Non-porphyrin Photosensitizers in Biomedicine

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1 Introduction

The modern development of photosensitizers in medicine began with the use of porphyrins and ultraviolet light to image neoplastic tissue during surgery. This, linked with the fact that some porphyrins were known to produce phototoxic species, made a good argument for the use of porphyrin derivatives in the targeted destruction of tumours and opened the way to the current clinical field of photodynamic therapy (PDT). An excellent review on the use and modern development of porphyrins and porphyrin-based photosensitizers appeared in this journal recently.[1] The current article concentrates on what one might consider to be the poor relations of the photosensitizer world, *viz.* the natural and synthetic non-porphyrin derived photosensitizers.

Photodynamic therapy was developed as a novel treatment for cancer. The idea of dual selectivity, *i.e.* a tumour-specific drug (the photosensitizer) used in conjunction with fibre optic targeted light delivery is intended to give a treatment modality free from the noxious side-effects encountered with conventional chemotherapy or radiotherapy. In addition, the variety of toxic species generated (directly or indirectly) by PDT at the tumour site may be of use in multi-drug resistant (MDR) tumour types – literally swamping the increased defence mechanisms present, or attacking the cells *via* novel routes. The phenomenon of MDR in cancer is quite often encountered and makes the disease even more refractory.

Haematoporphyrin derivative, as its name suggests, comes from a natural source, although its use in nature is not as a photosensitizing compound. There are many more examples of natural non-porphyrin photosensitizers which have evolved over millions of years – normally in plants for chemical defence against microbial or herbivorous attack. The isolation and elaboration of compounds such as the perylenequinone pigments (PQPs) and the furanocoumarins represent a major advance in this area of research.

The requirement for new photosensitizers arose from the fact that the first generation porphyrins used in PDT have several drawbacks. They exhibit poor light absorption properties in the near infra-red, the region of the spectrum giving the maximum penetration of light through tissue and thus being of most use in the treatment of tumours. Early porphyrin drugs were not single compounds but mixtures, thus making it difficult to establish structure–activity relationships and so improve the efficacy of treatment. In addition, from the time of early clinical trials, long-lived skin photosensitivity (typically < 3 months) was apparent. While this side-effect in no way compares with the immunosuppression and general malaise experienced by patients under treatment with conventional anti-cancer drugs, it is nevertheless a side-effect which, given the current efforts in drug design, should be removed in the near future. However, second generation porphyrins such as Temoporfin are also known to cause post-treatment skin photosensitivity.[1]

Mark Wainwright was born in Workington, Cumbria in 1962. After taking his Chemistry degree at Leicester University he moved to the University of Leeds where he undertook research into photoconductive polymers. After his PhD (1988), he remained in Leeds, joining the nascent Centre for Photodynamic Therapy under Professor Stan Brown, synthesising new photosensitizers for PDT. He is currently a Senior Lecturer in Organic Chemistry at the University of Central Lancashire and a Visiting Specialist in Medicinal Chemistry at the Royal Preston Hospital, with research interests in photodynamic anti-cancers and antibacterials.

2 New Photosensitizers

Synthetic photosensitizers certainly prefigured the use of porphyrins *etc.* in malignant disease. Raab's experiments with eosin and acridine for the photodestruction of paramecium at the turn of the century were followed closely by the first clinical application of PDT – the use of eosin against skin cancer – in 1903.[2] It is interesting also to recollect the early use of methylene blue (MB) and light in the treatment of inoperable cancers three years later.[3] The idea of porphyrin localisation and its use in tumour staining is also predated by the use of synthetic dyes such as the phenothiaziniums and benzo[*a*]phenoxaziniums, both of which groups (*e.g.* MB and Nile blue) have been investigated as agents for PDT but thus far have found little clinical use other than in their continuing application in pre-surgical vital staining and histology.

During the late 1940s a series of investigations was carried out on the efficacy of synthetic dyes on tumour destruction in animals.[4] The simple rationale behind the research lay in the field of vital staining and it is this work which has inspired much of the current activity on cationic photosensitizers.

The use of photosensitizers against other types of disease is a burgeoning field which again has its origins in the early days of chemotherapy. The use of *e.g.* toluidine blue in the eradication of the bacteria responsible for dental caries, or of the causative organisms in oral candidosis can be traced back to the clinical demonstration of such pathogens using vital stains. If a known synthetic photosensitizer such as toluidine blue can be seen to stain a pathogenic organism sufficiently, then it should be possible to destroy that organism *via* subsequent irradiation.

3 Physicochemical Properties

The process of the design and synthesis of new photosensitizers can start from simple ideas. For instance, in the preceding example, is it possible to synthesise more specific analogues? Can they be made more photoactive? More often than not, the answer to both of these questions is 'Yes!' However, a note of caution must be added. If the photoactivity of a compound is increased by, for example, halogen substitution, it should be remembered that other properties of the molecule will be altered, such as its degree of lipophilicity (normally such substitution will increase the log *P* of the compound, see following). This, in turn, may change the cellular uptake and localisation of the photosensitizer, and possibly its inherent or dark toxicity. As in any other idea of drug design, the various uptake and toxicity parameters must be optimised to give a therapeutically useful compound. It is still a constant surprise to the author that more analogues of commercially available dyes are not produced and tested.

Chemicals required for use as photodynamic agents will invariably end up in solution, either in the dosage form or as a consequence of physical metabolism. It is necessary for the medicinal chemist to be able to say how the dissolved photosensitizer will behave in the various pharmacological compartments in which it finds itself. This often comes down to the partitioning behaviour of

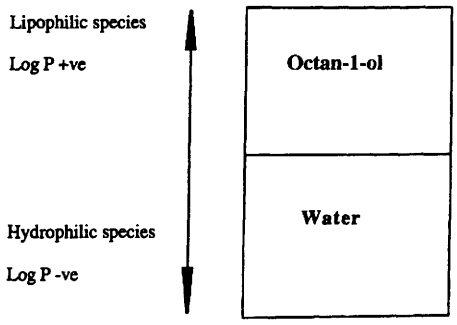


Figure 1 The partitioning behaviour of a photosensitizer

the compound between the water which is quite ubiquitous in the biological milieu and the various types of lipids encountered, for example, at the cell membrane or as part of the complex mixture of blood proteins, *i.e.* the hydrophilic/lipophilic balance of the drug. A simple *in vitro* measure of this behaviour is taken as the logarithm of the partition coefficient of the compound between a two-phase mixture, normally water and octan-1-ol (see Figure 1).

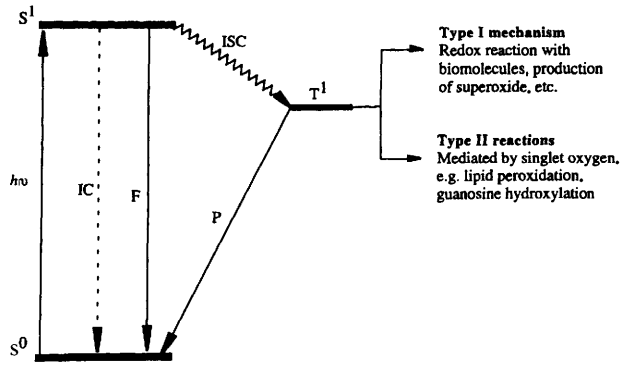
$$\log P = \log \{c_{Oct}/c_w\}$$

A problem particularly relevant to new photosensitizers based on commercial dyes arises here in that they are invariably used as charged species. Thus, dyes such as MB and toluidine blue are normally encountered as salts with the dye chromophore being the cation. In solution both dye cations are hydrophilic ($\log P = -0.9$). However, in biological systems toluidine blue is partially converted into a neutral form by deprotonation, and both dyes are liable to metabolic reduction which also renders them as neutral species. In each case the neutral form is highly lipophilic ($\log P > +3$). This behaviour is not apparent from simple water–octanol partitioning and so great care must be taken to avoid too much extrapolation from the results of such *in vitro* work.

The light absorption properties of putative new photosensitizers are also obviously of great importance (this is, in fact, the case with any drug: overlooking the photosensitizing activity of new agents can lead to needless side-effects such as skin sensitization). Visible and ultraviolet light are absorbed by various components of biological systems and this endogenous absorption may be critical in the use of photosensitizers if the wavelengths used for irradiation are too short. The natural pigment melanin has a wide absorption which covers most of the visible region, thus facilitating its protective capability against solar radiation. Haemoglobin has several absorption bands in the visible region. In addition, tissue scattering of light is important at shorter wavelengths. Thus, in tumour work, new photosensitizers are normally designed to absorb at long wavelengths – the normal range given is 600–1000 nm. This is known as the 'therapeutic window'. Photosensitizers which are useful against bacteria, viruses *etc.*, *i.e.* in external eradication, often absorb at wavelengths below 600 nm.

The efficacy of the photodynamic action associated with new photosensitizers should obviously be high if any advance is to be made on currently available agents. In terms of *in vitro* testing, certain performance indicators may be used, among which the singlet oxygen quantum efficiency (Φ_Δ) is often important, since it is intimately involved with the effectiveness of Type II processes (see Figure 2). It has often been demonstrated that the replacement of an atom in a lead structure with one of higher atomic number leads to an increase in the Φ_Δ value. This is known as the internal heavy atom effect.

In electronically excited molecules, spin–orbit coupling facilitates intersystem crossing (ISC, Figure 2), allowing otherwise forbidden changes in the spin state (*i.e.* singlet–triplet). Since the spin–orbit coupling constant is proportional to the fourth power of the atomic number of the element concerned, the presence of a 'heavy atom' (*e.g.* bromine or iodine) in a molecule enhances the degree of spin–orbit coupling. In terms of the compounds discussed here, this should lead to an increase in the triplet yield and improved photosensitizing activity.



Type I $^3Ps + Sub \rightarrow Ps^{+\cdot} + Sub^{-\cdot}$ (electron transfer)
$Sub^{-\cdot} + {}^3O_2 \rightarrow Sub + O_2^{-\cdot} \rightarrow HO^- + HO^\cdot$

Type II $^3Ps + {}^3O_2 \rightarrow {}^1Ps^+ + {}^1O_2$ (energy transfer)
$^1O_2 + Sub \rightarrow$ Sub-OOH (peroxides etc.)

(Where Ps = photosensitizer; Sub = substrate or solvent)

Figure 2 Modified Jablonski diagram showing the various photoprocesses involved on excitation of a dye molecule. Key: S^0 – singlet state, S^1 – first singlet excited state; ISC – intersystem crossing; T^1 – first triplet excited state; F – fluorescence, P – phosphorescence, IC – internal conversion.

It is, however, dangerous to take improved Φ_Δ values as a guarantee of high photodynamic activity – there are many other factors involved in moving from *in vitro* chemical testing to cell culture, and similarly to mammalian systems. Also, the reverse may be true: an apparent lack of photosensitizing ability does not necessarily mean that the compound undergoing testing will be ineffective in cells (*e.g.* Victoria blue, BO). A brief outline of Type I and II photoprocesses is given in Figure 2.

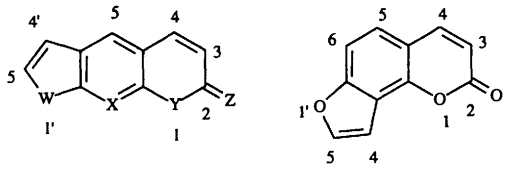
4 Naturally Occurring Photosensitizers and Their Derivatives

4.1 Psoralens

Psoralen derivatives have been used for thousands of years in the East and Middle East in the treatment of various skin disorders, but more recently the structure–activity relationships and sites of action of the psoralens have been elucidated and synthetic analogues prepared. The field of psoralen photomedicine, and in particular photophoresis, has become incredibly active in recent years, with a bewildering array of newly synthesised compounds as well as the *in vitro* testing of natural congeners and structural isomers of the furanocoumarin unit. A representative sample of the more promising compounds appears in Figure 3.

The concept of photophoresis is based on the selectivity of psoralen derivatives for malignant cells, such as the lymphocytes implicated in cutaneous T-cell lymphoma. Oral administration of the psoralen drug leads to its uptake by malignant T-cells in the bloodstream. Sequential removal of 0.5 l aliquots of blood, component separation and illumination of the white cell fraction with the appropriate wavelength of ultraviolet light leads to DNA damage *via* photoadduct formation and thus to direct cytotoxicity. Protein damage at the cell membrane leads to cell death or causes sufficient changes in cellular morphology such that the reintroduction of the white cell fraction into the bloodstream causes an autovaccination effect, *i.e.* the malignant cells are not recognised by the body's immune system and are thus destroyed.[5]

There are many examples in early photochemotherapy which suggest that simple psoralens cause cross-linking in DNA. This might be deleterious in the long term, and merits special attention if the disorder under treatment is non-malignant. Psoralen functionalisation in the pyrone ring can yield compounds which are unable to form cycloadducts *via* this ring due to steric factors. Since methylation in the furanocoumarin nucleus furnishes compounds, *e.g.*



Psoralens Angelicins

W	X	Y	Z	
O	CH	O	O	Psoralen
NH	CH	O	O	Pyrrolocoumarin
S	CH	O	O	Thienocoumarin
O	N	O	O	8 Azacoumarin
O	CH	NH	O	Furano-2 quinolone
O	CH	O	S	2-Thiofuranocoumarin
O	CH	O	Se	2 Selenofuranocoumarin

Figure 3 Psoralens, angelicins and bioisosteres

4,8,5′-trimethylpsoralen or 4,6,4′-trimethylangelicin, which cause DNA cross-linking,[6] the steric factor is obviously important The 3-ethoxycarbonyl analogue is sufficiently hindered to give rise only to mono-adducts with DNA

Psoralens can also cause hydroxylation of guanosine in nucleic acids, a mechanism often associated with the intermediacy of singlet oxygen However, in recent work with 3-ethoxycarbonyl-psoralen, which is efficient in causing guanosine hydroxylation, neither degassing of the reaction media nor the use of deuteriated water gave results consistent with a Type II mechanism It is possible, though, that the effective intercalation of the furanocoumarin chromophore with DNA may make it difficult for D_2O interaction to occur [7]

In many ways, the idea of psoralen – protein interactions has been overlooked in recent years, yet the widely reported psoralen – DNA adduct formation cannot explain the immunotherapeutic basis of the treatment [5]

Although it is suggested that psoralen photodamage to the cell membrane may be due to psoralen photoadducts with biomolecules other than nucleic acids,[5] this sort of damage can certainly be envisaged as occurring *via* a photodynamic rather than a photochemotherapeutic route, since the production of singlet oxygen by psoralen derivatives in solution is now well established If this is indeed the case, the ongoing efforts in new drug design and synthesis in this area, and particularly those involving increasing the Φ_Δ values are well justified

When a drug is established for the treatment of a particular disorder, analogues are synthesised as a matter of course in order to improve on the activity of the lead compound This is, in many cases, the *raison d'être* of the medicinal chemist There are two main reasons for the development of analogues of psoralen increasing the selectivity for malignant cells, or increasing the photoactivity at the target site, for example *via* the heavy atom effect Thus, many bioisosteres and structural isomers of the furanocoumarin nucleus have been isolated or synthesized *de novo* (see Figure 3) The replacement of the furan oxygen with sulfur or selenium gives rise to compounds having much improved photoactivity In addition, the 8-azapsoralens – *i e* analogues arising from replacement of carbon-8 with nitrogen – exhibit lower incidences of DNA cross-link formation The activity of 4,4′,5′-trimethyl-8-azapsoralen in terms of its inhibition of DNA synthesis in Ehrlich cells has been reported to be six times that of 8-methoxypsoralen (8-MOP) and it was efficacious in the clearance of psoriasis in recent clinical trials However, 8-MOP showed slightly higher activity than the azapsoralen [6] The evidence of a decreased incidence of cross-linking by the azapsoralen compared to that by 8-MOP should encourage further clinical testing

4.2 Anthracyclines

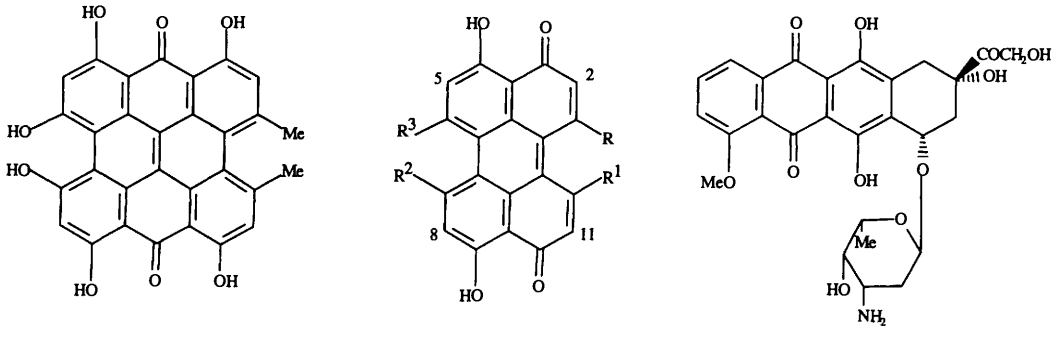
Insofar as the use of conventional anticancer drugs in PDT is concerned, it may be possible to exploit a favourable combination of tumour selectivity and reasonable light absorption properties to improve on typical antitumour activity Likely candidates fulfilling such criteria are members of the anthracycline group, such as Doxorubicin

Synthetic anthraquinone textile dyes are well known as causative agents in skin phototoxicity, *e g* 'bikini dermatitis' Drugs such as Doxorubicin (Figure 4) have also shown phototoxicity *in vitro*, although to a lesser degree than mainstream PDT agents Additionally, the λ_{max} values for such drugs are usually not much greater than 500 nm These factors may not be too important, since the current view on anthracycline PDT is to use the photoactivity as an additive therapy, and not as a single means of tumour destruction [8]

Although drugs like Doxorubicin show a reasonable selectivity for tumours, they also exert adverse effects on healthy cells, in particular cardiotoxicity The utilisation of the extra photodynamic activity associated with such drugs may make it possible to produce the same levels of antitumour activity at lower drug doses with a concomitant decrease in side effects The likely clinical inception of this type of combinative therapy must be given extra momentum by the fact that, through long clinical use, the pharmacology of the anthracycline drugs is by now very well understood

4.3 Hypericin and Hypocrellins

Herbivorous animals, for example domestic cattle, have long been known to suffer from a skin disorder (hypericism) after the ingestion of a photosensitizer contained in the weed, St John's Wort (*Hypericum perforatum*) Skin photosensitization occurs due to the



Hypericin Perylenequinonoids Doxorubicin

R	R¹	R²	R³	
R/R¹	$CH_2C(OH)MeCH(COMe)$	OMe	OMe	Hypocrellin A
R/R¹	$-CH_2CMe{=}C(COMe)$	OMe	OMe	Hypocrellin B
$-CH_2C(OH)Me$	$-CH_2C(OH)Me$	R²/R³	CH_2OCH_2	Cercosporin
$CH_2CHMeCOPh$	$CH_2CHMeOCO$-*p* C_6H_4OH	OMe	OMe	Calphostin C

Figure 4 Hypericin and the perylenequinonoid pigments

transport of the pigment hypericin in the animal's bloodstream to the epidermal capillaries and subsequent activation by sunlight Similar findings in China led to the use also of extracts of *Hypocrella bambusae* in the traditional treatment of skin disease The perylenequinonoid pigments (PQPs) and the related hypericin implicated here were investigated, and their favourable photoproperties – near infrared absorption and high singlet oxygen efficiency (typically Φ_Δ *ca* 0 8) – have promoted their *in vitro* testing for PDT

Hypericin, and the PQPs have been shown to be pharmacologically active in terms of malignant disease Their high log *P* values coupled with formal negative charge explain the lipoprotein binding and membrane targeting associated with the pigments In addition, the large pseudoplanar area makes them obvious candidates as antivirals – indeed hypericin is undergoing clinical trials in AIDS patients [9]

Increased interest in hypocrellins has arisen due to the fact that they have been found to be inhibitors of protein kinase C (PKC), a key enzyme in the proliferation of tumour cells Semi-synthetic approaches to new hypocrellin photosensitizers, involving their action against PKC were made starting from the known PQP, cercosporin *via* conjugate addition of, *e g* , thiophenol at positions 5 and 8 Calphostin C (see Figure 4) appears to be more active in PKC inhibition than any of the newly tested derivatives, and it is suggested that the increased photoactivity against PKC arises *via* the addition of cysteine residues in active sites of the protein (through -SH) at positions 5 and 8 of calphostin C Thus, when these positions are blocked, the photoactivity decreases [10]

Several new derivatives of the hypocrellins have exhibited higher photoactivity in cell culture The hypocrellin structure is such that the phenolic groups (positions 4 and 9) may be replaced by nucleophiles and alkylamino substitution has recently been shown to have dramatic effects on the phototoxic nature of the prepared derivatives Thus, the 4,9-bis(butylamino)-derivative of hypocrellin B has a much higher ϵ_{max} value than the parent compound, leading to far greater phototoxicity against EMT-6 cells in culture At the same time, the introduction of the butylamino side chains decreases the dark toxicity of the derivative relative to the parent thus yielding a compound reportedly having a high light/dark toxicity ratio ($\geq$ 167) This study has demonstrated the potential of the hypocrellin skeleton for functionalisation, leading to changes in physicochemcial properties and improved characteristics for PDT [11]

5 Synthetic Photosensitizers

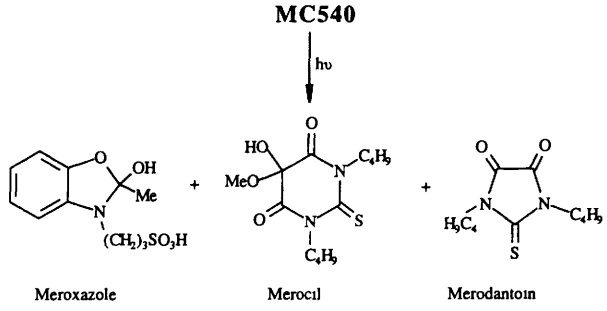
5.1 Cyanines

Cyanine dyes have been used as photosensitizers from the early days of the photographic industry In addition, work carried out by Carl Browning at the end of the First World War clarified some structure–activity parameters for a large series of the dyes as antibacterials The structure of cyanine dyes is such that analogue formation is straightforward, *e g via* variation in heteroatom, *N*-alkylation, length of polymethine chain *etc* , and has led to a large number of compounds being synthesised Research activity in this area has therefore been considerable

Generally, the λ_{max} for a given cyanine dye may be increased by lengthening the polymethine chain This may, however, lead to problems with photoisomerisation, a major deactivation route for cyanine dyes Increasing the chain length often yields dyes which are less chemically stable and causes decreased aqueous solubility A careful balance of these factors is thus required when designing likely candidates for testing as photosensitizers in biological systems

This work has resulted in the emergence of such dyes as Merocyanine 540 (MC540), EDKC (a kryptocyanine) and thence chalcogenapyrylliums as active agents in the photodynamic treatment of malignant disease Whereas EDKC and its congeners appear to have progressed little in recent years, MC540 is particularly useful against leukaemic cells and has undergone phase I clinical trials for the purging of autologous bone marrow grafts MC540 was in the vanguard of the synthetic non-porphyrin photosensitizers evaluated in the mid–late 1980s, and as such, the understanding of its mode of action – and therefore that of other cyanine photosensitizers – is now extensive

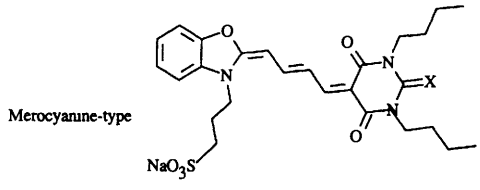
MC540 may be used in two different ways against target cells The conventional mode of action is that of membrane photooxidation, the lack of intracellular targeting reflecting the anionic character of the photosensitizer Conversely, the idea of preactivation of MC540 uses photoirradiation of the photosensitizer prior to its delivery to the tumour *etc* , the reaction of the dye with singlet oxygen yielding stable photoproducts such as merodantoin and merocil (Scheme 1) These photoproducts are known to act against topoisomerase II, an enzyme intimately involved in DNA replication, and thus to inhibit macromolecular synthesis by conventional chemotherapeutic routes One advantage of this technique lies in the lack of toxicity of the photoproducts to healthy cells, allowing attack on *e g* metastatic disease since no further light delivery is required [12]



Scheme 1 Preactivation photoproducts of MC540[12]

The structure of MC 540 allows wide scope for the design of analogous series This is necessary due to the low Φ_Δ value (0 002) of the dye On utilising the heavy atom effect in substituting selenium for sulfur at position 2 of the barbiturate moiety, a significant increase in the quantum yield of singlet oxygen is produced Photoisomerisation of the central double bond of the polymethine chain, a major deactivation pathway for MC540 is also absent in the seleno-analogue [13] Both of these factors obviously contribute to the increased Φ_Δ value *in vitro* (see Figure 5) However, photobleaching of the analogues in the cellular milieu under experimental conditions means that MC540 is still likely to be a better antileukaemic

Carbocyanines and related dyes have also been proposed as PDT agents As with MC540, their levels of activity suffer from photoisomerisation This problem has been approached in two ways the introduction of substituents into the *meso*-position of the



X	R	λ_{max}/nm	$\Phi_{\Delta(liposomes)}$	$\Phi_{\Delta(EtOH)}$
Merocyanines				
O (MC540)		535		0 001
S		555		0 002
Se		561		0 38
Dicarbocyanines				
S	Et	551	0 01	0 001
Se	H	574	0 08	0 014
O (DHOCl)	H			

Figure 5 Cyanine photosensitizers[13,14]

polymethine chain or the use of long chain alkyl groups on the 3,3′-positions While the former approach rigidifies the polymethine chain directly, it has been demonstrated in liposomes that the long chain *N*-alkyl substituents cause physical anchoring and thus rigidification of the molecule within the lipid bilayer [14] In addition, Φ_Δ values may be increased *via* the heavy atom effect (see Figure 5)

Compared to MC540, the different charge on the carbocyanines obviously has ramifications regarding sites of action Thus, whereas MC540 acts at the plasma membrane, cationic cyanines may be internalised, typically in the mitochondria However, as more examples are synthesised, new targets are being identified For example, 3,3′-dihexyloxacarbocyanine iodide (DHOCI – see Figure 5) causes specific photodamage to microtubules [15] Such results are very promising, given the great cellular sensitivity of the target site

5.2 Phenothiazinium Photosensitizers

The phenothiazinium dye methylene blue (MB) has been used extensively for over a century as a vital stain It is widely employed in the clinical diagnosis of a variety of diseases and as a tumour marker in surgery Its use as a PDT agent is not widespread, which might be surprising, given the extensive commercial use of MB as a photosensitizer, were it not for the facile reduction of the dye in the biological milieu MB is reduced to leuco methylene blue (LMB) by the ubiquitous cellular coenzymes, NADH and $FADH_2$ In point of fact, the MB → LMB conversion forms the basis of the tuberculin test in milk

The reduction of MB by tumour cells means that the efficacy of photosensitization is decreased LMB is colourless, and is thus inactivable by the long wavelength light used in PDT In addition, the pK_a value of LMB is low (5 8) compared with MB, leading to a low level of ionisation of the reduced species (31% ionised at pH 7 3) High ionisation is essential for efficient DNA intercalation, and photodamage to DNA is thought to be important in the photocytotoxicity of MB

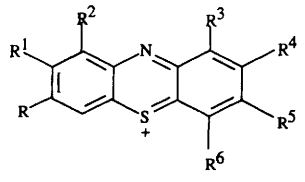
There are several closely-related commercial analogues of MB (Figure 6) the demethylated azure stains and thionin, and nuclear-substituted derivatives such as toluidine blue O (TBO), methylene green and Taylor's blue The majority of biological and clinical work has involved MB and TBO, once again reflecting their widespread use in vital staining

Although, as mentioned above, MB is not yet widely used in clinical PDT, its efficacy in the treatment of several tumour types has been demonstrated, both in animals and recently in patients Recurrent inoperable oesophageal tumours have been treated with locally delivered MB and were illuminated with a modified optical fibre intraluminally, giving tumour eradication [16] In these cases, the photosensitizer was injected directly into the tumour, thus further minimising side-effects

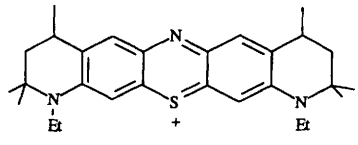
One of the reasons for the apparent reluctance in the use of MB–PDT could be the dark toxicity associated with the photosensitizer To a certain extent this may be overcome by physical selectivity – *i e* applying the photosensitizer directly to the tumour site, as in the preceding case This also explains the trial use of MB in bladder cancer since the photosensitizer solution can be instilled into the bladder cavity quite simply

Extra selectivity in anticancer drugs can be achieved using antibody labelling This has been attempted with MB by the synthesis of side-chain maleimido- and succinimido- derivatives The resulting MB–protein conjugates are reported to be slightly less photoactive *in vitro* than the parent compounds [17]

The above example aside, there are few reports of novel synthetic (*i e* non-commercial) analogues of MB This may be due to the misconception that such dyes may only be produced using archaic recipes from the late 19th century dye industry While it is true that the more complex (ring-substituted) derivatives are best synthesised *via* dichromate oxidation of diaminoarylthiosulfonic acids, the simpler 3,7-bisamino-analogues of MB are easily obtained through 5-phenothiazinium iodide[18] (Schemes 2 and 3), and using this method, it is possible to achieve differing amino character However, the utility of the 'old' method is shown by a recent example, prepared quite simply from 1 ethyl-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline The reduced quinoline derivative can be thought of as an elaboration of the *N,N*-dimethylaniline used routinely in the industrial preparation of MB itself The resulting

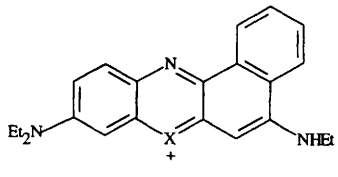


R	R^1	R^2	R^3	R^4	R^5	R^6
Me_2N	H	H	H	H	NMe_2	H
Me_2N	H	H	H	H	NH_2	H
Me_2N	H	H	H	H	NHMe	H
MeNH	H	H	H	H	NH_2	H
H_2N	H	H	H	H	NH_2	H
Me_2N	H	H	H	H	NMe_2	NO_2
EtNH	Me	H	H	Me	NHEt	H
Me_2N	H	Me	Me	H	NMe_2	H
Me_2N	H	H	H	Me	NH_2	H



MBD

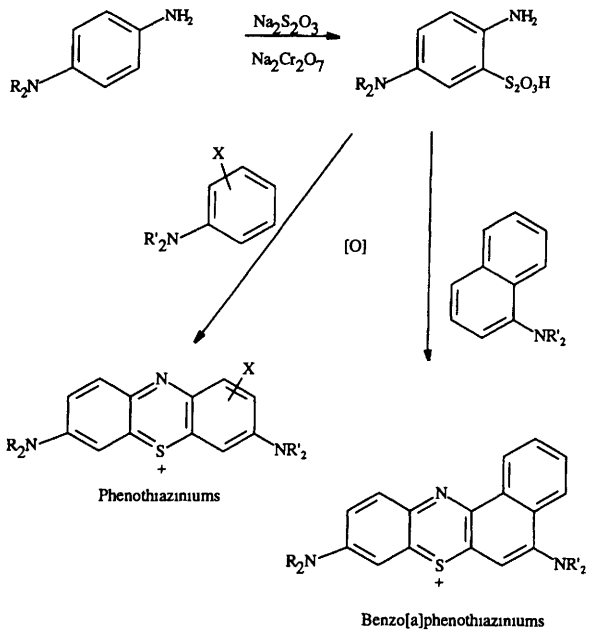
	λ_{max}/nm
Methylene Blue	661
Azure A	633
Azure B	648
Azure C	616
Thionin	598
Methylene Green	656
New Methylene Green	630
Taylor's Blue	649
Toluidine Blue	626



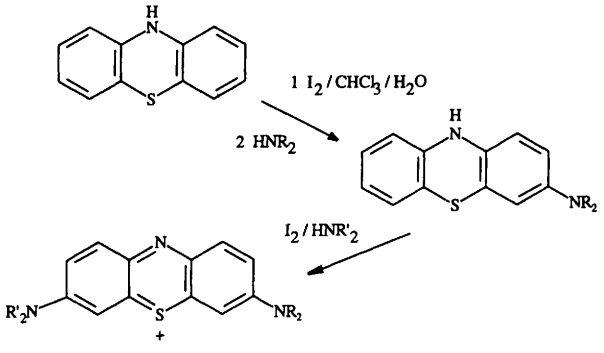
X	λ_{max}/nm	Φ_Δ
O[a]	632	0 005
S	652	0 025
Se	659	0 650

Replacement of NHEt with NH_2 = Nile Blue A
Replacement of NHEt with H and NEt_2 with NME_2 = Meldola s Blue

Figure 6 Phenothiazinium and Nile Blue derivatives[25]



Scheme 2 Synthesis of derivatives Methylene Blue and Nile Blue



Scheme 3 Synthesis of Methylene Blue derivatives from phenothiazine[18]

pentacyclic MB derivative (MBD – see Figure 6) showed selective uptake by a fibrosarcoma in rats but was not taken up by metastatic neoplastic cells [19]

The use of MB against viruses is a rapidly expanding area The photoactivity of the dye against highly infective enveloped viruses such as HIV and hepatitis C is important because of its application in the disinfection of donated blood The disinfection technique is quite straightforward the long wavelength absorption of MB means that blood mixed with a small amount of photosensitizer may be illuminated while still in the 'blood bag' Although MB can inactivate free viruses without collateral damage to other blood components such as proteins, platelets *etc* , there is a problem in that intracellular viruses remain unaffected The answer may lie in a PDT/photochemotherapy approach – *i e* using MB to eradicate free viruses in the sample in conjunction with a psoralen-type agent for intracellular viruses [20] This method would obviously require more sophisticated light delivery

5.3 Toluidine Blue (TBO)

Similar in structure to MB, TBO has similar properties as regards DNA intercalation, although the λ_{max} of TBO is rather shorter at 626 nm (in water), which obviously makes it less attractive in terms of light penetration in tissue However, TBO has found widespread use in the diagnosis of oral disease, since it is a selective stain for oral cancer and also for various oral pathogens This has led to an investigation into photosensitizer-mediated photodestruction of bacteria and other microbes pertinent to modern dentistry by Michael Wilson and coworkers at the Eastman Dental Institute in London

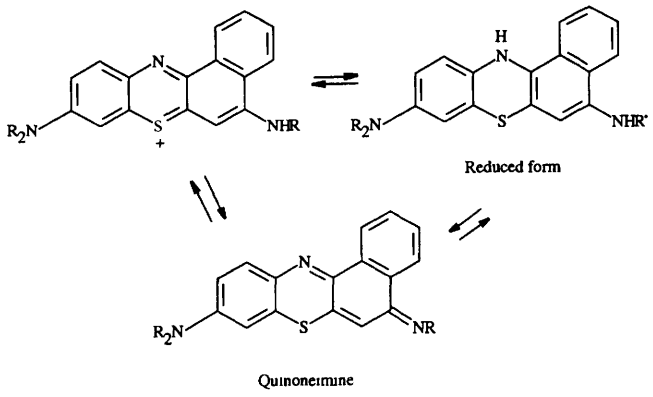
The presence in dental plaques of bacteria leads to the erosion and disease of the oral surface involved – either tooth enamel (dental caries) or gum tissue (gingivitis) Chronic disease requires the removal of a large amount of enamel and dentine or tissue in order to eradicate the bacteria The use of a pathogen-specific photosensitizer and low-power laser light to destroy the bacteria would thus entail less removal of healthy tissue This phototoxicity has been demonstrated by TBO for a wide range of oral pathogens such as *Streptococcus mutans* and *Lactobacillus casei* [21] The technique is also of use against oral *Candida* stains – important pathogens in terms of the treatment of AIDS patients – and has been demonstrated against the clinically important methicillin-resistant *Staphylococcus aureus* (MRSA) [22] MRSA infection, particularly in post-operative situations (biofilm infestation of implants, catheters *etc*) is an increasing cause of morbidity Another refractory stain of pathogens, *Helicobacter*, which has in recent years received a good deal of attention due to its reputed involvement in gastric ulcers and cancer, is also susceptible to photodynamic treatment using TBO [23]

Although the photokilling of bacteria and yeasts has been widely reported, the clinical importance of this work is that it has been demonstrated in biofilms, since this is the most likely pathogenic presentation The reason that drug resistance is overcome using the photodynamic approach must surely be the same as in resistant tumour cells – a different type of toxicity means that the mechanisms available to combat it are absent Other types of photosensitizer have been shown to be effective in this respect, such as aluminium disulfonated phthalocyanine, however this is less effective than TBO [21] The high activity of TBO and MB against yeasts relative to established tumour PDT agents such as aluminium disulfonated phthalocyanine and dihaematoporphyrin ester was evident in the *in vitro* photodestruction of *Candida albicans* [24] This is perhaps unsurprising in the latter case since tumour phototoxicity due to dihaematoporphyrin ester is normally associated with its localisation in tumour vasculature, not intracellularly

5.4 Nile Blue Analogues

Nile blue is taken up extremely well by tumour cells, thus making it an excellent tumour marker It is actually a benzo[*a*]phenoxazinium salt synthesised from 5-(*N*,*N*-diethylamino)-2-nitrosophenol and 1-naphthylamine Simple analogues with different amine functionality in the 5- and 9-positions may be obtained easily from Meldola's blue (Figure 6), or derivatives thereof Nile blue and several of its analogues were examined as potential photosensitizers for PDT, but very low singlet oxygen efficiencies preclude their usage The reason for the poor efficiency is known to be the low occurrence of intersystem crossing (see Figure 2) This has been remedied by introducing heavy atoms into the chromogen Indeed, the recent work on benzo[*a*]phenoxazinium chalcogen analogues stands as an excellent example of the heavy atom effect (see Figure 6)

Nile blue analogues containing sulfur at position 7 are synthesised *via* a similar method to that for MB except that the 4-dialkylaminoaniline-2-thiosulfonic acid is isolated, purified and then oxidised (dichromate) together with a 1-aminonaphthalene (Scheme 2) The nile blue derivatives so produced were found to have greatly increased singlet oxygen efficiencies, and also to be lysosomotropic – *i e* to accumulate preferentially in lysosomes The presence of the 9-amino (in later derivatives, 9-ethylamino) group allows the formation of the neutral quinoneimine species at physiological pH (see Scheme 4), thus facilitating crossing of the lysosomal membrane Once inside the organelle, lower pH causes protonation of the quinoneimine, blocking the reverse passage Photodestruction of the lysosomes releases hydrolytic enzymes into the interior of the cell, thus mediating cell death The activity of 9-ethylamino derivatives was further enhanced by inclusion of selenium at position 7, again increasing the singlet oxygen efficiency In the cultured cells used (EMT-6 mouse mammary sarcoma) the seleno-analogue was 1000 times more phototoxic than photofrin II In addition it has been shown that reduction and indeed photoreduction of the photosensitizers occurs in the absence of oxygen, yielding the neutral leuco species (Scheme 4) [25]



Scheme 4 Pharmacologically relevant species from Nile Blue derivatives[25]

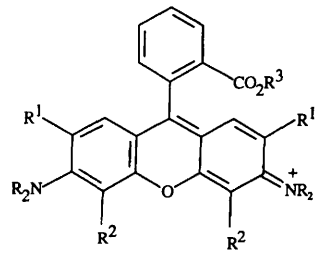
In animal tumour models, 5-ethylamino-9-diethylaminobenzo[*a*]phenothiazinium chloride (EtNBS) has been shown to be an efficient PDT drug The reasons for this lie, most probably, in the redox activity of the compound in the tumour and in pharmacologically different compartments such as the skin Thus, subcutaneous administration of the drug followed by photoirradiation gave tumour destruction without subsequent skin photosensitization because of differences in distribution (eight hours post-injection EtNBS was present in the ratios 4 1 tumour surrounding muscle and 8 1 tumour skin) and also because of the differing metabolic rates of the organs involved The tumour contained mainly oxidised EtNBS whereas the drug is evidently reduced to the inactive leuco-EtNBS in the skin In addition, the lysosomal selectivity of EtNBS ensures that it is present *inside* the tumour cells, thus leading to efficient tumour kill, rather than destruction of its vascular support [26]

The first reported photodestruction of larger-scale murine tumours (diameter 8–10 mm) has been achieved using a combination of EtNBS and a benzoporphyrin derivative (BPD–MA) It is thought that the PDT effect of the two drugs is synergistic rather than additive, since increasing the light dose or the concentration of either of the drugs singly had no significant effect (and indeed doubling the dose of BPD–MA caused death in three-quarters of the animals tested) Examination of the localisation of the drugs showed that BPD–MA was quite diffusely localised throughout the tumour, but mainly at the cell membranes, and so did not compete with EtNBS [27]

5.5 Rhodamines

The discovery that the commercial dye Rhodamine 123 (Rh123) is taken up specifically by mitochondria, together with its known use as a fluorescent indicator led to its use as a fluorescent probe in subcellular studies Subsequently, and with the modern evolution of PDT, this was extended to the investigation of Rh123-treatment of animal tumours Rh123 has a high fluorescence quantum yield (Φ_f = 0 9) and not surprisingly shows considerable dependence for cell-killing on high levels of tumour cell oxygenation [28] Analogue preparation has therefore been carried out to improve on this situation

The 4,5-dibrominated analogue of Rh123 is simply prepared *via* direct bromination at room temperature Differing ester functionality has also been included in recent work to improve cellular photosensitizing ability (see Figure 7) although there is a concomitant increase in dark toxicity with the longer chain (butyl) ester, presumably due to the higher lipophilicity of this compound leading to a lower rate of efflux from cells [29] Tetrabromorhodamine 123 (TBR123), having two more bromines attached to the parent nucleus is, not surprisingly, considerably more lipophilic and is found in hydrophobic regions of malignant cells [30] Interestingly, when used against multidrug resistant (MDR) cell lines, photoinactivation of the cells was not observed, but the cellular detoxification agent P-glycoprotein was inhibited, allowing increased uptake of other toxic compounds such as Rh123 or the conventional anticancer drug,



	R	R^1	R^2	R^3	Φ_Δ
Rh123	H	H	H	Me	0 01
DBR123	H	H	Br	Me	0 47
TBR123	H	Br	Br	Me	0 70

Figure 7 Rhodamines

Daunorubicin This points to the use of such compounds as TBR123 in the adjuvant therapy of MDR disease, either with standard chemotherapeutic agents or with other photosensitizers

The main drawback in the use of rhodamines for the PDT of solid tumours is the relatively short wavelength of absorption available Rh123 absorbs light at just above 500 nm This value may be increased by the usual means, *e g* alkyl substitution at the amino groups (rhodamine 6G, λ_{max} 528 nm, rhodamine B, λ_{max} 543 nm) or by direct halogenation of the chromogen (*e g* TBR123) As mentioned previously, this alteration of the parent compound may decrease uptake or introduce unwanted dark toxicity However, their proven *in vitro* activity against malignant cells strongly indicates the use of rhodamines in the eradication of leukaemic cells from bone marrow extracts in preparation for transplantation [29]

5.6 Triarylmethane Photosensitizers

The antimicrobial activity of the cationic triphenylmethane dyes is well established and several examples were among those examined for antitumour activity in the 1940s [4] The structure of the triphenylmethanes is such that the energy accrued from photoexcitation can be dissipated easily *via* internal conversion (see Figure 2), thus making it difficult to examine parameters such as singlet oxygen efficiencies However, this has not precluded examination of triarylmethanes for use in PDT and related areas

The Victoria blues are well-known commercially available dyes, and are similar in structure to crystal violet, one of the phenyls being replaced by a 1-naphthyl group with a secondary amino function at position 4 (see Figure 8) The Victoria blues were included in the

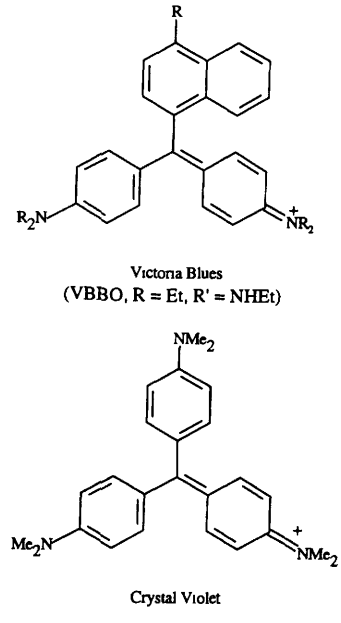


Figure 8 Triarylmethanes

Lewis study[4] and exhibited uptake by mammalian tumours This, combined with its long wavelength absorption (612 nm) justified a re-examination of VBBO as a photosensitizer Although no evi dence of singlet oxygen production has been found in *in vitro* tests, electron paramagnetic resonance has been used to demonstrate that the dye can photosensitize the production of superoxide [31] VBBO has been shown to be highly photoactive against several tumour cell lines, with a low level of dark toxicity, the combination of delocalised positive charge and high lipophilicity ensuring mitochondrial uptake The dye also exhibited very high photoactivity against two human leukaemic cell lines, with 99% cell killing at a photosensitizer dose of 0 1 μmol dm^{-3} [32]

In terms of structure–activity relationships in the Victoria blue series, the naphthyl moiety is important for photoactivity, since triphenylmethane dyes are generally inactive in this respect Also, compounds containing only tertiary amino functionality are less active both in terms of uptake and photosensitizing ability, thus suggesting that the secondary amino group is involved in drug action, as is the case with the Nile blue derivatives (*vide supra*) The presence of the secondary amino group in the commercial Victoria blues allows simple conversion of the dye cation to the neutral quinoneimine species, the Homolka base, and this has been used to explain the high activity of VBBO compared with analogues having all-tertiary amino character [33]

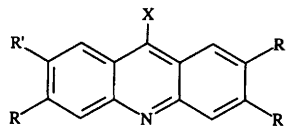
5.7 Acridines

Acridines are among the most widely investigated heterocyclic compounds in the modern history of chemistry Their use extends, once again, from Ehrlich's pioneering work in the late nineteenth century, through the use of simple aminoacridines in wound anti sepsis during the latter part of World War One and the widely used antimalarial Mepacrine, up to conventional present day anticancers such as Amsacrine Acridines were also among the first synthetic heterocyclic dyestuffs

In terms of photosensitizing activity, acridine was shown to have a photodynamic effect by Raab nearly a century ago, as has been mentioned The aminoacridines have long been used as fluorescent probes, their cellular 'targets' depending, unsurprisingly on structure Thus, proflavine is an excellent nucleic acid probe, whereas its bis(dimethylamino)-analogue acridine orange accumulates in lysosomes Alkylation of the ring nitrogen in acridine orange with an ethyl group or larger gives rise to mitochondrial localisation

Given the long medicinal and dyestuffs pedigree of the acridines – and it is known that the DNA strand-breaking activities of a number of commercially available intercalating acridines are enhanced on photoirradiation[34] – it is strange that this combination has not yet given rise to much activity in the field of PDT Until recently only acridine orange had been shown to cause tumour photodestruction in animals, using an argon laser [35] Because of the highly fluorescent nature of such aminoacridines, it might be expected that singlet oxygen efficiencies are low, and indeed there appears to be a strong oxygen tension dependence However, lysosomal destruction has been demonstrated using acridine orange and blue light in cultured tumour cells [36]

For the photodynamic therapy of larger tumours *in vivo*, the short wavelengths of absorption of commercial acridines such as acridine orange, proflavine, acridine yellow *etc* might be problematical, since they fall far short of the therapeutic window The extension of acridine absorbance into the near infrared may be achieved by cyanine synthesis *e g* 9-styrylacridines (see Figure 9) By the facile condensation of 9-methylacridine with *N,N*-dialkylaminobenzaldehyde derivatives, so-called acidochromic dyes are produced These, in addition to absorbing light at *ca* 700 nm, can exist in neutral or protonated (N-10) forms at approximately physiological pH, since they have pK_a values of *ca* 5 The theory behind the use of such compounds in tumour work uses the pH drop in tumours This would give a higher degree of protonation inside the tumour, thus adding to the selectivity of the system, since the protonated dye is the photoactive species and has an absorption which is bathochromically shifted by *ca* 200 nm Extrapolating to the idea of tumour treatment *in vivo*, this would mean that peritumoural



	R	R′	X	λ_{max}/nm[a]
Proflavine	NH_2	H	H	456
Acridine Orange	NME_2	H	H	489
Acridine Yellow	NH_2	Me	H	442
Styryl[b]	H	H	*p*-$Me_2NC_6H_4CH$=CH	618

[a] EtOH/HCl
[b] 436 nm in neutral EtOH[37]

Figure 9 Acridines

tissues would contain mainly neutral dye which would of course remain unaffected by near infrared light The only drawback to this (preliminary stages) work is that the phototoxicity of the acridines so far synthesised is likely to be low (< 5% of the photosensitizing activity of MB *in vitro*) [37]

6 Conclusions

Thus far, the majority of photosensitizers used in cancer PDT clinical trials have either been porphyrins or their derivatives This is due mainly to the fact that modern PDT has evolved from the naturally derived porphyrins such as HpD and DHE It is not surprising, therefore, that the second generation photosensitizers are based on porphyrins and phthalocyanines

Whilst it has been proposed that established, conventional chemotherapeutic agents such as the anthracyclines have sufficient photosensitizing ability to allow their use in clinical PDT, the availability and well-established chemistry of commercial non-porphyrin dyes and photosensitizers has also led to their testing as PDT agents *in vitro* Using commercial and natural photosensitizers as lead compounds, a great deal of design and development has been carried out by various groups in synthesising improved derivatives Several candidates have shown higher levels of activity than porphyrin-derived materials in cell culture and in animal testing (*e g* the benzo[*a*]phenothiazinium derivatives) In addition, a wide range of vital intracellular targets has been demonstrated, for example the use of DHOCI against microtubules, which should lead to higher therapeutic efficiencies than conventional membrane phototoxicity So far, however, clinical usage of such new compounds has been, at best, minimal

In terms of alternative targets to cancer, *e g* bacteria and viruses, there is less rationale for the use of porphyrin species The field of vital staining, which has developed over the past century, utilises many commercially available dyes As many of these dyes exhibit photosensitizing activity, it is logical to suggest their use in photodynamic pathogen eradication It is to be hoped that the demonstration of the photosensitizing capability of the non-porphyrin dyes against microbial and viral targets will lead to increased interest from industry

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