Rational design of phenothiazinium derivatives and photoantimicrobial drug discovery

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Abstract

While the model for conventional antimicrobial drug discovery is based securely on singular modes and sites of action, those associated with phenothiazinium photoantimicrobial candidates are both multifactorial *and* variable, resulting from oxidation events due to reactive oxygen species (ROS). The effective counteraction of such species and their variable targets is clearly problematic from the point of view of microbial resistance mechanism development, and offers considerable opportunity for the use of these agents in local infection control. However, this also means that drug development cannot be carried out using similar methods to those employed for conventional agents. Furthermore, these multifactorial photoantimicrobial agents are truly broad-spectrum since they are active against bacteria, fungi, viruses and protozoa, again at variance with the targeting of conventional, single-class antimicrobials.

This review concentrates on the use of the phenothiazinium class as exemplar photoantimicrobials, due to their pre-eminence in the field and considers the various criteria required for successful activity against microbes. These include alicyclic fusion, chalcogen substitution, benzo[*a*] fusion and the heavy atom effect, to decrease aggregation, improve ROS production and extend absorption wavelength, as well as conventional approaches, such as increasing cationic character to improve microbial selectivity/targeting.

Keywords: drug resistance; methylene blue; phenothiazinium; photoantimicrobial; photosensitiser design.

1. Introduction

When Alexander Fleming made his fortuitous discovery concerning the observable antistaphylococcal behaviour of *Penicillium* mould exudate, his thought was, reportedly, that what was occurring was enzymic lysis of the bacterial target [1], and the penicillin class of antibacterial agents had been a clinical reality for a number of years before an accurate biochemical understanding of its action was revealed. This class was therefore in widespread clinical use purely on the demonstrable bases of antibacterial efficacy and low human toxicity.

The huge impact of the penicillins on infection control paved the way for the discovery of other major classes of antimicrobial drugs during the 'golden age'. However, in most cases, the cellular targets for modern therapeutics are the same as those discovered for the corresponding lead compounds, i.e. the drug target is determined from the original evidence of activity, rather than being chosen from a study of target cell biochemistry, morphology etc. – for example, the original chlortetracycline and the 21st Century glycylcyclines all act at the bacterial ribosome [2]; penicillin V and the carbapenems all act against bacterial peptidoglycan crosslinking [3]. Such an approach might be termed the 'received target' paradigm.

In addition, few, if any, of the antibiotics discovered using this approach, or the semisynthetics derived from them, have more than a single site/mode of action, which is clinically problematic given the speed of microbial evolution, and is the reason that single-mode-of-action antimicrobials generally have limited useful lifetimes. Indeed, Fleming himself alluded to the likelihood of penicillin resistance without drug conservation in his Nobel lecture [4].

In attempting to address the problem of rapid and increasing microbial drug resistance, the UK Government's published Antimicrobial Research (AMR) Strategy, and the more recent O'Neill Report make several mentions of 'novel approaches' to the treatment and prevention of infections [5,6]. One potential approach proposed – for several years now - as a partial replacement/conservation aid for conventional agents is that of light-activated or *photo*antimicrobials [7]. However, due to broad-spectrum activity, non-specific modes of action and the requirement for light activation, the design of such agents differs considerably from that of conventional, pharma industry-produced antimicrobials.

2. Photoantimicrobial Chemotherapy (PACT [8])

The cell-killing effect of acridine and the synthetic xanthene dye eosin, in combination with light, was discovered by Raab in 1900 [9]. The organism under study was *Paramecium caudatum*, a single-celled organism, and the phenomenon was shortly termed 'Photodynamy' (light causing action). Raab's experimentation was of little utility in terms of drug design, given that the acridine and eosin used belong to quite different chemical classes (acridine and xanthene, respectively), absorb light in separate regions of the spectrum (ultraviolet/visible) and produce different oxidative cell-killing agents (Type I/Type II photosensitisation routes – see below). However, the salient point was that this was the first reported laboratory demonstration of the photodynamic effect.

During the first few decades of the 20th Century, much of the chemical industry was based around dye synthesis, and dyes were certainly tested widely in applications other than textile colouration.

Pioneering work by Ehrlich, Koch and others had demonstrated the use of synthetic dyes for sample staining in microscopy and had allowed the quantum leap to the concept of selective toxicity before Raab's photodynamic breakthrough. Ehrlich, in particular, made great strides towards a dye-based chemotherapeutic approach, including early notions of structure-activity relationships before the First World War [10]. Indeed, 'Flavine therapy' of battlefield injuries - based on the direct application of cationic dyes (e.g. acriflavine and brilliant green) – evolved from work carried out by Browning with Ehrlich before the conflict [11].

Methylene blue is a cationic dye, based on the oxidised phenothiazine heterocycle (Figure 1), and used in Guttmann and Ehrlich's clinical malarial cures in 1891 [12]. The lack of toxicity of this dye and the burgeoning interest in chemotherapy led to its use in a range of diseases [13]. However, its photoantimicrobial potential was not reported until – ironically - the same year as Fleming's epoch-making publication [14]. However, between 1928 and the clinical introduction of IG Farben's Prontosil in the late 1930s, methylene blue had been shown to be bactericidal, virucidal and antiprotozoal on illumination [14,15].

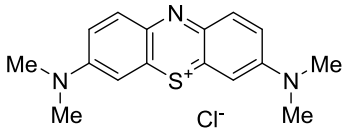


Figure 1. Methylene blue chloride

Given the miracle drug status accorded the penicillins and ensuing antibiotics discovered/developed in the mid-20th Century, it is not surprising that there was scant further contemporary development of photoantimicrobials. Such a *renaissance* resulted from the development of significant clinical microbial drug resistance during the 1980s. Modern photoantimicrobial drug discovery has thus been in progress for around a quarter of a century [16], mainly in academia.

3. Photoantimicrobial Action

As noted, photoantimicrobials act via the absorption of light energy, producing reactive oxygen species as a result which represent a cytotoxic threat to simple microbial cells. The confluence of photoantimicrobial, target cell, oxygen and light furnishes the basic conditions for cytotoxicity in practice, but consideration needs to be given to photoantimicrobial structure design to ensure efficient ROS production *in vivo*.

Mechanistically, photoantimicrobial action occurs in the same way as that of the related clinical anticancer approach, photodynamic therapy (PDT). On a molecular level, this involves the absorption of light of a specific wavelength (i.e. depending on the chromophore involved) to enable the promotion of a paired ground state electron to the singlet excited state (HOMO →LUMO, spins still paired, Figure 2). Given a long-lived singlet excited state, spin inversion yields the more reactive (unpaired) triplet excited state, and it is usually this which reacts with its environment, either

through electron transfer with the molecular environment or energy transfer to ground state triplet oxygen (Type I or Type II photosensitisation pathways respectively). The former results in the formation of the hydroxyl radical ($HO^{\cdot}$) and the superoxide anion (O_2^{-}), while the latter produces singlet oxygen ($^1O_2$) (Figure 2).

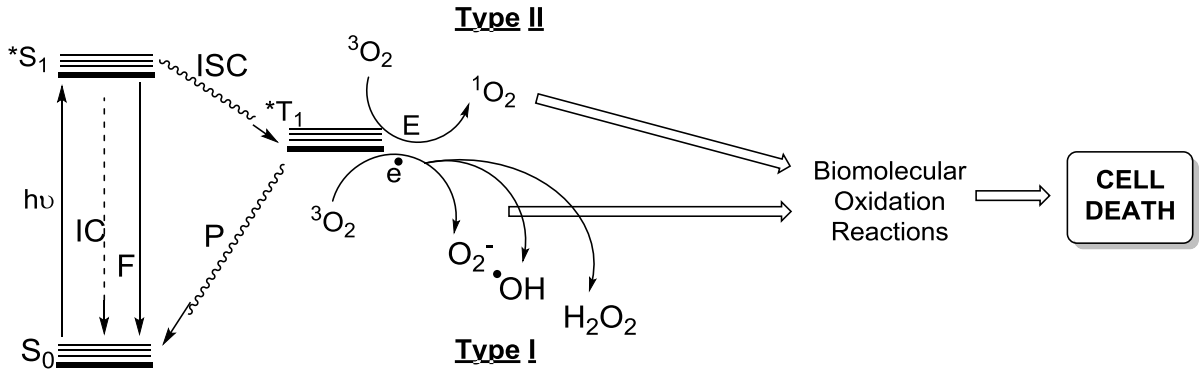


Figure 2. Photosensitisation pathways. Key: S_0 – singlet ground state; $*S_1$ – singlet excited state; T_1 – triplet excited state; hυ – light energy; IC – internal conversion; F – fluorescence; P – phosphorescence; ISC – intersystem crossing.

Due to the oxidising and highly reactive nature of these species, they do not persist for extended periods - for example, $^1O_2$ decays to its non-toxic ground state on a sub-microsecond scale. However, each will cause oxidation across a range of biomolecules involved in all parts of the cell, and close proximity of the photoantimicrobial to its target – whether internal or external – is thus normally sufficient to lead to cell death.

4. Designing an effective photoantimicrobial

For clinical use, a photoantimicrobial clearly requires a dual design strategy. Unlike conventional agents, the photosensitisation requirement means that, alongside microbial selectivity, light absorption must be included as an essential component, and this must result in efficient conversion to ROS. The key to photosensitisation lies in the production of a sufficient population of triplet excited state molecules (Figure 2). This is supplied by a relatively long-lived singlet excited state, as exhibited by several lead structures such as the phenothiazinium derivative methylene blue [17]. Furthermore, since excitation is dependent on light absorption, in the case of photoantimicrobials consideration must also be given to the circumvention of endogenous absorption, e.g. due to proteins, blood and melanin present at the infection site (Figure 3). Red- or near infrared absorption is generally accepted as a means to this end, and this again requires further consideration in terms of overall drug design.

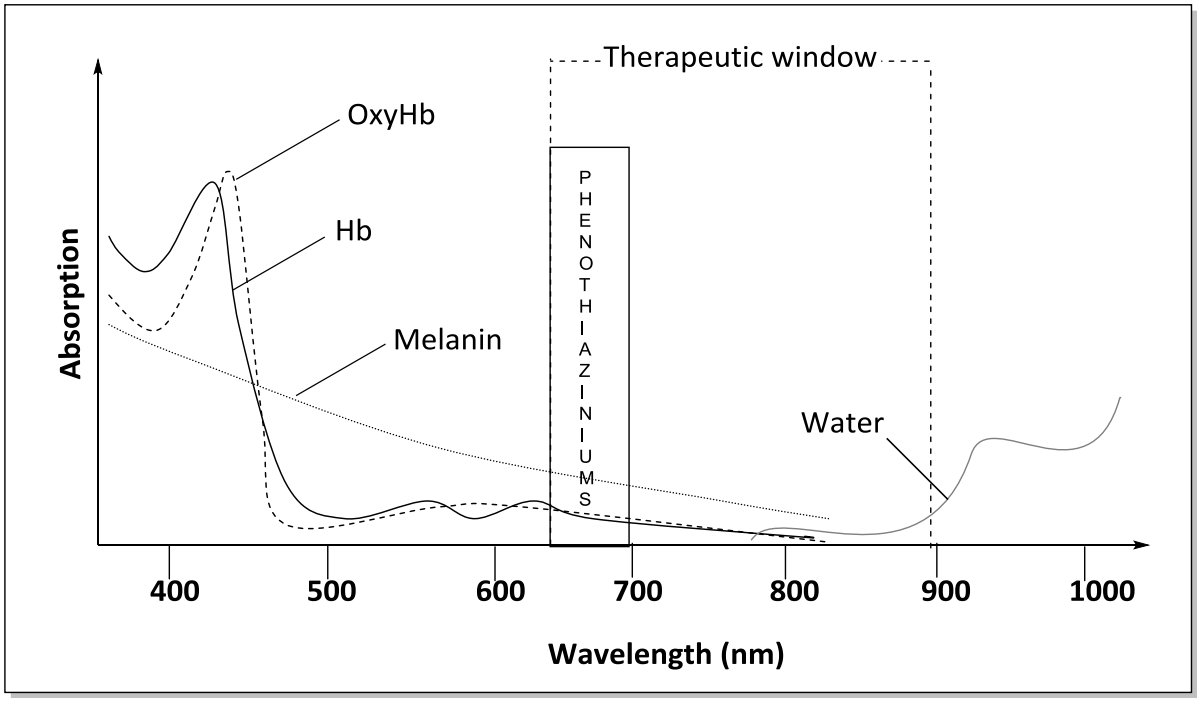


Figure 3. Pertinent endogenous absorption spectra, the therapeutic window and current useful phenothiazinium absorption range. Hb – haemoglobin; OxyHb – oxyhaemoglobin.

As noted above, the exquisite selectivity associated with conventional single-mode-of-action antimicrobials actually *guarantees* resistance development via selective evolutionary pressure. With the multifactorial modes of action of photoantimicrobials, however, there is little requirement for targeting at the biomolecular level, and a much more general selectivity between host and target cells is preferable (see *Microbial Selectivity*, below).

4.1 Microbial Selectivity

Photoantimicrobials are truly broad-spectrum agents. This offers advantages in the treatment of e.g. local bacterial infections due to mixed strains, or mixed Gram-positive / Gram-negative species, or of unknown aetiology. Such a definition would be used for conventional antibacterial agents in the pharmaceutical sector. However, for cationic photoantimicrobials this may be extended to the combined therapy of primary and secondary infections where the primary and secondary causes are of separate class – e.g. opportunistic bacterial colonisation of tissue damaged by initial fungal incursion, and similarly for infection due to emerging or unknown pathogens, whether these are of bacterial, fungal, viral or protozoal origin.

For such activity the design model is, perhaps not surprisingly, similar to that of the original therapeutic dye work carried out by Ehrlich *et al.*, i.e. uptake by microbial cell types - of whichever class - but not by the host. Clearly there are various parameters which might be set in terms of molecular design to ensure such a distribution, such as molecular charge, hydrophilic-lipophilic balance or degree of molecular planarity. While this is obviously far more straightforward an approach than those employed by modern pharmaceutical research, it remains an effective process for photodynamic drug design and discovery.

Photosensitisers with net positive charge are established as the premier class of broad-spectrum agents, due to high activity against both Gram classifications. This is not the case for electronically neutral analogues or those possessing overall negative charge, which tend to be considerably less effective against Gram-negative bacteria [18]. Given the significant problems engendered by Gram-negative infections in modern healthcare, this is an important point. Efficacy against fungi, viruses and protozoa cannot be simply differentiated in this way.

The huge difference in complexity between mammalian and microbial cells explains the relatively simple and rapid binding, uptake and overall greater efficacy of cationic photoantimicrobials. For example, methylene blue binds to anionic residues in the Gram-positive bacterial cell wall, such as teichoic acids [19], while at the outer membrane of a Gram-negative cell, it will displace calcium or magnesium ions which aid in stabilising the polar head groups of the outer membrane, thus allowing ingress of the photoantimicrobial, i.e. a self-promoted uptake mechanism [20].

It should be recalled, also, that the envisaged administration of photoantimicrobial agents and their subsequent illumination is via topical or otherwise local application – i.e. the site of infection is physically targeted at administration (whether externally or internally using endoscopy/fibre optics). The applied photosensitisers are thus far less exposed to distributional and metabolic factors than systemically-administered conventional drugs. Similarly, there is far lower potential for damage to the host's indigenous microflora.

4.2 Chemical Considerations

Given the low level of specification required in terms of structure, effective photoantimicrobials need only to consist – at base level - of a chromophore or chromophore-auxochrome combination which allows long-wavelength light absorption and efficient ROS production, and net molecular positive charge. The phenothiazinium class of cationic or basic dyes provided the initial lead compounds here, e.g. methylene blue and the related toluidine blue (Figure 4). To these have been added *de novo* cationic derivative series of the phthalocyanine, porphyrin and cyanine classes – all containing strong red light absorbers. However, there is far greater evidence and rationale for the original class, given not only its use in microbial staining for the past 150 years, but also its continuing clinical – local and systemic - applications in intraoperative staining and the treatment of methaemoglobinaemia [21].

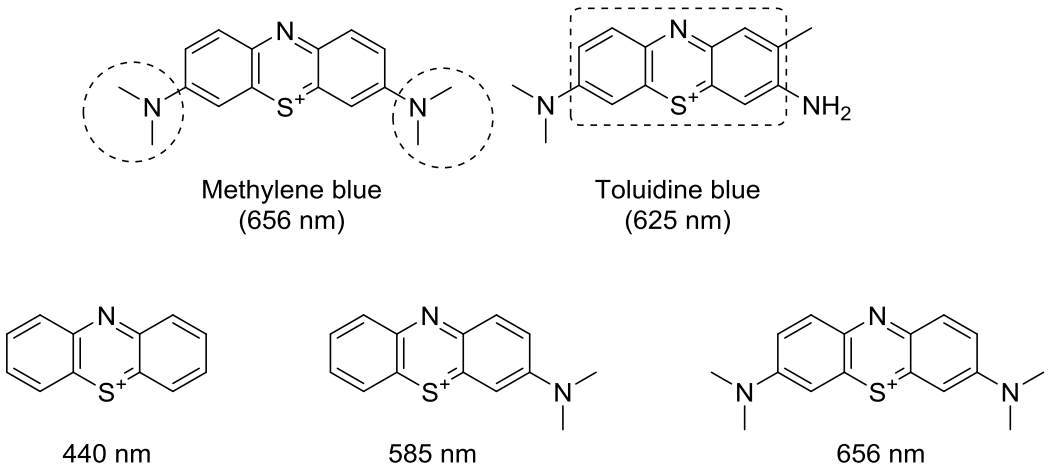


Figure 4. Lead phenothiazinium photoantimicrobials, identifying auxochromic (methylene blue) and chromophoric (toluidine blue) moieties, and the importance of the auxochrome in promoting long-wavelength absorption (λ_{max} values measured in methanol).

4.3 Phenothiazinium Derivatives - Photoproperties

Both methylene blue (λ_{max} = 660 nm in aqueous solution) and toluidine blue (625 nm) exhibit intense light absorption in the red region of the spectrum. In terms of the avoidance of interference from endogenous absorbers, this is sufficient, given that haem absorption reaches a minimum at around 630 nm (Figure 3). However, considerable efforts have been made in extending phenothiazinium absorption further into the red region (*bathochromic* shifting) due to the clinical desirability of increased tissue penetration of light, which reaches a maximum between 800-900 nm [22].

4.3.1 Aggregation

Photosensitisation relies on initial efficient interaction of incident light with the sensitiser molecule. Interference with this process is therefore undesirable. As noted above, in practice such interference occurs when incident light is absorbed by molecules in the environment of the photosensitiser, i.e. endogenous absorption, but photosensitiser aggregation may also problematic.

Aggregation is a common phenomenon associated with planar molecules, and is thus a well-understood feature in dye chemistry. Clearly, the greater the degree of molecular planarity, the easier it is for stacking interactions to occur, and these will usually increase with concentration. Molecules within the body of the aggregate are not reached by incident light, thus photosensitisation is only possible at the surface. Similarly, energy transfer to oxygen is less likely within the tight confines of the aggregate. Furthermore, most aggregated photoantimicrobials exhibit an absorption band at lower wavelengths than the λ_{max}. This may bring the absorption into competition with endogenous species, as mentioned previously.

It is thus important to design photosensitiser molecules which exhibit low aggregational behaviour. This is normally achieved via the inclusion of chemical groups which are either bulky and/or charged,

thus causing intermolecular repulsion. In addition, monomeric species, as opposed to oligomeric or aggregated species, could be expected to be taken up or to bind to biomolecules more effectively due to smaller molecular volume.

4.3.2 Auxochromic Derivatives

Clearly auxochromic alteration+ can affect various properties of colourant molecules, given that there are both associated steric and electronic factors. Thus, for example, in the present case, altering the auxochromic character might cause wavelength shifting as well as altered ROS production, aggregation and hydrophilic/lipophilic balance.

As noted above, interference by endogenous molecules in light absorption processes can be detrimental to photoantimicrobial performance, requiring design to produce molecules which absorb beyond endogenous range.

Using the 3,7-diaminophenothiazinium (thionin) skeleton as a basic framework, it can be seen that consecutive amino substitution with methyl groups causes regular bathochromic shifting of the λ_{max} up to methylene blue.

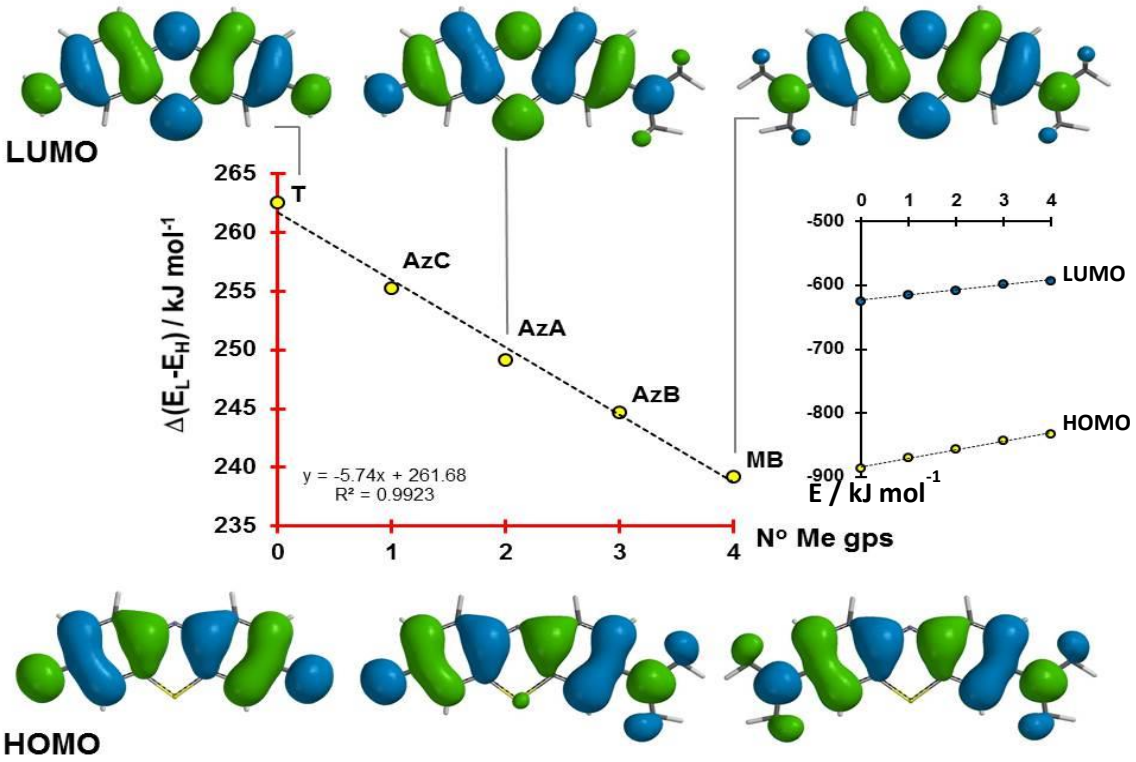


Figure 5. HOMO/LUMO and orbital energy dependence on extent of *N*-methyl substitution. Key: T – thionin; AzC – azure C; AzA – azure A; AzB – azure B; MB – methylene blue.

Figure 5 shows the highest-occupied molecular orbitals (HOMO, lower) and lowest unoccupied molecular orbitals (LUMO, upper) of thionine, azure A and methylene blue from left to right respectively. The small graph on the right shows these orbital energies as a function of the number of *N*-methyl substituents and the main graph the difference in energy between LUMO and HOMO ($\Delta(E_L-E_H)$), again as a function of the extent of *N*-methyl substitution.

Overall it can be seen that orbital density is weakly transferred from 'out' to 'in' comparing HOMO and associated LUMO, with significant enhancement of orbital density on the N and S atoms in the LUMO and associated decreases in orbital density associated with *N*-methyl groups in the HOMO.

N-Methyl substitution leads to a systematic increase in *both* HOMO and LUMO orbital energies. This can be rationalised by the increasing number of nodes appearing in the both orbital sets as *N*-methyl substitution is increased from left to right above. Furthermore, there is greater orbital density on the *N*-methyls in the HOMOs than LUMOs leading to a greater sensitivity of the HOMOs than LUMOs energy to such substitution. In other words, the anti-bonding character of the HOMOs is more sensitive to *N*-methyl substitution than in the LUMOs and therefore the energy dependence is greater in the former than the latter.

In short, assuming that the lowest energy electronic transition is 100% HOMO->LUMO in character, then the HOMO-LUMO energy gap reduces upon increasing the extent of *N*-methyl substituent

primarily due to the systematic raising in energy of the HOMO to a greater extent than that of the LUMO due to variation in the significance of hyperconjugation effects in the two orbitals.

Vertical excitation energy is a function of the HOMO/LUMO energy gap and changes in electrostatic, **Q**, and exchange interaction, **K**, energies resulting from the single electronic occupancy of both orbitals in the vertical E/S geometry; $E^{abs}{}_{V} = \Delta + \mathbf{Q} + \mathbf{K}$. Δ and **K** are always positive energy contributors to vertical S_1 energy, **Q** may be positive or negative. Assuming that **Q** and **K** are the same for all structures in the group, there ought to be a correlation between $\Delta(E_L\text{-}E_H)$ and λ_{max} / $E^{obs}{}_{max}$ observed in the absorption spectrum. A graph of ΔE_{LH} versus $E^{obs}{}_{max}$ for all commercially-available MB derivatives is given below (Figure 6).

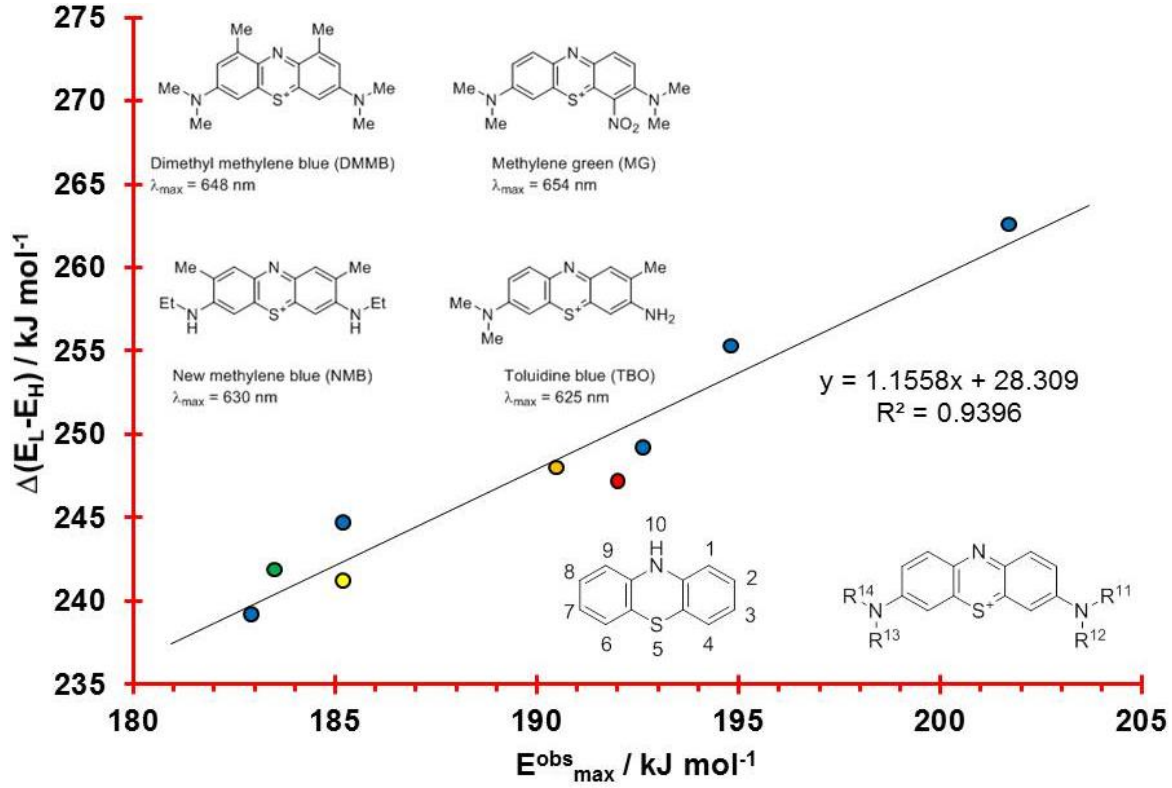


Figure 6. Plot of ΔE_{LH} versus $E^{obs}{}_{max}$. dark blue = *N*-Me substituents, green = MG, orange = NMB =, yellow = DMMB and red = TBO. The darker blue from left to right correspond to 4, 3, 2, 1, 0 *N*-methyl groups respectively.

While Figure 6 clearly shows a sound correlation in terms of λ_{max}, this approach is of less utility in predicting the ability of putative molecules to produce singlet oxygen.

Auxochromic variant synthesis

The use of iodine or bromine oxidation in phenothiazinium synthesis has allowed considerable range extension compared to earlier work, for example by Taylor, which was perforce limited both by reactant aqueous solubility and stability to the strong oxidising agents employed (usually chromium(VI)) [24]. Iodine oxidation of 10*H*-phenothiazine produces the oxidised phenothiazinium

chromophore which is reactive to nucleophiles at both positions 3- and 7-, and can be attacked sequentially. This allows the synthesis of both symmetrically and asymmetrically-substituted derivatives [23]. Similarly, bromine oxidation furnishes 3,7-dibromophenothiazinium bromide, in which the bromines can be replaced by amines, although this route is more suitable for the production of symmetrical derivatives. Both approaches are carried out in organic solvents and thus allow the inclusion of long-chain auxochromes. General synthetic routes to phenothiazinium derivatives are shown in Figure 7.

Principally in this class of photoantimicrobials, absorption of light at increased wavelength (i.e. usually further into the red region) has been achieved by greater electron release from the auxochromic amino groups at positions 3- and 7-of the phenothiazinium chromophore, normally by increasing the alkyl group size. This is shown for the series of simple methylene blue homologues first fully published by Mellish *et al.* [25] (Table 1, synthesised via route (a), Figure 7). Importantly, such substitution also retains the capacity for efficacious ROS production. Extension of this idea to auxochromic *N*-aryl or *N*-heteroaryl substitution also allows significant bathochromic shifting [26], but in this case decreases ROS production to minimal levels, and so is of little utility in development. Aromatic inclusion without a concomitant loss of ROS production capability has been achieved using benzylamine derivatives to provide the auxochromic moiety (Route (a), Figure 7). This also allows the potential for photosensitiser functionalisation – e.g. for polymer attachment - without deleterious effects on chromophore performance [26].

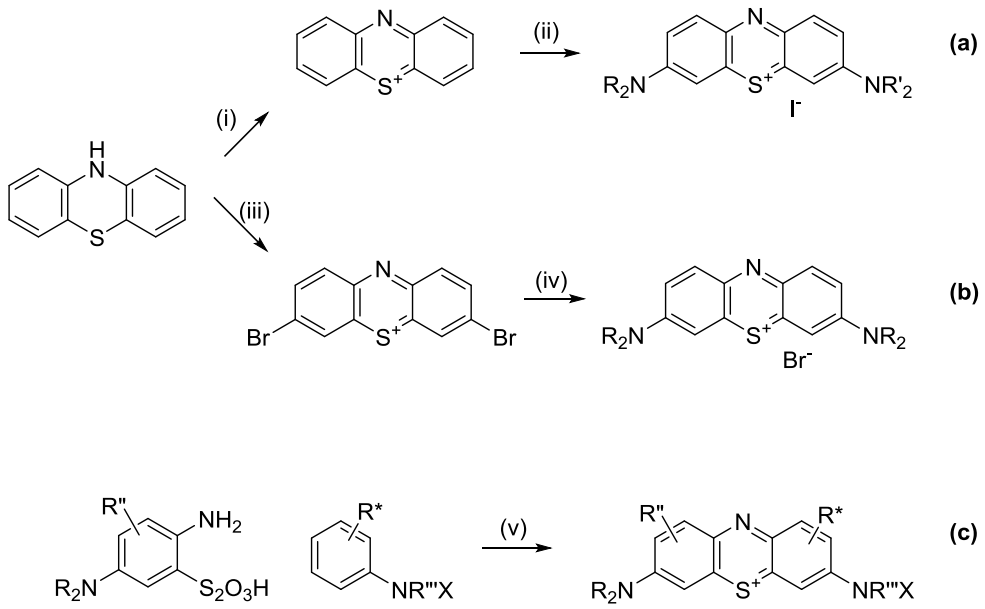
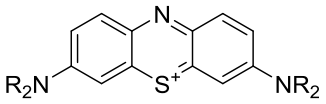


Figure 7. Basic synthetic routes to phenothiazinium derivatives. Key: (i) iodine; (ii) HNR_2 (providing symmetrical derivatives), or HNR_2, followed by HNR'_2 (providing asymmetrical derivatives); (iii) bromine; (iv) HNR_2; (v) mild oxidant, e.g. silver(I) carbonate. Auxochromic substituent groups are usually alkyl, aryl, aralkyl or substituted derivatives thereof; R"' and X may be H. Chromophoric substituents R" / R* are usually electron-releasing. This approach is also used to produce benzannelated / dihydropyrrolo- / tetrahydropyrido-derivatives (tetra- and pentacyclic molecules, see below).



R	λ_{max} (nm)[a]	Log ε_{max}[a]	Rel.$^1O_2$[a]	LogP
Me	657	4.88	1.00	-0.1
Et	664	4.87	0.55	+0.8
n-Pr	669	4.75	0.59	+1.1
n-Bu	671	4.84	0.61	+1.3
n-Pe	672	4.69	0.26	+1.6
n-Hx	673	4.55	0.21	+1.8

Table 1. Simple homologous series of photosensitisers based on methylene blue. [a] – measured in methanol [25].

Auxochrome decay

Conversely, *N*-demethylation of methylene blue is a straightforward oxidative process, as with other *N*-methylated dye or drug molecules. This has been understood almost from the initial experiments with the parent compound in the 19th Century, since it was, at the outset, undoubtedly a mixture of methylated derivatives due to the oxidative nature of its preparation. As shown in Figure 8, successive demethylation of the parent produces five further derivatives, all of which are photosensitisers. However, the loss of auxochromic methyl groups also causes a drop in absorption wavelength, due to decreased electron release from the demethylated auxochrome. This, as noted above, brings into play competition for excitational energy with endogenous absorbers such as haem and melanin.

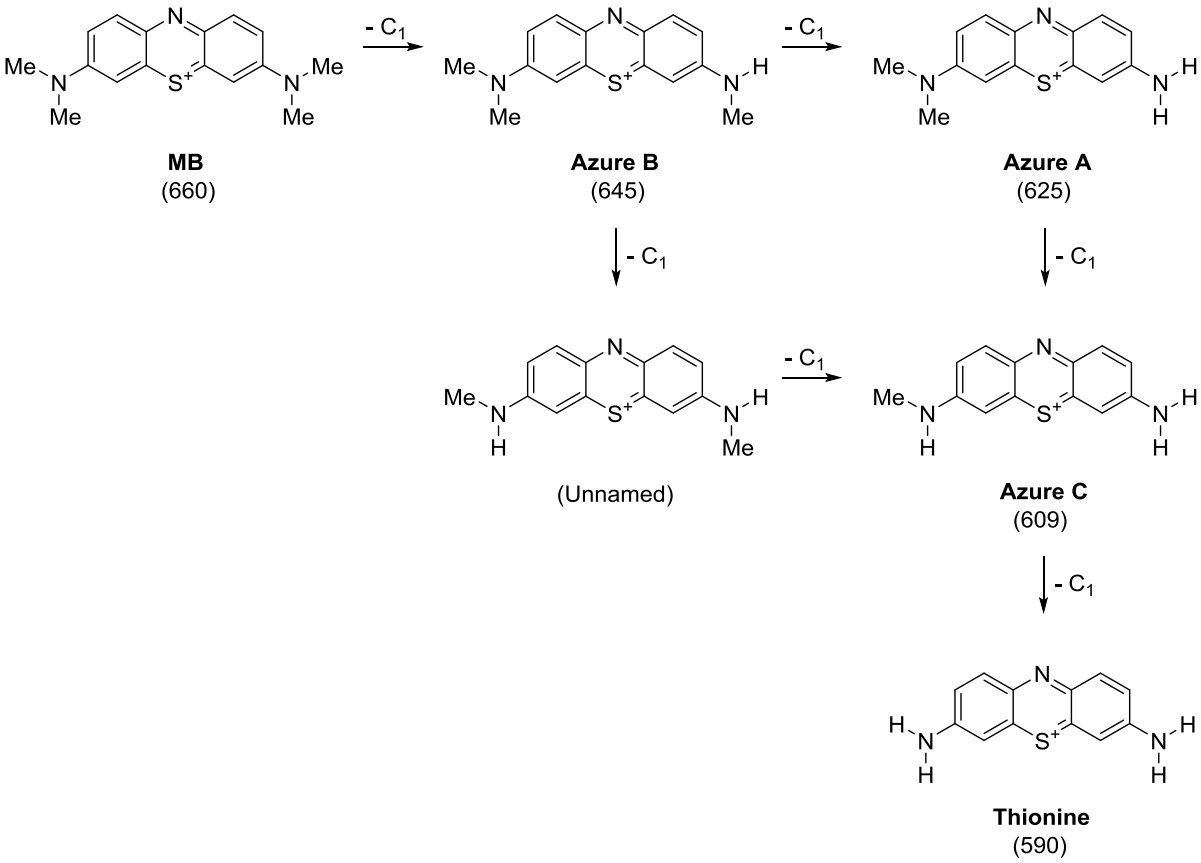


Figure 8. Demethylation in the methylene blue series and maximum aqueous wavelength of absorption (nm).

From a design viewpoint, facile demethylation is problematic. One of the principal legislational barriers in photoantimicrobial development is compound purity. Consequently, an agent which decays into a mixture will be required to show efficacy/toxicity profiling of each of the daughter products. As a result, despite the clinical acceptance of methylene blue for conventional human use, it is may not always escape this requirement as a photoantimicrobial agent.

While such dealkylation is less likely in higher homologues, these are, by definition, new compounds without prior clinical use and are therefore subject to full drug legislative requirements.

Auxochrome functionalisation is also useful in altering solubility – i.e. via the increase in hydrophilic or hydrophobic character. This is particularly simple with the advent of the halogen oxidation routes (Figure 7 (a)/(b)), since this also allows asymmetric substitution with respect to the incoming amines, for example 3-(long chain dialkylamino)-7-diethanolamino or similar.

This approach has been extended to include extra amino functionality as a part of the auxochromic side chain, both by the current author [27], Maisch [28] and Boyle [29], usually employing route (a) shown in Figure 7. Given that this produces derivatives with extra cationic sites in the side chain, it is attractive from the point of view of designing molecules for antimicrobial attack, for example at the outer membrane of Gram-negative bacteria. Ironically, such a pattern of functionality is apparent in (conventionally) antimalarial methylene blue derivatives produced under the IG Farben umbrella in

the 1930s [30], although such examples were limited by the strong oxidant/aqueous media conditions noted above (route (c), Figure 7). Side-chain amino functionality has also allowed the extension to dimeric examples, i.e. where the distal amino group provides a site for nucleophilic attack on the oxidised monoauxochromic precursor (Figure 7). Hypothetically at least, this type of photosensitiser would be able to act as a 'hairpin' in attacking – typically - viral DNA in blood product photodisinfection protocols [31].

4.3.3 Chromophoric Alteration

The central functionalisation of phenothiazinium photosensitiser structures is complicated by the requirement for amino moieties at positions 3- and 7-. In addition, direct functionalisation often damages *in situ* substitution patterns, leading to a loss of activity. However, chromophore methylation of both lead compounds, methylene blue and toluidine blue, has been reported, such syntheses utilising methylated starting materials, via route (c), Figure 7 [32-34].

The position of the methyl (or other alkyl group) in the precursory aniline is important, since rotation of the amino moiety to coplanarity with the aromatic nucleus is required in order to facilitate the oxidation reaction which furnishes the required phenothiazinium chromophore (Figure 7). Clearly, close group proximity causes inhibition of this rotation and blocks the reaction. Thus, in the case of methylene blue, chromophore methylation is only possible at positions 1- and 9- (Figure 9), due to steric interference by the dimethylamino auxochromes [32]. 1,9-Dimethylmethylene blue (DMMB, Taylors's Blue, Figure 9) is a powerful photosensitiser against both microbial and mammalian cells, thus its utility in clinical terms may be limited by host toxicity problems. The similarly powerful new methylene blue (NMB, Figure 9) also suffers from toxicity problems, but is structurally interesting as it exhibits 2,8-dimethylation, allowed due to monoethylated amino auxochromes (Figure 9 – auxochrome rotation also allows potential 4,6-dialkylation). Clearly, on a structural basis, NMB is mis-named.

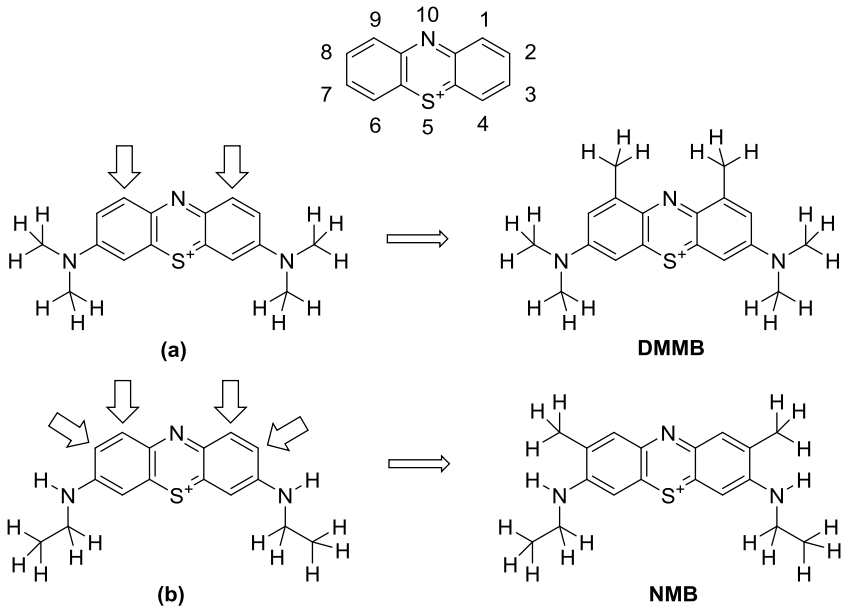
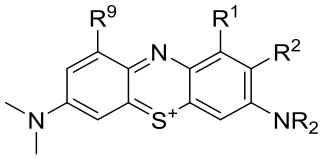


Figure 9. The phenothiazinium nucleus, steric auxochromic hindrance and alkylation pattern. Arrows denote potential alkylation sites for the given auxochromic dispositions in methylene blue (a) and the 3,7-di(ethylamino) congener, (b).

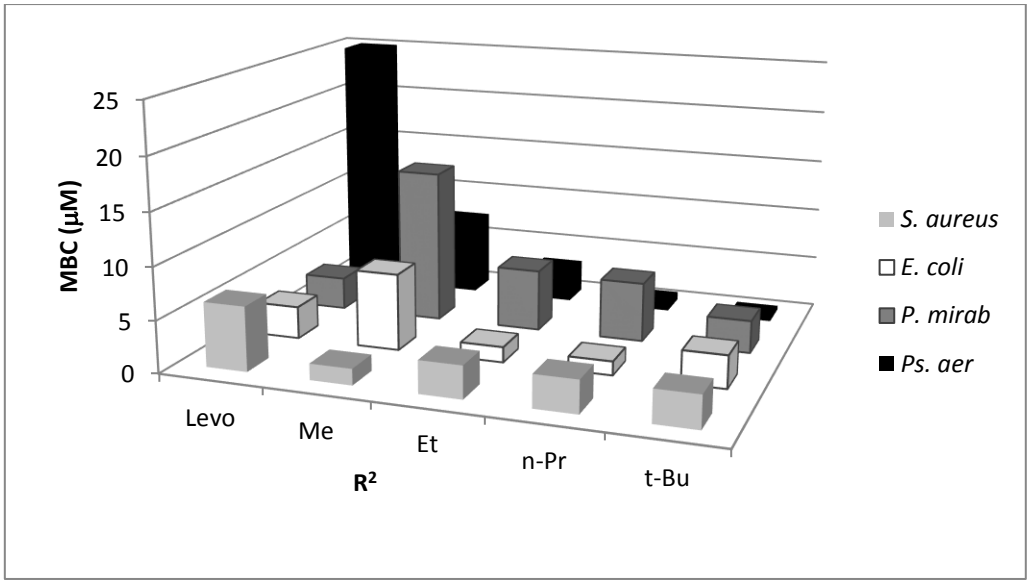
Functionalisation of the toluidine blue nucleus is somewhat more straightforward due to the absence of any auxochromic alkylation at C-3, and several mono-, di- and trimethylated derivatives have been produced [34]. However, as with DMMB above, one effect of methylation is a decrease in λ_{max} from the parent which, in the case of toluidine blue, means that the resulting derivatives would be in greater competition with endogenous absorbers in the clinical situation.



PS	R	R^1	R^2	R^9	λ_{max} (nm)
MB	Me	H	H	H	656
1-MMB	Me	Me	H	H	655
DMMB	Me	Me	H	Me	650
Azure A	H	H	H	H	633
TB	H	H	Me	H	626
1-MTB	H	Me	Me	H	616
9-MTB	H	H	Me	Me	615

Table 2. Depression of λ_{max} with chromophore methylation (measured in methanol)

Given that, as a photoantimicrobial, toluidine blue already represents successful chromophore methylation – it could alternatively be named 2-methylazure A (see Table 2) – varying chromophoric substitution at position 2 has also been investigated (Figure 10 [35]). In order to maintain photoactivity, hydrocarbon replacements were made, including simple alkyl and aryl. All replacements led to increased photoantimicrobial activity compared to the parent compound and increased λ_{max} to such an extent that the series approaches the methylene blue derivatives.



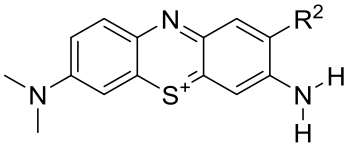


Figure 10. Photobactericidal activity of simple toluidine blue derivatives compared to conventional levofloxacin (Levo). MBC = minimum bactericidal concentration. Key to bacterial species: *S. aureus* – *Staphylococcus aureus* (Gram +ve bacterium)*; E. coli* – *Escherichia coli* (Gram –ve); *P. mirab* – *Proteus mirabilis* (Gram –ve); *Ps. aer* – *Pseudomonas aeruginosa* (Gram –ve) [35].

The increased photoantimicrobial activity in the series, relative to the parent compound, may be linked to the decreased aggregation exhibited by the derivatives, as can be seen in Figure 11 for the *tert*-butyl analogue. Its activity against the problematic Gram-negative pathogen *Pseudomonas aeruginosa* (MBC =390 nM) is extremely impressive, particularly when viewed against that of the conventional fluoroquinolone antibacterial drug levofloxacin.

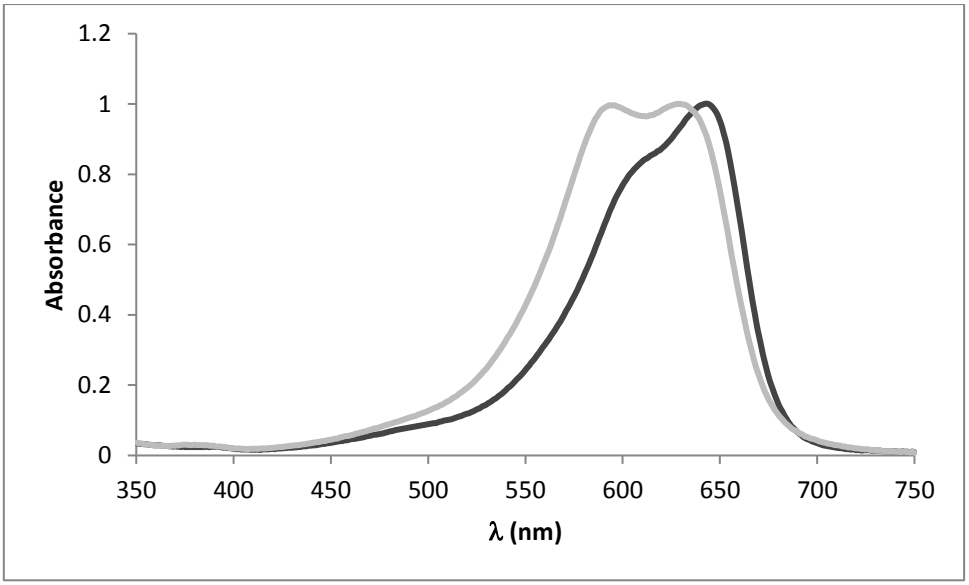


Figure 11. Aqueous visible spectra for toluidine blue (pale grey curve) and its 2-*t*-butyl analogue (black), showing the more pronounced aggregate peak at lower wavelength for the parent compound.

4.3.4 Annelation

Chromophoric ring fusion has also been exemplified in the phenothiazinium series as a source of novel photosensitisers, utilising both aromatic and alicyclic annelative routes, in both cases leading to further red-shifting. This is shown for simple derivatives in Figure 12.

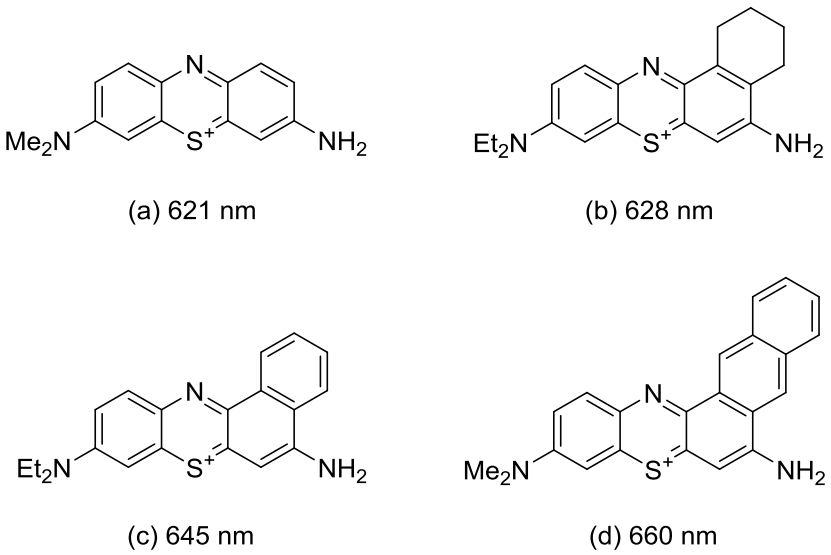
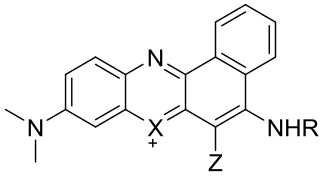


Figure 12. Wavelength shifting in annelated derivatives. Wavelengths measured in methanol: (a) Azure A; (b), (c) [36], (d) [37].

The benzo[*a*]phenoxazinium derivative Nile blue has been an established histopathological stain for around a century. Due to demonstrable tumour staining *in vivo*, it was used by Foley as an alternative lead to the ubiquitous haematoporphyrin derivative in anticancer photosensitiser discovery in the late 1980s. However, as the ring oxygen-inclusive chromophore produces no measurable singlet oxygen, lower period chalcogens were tested, and both sulphur and selenium incorporation proved fruitful. Similarly, ring bromination or iodination of the benzo[*a*]phenoxazinium provided photosensitisers (Table 3). The derivative EtNBSe (Table 3) has shown considerable potential as a photoantimicrobial agent [38].



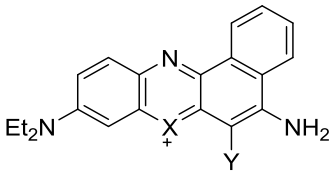
NBD

	Z	**X**	**R**	**λ_{max} (nm)[a]**	**Relative $^1O_2$ yield[a]**
Methylene blue	-	-	-	656	1.00
NBA [39]	H	O	H	623	0.01
NBDI [40]	I	O	H	642	0.08
NBS [39]	H	S	H	645	0.06
EtNBSe [41]	H	Se	Et	661	1.77

Table 3. Benzo-fused azine photosensitisers (NBD) and comparison with methylene blue. [a]- measured in methanol.

The employment of lower period elements in dyes is an established method of stabilisation of the singlet electronic excited state (Figure 2) which, as previously stated, normally increases the probability of triplet state population (via intersystem crossing) and reaction / energy transfer. This is generally known as the *Heavy Atom Effect*. Interestingly, however, neither the homologous series of seleno-methylene blue derivatives [42] nor iodomethylene blue [37] showed significant increases, either in λ_{max} or in singlet oxygen yield.

However, in terms of molecular design, the presence of heavy atoms will also alter the hydrophilic/lipophilic balance and thus the distribution and uptake of the resulting compounds at the biological target, as can be seen with respect to compound lipophilicity (as Log P) in Table 4.



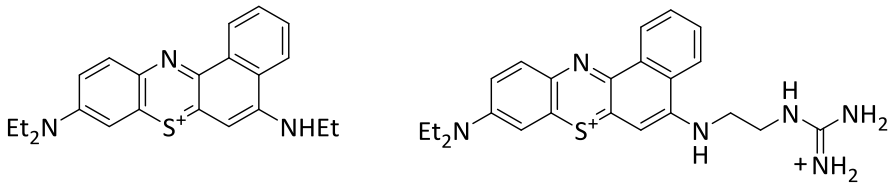
	X	Y	λ_{max} (nm)	$^1O_2$ eff.	Log*P*
NBA	O	H	623	0.005	+2.23
NBA-6I	O	I	642	0.036	+3.75
NBS	S	H	645	0.024	+2.55
NBS-6I	S	I	660	0.170	+3.70

Table 4. Heavy atom inclusion for improved photoproperties and variation in Log P for benzo[*a*]phenothiazinium derivatives [40,43]

Regarding photoantimicrobial activity, as with the anticancer application, the benzo[*a*]phenoxazinium derivatives are photodynamically inactive due to poor conversion to the triplet excited state. Consequently, for the short 5-ethylamino series, broad-spectrum efficacy followed the expected trend, with the ring-selenium analogue (EtNBSe, Table 3) being the most active and having the greatest triplet yield [44].

The high activity of EtNBSe has also been demonstrated against *Leishmania major* [45], again demonstrating improved uptake and activity compared to both the sulphur analogue and the anionic porphyrins utilised for comparison. Such work is also notable in that it demonstrates a fitting avenue for the employment of the photoantimicrobial approach, viz. tropical skin disease, offering rapid and relatively inexpensive therapy in less affluent areas. Also fitting, of course, is the fact that Ehrlich's own efforts in early chemotherapy utilised dye-based molecules against the scourge of tropical disease [46].

In terms of specifically targeting microbial cells via structural alteration, little has been essayed. However, work testing the "charge hypothesis" – i.e. the greater efficacy of cationic species as broad-spectrum agents - was carried out on a short series of Nile Blue (EtNBS) analogues by Vecchio et al. Unsurprisingly, the incorporation of a guanidine residue as part of an auxochromic side chain (EtNBS-G, Figure 13) produced the most active compound of the series, the permanent extra positive charge so endowed producing high activity against both Gram-positive and Gram-negative bacteria [47].

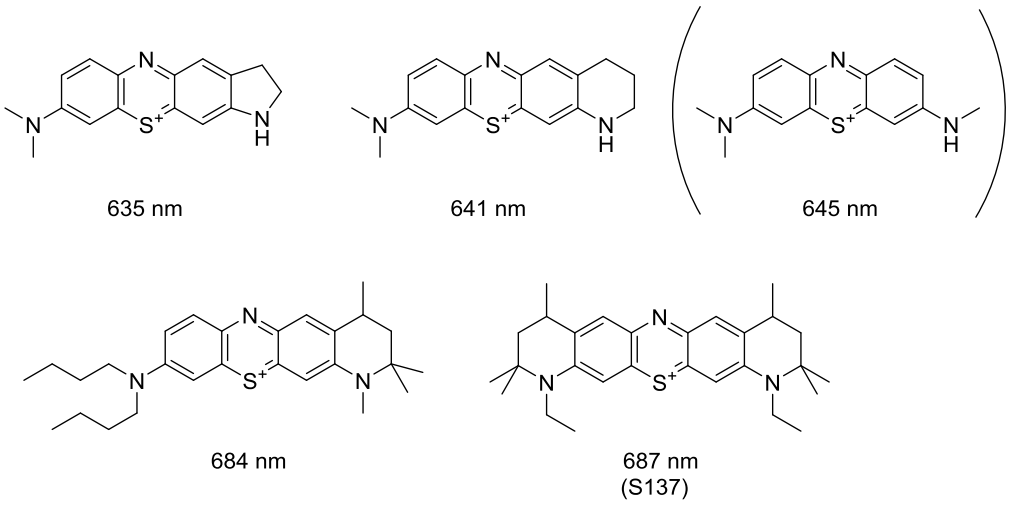


EtNBS EtNBS-G

Figure 13. Photoantimicrobial Nile Blue derivatives

4.3.4.1 Auxochrome rigidification - tetrahydropyrido[3,2-*b*]- and dihydropyrrolo[3,2-*b*]phenothiazinium derivatives

Fused alicyclic ring derivatives may be thought of as a variation on annelation as covered above. However such examples are synthesised from reduced indoles or quinolines and combine the advantages of both ring fusion and alkyl group size increase. In addition, as the auxochromic nitrogen is part of the ring system, the auxochrome is therefore rigidified. Due to this, such (pentacyclic) derivatives represent the longest wavelength-absorbing conventional (i.e. amino auxochrome) phenothiazinium derivatives reported thus far, λ_{max} values approaching 700 nm (Figure 14) [48]. The simplest derivatives for five- and six-membered fusion are shown in Figure 14, the six-membered (tetrahydropyrido-) system being slightly bathochromically shifted relative to the smaller ring fusion.



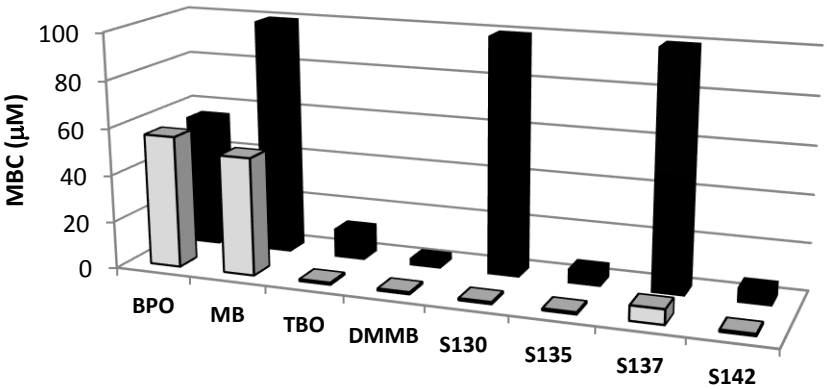
635 nm 641 nm 645 nm

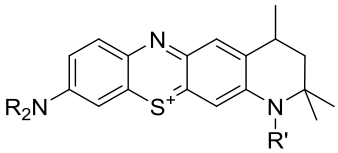
684 nm 687 nm (S137)

Figure 14. Variation in λ_{max} (measured in water) between five- and six-membered ring-fused phenothiazinium derivatives (azure B included in parentheses for comparison).

As expected, the higher hydrocarbon content furnished by double ring fusion produced derivatives of higher lipophilicity/lower aggregation and this was further increased by alkyl ring substitution. In terms of photoantimicrobial efficacy, derivatives such as S137 and its congeners (Figure 14) are highly active, moreso, indeed than the powerful, established familial photosensitisers dimethyl methylene blue and new methylene blue, achieved via strong interaction with/disruption of membrane structures [49]. An example of such high activity may be seen in Figure 15, where this is

demonstrated against *Propionibacterium acnes*, and relative to the 'industry standard', benzoyl peroxide [50]. The properly photoantimicrobial nature of high-performing derivatives, such as S137, has been evidenced via similar efficacy against pathogenic species of *Candida* and *Trichophyton* spp. [51, 52]. Clearly, in both instances, such activities are encouraging, given the ease of application of photosensitiser and light to skin and nails.





R = Me, R' = Et, **S130**
R = Et, R' = Et, **S135**
R = Bu, R' = Me, **S142**

Figure 15. Photoantimicrobial activities of tetra- and pentacyclic derivatives vs. *Propionibacterium acnes*. BPO – benzoyl peroxide; MB – methylene blue; TBO – toluidine blue; DMMB – dimethyl methylene blue; S137 structure as Figure 14; pale grey columns – light activation; black columns – no illumination.

4.4. Photoantimicrobial Progress

Testing or screening potential drug candidates is an essential step in the design/development process. Clearly the function of photoantimicrobials is to kill or otherwise inactivate the microbial target and this is performed via the intermediacy of reactive oxygen species. While it is simple to measure the production of ROS spectrophotometrically, from a design viewpoint this is often a redundant step given that differences in solvent, oxygenation and – importantly – biomolecular binding and aggregation affect light absorption and any subsequent photosensitisation process. It is therefore much more efficient, in terms of molecular ranking, to carry out photosensitisation testing against a realistic microbiological target. A candidate photosensitiser may produce singlet oxygen with great efficiency in spectrophotometric testing, but exhibit little interaction with, or uptake by, the microbial target, thus being unable to focus its photosensitising potential effectively. Consequently, the process of photoantimicrobial ranking is somewhat streamlined by testing directly with the pertinent target.

Perhaps surprisingly, despite its long use in medicine and consequent presence as the obvious lead compound in this research area, methylene blue is far from being the perfect photoantimicrobial agent. As noted previously, there are various criteria to take into account in designing active agents, one of which is the hydrophilic/hydrophobic balance. MB (Log P = -0.1) is very hydrophilic, and this can inhibit its passage into cells. Conversely, the lack of uptake (or slow uptake, relative to the microbial target) by mammalian cells is obviously an advantage from a host toxicity viewpoint. It is also highly aggregated, due to its simple, planar structure and this may, again, inhibit the photodynamic process, as discussed above.

This is not to say that MB is a *bad* photoantimicrobial agent. However, it is the lead compound in the field mainly due to its long use and safety, rather than a sparkling reputation for photodynamic performance. Many novel candidates have been synthesised which have been reported to be far superior in initial and further screening against relevant biological targets [49], and there is now a sound understanding of the structure-function relationships for this class of photosensitisers (Figure 16). The fact remains, however, that regulatory bodies are more concerned with safety than with high performance.

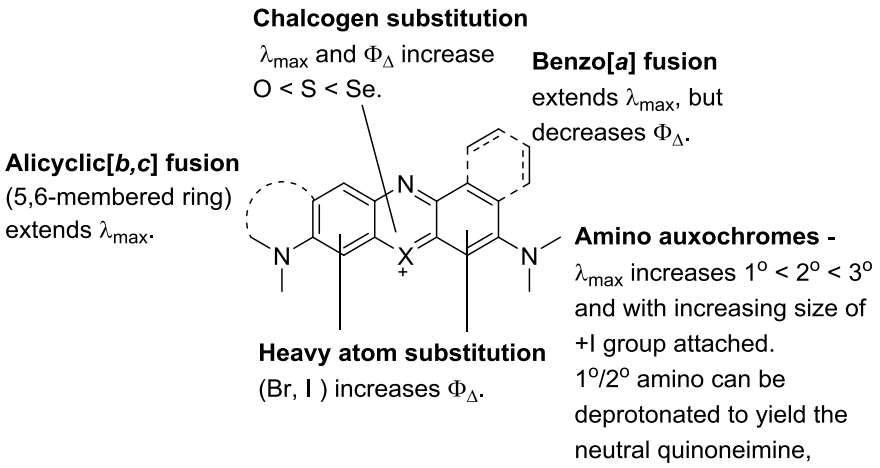


Figure 16. Overview of structure-function in phenothiazinium-based photoantimicrobials

Consequently, there are other hurdles to overcome before clinical acceptance is achieved, principally involved with drug safety. While academic groups and small, spin-out companies are able to carry out a considerable amount of pre-clinical testing, there is usually a huge funding gap between this and patient trialling [16]. Given that supplying this gap appears most unappealing either to the pharmaceutical industry or healthcare concerns, it is surprising that there is a similar lack of appetite from venture capitalists. The risk is high, certainly, but the marketplace for effective new anti-infectives has not been so ripe for exploitation since before the original sulphonamide breakthrough in the 1930s [53].

Furthermore, the local application of both the photoantimicrobial and light automatically suggests skin infections as the primary targets. In turn this leads not only to the 'conventional' skin and soft tissue infections such as burns, ulcers and surgical wounds seen in healthcare in the industrialised

nations, but also tropical infections such as leishmaniasis, trypanosomiasis and Buruli ulcer. Clearly there are other localised infected presentations inside the body which might still be reached using fibre optics.

Blood product photodecontamination has been in use for over 20 years [54], plasma being treated with methylene blue to remove infective agents in several regions (including Europe). However, toxicity concerns – both in terms of cellular component damage and potential host toxicity - have inhibited the development and introduction of improved photoantimicrobial agents to such an extent that research in this area might now be considered a backwater. Once again, this is surprising when the size of the blood products industry worldwide is taken into account.

5. Conclusion

The renaissance of research into photosensitising compounds suitable for infection control has been in existence for approximately a quarter of a century. The impetus for this was, and remains, the ceaseless global increase in conventional antimicrobial resistance, and the phenothiazinium class of photosensitisers remains the principal model, with the lead compound methylene blue being the 'first in class' clinical entry.

Structure-function and, to a lesser extent, structure-activity relationships have been developed for this class of agents and the design and synthesis of novel congeners is relatively straightforward, with the main difficulty now being the movement of long wavelength absorption into the near-infrared region to assist in tissue penetration for deeper infected presentations.

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