without obstruction or perforation. This is supported by a recent case report (McCaughan *et al.*, 1991). Intraductal PDT using DHE as the sensitiser was administered to a woman, who had histologically proven adenocarcinoma of the common bile duct. The patient has had seven PDT treatments over the course of 4 years, with no jaundice and continues in relatively good health.

There is a long way to go before PDT could become relevant in the treatment of pancreatic cancer in man. PDT can only destroy small volumes of tumour tissue, and it would be essential to use it in conjunction with other techniques (e.g. surgery) for removing the main bulk of a tumour. It may be possible to apply PDT to the tumour bed to destroy remaining areas of tumour after resection of a lesion which is macroscopically limited to the pancreas. Our results suggest it would be safe to treat all the surrounding tissues, with the exception of the duodenum. The other major challenge for the clinical use of PDT is to know which areas to treat. PDT is a local treatment, and although the sensitiser is given systemically, tissue effects will only be produced where light is applied. It will be a diagnostic challenge to find out how far the tumour has spread. However, for normal tissues that are not sensitive to PDT damage or which recover from PDT satisfactorily, it should be safe to deliver light to them. PDT will be of most value when relatively large surfaces of normal tissue can be treated to pick up all the small areas of tumour that are not easily detectable.

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Fluorescence distribution and photodynamic effect of ALA-induced PP IX in the DMH rat colonic tumour model

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Summary Aminolaevulinic acid (ALA) is the first committed step in haem synthesis. In the presence of excess ALA the natural regulatory feedback system is disrupted allowing accumulation of protoporphyrin IX (PP IX) the last intermediate product before haem, and an effective sensitiser. This method of endogenous photosensitisation of cells has been exploited for photodynamic therapy (PDT). We have studied the fluorescence distribution and biological effect of induced PP IX in normal and tumour tissue in the rat colon. Fluorescence in normal colonic tissue was at a peak of 4 h with a rapid fall off by 6 h. The fluorescence had returned to background levels by 24 h. All normal tissue layers followed the same fluorescence profile but the mucosa showed fluorescent levels six times higher than the submucosa, with muscle barely above background values. At 6 h the ratio of fluorescence levels between normal mucosa and viable tumour was approximately 1:6. At this time laser treatment showed necrosis of normal mucosa and tumour with sparing of normal muscle. There was good correlation between the fluorescence distribution and the biological effect of ALA-induced photosensitisation on exposure to red light. ALA may be superior to conventional sensitisers for tumours that produce haem as the PP IX is synthesised in malignant cells while the other sensitisers mainly localise to the vascular stroma of tumours. There is also a greater concentration difference between the PP IX levels in tumours and in normal mucosa and normal muscle than with the other photosensitisers raising the possibility of more selective necrosis in tumours.

Photodynamic therapy (PDT) is concerned with the use of light to activate a photosensitising drug which results in the generation of cytotoxic species. Much work has been undertaken in this field with the same major problems becoming apparent. Although PDT has been based on the selective retention of photosensitising drugs by tumours this is clearly not the case with a maximal ratio of 3:1 tumour to normal tissue with the commonly used photosensitisers (Tralau *et al.*, 1987). At 48 h after an intravenous injection of aluminium sulphonated phthalocyanine (AlSPc) colonic tumours contained approximately twice as much sensitiser as normal colon. Fluorescence microscopy studies were performed on the same specimens showing significant photosensitiser accumulation in tumour stroma whereas tumour cells and normal mucosa contained similar amounts. Therefore selectivity between normal and tumour tissue is minimal and following exposure to red light considerable normal tissue damage is experienced where tumour tissue invades normal tissue (Barr *et al.*, 1991). New strategies are required to enhance PDT selectivity. Sensitiser photodegradation may be exploited to improve selectivity as demonstrated by Barr *et al.* (1990), who used low dose sensitisation with AlSPc in the colonic tumour model, although with this method the extent of tumour necrosis was considerably reduced. A further problem experienced with the best known clinical photosensitiser haematoporphyrin derivative (HpD) is the extended skin photosensitivity which may persist for months (Zalar *et al.*, 1977).

Skin photosensitivity and other side effects of porphyrin sensitisation are experienced by patients suffering from hepatic porphyria, with worsening health after treatment with barbiturates. These observations led to the discovery that administration of certain drugs and chemicals into a normal animal produced symptoms which mimicked those of hepatic porphyria and the condition is known as chemical porphyria. This work has been the subject of several review articles (Drabkin, 1963 and Granick, 1965). Chemical porphyria, although generally used as an experimental model for understanding the porphyria disease states, has now been suggested as a novel means of endogenous sensitisation for PDT (Divaris *et al.*, 1990). This approach involves the administration of 5-aminolaevulinic acid (ALA) which results in endogenous photosensitisation both in cultured cells (Malik & Djaldetti, 1979) and in whole animals (Sima *et al.*, 1981). ALA is present naturally in mammalian cells since it is the first committed intermediate in the haem biosynthesis pathway. Through the introduction of an excess of ALA either *in vitro*, or *in vivo*, the regulatory feedback system is overloaded causing an accumulation of porphyrin precursors to haem, particularly protoporphyrin IX (PP IX), an active *in vitro* photosensitiser. Divaris *et al.* (1990) have performed *in vivo* studies which showed that after intraperitoneal administration of ALA to mice PP IX accumulated in the skin in sufficient amounts to cause photosensitised damage on exposure to light. This group has also shown that the urothelium of the bladder and the endometrium in the uterus become highly sensitised following intraperitoneal administration of ALA, whereas the underlying layers in these organs exhibited relatively little sensitisation which could enable selective destruction of superficial cancers in the urothelium and endometrium without causing perforation to the bladder or uterus. Some promising results have recently been obtained in a clinical trial after topically applied ALA, which achieved a 90% complete response rate in the treatment of basal cell carcinomas (Kennedy *et al.*, 1990). It should be noted that direct administration of PP IX itself has been limited by the poor water solubility of this particular porphyrin.

The experimental studies presented in this paper have been undertaken on another hollow organ, the colon, using intravenous administration of ALA. The aim was to investigate and quantify the phorphyrin fluorescence in rat colonic tissue, both normal and tumour, and to determine microscopically which sites exhibited porphyrin accumulation as a function of dose and time after administration. These tissues were then treated with laser light for assessment of photosensitising activity and selectivity of damage. A comparison was made between the fluorescence distribution and the biological effect of ALA-induced photosensitisation.

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Materials and methods

5-ALA was obtained from Sigma Chemical Company (UK), with a purity of 98%, dissolved in phosphate buffered saline at a concentration of 80 mg ml^{-1} and used within 24 h. ALA was administered intravenously in doses of up to 200 mg kg^{-1} via tail vein injection to Wistar rats (weighing 125–200 g) under general anaesthetic from intramuscular Hypnorm (Fentanyl and fluanisone, Jansen Pharmaceuticals Ltd.). Colonic tumours were chemically induced in male Wistar rats using dimethylhydrazine (DMH) (Aldrich Chemical Company, UK) as reported by Filipe (1975).

Fluorescence microscopy studies

Imaging and quantification of fluorescence in sections of normal and tumour tissue were achieved using fluorescence microscopy. At 5 and 30 min and 1, 2, 3, 4, 5, 6, 8, 17 and 24 h following administration of ALA the animals were killed and normal, or tumour, tissue removed from the colon and immediately frozen in isopentane (BDH Ltd., UK) cooled in liquid nitrogen. Frozen sections of 10 μm thickness were cut (Cryostat E microtome, Reichert Ltd.) and stored at − 70°C. Tissue sections were prepared and imaged with a minimum of light exposure to avoid bleaching of porphyrin.

The fluorescence microscope (Olympus IMT-2) was attached to a charged-coupled device (CCD) camera system (Wright Instruments Ltd., model 1). This photometric imaging system is highly sensitive with comparable detection efficiency to photomultipliers and has been fully described in previous studies on AlSPc fluorescence imaging, Chan *et al.* (1989), Chatlani *et al.* (1991), Barr *et al.* (1988). Fluorescence was excited using an 8 mW helium neon laser operating at 632.8 nm, with the output directed through a liquid light-guide (via a 10 nm bandpass filter to remove extraneous light) onto a dichroic mirror in the epi-fluorescence microscope which incorporated phase-contrast attachments. Imaging of porphyrin fluorescence has, in contrast to this work, usually employed excitation near 400 nm corresponding to the intense Soret band absorption. However, excitation at these shorter wavelengths gives rise to considerable background luminescence from the microscope optics and much higher tissue autofluorescence and therefore offers no clear-cut advantage to the excitation wavelength employed here which, moreover, is very close to the selected therapeutic wavelength of 630 nm. Fluorescence was detected in the range 660 to 710 nm, using a combination of bandpass (Omega Optical Inc.) and longpass (Schott RG655) filters. The CCD sensor (578 × 385 pixels, model P8603, EEV Ltd.) was cryogenically cooled, and imaging operations and processing were controlled by an IBM AT/PC clone. Both false colour coded or black and white images were generated by the computer.

Fluorescence was quantified digitally (software provided by Wright Instruments Ltd.) using box superimposition on the image to give an average number of counts per pixel. Specific tissue layers and/or whole images were analysed using this technique to determine the relative intensities of porphyrin fluorescence, after making small corrections for autofluorescence, luminescence from the epi-fluorescence optics, and a computer generated off-set. After fluorescence imaging, the sections were fixed and stained with haematoxylin and Van Gieson's (HVG) stains and the same microscopic areas were then photographed for confirmation of histology.

Fluorescence spectroscopy

Fluorescence emission spectra were observed *ex vivo* from normal rat colon strips showing ALA-induced sensitisation. Porphyrins exhibit characteristic fluorescence profiles and this provides a means of confirming the presence of (and possibly distinguishing) the porphyrin species synthesised at a given time after introduction of ALA. Normal rats were given a 200 mg kg^{-1} dose of ALA and killed at times corresponding to those for the fluorescence microscopy studies and their colon immediately removed. Strips of colon were opened, cleaned and mounted flat on glass slides with the mucosal surface uppermost. The slides were placed in a fluorimeter (Perkin-Elmer LS 5B) at a 30 degree angle to the excitation beam in order to minimise scattered light which was further attenuated by a longpass filter (OG550, Schott) mounted on the emission port. The emission spectra (uncorrected) of each sample was then recorded using an excitation wavelength of 514 nm which enabled examination of the complete band of fluorescence extending from approximately 600–700 nm. Control strips were also examined to enable quantification and exclusion of autofluorescence and scattered light from unsensitised colon.

Phototherapy studies

The effect of light on normal and tumour tissue after i.v. administration of 200 mg kg^{-1} ALA was studied. The light source used was a copper vapour pumped dye laser (Oxford lasers Ltd.) which was set to deliver 50 J (100 mW for 500 s) from a 200 μm fibre at a wavelength of 630 nm which corresponds to a porphyrin absorption band. Laser treatments were performed at laparotomy with the fibre just touching the normal colonic mucosa, or inserted into the apex of the tumour at a depth of 1 mm. Normal colonic tissue was treated at 5, 20, 30 and 40 min and 1, 2, 3, 4, 5, 6 and 24 h, and tumour was treated at 6 h. Control animals that had not been given ALA were treated as before to quantify any thermal damage. All animals were killed 72 h later when mucosal damage, when present, was at a maximum (Barr *et al.*, 1987). To assess necrosis macroscopically at any treated laser site both the greatest and the smallest diameter of damage were recorded and the mean diameter calculated. Presence of necrosis was confirmed microscopically.

Results

Fluorescence microscopy and photometry

Initial measurements of ALA-induced fluorescence using the CCD imaging system were made on normal colonic tissue sections taken at 3, 6 or 24 h as a function of ALA dose: 50, 100, 150 or 200 mg kg^{-1} respectively. For these times greatest fluorescence was found at 3 h with background levels being reached by 24 h. After the 200 mg kg^{-1} dose there was still significant sensitisation at 6 h and this dose was therefore considered to be the most useful therapeutically as it provided a longer range of times available for PDT treatment. Sections of normal colon were examined over a greater range of time points (5 and 30 min, 1, 2, 3, 4, 5, 6, 8, 17 and 24 h, post-administration with 200 mg kg^{-1}, and the fluorescence levels in the mucosa, submucosa and muscle layers were determined separately. The results are shown in Figure 1. At

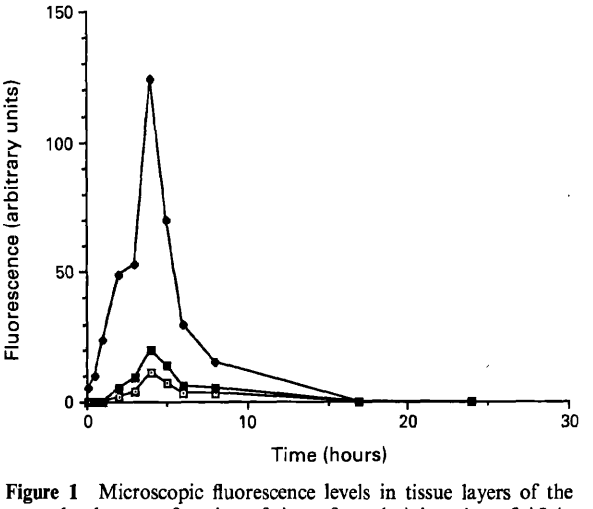

Figure 1 Microscopic fluorescence levels in tissue layers of the normal colon as a function of time after administration of ALA. –□– muscle, –◆– mucosa, –■– submucosa.

30 min, relatively low fluorescence was observed in the mucosa, with no discernible fluorescence in submucosa and muscle. Fluorescence levels increased rapidly reaching a maximum in all layers at 4 h, thereafter returning to background levels at 17 h. All layers showed comparable fluorescence profiles *vs* time although fluorescence levels varied considerably according to the tissue site. At all times the mucosa exhibited the highest fluorescence, approximately six times greater than submucosal fluorescence, with muscle barely above background levels.

Figure 2 shows the fluorescence images of normal (a) and tumour tissue (c) 6 h after introduction of 200 mg kg^{-1} ALA together with the subsequent photographs of the same sections stained with an HVG stain ((b) and (d)). There is specific sensitisation of tumour cells with tumour stroma exhibiting a three times lower average fluorescence level. At this time (6 h post-administration) we estimate that the tumour glands gave on average a fluorescence ratio of approximately 6:1 to normal mucosal glands, 30:1 to normal submucosa and 60:1 to normal muscle. Confirmation of the

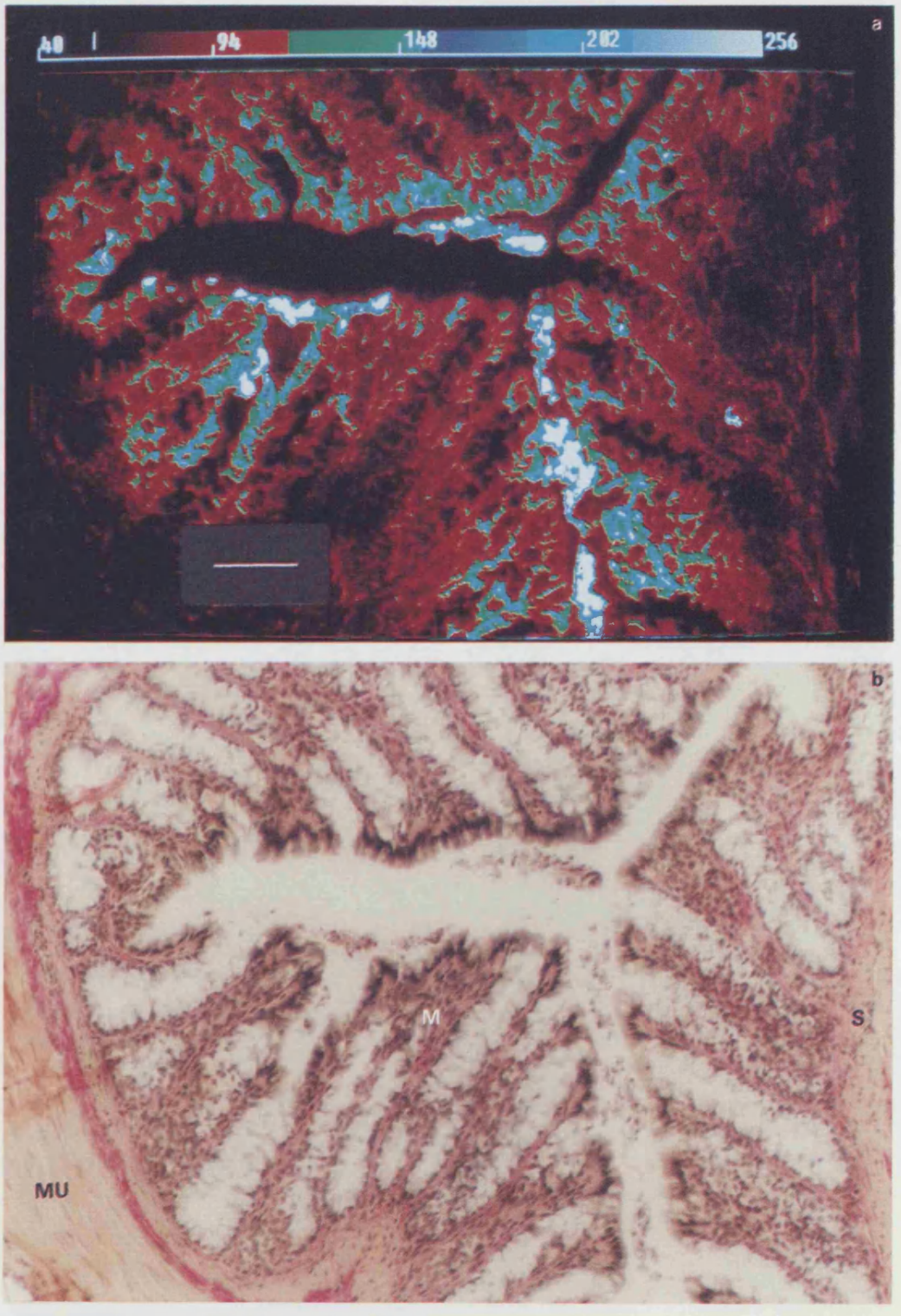

Figure 2 (continued overleaf)

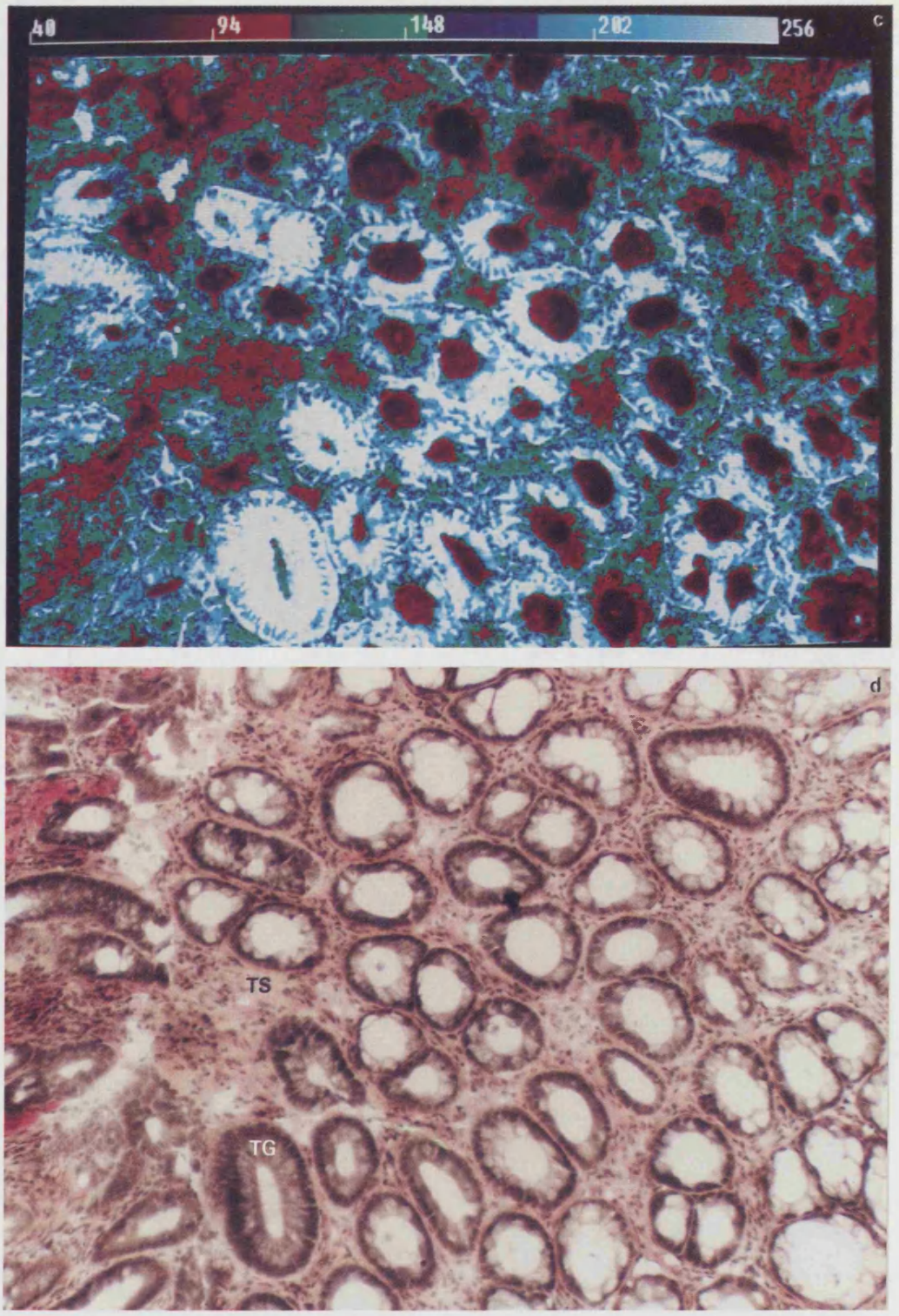

Figure 2 Fluorescence images of normal **a**, and tumour tissue **c**, 6 h after introduction of 200 mg kg^{-1} ALA together with the subsequent photographs of the same sections stained with an HVG stain (**b**, normal mucosa (M), muscle (MU) and submucosa (S) and **d**, tumour stroma (TS) and tumour gland (TG)). Scale: white bar represents 80 μm. The false colour code begins at 40 counts to allow for background fluorescence.

specificity of sensitisation of tumour cells was obtained by re-imaging Figure 2c using a higher power and is depicted in Figures 3a and b, which also show that sensitisation is restricted to extra-nuclear sites. At all times studied, blood vessels exhibited low fluorescence levels and this is shown in Figure 4a and b. This figure is of images from normal colon 4 h post-administration demonstrating that blood vessel sensitisation is comparable to that of the surrounding connective tissue, in contrast to the intense mucosal fluorescence.

Fluorescence spectroscopy

The fluorescence emission spectra from normal rat colon strips (excitation wavelength 514 nm) 30 min and 6 h after ALA-induced sensitisation and unsensitised control colon are shown in Figure 5. The spectra from the sensitised colons differ in intensity of fluorescence but appear to exhibit the same spectral profile with maxima at approximately 636 and 704 nm; both spectra are considerably more intense than the

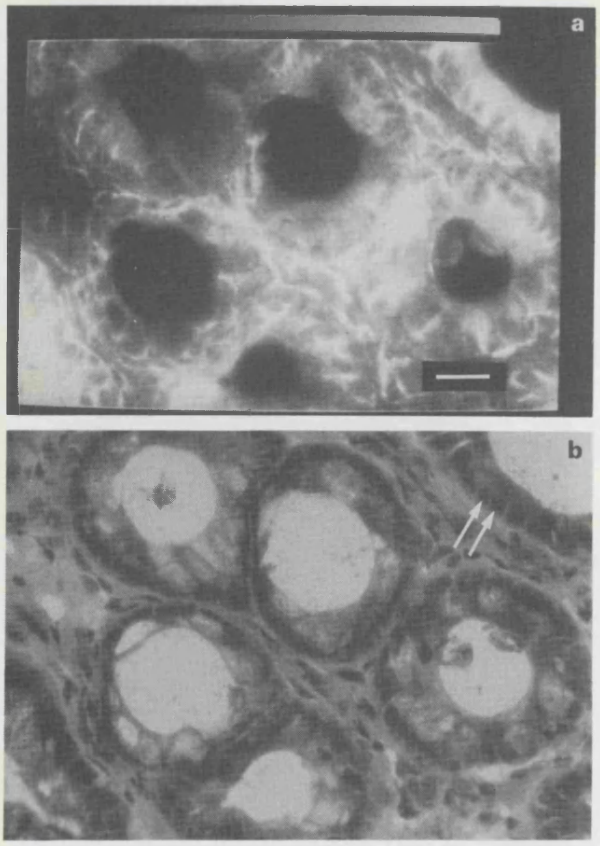

Figure 3 High power image of Figure 2c shown in **a** with subsequent stained section **b**. Arrows in **b** indicate two typical tumour nuclei depicting non-fluorescence of these structures. Scale: white bar represents 20 μm.

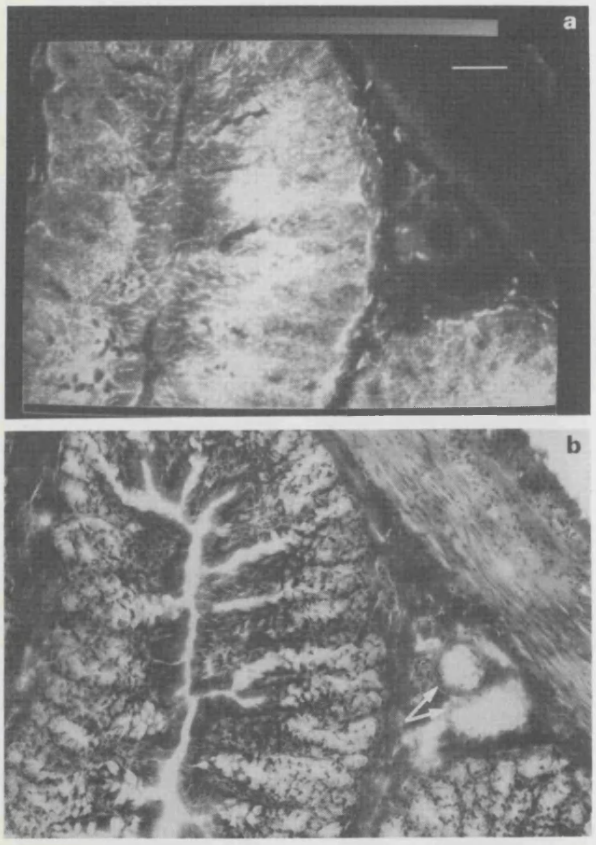

Figure 4 Fluorescence image of normal colon **a**, 4 h after introduction of 200 mg kg^{-1} ALA together with the subsequent stained section **b**. Arrows in **b** indicate blood vessels. Scale: white bar represents 80 μm.

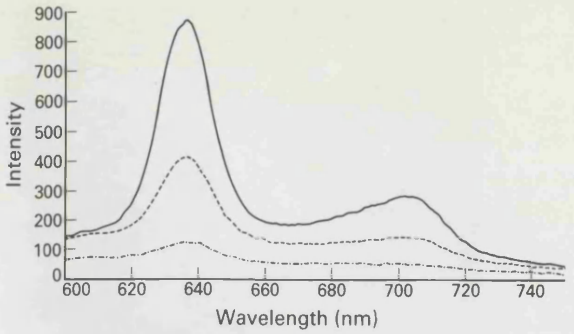

Figure 5 Fluorescence emission spectra (excitation wavelength 514 nm) from normal colon strips 6 h —— and 30 min – – – after 200 mg kg^{-1} ALA, and unsensitised control tissue –.–.–.

control spectrum which apart from scattered light may also contain a contribution from endogenous porphyrin species. Further spectra were recorded at 1 and 2 h which gave the same maxima, and excitation spectra recorded from 500–650nm with detection set at 700 nm were also consistent with PP IX (data not shown). The results are essentially identical to the *in vivo* spectra obtained by Pottier *et al.* (1986) from skins of mice injected i.p. with ALA, who assigned the spectrum solely to protoporphyrin IX. The apparent small red-shift compared to the spectrum in dimethyl sulphoxide solution (maximum at 629 nm) was ascribed to binding with protein substrates as PP IX bound to human serum albumin shows a fluorescence maximum at 635 nm (Lamola *et al.*, 1981).

Phototherapy studies

The mean diameter of necrosis in normal colonic mucosa was measured as a function of time after administration of ALA and results are shown in Figure 6. These results show broad correlation with the corresponding mucosal fluorescence measurements from the CCD imaging studies with greatest damage also found at 4 h (mean diameter 9.1 ± 1.5 mm) with no visible necrosis at 24 h. The notable exception was at 30 min where considerable necrosis was seen (mean diameter 7.2 ± 1.0 mm) even though mucosal fluorescence was more than an order of magnitude lower than at 4 h.

Histological examination was undertaken on all specimens showing macroscopic necrosis 72 h after laser treatment. At all treatment times after introduction of ALA, an acute inflammatory infiltrate, mainly comprising of monocytes and polymorphonuclear neutrocytes, was seen throughout all tis-

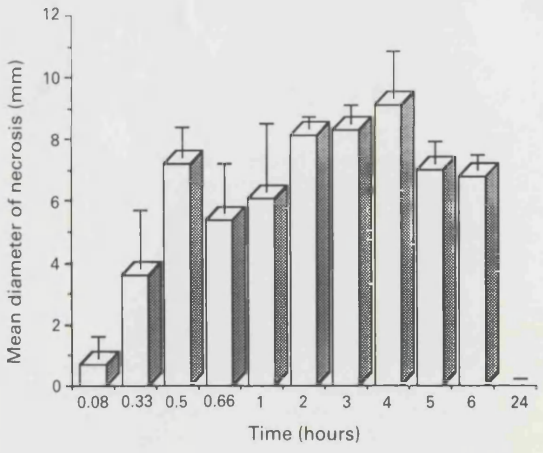

Figure 6 Diameter of necrosis in normal colonic mucosa measured as a function of time after administration of ALA. Each point represents the mean (± 1s.d.) of five animals.

sue layers of the colon but there was little evidence of muscular layer necrosis in the specimens examined. This relative sparing of the muscle layer of normal colon is shown in Figure 7a treated at 6 h, with the corresponding high power image b. Tumour tissue treated at the same time shows obvious necrosis (Figure 8).

Discussion

A new means of sensitisation for PDT has been studied which involves the introduction of 5-aminolaevulinic acid (ALA) to induce endogenous porphyrin sensitisation. We have investigated the microscopic distribution and spectroscopy of ALA-induced porphyrin fluorescence at various time intervals after the introduction of ALA, in both normal and tumour tissue in the rat colon. It has been shown that the fluorescence spectrum in normal colon is consistent with that of protoporphyrin IX and using quantitive fluorescence microscopy that the fluorescence is mainly localised in the normal mucosa, with approximately six times less fluorescence in submucosal tissue and with muscle barely exceeding background fluorescence levels. We have found that at 6 h there is a considerable differential between porphyrin fluorescence in tumour and normal colon. At this time colon tumour cells exhibit specific fluorescence with average levels

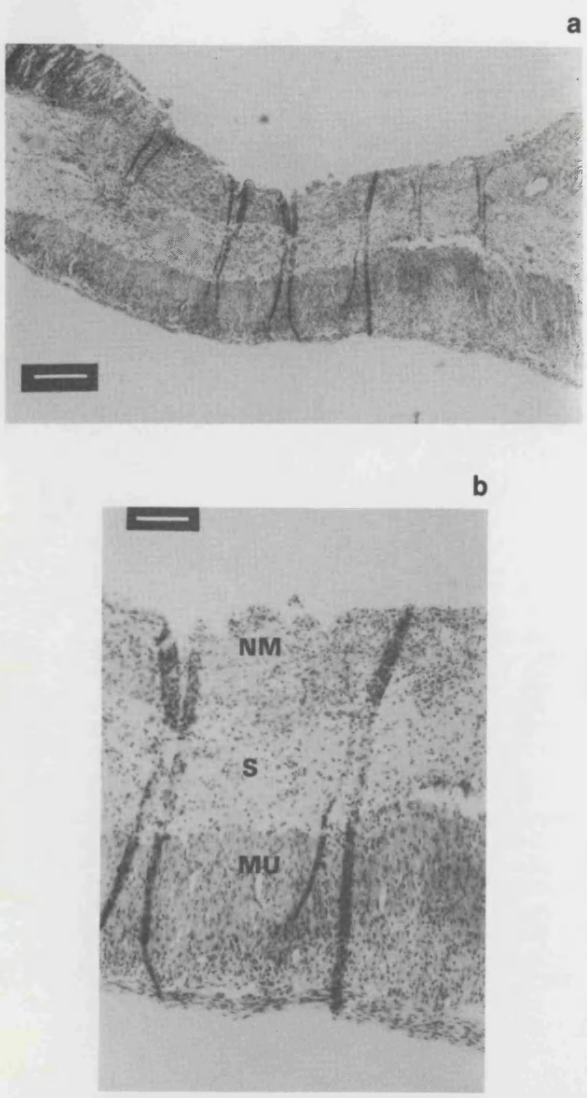

Figure 7 Histological section of normal colon treated 6 h after 200 mg kg^{-1} ALA showing necrotic mucosa (NM), inflammatory infiltrate in the submucosa (S) and relative sparing of the muscle layer (MU). Scale: white bar represents 200 μm in **a** and 80 μm in **b**.

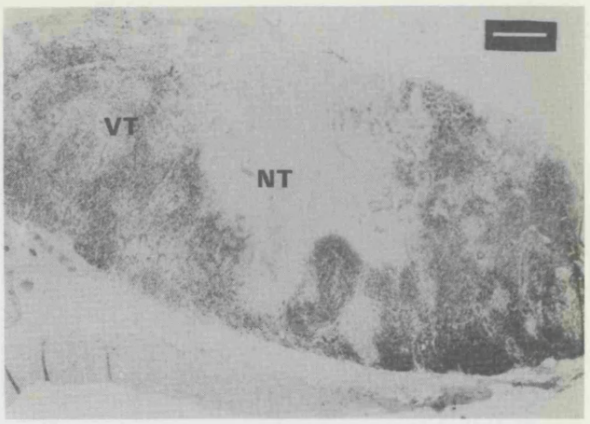

Figure 8 Histological section of colonic tumour treated 6 h after 200 mg kg^{-1} ALA showing necrotic tumour (NT) and viable tumour (VT) beyond the treatment site. Scale: white bar represents 200 μm.

that are approximately six times higher than normal mucosa, 30 times higher than normal submucosa, and 60 times higher than normal muscle levels. Our fluorescence spectroscopic and pharmacokinetic data are consistent with previous work by Pottier *et al.* (1986) who have likewise shown that sensitisation by PP IX occurs rapidly reaching a maximum near 3 h in the skin and declining to background levels by 24 h.

The assumption that localisation and intensity of microscopic fluorescence correlates with PDT induced damage has generally been confirmed with our phototherapy studies. The mean diameter of necrosis in normal colon (allowing for the geometry of the light delivery) correlated with the relative porphyrin fluorescence levels between 5 min and 24 h, except at 30 min (discussed below). Additionally, necrosis in all specimens has been restricted to the colonic mucosa with muscle remaining viable. Divaris *et al.* (1990) have shown previously that after systemic administration of ALA to mice the intensity of the ALA-induced PP IX fluorescence in skin also correlated with the amount of phototoxic damage. With phototherapy it has proved possible to kill tumour tissue with ALA-induced sensitisation. Laser irradiation at 630 nm of DMH-induced colonic tumour has shown tumour cell kill at 6 h post-administration when necrotic damage to normal colon is limited to mucosa (which is capable of healing through regeneration – Barr *et al.*, 1987). It is reassuring that necrosis does not extend into the muscle layer, although there is evidence of an inflammatory infiltrate. Previous work has shown that the mechanical integrity of the colon can be maintained even if there is muscle necrosis, probably due to a lack of any deleterious effect on the submucosal collagen (Barr *et al.*, 1987), although experiments on bladder show that even if there is histological evidence of muscle healing by regeneration, the muscle function may be permanently damaged (Pope & Bown, 1991) and so clearly it is better that there is no muscle damage. Tumours were only examined and treated 6 h after administration of ALA, and in due course experiments should be carried out to look at other time intervals, but this time does show considerable potential for selective tumour treatment. We have also demonstrated a significant advantage of ALA-induced porphyrin over conventional sensitisers in that the level of sensitisation returns to background values in both normal and tumour tissue by 24 h. This suggests that continued tissue sensitisation post-treatment will not be a problem.

An unexpected result was found when treating normal tissue 30 min after introduction of ALA. A relatively low fluorescence reading was obtained in the mucosa at this time with no discernible fluorescence in the submucosa and muscle layers, so we would have predicted little, if any, damage after light treatment. However, significant photodamage was found with a diameter of necrosis of 7.2 ± 1.0 mm, which

could be observed histologically as comprising necrotic mucosa and a particularly heavy inflammatory infiltrate in the submucosa and muscle layers. The mechanism of photodamage in Friend erythroleukaemic cells from the use of ALA has been shown to be a combination of the cellular location and the chemical nature of the photosensitiser at a particular time (Malik & Lugaci, 1987). They have reported that endogenous porphyrins initially accumulated in the mitochondria and then translocated to other photosensitive sites within these cells. A possible explanation of our anomalous *in vivo* results would be that 30 min after ALA administration the induced porphyrin (in this case PP IX) is located at particularly photosensitive sites, such as the mitochondria, and that thereafter the porphyrin is located in other less photosensitive sites in the cell. Another explanation is that other photoactive porphyrins (e.g. uroporphyrin) were present at the shorter times, but given the consistency of the fluorescence spectra recorded from 30 min to 6 h we believe this to be unlikely, although we can offer no explanation for the specific production of PP IX *in vivo* which is contrary to the *in vitro* results. Chromatographic analysis of the porphyrin content may provide more definitive conclusions.

Interesting comparisons can be made with our recent studies of AlSPc sensitisation of normal rat colon (Chatlani *et al.*, 1991), which showed that AlSPc fluorescence was particularly high in and adjacent to blood vessel walls, in complete contrast to the results presented here. The mechanism of photodamage may therefore differ profoundly between these two methods of sensitisation. ALA-induced sensitisation would appear to rely on direct cell kill whereas the mechanism with AlSPc relies primarily on vascular effects. This conclusion may have the most far reaching implications for ALA. With the current photosensitisers which localise mainly to the vascular stroma of tumours and normal areas, it is only possible to destroy small numbers of tumour cells infiltrating normal areas by also necrosing the normal tissue (Barr *et al.*, 1991). In contrast, the combination of tumour to normal selectivity and synthesis of PP IX in individual malignant cells may make it possible to kill infiltrating cells without so much damage to normal structures. Naturally, this is only applicable to tumours than can synthesise PP IX from ALA, but could be extremely valuable for sterilising the resection margins after tumour surgery. Since PDT after ALA has shown a high degree of selectivity of necrosis to the normal colonic mucosa, this treatment could have a particular application for intramucosal carcinomas of hollow organs, and also potentially an application in peritoneal endometriosis.

In summary, this new method of sensitisation has several advantages over conventional exogenous photosensitiser administration. Sensitisation is rapid with near maximal tissue levels of PP IX being reached at 3–4 h. Photosensitisation of tissues including skin (Pottier *et al.*, 1986) is likely to be comparable in intensity to that after HpD, but would only be expected to last 24 h instead of several weeks. The absorption peak of PP IX in the red part of the spectrum is low (comparable to that for HpD, and much lower than that for AlSPc), but this just means that more light is required. The real bonus is the direct sensitisation of individual malignant cells, and if this can be exploited, the potential for the technique is very considerable.

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Photodynamic therapy of the normal rat stomach: a comparative study between di-sulphonated aluminium phthalocyanine and 5-aminolaevulinic acid

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Summary Dysplasia in the upper gastrointestinal tract carries a risk of invasive malignant change. Surgical excision of the affected organ is the only treatment available. Photodynamic therapy has been shown to be promising in the treatment of early and superficial tumours and may be useful for the ablation of dysplastic mucosa. Because of the diffuse nature of the disease, such treatment would necessarily involve destruction of large areas of mucosa and it is desirable to confine its effect to the mucosa in order that safe healing can take place. By means of photometric fluorescence microscopy, we have studied the pattern of photosensitisation in the normal rat stomach using di-sulphonated aluminium phthalocyanine (AlS_2Pc) and 5-aminolaevulinic acid (ALA) as photosensitisers. AlS_2Pc resulted in a panmural photosensitisation of the gastric wall with the highest level encountered in the submucosa. The mucosa and muscularis propria were sensitised to equal extent. Following light exposure, a full thickness damage resulted. ALA is a natural porphyrin precursor and exogenous administration gave rise to accumulation of protoporphyrin IX (PPIX) in the cells. The resultant pattern of photosensitisation was predominantly mucosal and its photodynamic effect was essentially confined to the mucosa. ALA produced a selective photosensitisation of the gastric mucosa for its photodynamic ablation with sparing the underlying tissue layers.

High grade epithelial dysplasia of gastric type mucosa whether in the stomach or oesophagus carries a risk of invasive malignant change (Farrands *et al.*, 1983; Skinner *et al.*, 1983; Offerhaus *et al.*, 1984; Schmidt *et al.*, 1985; Hamilton & Smith, 1987; Atkinson, 1989). In the stomach, dysplastic changes have been described in association with previous gastric surgery (Schrumpf *et al.*, 1977; Farrands *et al.*, 1983; Offerhaus *et al.*, 1984 & 1989), as well as gastric polyps (Aste *et al.*, 1986). In its severe form, the malignant potential is high (Ming *et al.*, 1984). In the oesophagus this condition often arises from Barrett's epithelium. There is no satisfactory treatment for this condition apart from surgical excision of the affected region of the organ concerned if this risk is significant (Skinner *et al.*, 1983; Ferrands *et al.*, 1983; Dent, 1989; Atkinson, 1989). Surgery is a major procedure and often not a viable option. Thermal laser ablation carries the risk of viscus perforation (Barr *et al.*, 1987*b*) and is not practical because of the diffuse and multicentric nature of dysplasia. Photodynamic therapy (PDT) of tumours involves the local activation of a preadministered photosensitiser by light of a specific wavelength matched to the absorption characteristic of the photosensitiser used. The activated photosensitiser subsequently give rise to production of cytotoxic singlet oxygen species (Weishaupt *et al.*, 1976). The mechanism of cell kill is by chemotoxicity and there is less likelihood of viscus perforation (Barr *et al.*, 1987*b*). PDT has been used to treat gastrointestinal tumours (Kato *et al.*, 1986; Jin *et al.*, 1987; Patrice *et al.*, 1990; Krasner *et al.*, 1990). Because of limited transmittance of light in tissue, it has been shown to be most effective in the treatment of small and early tumours (Kato *et al.*, 1986; Jin *et al.*, 1987; Krasner *et al.*, 1990). Epithelial dysplasia is an early superficial form of neoplastic lesion which is confined only to the mucosa and PDT may be a useful treatment modality for this condition. Due to its diffuse nature, treatment would necessarily involve destruction of large areas of mucosa. It is thus essential to limit the photodynamic effect to the mucosa to minimise complication and enhance prompt and safe healing. Photodynamic effect is local and only occurs when the products of the local light dose and photosensitiser concentration exceeds a threshold (Cowled & Forbes, 1985). In addition, because of photodegradation of photosensitiser by the activating light, the local tissue concentration of photosensitiser has to exceed a particular level irrespective of light dose before photodynamic action can take place (Potter *et al.*, 1987). When a sufficient differential photosensitiser distribution exist between mucosa and underlying structure, it is possible to produce a predominant photodynamic effect in the mucosa by keeping the concentration of photosensitiser in the mucosa above this threshold while that in the underlying tissue below it. This approach has been adopted experimentally in the bladder, and the resultant photodynamic effect causes minimal disruption of both the anatomical and functional integrity of the bladder while still achieving the desired objective of complete mucosal ablation (Pope & Bown, 1991*a*).

We have previously reported the efficacy of aluminium sulphonated phthalocyanine (AlSPc) as a photosensitiser in both normal tissue and tumours (Barr *et al.*, 1987*a*; Tralau *et al.*, 1987; Barr *et al.*, 1990) as well as its advantages over haematoporphyrin derivative with respect to cutaneous phototoxicity (Tralau *et al.*, 1989). We have further observed that PDT using AlSPc does not compromise the mechanical strength of the normal colon (Barr *et al.*, 1987*b*). Most of the PDT studies using AlSPc have been carried out using a mixture of compounds with different degree of sulphonation (range of 1 to 4 sulphonated groups with the average being 3.2). From recent studies, di-sulphonated phthalocyanine has been shown to be a more potent photosensitiser both *in vitro* and *in vivo* (Paquette *et al.*, 1988; Berg *et al.*, 1989; Chan *et al.*, 1990; Chatlani *et al.*, 1991). For these reasons, we have chosen to use the di-sulphonated aluminium phthalocyanine (AlS_2Pc) for this study.

ALA in itself is not a photosensitiser. It is a natural porphyrin precursor and its synthesis by living cells is the first committed step which will eventually lead to haem formation. In this biosynthetic pathway, the rate limiting step is that involved in the synthesis of ALA which is controlled by a regulatory feedback inhibition (Marriott, 1968; Rimington, 1966). By administering a large quantity of exogenous ALA

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both to *in vitro* systems as well as whole animals, it has been shown that the natural regulatory mechanism can become overloaded and as a result, porphyrin intermediates of the biosynthetic pathway, particularly protoporphyrin IX (PPIX), accumulate (Malik & Djaldetti, 1979; Sima *et al.*, 1981). PPIX is a potent photosensitiser. Malik & Lugaci (1987) and Kennedy's group (Divaris *et al.*, 1990) have shown that enough PPIX can be synthesised this way to produce a photodynamic effect both *in vitro* and *in vivo*. More recently, exogenous ALA applied topically has been shown to be effective in the photodynamic treatment of various cutaneous cancers (Kennedy *et al.*, 1990; Wolf & Kerl, 1991).

Although in theory all nucleated cells exhibiting aerobic metabolism capable of haem synthesis are liable to become photosensitised, Divaris found that following administration of exogenous ALA to mice, there was a marked difference in the level of photosensitisation in the various tissue structures in the skin as studied by fluorescence microscopy. The epidermal cells and cells of the pilosebaceous apparatus were markedly fluorescent as compared to the dermis (Divaris *et al.*, 1990). The same group has also reported that in the bladder and uterus, ALA administration resulted in preferential photosensitisation of the mucosa and endometrium over the other underlying structures of the respective organs. These findings prompted us to investigate ALA as a possible photosensitiser for photodynamic ablation of gastric mucosa.

Materials and methods

Photosensitiser

AlS_2Pc was purified and analysed using high performance liquid chromatography (HPLC) at the Department of Chemistry, Imperial College of Science Technology and Medicine. The di-sulphonated fraction of AlS_2Pc was separated from a mixture prepared by the oleum sulphonation of aluminium phthalocyanine chloride, using reverse phase liquid chromatography. This fraction contained a range of di-sulphonated isomers dominated by the most hydrophobic component which comprised $60 \pm 5\%$ of its integrated HPLC (Ambroz *et al.*, 1991). The photosensitiser was made up in 0.1 molar sodium hydroxide and phosphate buffered saline and administered intravenously via the tail vein. ALA was obtained as a hydrochloride (formular weight = 167.6) in 98% pure powder form from Sigma Chemical Company Limited (Poole, UK). It was dissolved in phosphate buffered saline for administration.

Animals

All studies were performed on female Wistar rats supplied by the Imperial Cancer Research Fund. Their age ranged from 4 to 8 weeks and their weight ranged from 100 g to 200 g. Injections of photosensitisers were carried out under intramuscular Hypnorm (fentanyl and fluanisone) anaesthesia. The concentration of photosensitiser was adjusted to maintain the volume of injection between 0.3–0.5 ml to ensure accurate injection. Photodynamic therapy was carried out during laparotomy under intramuscular Hypnorm and diazepam anaesthesia.

Distributions of photosensitisers in the stomach

This was studied by means of fluorescence microscopy and photometry. After administration of photosensitiser, animals were killed at a range of times from 15 min to 2 weeks. A small disc of stomach wall was excised from the glandular stomach along the greater curvature just distal to the limiting line and immediately frozen by submerging in a bath of isopentane (2-methylbutane) prechilled in liquid nitrogen. The snap frozen tissue samples were then stored in liquid nitrogen until sectioned. Tissue blocks were mounted on OCT medium (tissue tek II embedding compound, BDH) and 10 μm sections were cut using a Cryocut E microtome (Reichert-Jung). The slides were stored in a freezer at – 20°C and only allowed to thaw just prior to fluorescence microscopy. An inverted microscope (Olympus IMT-2) with epifluorescence and phase-contrast attachments was used as described previously (Chan *et al.*, 1989). Fluorescence excitation came from an 8 mW helium-neon laser (632.8 nm). The beam was delivered by a liquid light guide and through a 10 nm band-pass filter centred at 633 nm to remove extraneous light onto the dichroic mirror (Omega Optical Inc.) for epifluorescence study. The phthalocyanine fluorescence was detected between 665 and 700 nm using a combination of band-pass (Omega Optical Inc.) and long-pass (Schott RG665) filters. The fluorescence signal was detected by a highly sensitive cryogenically cooled CCD (charge-coupled device) camera (Wright Instruments, model 1, resolution 400×600 pixels) fitted to the microscope. This signal was processed by an IBM personal computer into a falsely colour-coded microscopic image of the section depicting the mean signal counts per pixel. The software also allowed quantitative analysis of the signal by calculating the mean fluorescence count and its standard deviation within any chosen area on the fluorescence image. Using a ten times objective of the microscope, a view of the entire cross section of the stomach was included. The mucosa, submucosa and muscularis propria were usually readily discernable on the fluorescence image. Three representative areas over each tissue layer at least 100×100 pixels in size were chosen for analysis on each section. Conventional light microscopy of the stained serial section of the specimen also helped to enable accurate identification of the various microscopic structures. As PPIX and AlS_2Pc have very different fluorescence efficiency using 633 nm excitation, exposure time of the specimen to the exciting laser light was set to produce a comparable range of measurements (7.5 seconds for AlS_2Pc and 25 seconds for ALA). Fluorescence was measured arbitrarily as counts per pixel (20 photoelectrons per count; quantum efficiency = 0.5 at this wavelength). The longer exposure time used for ALA resulted in higher tissue autofluorescence than that for AlS_2Pc. All fluorescence measurements were corrected for their respective autofluorescence (as measured on control specimens) of each respective layer of tissue with the respective exposure time for each photosensitiser. After fluorescence microscopy, specimens were fixed in formalin and stained with haemotoxylin and eosin. Both the falsely coloured coded fluorescence image and the light microscopic image of the subsequently stained section were photographed for comparison (Figure 3a, 3b, 4a and 4b). All studies with AlS_2Pc were carried out using 5 mg kg^{-1} (6.5 μmol kg^{-1}) of AlS_2Pc. As the conversion of ALA to the photoactive PPIX is dose dependent, a range of doses (20 mg kg^{-1} (0.119 mmol kg^{-1}), 100 mg kg^{-1} (0.597 mmol kg^{-1}) and 200 mg kg^{-1} (1.193 mmol kg^{-1}) were employed.

Photodynamic therapy

The light source used was a pulsed (12 kHz) copper vapour pumped dye laser (Oxford Lasers). In the AlS_2Pc group, the output was tuned to 675 nm (peak absorption for AlS_2Pc) and delivered via a 200 μm fibre threaded into the stomach through the forestomach and held just touching the mucosa of the glandular stomach. The fibre was maintained at approximately 90° to the mucosal surface. The rest of the abdominal viscera were shielded from forward light scatter by interposition of a piece of opaque paper. Only one point was treated in each animal. Power output from the fibre tip was 50 mW and the total irradiation time 1000 sec giving a total energy delivery of 50 J per animal. In one sub-group, all animals were sensitised with 5 mg kg^{-1} of AlS_2Pc and then exposed to laser light at a range of times from 1 to 48 h following sensitisation. In the other sub-group, animals were sensitised with AlS_2Pc at a range of doses from 0.5 mg kg^{-1} to 5 mg kg^{-1} and then exposed to laser light 2 h later. In the ALA group, the laser was tuned to 630 nm and the same power and exposure time were used. Two sub-groups of

animals were treated with ALA photosensitisation. In one sub-group, all animals were given 200 mg kg^{-1} of ALA and light exposure was effected at a range of time from 30 min to 8 h. In the other sub-group, animals were sensitised with different doses of ALA (1 mg kg^{-1}, 5 mg kg^{-1}, 20 mg kg^{-1}, 100 mg kg^{-1}, 200 mg kg^{-1} and 400 mg kg^{-1}) and then exposed to laser light at the time of peak photosensitisation of the respective doses as determined from fluorescence photometry. Fluorescence photometry was not carried out with 1 mg kg^{-1} and 5 mg kg^{-1} of ALA because with these doses, the fluorescence yield was too low relative to the background tissue fluorescence to provide sufficient contrast for detection using our system. Control unsensitised animals were irradiated using similar parameters to exclude thermal effects. Treated areas were marked with two silk sutures placed along the greater curve at 1 cm proximal and 1 cm distal to the point of contact of the laser fibre to ease subsequent identification. Animals were allowed to recover and kept in standard laboratory conditions until sacrificed at 72 h. On killing the animal, the stomach was immediately excised and opened along the lesser curve for macroscopic inspection. The specimens were laid out on a piece of card and the size of the PDT induced lesions were determined by taking the mean of the longest diameter and the broadest diameter of the lesion (Barr *et al.*, 1987*a*). The specimen was then fixed in formalin and prepared for conventional light microscopy.

Results

Fluorescence photometry

Fluorescence spectroscopy using a Perkin-Elmer LS-5B spectrofluorimeter (excitation at 400 nm) of an *ex vivo* specimen of stomach from a rat sensitised with 200 mg kg^{-1} of ALA was carried out and the spectrum obtained was found to be consistent with the fluorescence emission spectrum of PPIX as also found by Divaris *et al.* (1990). Following administration of 5 mg kg^{-1} of AlS_2Pc, fluorescence reached a peak at 1 h and rapidly declined in the first 48 h (Figure 1a). By 2 weeks, the fluorescence signal approached that of the control specimen. At all time points, the highest uptake of AlS_2Pc was seen in the submucosa. Mean uptake by the submucosa was approximately twice that of the mucosa and muscularis propria. With 200 mg kg^{-1} of ALA, the fluorescence signal in the mucosal layer rose rapidly to a peak at 3 h while signal over the other layers rose much less (Figure 1b). Peak fluorescence was achieved earlier with the 20 mg kg^{-1} of ALA as compared to higher doses and the trend suggested an earlier fluorescence peak with 100 mg kg^{-1} as compared to 200 mg kg^{-1} (Figure 2). Although the level of maximum fluorescence increased with the dose of ALA administered, this relationship was not a linear one and the peak fluorescence level achieved with 200 mg kg^{-1} of ALA was only marginally higher than that with 100 mg kg^{-1}. Fluorescence declined very rapidly and almost reaching background level by 6 to 8 h.

The microscopic distribution of fluorescence after administration of 5 mg kg^{-1} of AlS_2Pc is represented in Figure 3a and b. Highest levels of fluorescence were seen in the submucosal layer and particularly around blood vessels. Fluorescence levels in the mucosa and muscularis were comparable and both lower than that found in the submucosa. With ALA however, the resultant PPIX fluorescence was predominantly over the mucosa (Figure 4a and b) with very little fluorescence seen over the submucosa and muscularis propria. At higher magnification, AlS_2Pc fluorescence was highest around the periphery of the epithelial cells in the mucosa suggesting that AlS_2Pc was largely extracellular (Figure 5a).

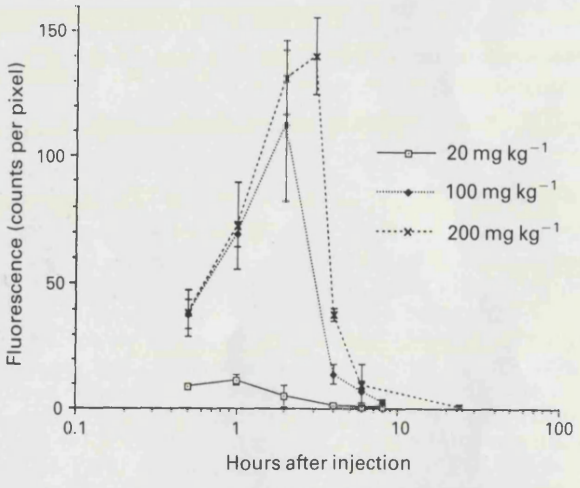

Figure 2 Mean level of fluorescence (± s.d.) of the gastric mucosa after intravenous administration of 20 mg kg^{-1}, 100 mg kg^{-1} and 200 mg kg^{-1} of ALA as a function of time. All values have been corrected for tissue autofluorescence. Value at each time point represents the mean in three or four animals.

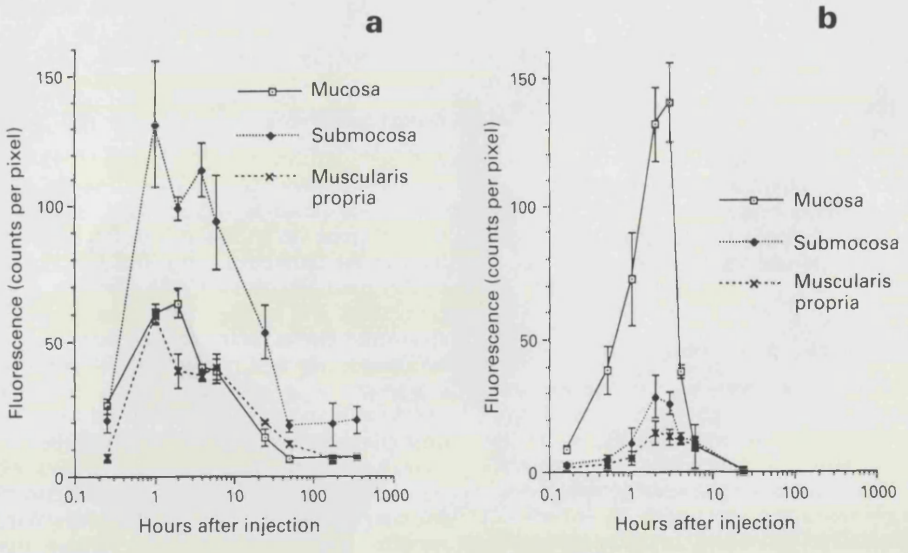

Figure 1 Mean level of fluorescence (± s.d.) of the layers of the gastric wall after intravenous administration of 5 mg kg^{-1} of AlS_2Pc **a**, or 200 mg kg^{-1} ALA **b**, as a function of time. All values have been corrected for tissue autofluorescence. Value at each time point represents the mean in three or four animals.

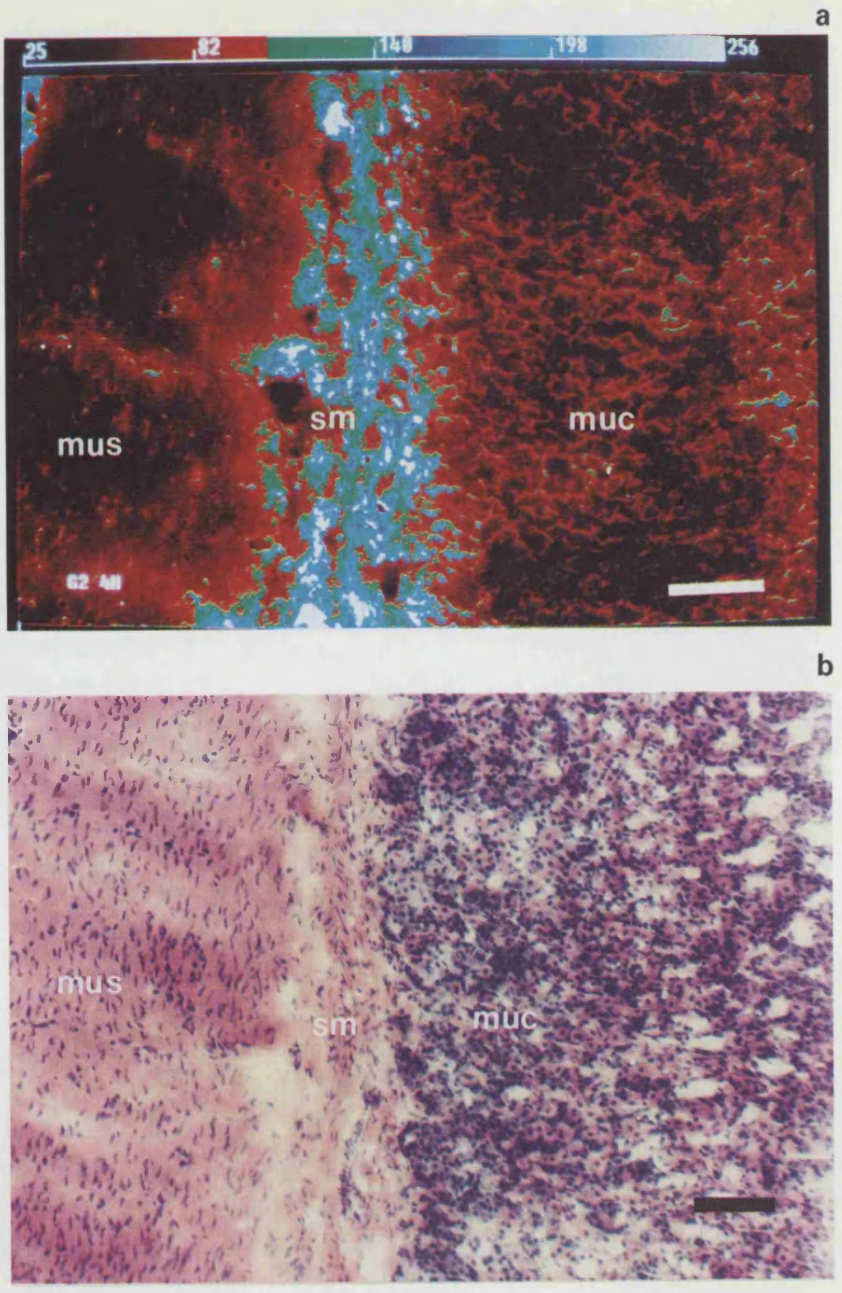

Figure 3 **a**, Fluorescence image of a frozen section of gastric wall 4 h after intravenous administration of 5 mg kg^{-1} of AlS_2Pc. The upper colour bar represents the fluorescence scale (Black = 25 counts per pixel; White = 256 counts per pixel). Scale: the bar in the right bottom corner represents 100 μm. (muc = mucosa; mus = muscularis propria; sm = submucosa). **b**, Micrograph of section in **a** after H-E staining showing the corresponding mucosal, submucosal and muscular layer. Scale: the bar in the right bottom corner represents 100 μm. (muc = mucosa; mus = muscularis propria; sm = submucosa).

ALA induced PPIX fluorescence was, however, intracellular in location and mainly perinuclear (Figure 5b).

The relative level of fluorescence of the mucosa and muscularis propria achieved following administration of both compounds differed markedly. With AlS_2Pc, apart from immediately following administration when a significant quantity of photosensitiser was still in the intravascular compartment, the ratio of fluorescence between the mucosa and the muscularis propria remained at approximately 1 throughout. In contrast, after administration of ALA, the ratio of fluorescence level between mucosa and muscle varied with time (Figure 6). With all doses of ALA, this ratio rose to a peak in excess of 14 one hour after administration. With 20 mg kg^{-1}, of ALA, this ratio fell back rapidly to almost unity at 2 h. When higher doses of ALA were given, the high fluorescence ratio was sustained over a longer duration and with the dose of 200 mg kg^{-1}, did not reach unity until 8 h after administration.

Photodynamic therapy

No macroscopic lesion was seen in the control groups although on close microscopic scrutiny, a small area of mucosal necrosis comparable to the diameter of the fibre could be found. In contrast, photodynamic lesions were macroscopically obvious if present. After photosensitisation with 5 mg kg^{-1} of AlS_2Pc, maximum damage occurred when animals were exposed to light between 1 and 3 h after sensitisation (Figure 7a) which correlated relatively well with the time peak of AlS_2Pc fluorescence. Using 200 mg kg^{-1} of ALA, apart from a lesser extent of damage produced when light exposure occurred at $\frac{1}{2}$ hour after administration, the extent

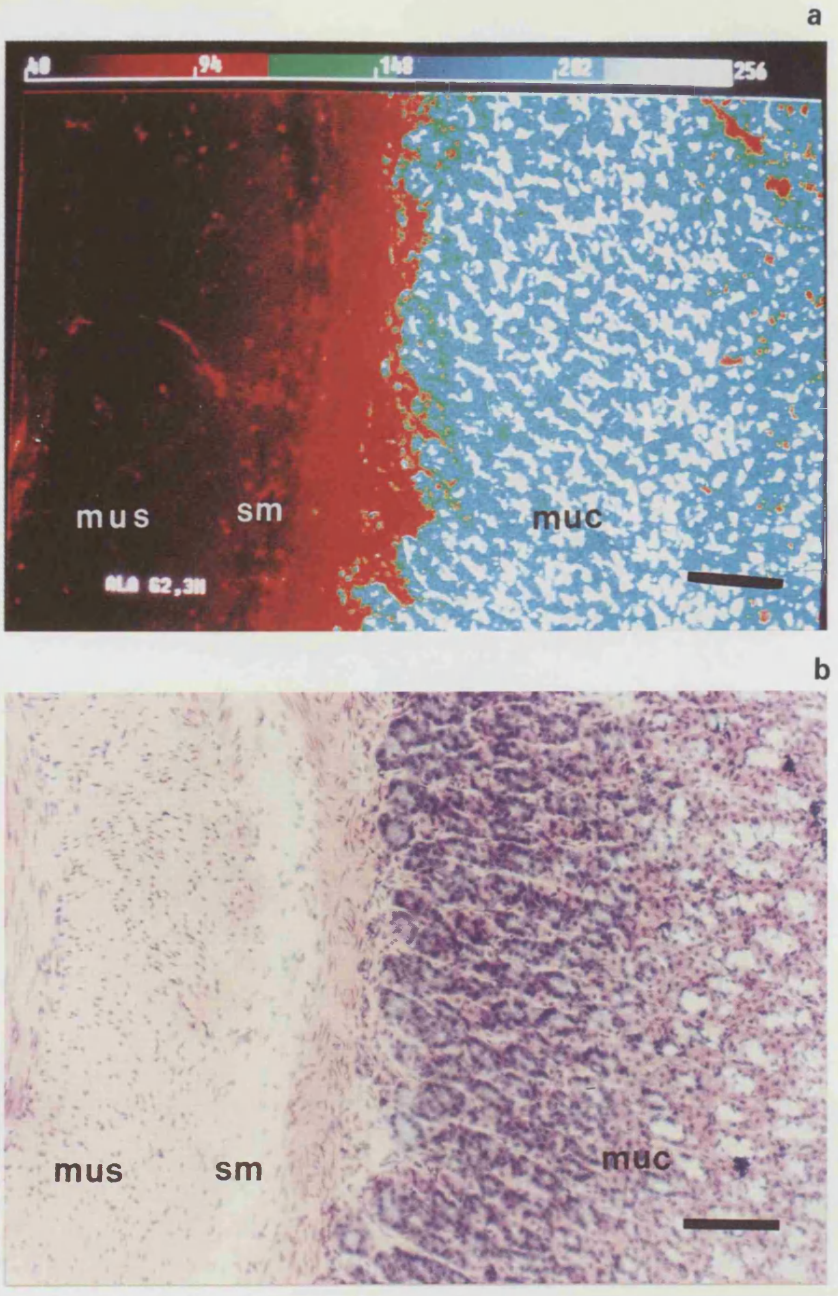

Figure 4 **a**, Fluorescence image of a frozen section of gastric wall 3 h after intravenous administration of 200 mg kg^{-1} of ALA. The upper colour bar represents the fluorescence scale (Black = 25 counts per pixel; White = 256 counts per pixel). The mucosal layer is brightly fluorescent while fluorescence levels in all the other layers of the gastric wall are little above background. Scale: the bar in the right bottom corner represents 100 μm. (muc = mucosa; mus = muscularis propria; sm = submucosa). **b**, Micrograph of section in **a** after H-E staining showing the corresponding mucosal, submucosal and muscular layer. Scale: the bar in the right bottom corner represents 100 μm. (muc = mucosa; mus = muscularis propria; sm = submucosa).

of damage appeared to plateau off subsequently when light exposure occurred between 1 and 6 h after administration (Figure 7b).

The extent of photodynamic damage produced with AlS_2Pc photosensitisation varied with the dose administered. No photodynamic lesion was produced at the sensitiser dose of 0.5 or 1 mg kg^{-1}. Above 1 mg kg^{-1}, the size of the lesion produced appeared to correlate with increasing dose of AlS_2Pc. This dose effect was not seen with ALA photosensitisation. No PDT damage was evident when the animals were sensitised with 1 mg kg^{-1} or 5 mg kg^{-1} of ALA. However, the mean diameter of the PDT lesion remained relatively constant despite a 20 times increase in the dose of ALA given from 20 mg kg^{-1} to 400 mg kg^{-1} (Figure 8). The threshold dose of ALA for photodynamic effect in the stomach lies between 5 mg kg^{-1} and 20 mg kg^{-1}.

Histology at 72 h after PDT with 5 mg kg^{-1} of AlS_2Pc showed a full thickness necrosis of the gastric wall with widespread infiltration of acute inflammatory cells (Figure 9a). The same transmural necrosis was seen with doses of 3 mg kg^{-1} and 2 mg kg^{-1}. Two weeks after PDT, the dead tissue has been demolished either by sloughing or resorption leaving a full thickness defect which was bridged by extensive deposition of scar tissue and new collagen on the serosal aspect. The mucosal defect was re-epithelialised initially with mucus secreting glandular epithelium but by 16 weeks after PDT, there were parietal cells and smooth muscle regeneration. No microscopic evidence of photodynamic damage was

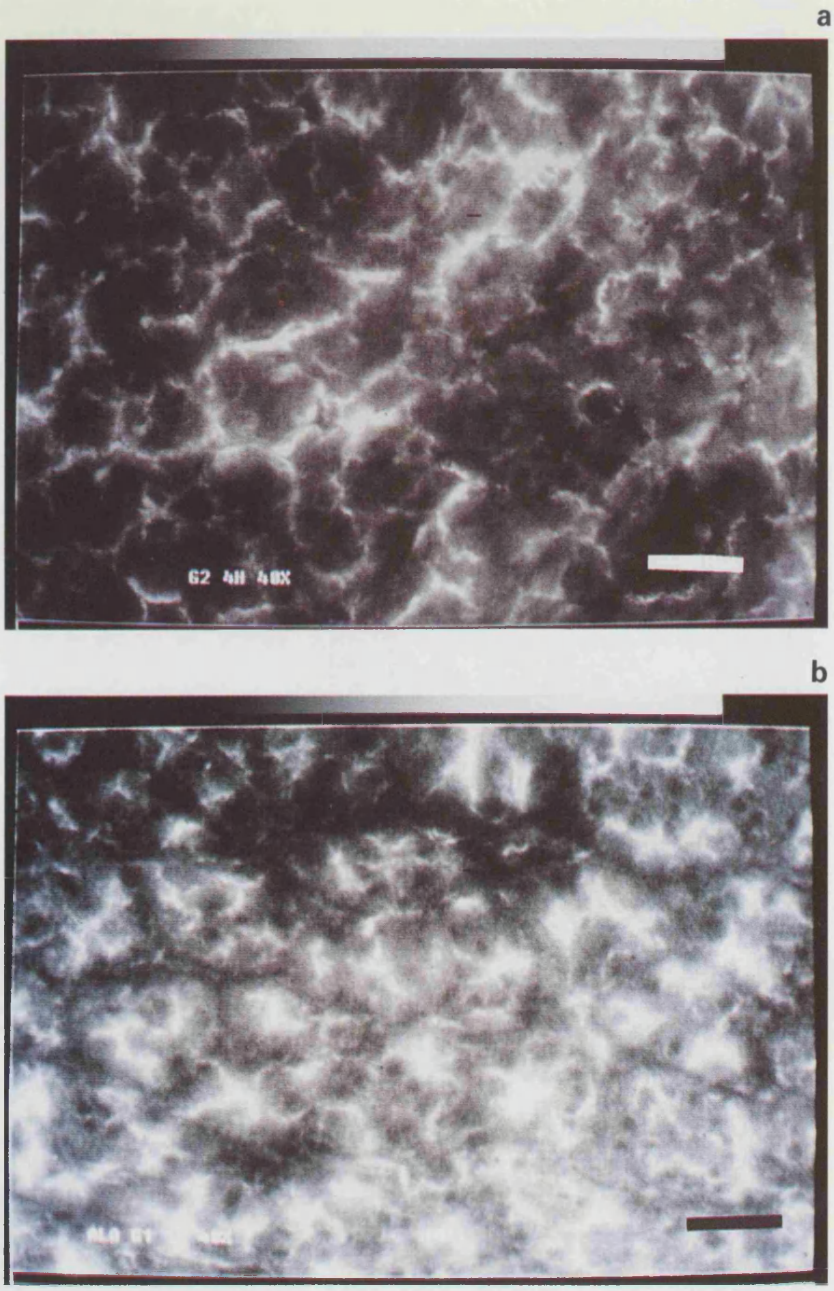

Figure 5 **a**, Grey scale fluorescence micrograph of a frozen section of gastric mucosa 4 h after intravenous administration of 5 mg kg^{-1} of AlS$_2$Pc showing high level of fluorescence mainly around the periphery of the epithelial cells. Scale: the bar in the lower right corner represents 25 μm. **b**, Grey scale fluorescence micrograph of a frozen section of gastric mucosa 1 h after intravenous administration of 200 mg kg^{-1} ALA showing high level of fluorescence mainly inside the cells but outside the nuclei. Scale: the bar in the lower right corner represents 25 μm.

seen with 1 mg kg^{-1} of AlS$_2$Pc. The photodynamic effect of ALA photosensitisation was predominantly confined to the mucosa with extensive necrosis of mucosal epithelial cells. There was some damage to the muscularis propria when a dose of 200 mg kg^{-1} was used but the layer remain viable. With the dose of 20 mg kg^{-1} however, the submucosa and muscularis were hardly affected (Figure 9b) and the resultant healing involve minimal scar tissue formation.

Discussion

Dysplasia in the alimentary tract is a difficult clinical problem. Although its malignant potential is well known, many clinicians are reluctant to advise excisional surgery in the absence of invasive malignant change. PDT may be an effective but less invasive treatment for this condition. The ideal goal would be the selective destruction of only the dysplastic mucosa. Barr *et al.* (1990) had shown that with AlSPc truly selective tumour necrosis (i.e. necrosis of tumour tissue but not the adjacent normal tissue from which the tumour arise) could be produced by judicious manipulation of treatment parameters in such a way that the photosensitiser concentration in the normal tissue fell below the photodynamic threshold while that in the tumour tissue remained above it. The volume of tumour necrosis produced was however very small. This was due to the small therapeutic ratio of conventional photosensitiser (2:1) between tumour and adjacent normal tissue (Tralau *et al.*, 1987). In addition, this true selectivity could only be applied

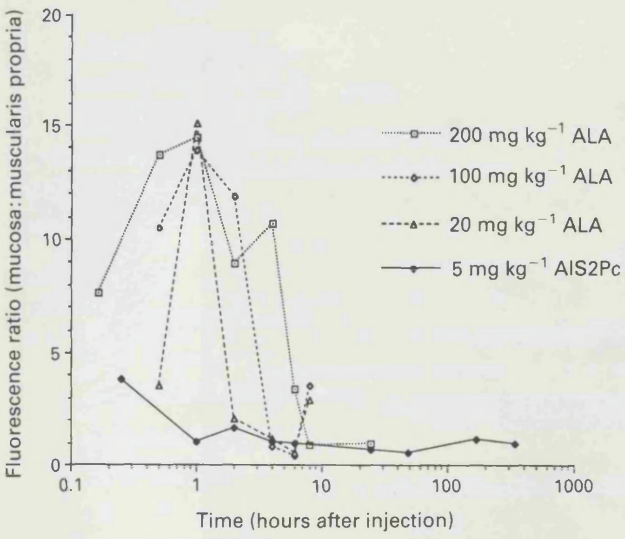

Figure 6 Ratio of fluorescence levels between the mucosa and muscularis propria in the rat stomach at the various time intervals after intravenous injection of 5 mg kg^{-1} of AlS_2Pc and 20 mg kg^{-1}, 100 mg kg^{-1} and 200 mg kg^{-1} of ALA.

to part of the tumours treated, and did not apply to the region where the tumour was invading normal tissue (Barr *et al.*, 1991). This was because there was always more photosensitiser in the stroma than malignant cells and shut down of small vessels would inevitably damage normal tissue along with tumour. Although some workers had shown qualitatively that haematoporphyrin derivative could localise in benign tumours (Dal Fante *et al.*, 1988; Gregorie *et al.*, 1968), carcinoma *in situ* (Cortese *et al.*, 1979; Gray *et al.*, 1967) and severe dysplasia (Benson *et al.*, 1982), quantitatively this differential was not likely to exceed that seen between established tumour and its normal tissue. Clearly, without a substantial therapeutic ratio between dysplastic and normal tissue, true selective ablation of dysplastic mucosa would be impractical clinically. Selectivity between mucosa and normal muscle is likely to be far more important than selectivity between dysplastic mucosa and normal mucosa. As long as large areas of mucosal defect heals safely with healthy epithelium, the net result will be selective destruction of dysplastic mucosa. The importance of preservation of underlying muscle in photodynamic mucosal ablation can be seen in the bladder where it has been shown that when photodynamic damage is localised to the mucosa, the underlying muscle layer retains its structural as well as functional integrity (Pope & Bown, 1991*a*). The aim of this study is to produce selective mucosal necrosis with sparing of the underlying layers and study the healing that takes place after such a specific injury.

In the first part of this study, we have demonstrated some of the pharmacokinetics of AlS_2Pc in the normal stomach. We have established the threshold photodynamic dose of AlS_2Pc as well as the time of maximum photosensitisation.

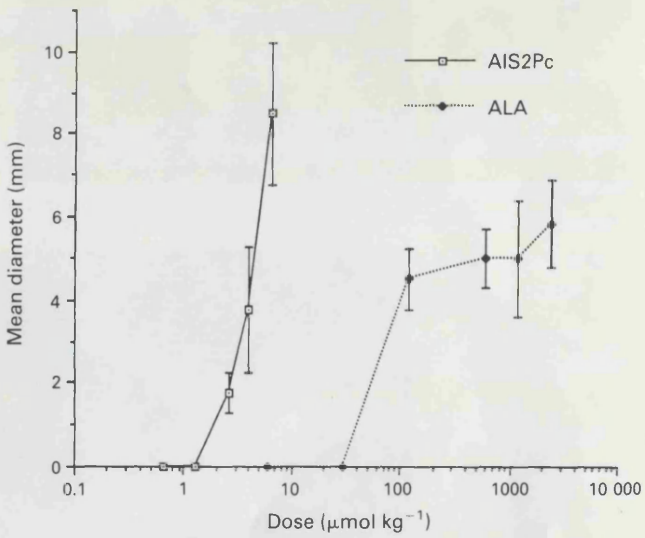

Figure 8 Mean diameter (± s.d.) of the photodynamic damage (50 mW × 1000 s, 675 nm wavelength for AlS_2Pc and 630 nm wavelength for ALA) in normal glandular gastric mucosa as a function of the administered dose of photosensitisers. All animals were exposed to light at the time of peak fluorescence for the respective photosensitisers as determined from fluorescence photometry. Each value represents the mean in two to three animals.

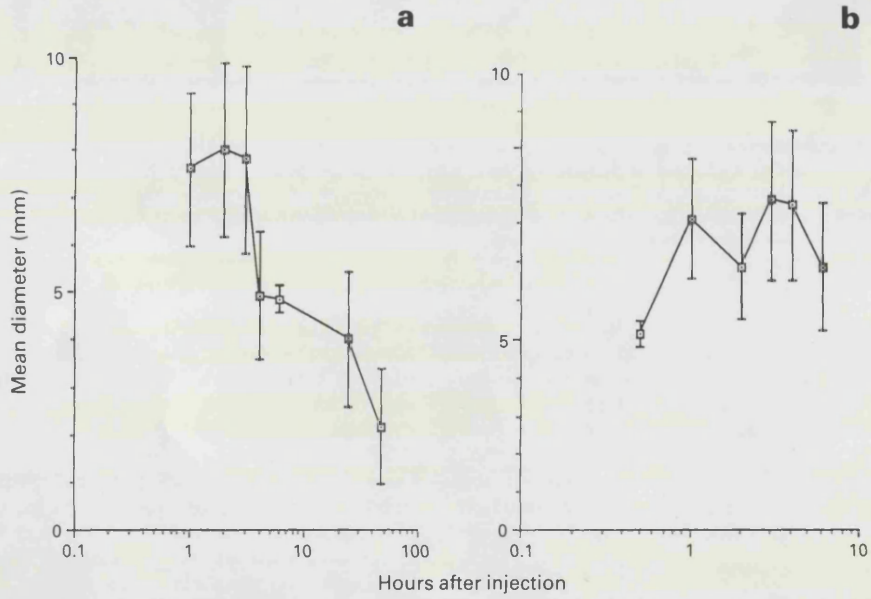

Figure 7 **a**, Mean diameter (± s.d.) of gastric ulcer produced in the normal glandular stomach mucosa after light exposure (675 nm, 50 mW × 1000 s) at the various times following sensitisation with 5 mg kg^{-1} AlS_2Pc. Each value represents the mean of diameters in four animals. **b**, Mean diameter (± s.d.) of gastric ulcer produced in the normal glandular stomach after light exposure (630 nm, 50 mW × 1000 s) at the various times following sensitisation with 200 mg kg^{-1} ALA. Each value represents the mean of diameters in four animals.

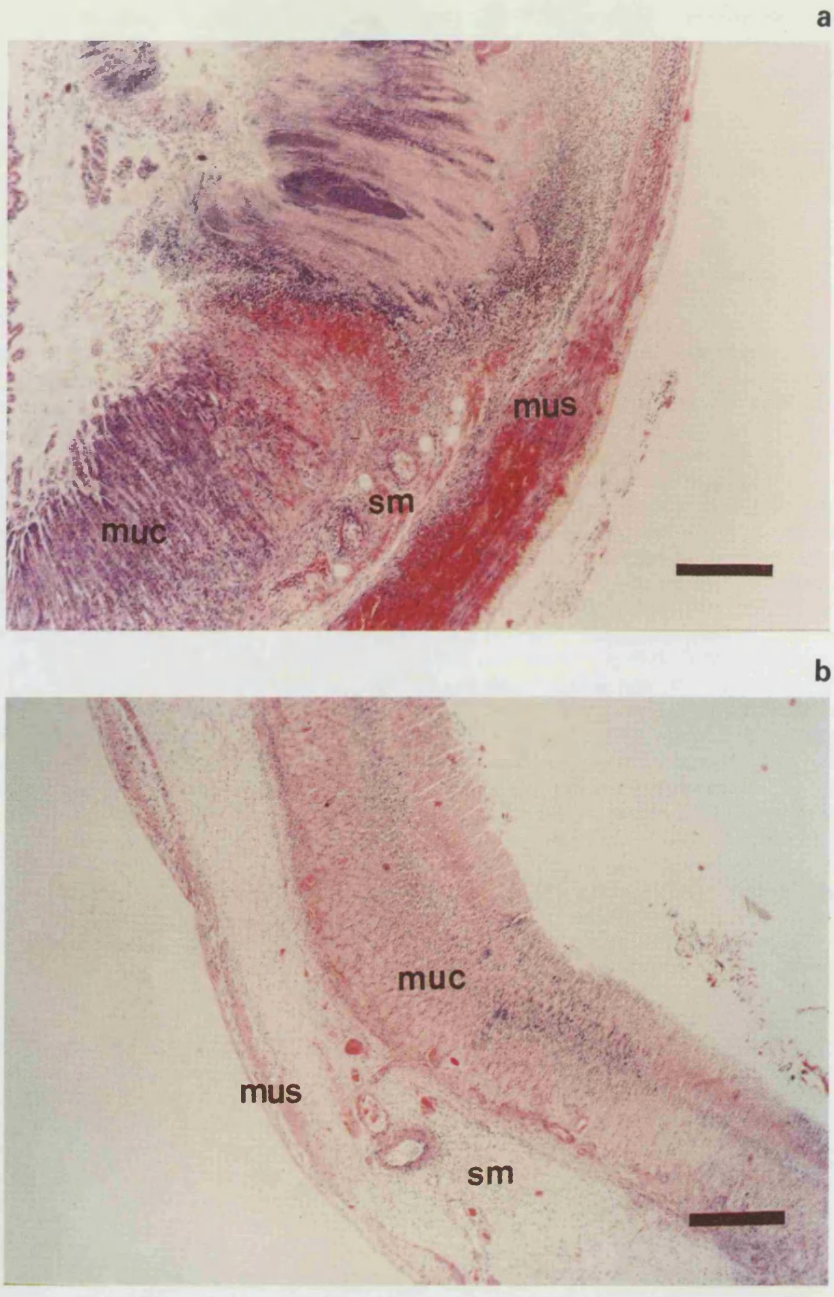

Figure 9 **a**, Micrograph of a H-E stained section of a typical lesion 3 days after photodynamic therapy with 5 mg kg^{-1} AlS$_2$Pc (50 mW red light at 675 nm for 1000 s 2 h after administration). The section shows extensive full thickness necrosis with widespread infiltration of acute inflammatory cells. Scale: the bar in the lower right corner represents 250 μm. (muc = mucosa; mus = muscularis propria; sm = submucosa). **b**, Micrograph of a H-E stained section of a lesion in the normal rat stomach produced 3 days after photodynamic therapy with ALA photosensitisation. 20 mg kg^{-1} of ALA was injected intravenously and the stomach exposed to 50 mW red light at 630 nm for 1000 s 1 h after administration. The section shows extensive mucosal necrosis with widespread infiltration of acute inflammatory cells. The submucosal and muscular layer appear intact. Scale: the bar in the lower right corner represents 250 μm. (muc = mucosa; mus = muscularis propria; sm = submucosa).

Our group has previously shown that AlS$_2$Pc distribution in tissue is dependent on the level of sulphonation (Chatlani *et al.*, 1991). The use of the more hydrophobic di-sulphonated preparation in this study was an attempt to localise AlSPc to the more cellular mucosal layer. However, what we found was sensitisation of the full thickness of the stomach wall. We were somewhat surprised to see little differential distribution between the mucosa and the muscularis propria as significant preferential mucosal sensitisation had been observed in bladder wall due to longer retention of AlSPc in the mucosa (Pope *et al.*, 1991*b*). *In vitro* studies had demonstrated that AlS$_2$Pc was readily taken up into cells in culture and the degree of this uptake increases linearly with lipophilicity. (Berg *et al.*, 1989). The subcellular localisation of AlS$_2$Pc *in vivo* after intravenous administration is unknown, althought this study demonstrates that it is predominantly peripheral around the gastric epithelial cells and probably extracellular. Parietal cells of gastric mucosa secrete acid. As hydrogen ions are secreted into the gastric lumen, bicarbonate and hydroxyl ions are transported across the basement membrane into the interstitium in order to preserve cellular neutrality. The resultant higher pH of the extracellular fluid can reduce the lipid solubility of the AlS$_2$Pc leading to a reduction in the AlS$_2$Pc partitioning between the

intra and extracellular compartment (Berg *et al.*, 1989). This could explain the extracellular location of AlS_2Pc in the gastric mucosa and the lack of preferential retention of AlS_2Pc by the mucosa as compared to the muscular layer in contrast to findings in other hollow organs such as the bladder as discussed above. In the light of the distribution of AlS_2Pc in the normal stomach wall, its panmural photodynamic effect is perhaps not surprising.

Following administration of exogenous ALA, accumulation of PPIX is dependent on the differential rate of its synthesis versus its conversion to haem by the various tissues. The greater accumulation of PPIX seen in the mucosal cells can either be due to a differentially larger PPIX synthesising capacity or a lower conversion capacity of PPIX to haem by mucosal cells as compared to the smooth muscle and connective tissue cells. Haem forms an integral part of various haem proteins which include haemoglobin, myoglobin, cytochromes and catalase. As haem requirement by different cells varies, the capacity for its synthesis can be expected to vary with different tissues. Sardesai and his colleagues (1964) measured the extracted porphyrins from various tissue following incubation with ALA and obtained the highest level of extracted porphyrins from nucleated chicken erythrocytes, especially haemolysed. In non haemopoietic tissue, higher levels were obtained from liver, and in decreasing order, kidney, brain and heart. They suggested that the porphyrin synthesis observed correlated with the rate and quantity of haem protein synthesis by the various tissues. Although porphyrin metabolism in other tissues has not been completely elucidated and the precise determinants for the larger capacity of porphyrin synthesis and accumulation in some tissues remains unclear, it is notable in the alimentary tract that the mucosa has the highest rate of turnover and is probably most metabolically active. By inference, neoplastic tissue may prove to have an even higher capacity in this respect. Using the techniques of tissue explant culture, Navone *et al.* (1990) were able to show that human breast cancer showed a 20-fold enhancement of enzymatic activity for porphyrin synthesis from ALA as compared to normal breast tissue between the stages of porphobilinogen and coproporphyrinogen formation. For purposes of selective PDT of tumours, however, the absolute quantity of the photoactive PPIX accumulated in the respective tissues is a more important end point. Preliminary studies on a colonic tumour model in rats showed that significantly more PPIX was synthesised in the tumour cells than in normal tissue (Bedwell *et al.*, 1992).

The histological location of photodynamic damage with both ALA and AlS_2Pc appears to reflect the distribution of photosensitiser across the gastric wall as observed with fluorescence microscopy. The mechanism of cytotoxicity is, however, different. There is good evidence to suggest that the photodynamic effect on cells from AlS_2Pc is secondary to damage to the microvasculature (Nelson *et al.*, 1988; Milanesi *et al.*, 1987). With ALA however, the synthesised PPIX is intracellular and the mechanism of cell death results from direct cellular photosensitisation. This is advantageous as this latter mechanism is less likely to disrupt the supporting tissue and vascular structure of the mucosa and this may lead to better healing with less scarring. Applying this to small nests of tumour cells, it would be possible to eradicate these without effecting adjacent normal cells if differential photosensitisation exists whereas with AlS_2Pc, damage to small vessels would necessarily result in necrosis of the entire field where these nest of tumour cells reside.

The kinetics of photosensitisers from the two substances is quite different. AlS_2Pc is an exogenous photosensitiser and its resultant phototoxicity is dose related. ALA, however, is not a photosensitiser in its own right. Phototoxicity requires its conversion to PPIX. In the presence of infinite capacity for this conversion, the yield of PPIX at any time point should correlate with the amount of ALA supplied. As each cell has a finite capacity for conversion of ALA to PPIX, when this capacity becomes saturated anywhere along its biosynthetic pathway, the time and dose correlation is lost (Figure 2). The lack of correlation between the time of maximum photodynamic effect and that of maximum tissue levels of PPIX also contrasts with the finding with AlS_2Pc. This might be due to production of other porphyrin species which are good photosensitisers but not well detected by our system. Alternatively, the intracellular location of protoporphyrin IX to sensitive organelles at certain times during the biosynthetic process might also cause an increased sensitivity of the cells to photodynamic effect during those times. Unlike AlS_2Pc, the extent of photodynamic effect of ALA failed to show a correlation with dose above its threshold photodynamic dose. There were several possible explanations. Firstly, a substantial amount of ALA is excreted unchanged in the urine in the first few hours after exogenous administration (Berlin *et al.*, 1956) and this excretion is likely to be dose related. Secondly, the PPIX biosynthetic pathway could have been saturated by a relatively low dose of exogenous ALA. Finally, the different site and mechanism of photodynamic action with ALA induced photosensitisation could result in a different dose response relationship.

As shown in our study, ALA provides a significant therapeutic ratio between the mucosa and muscle (in excess of 10:1). This means that with appropriate treatment parameters extensive mucosal necrosis can be produced with sparing of the underlying muscle layer. We have also shown that photodynamic destruction of normal mucosa is followed by complete regeneration of normal healthy mucosa. The next step, using a suitable animal model, will be to investigate the healing following photodynamic ablation of dysplastic mucosa. Although we confined our current experiments to the production of focal lesions, large areas of selective mucosal necrosis should be producible in the stomach with appropriate light delivery. The observation of late parietal cells regeneration in the regenerated mucosa following PDT is encouraging as it suggests that full mucosa secretory function can be preserved but safe healing of large areas of gastric mucosal necrosis will still require experimental validation. ALA has the added advantage of causing only short lived photosensitisation (Divaris *et al.*, 1990) and hence cutaneous phototoxic side effects are unlikely after 24 h. This should open up new possibilities for treatment of large tumours with multiple treatment sessions. Thus a certain volume of tumour can be necrosed with each treatment, exposing deeper fresh tumour for further PDT.

In conclusion, we have shown that by using parenterally administered ALA, we are able to produce a selective photosensitisation of gastric mucosa with sparing of the other tissue layers of the stomach and further studies using appropriate gastric tumour models are now warranted.

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secondary to non-respiratory weakness, and her respiratory rate remained steady.

The patient was told that we would be using a drug to improve muscle strength, and guanoxan was initiated with a 5 mg test dose. There were no side-effects and the drug was continued. The patient did not know exactly when the drug was introduced but she spontaneously remarked the same evening that "something has happened". Her PEFR rose to 200 l/min and the dysphagia had improved. Guanoxan was increased to 15 mg twice daily with continued improvement in swallowing and PEFR (figure). The patient had mild bowel looseness at the top dosage. The drug was gradually withdrawn over 2 weeks. Type B botulinus toxin was isolated from her remaining pot of hazelnut yoghurt.

Guanidine is the drug of choice for muscular weakness of botulism. 4-aminopyridine is less widely used; its side-effects tend to be more severe. With guanidine side-effects may limit the dosage and the drug comes in an oral preparation only and absorption can be compromised by paralytic ileus.[3] The main cause of mortality in cases of botulism in the developed world is complications of supportive therapy. Guanoxan (whose molecule contains a guanidine moiety) allowed our patient to drink earlier than she would have been able to do otherwise and nasogastric or parenteral feeding were not needed; nor, with the improvement in PEFR, was ventilatory support. The general improvement in her condition permitted earlier mobilisation so avoiding the complications of prolonged bed rest. Guanoxan or guanidine should be considered early in the management of suspected botulism. To wait for laboratory confirmation will increase the probability of supportive procedures being required.

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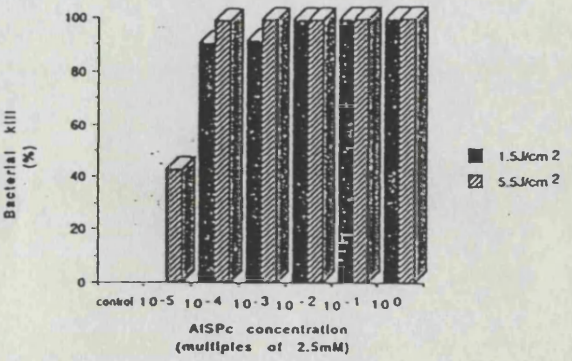
In vitro killing of *Helicobacter pylori* with photodynamic therapy

SIR,—*Helicobacter pylori* is closely associated with gastritis and peptic ulceration, and eradication of this organism results in lower recurrence rates after ulcer healing. However, even combinations of bismuth salts and antibiotics are far from perfect.[1,2] Since *H pylori* colonises endoscopically accessible parts of the upper gastrointestinal tract, it might be amenable to local endoscopic therapy. Photodynamic therapy (PDT) produces local tissue necrosis with light after administration of a photosensitising agent. The technique is of interest in tumour therapy but there have also been reports on the use of PDT to kill bacteria.[3,4] We have tested PDT against *H pylori* in vitro as a first step to assess the feasibility of destroying *H pylori* in vivo without unacceptable damage to normal mucosa.

H pylori was isolated from biopsy tissue taken from the antrum of six patients with gastritis and stored in glycerol broth at −70°C. Strains were subcultured onto brain/heart infusion agar containing 10 μg/ml amphotericin and incubated under microaerobic conditions. All six strains had similar sensitivity to PDT so just one was used subsequently.

The photosensitiser used was aluminium sulphonated phthalocyanine (AlSPc) (Ciba-Geigy). A 3-day agar culture of *H pylori* was harvested into saline and resuspended for 4 h in photosensitiser in aqueous solution at concentrations of 2·5 mmol/l to 25 nmol/l. 50 μl was then plated onto agar and exposed to red light at 675 nm from a copper vapour pumped dye laser (Oxford Lasers) to give light doses of 1·5 and 5·5 J/cm². Plates were incubated for 5 days and colonies were counted. Independent confirmation of uptake of AlSPc was achieved by fluorescence microscopy on *H pylori* cultures exposed to AlSPc for 4 hours at 25 μmol/l.



% kill of *H pylori* at two laser energies and after varying AlSPc concentrations.

The minimum inhibitory concentration (MIC) of bismuth subcitrate was measured for all six strains by standard plate assay techniques. Again there was no difference between strains, so further studies were done on the strain used for the main experiment. Bismuth subcitrate at a concentration 25% of the MIC was added to *H pylori* exposed to AlSPc at 2·5 μmol/l for 4 h and then exposed to light at 0·3, 0·6, or 0·9 J/cm² (energies sufficient to give partial but not complete kill if bismuth has not been added).

Red laser light at 1·5 J/cm² killed 100% of *H pylori* at photosensitiser concentrations down to 25 μmol/l; with 5·5 J/cm² there was 100% kill down to 2·5 μmol/l. On control plates (light only, sensitiser only, or no treatment) no bacterial cell kill was observed.

The MIC bismuth subcitrate was 16–32 mg/l. When sensitised *H pylori* were treated with bismuth subcitrate at 4 mg/l before laser therapy the PDT effect was abolished and colony growth was the same as that seen on control plates.

After an intravenous dose of 5 mg/kg AlSPc in rats the peak concentration in the colon is 5 μg/g.[5] A light dose of 50 J delivered from a fibre just touching the mucosa produced local necrosis which healed safely by regeneration of normal colon; light doses as low as those used in the above experiments had no effect. Fluorescence microscopy studies show that peak mucosal concentrations of 50 μmol/l or so were reached after administration of this dose of AlSPc. We achieved 100% *H pylori* kill at half this concentration even with a light dose of only 1·5 J/cm². Gastric mucosa, if it reacts similarly to colonic mucosa, is likely to remain intact when PDT is used to kill *H pylori*. It is unlikely that the intravenous route will deliver AlSPc to *H pylori*, so the key to this technique is whether *H pylori* will take up the photosensitiser in vivo after oral administration. The inhibitory action of bismuth on PDT could perhaps be avoided by giving bismuth after rather than before PDT. These preliminary findings raise the possibility of using PDT with or without antimicrobial drugs to eradicate *H pylori* from the stomach.

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