Available online at www.sciencedirect.com
ScienceDirect
Journal of Photochemistry and Photobiology B: Biology 86 (2007) 45–58
Journal of Photochemistry and Photobiology B:Biology
www.elsevier.com/locate/jphotobiol

Review

Phenothiazinium derivatives for pathogen inactivation in blood products

Mark Wainwright [a,*], Harald Mohr [b], Wolfram H. Walker [c]

[a] School of Pharmacy and Chemistry, James Parsons Building, Liverpool John Moores University, Liverpool L3 3AF, UK
[b] Blood Center for the German Red Cross Chapters of NStOB, Institute Springe, 31832 Springe, Germany
[c] MacoPharma International GmbH, Robert-Bosch-Str. 11, 63225 Langen, Germany

Received 6 May 2006; received in revised form 29 June 2006; accepted 4 July 2006
Available online 18 September 2006

Abstract

Phenothiazine-based photosensitisers have been employed in photoantimicrobial research for nearly 80 years, both as lead and novel compounds. However, the main structural variations have mainly involved the auxochromic side chains and little has been reported concerning either peripheral substitution or structures with chromophores other than those of the phenothiazinium or annelated benzo[*a*]phenothiazinium type. In terms of application, the phenothiazinium series has featured commonly in cytology and cytopathology, as well as in haematological staining.

The current work covers the evolution of improved photosensitisers based on the phenothiazine ring system, with particular reference to the field of pathogen inactivation, and the structural alteration of lead compounds such as methylene blue and Nile blue to yield improved photosensitisers for this important aspect of blood product safety.

Keywords: Benzo[*a*]phenothiazinium; Blood products; Pathogen inactivation; Phenothiazinium; Platelets; Plasma; Red blood cells

Contents

1. Introduction . . . 46
   1.1. Why are phenothiazinium photosensitisers ideal for PACT? . . . 46
   1.2. Basic design – what makes an effective photosensitiser molecule? . . . 48
      1.2.1. Compound purity . . . 48
      1.2.2. Singlet oxygen generation . . . 49
      1.2.3. Selectivity . . . 49
      1.2.4. Absorption wavelength . . . 50
      1.2.5. Metabolism/cellular breakdown . . . 50
      1.2.6. Holistic consideration . . . 50
2. Blood products . . . 50
   2.1. Blood fractions . . . 50
   2.2. Pathogen inactivation . . . 51
   2.3. Photodynamic inactivation . . . 52
   2.4. Thionin/light + low-dose UVB for the decontamination of platelet concentrates . . . 53
3. Phenothiazine derivatives . . . 53

* Corresponding author. Tel.: +44 151 231 2039.
E-mail address: mark_wainwright@hotmail.com (M. Wainwright).

1011-1344/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotobiol.2006.07.005

3.1. Phenothiazinium photosensitisers . . . 53
3.1.1. Use in pathogen eradication in blood products . . . 53
3.2. Phenothiazones . . . 54
3.2.1. Use in pathogen eradication . . . 54
3.3. Benzo[*a*]phenothiazinium derivatives . . . 55
3.4. Other annelated derivatives . . . 56
4. Conclusion . . . 56
5. Abbreviations . . . 56
References . . . 56

1. Introduction

Methylene blue (Fig. 1) has been used as a lead compound in conventional antimicrobial research for over a hundred years. Since the first publication of its efficacy as an antimalarial compound in 1891, it has also been employed as an antibacterial, e.g., in local antisepsis and against tuberculosis [1–3] and its low toxicity in man is reflected in its current clinical use in methaemoglobinaemia [4]. While its long usage may be explained by the scarcity of active compounds available early in the last century, it continues to be used as a lead antimicrobial compound, e.g., in recent antimalarial research [5–7]. However, it is unlikely that methylene blue is the optimum compond among phenothiazine derivatives for antimicrobial purposes in all cases. Unsurprisingly, there are few records covering the range of compounds examined before the Second World War, but the number is likely to be limited, as in recent years, to alteration of the auxochromic amino groups (Fig. 2). In addition, organised screening of compounds for photodynamic activity was not carried out at that time.

Phenothiazinium synthesis is well established, having been reported as early as 1876. However, the reagents required to furnish the oxidised character of the chromophore have often deterred approaches to more complex molecules, e.g., having ring functionalisation/elaboration beyond the 3,7-*bis*-auxochromic groups. Workers in this area have normally relied on functionalisation of the *N*-alkyl moieties for novelty, as this may be carried out after chromophore construction (e.g., the anticancer *bis*-nitrogen mustard analogue of methylene blue, Fig. 3 [8]).

In addition many groups investigating photoantimicrobial applications have used only standard photosensitisers such as methylene blue and toluidine blue (Fig. 1) without attempting to synthesise more active analogues. Logically, in terms of medicinal chemistry, there is little justification for the acceptance of either of these compounds as the *sine qua non* among phenothiazinium derivatives. Indeed, a few more adventurous groups have synthesised both more active and more toxic analogues, as would be expected in modern drug discovery programmes [9,10].

Since the renaissance in photodynamic therapy (PDT) as a novel approach to cancer treatment 20 years ago, many new compounds have appeared in the literature, representing a wide range of chromophoric types. Among these, photosensitisers based on the phenothiazinium nucleus represent a major contribution [11], with benzannelation furnishing new groups of compounds, although again usually within the limits of auxochromic variation (Fig. 2) [12]. However, in the field of photoantimicrobial chemotherapy (PACT [13]), it has become apparent that cationic photosensitisers producing significant yields of singlet oxygen are of premier importance, particularly in terms of the scope of useful activity, e.g., against *both* Gram-positive and Gram-negative bacteria [14], as well as viruses and yeasts [13]. Within this group, the phenothiaziniums and their congeners are ideally placed to act as lead compounds in terms of drug discovery.

1.1. Why are phenothiazinium photosensitisers ideal for PACT?

Among the aniline dyes invented in the nineteenth century, phenothiazine derivatives including methylene blue, the azures and toluidine blue were quickly adopted for their utility in biological staining. It is no surprise that these dyes were subsequently thoroughly investigated in various areas of biomedicine [15].

Since methylene blue and its close congeners are selectively taken up by microbial cells (i.e., in the presence of animal cells), the fact that most reported phenothiazinium salts are also photosensitisers producing significant yields of singlet oxygen should be sufficient reason for their widespread examination in PACT. That this has not been the case perhaps reflects the dominance of porphyrin-based photosensitisers from the related anti-cancer field of photodynamic therapy (PDT). There is, of course, far less rationale for the use of anionic porphyrins in PACT, particularly where there is a need for broad-spectrum antimicrobial activity.

It has been shown recently that cationic photosensitisers are much more efficient as broad-spectrum antibacterials than are their anionic or neutral congeners [16,17]. This is due to the greater complexity of the Gram-negative cell wall, which is less permeable to anionic species, but is disrupted by cationics [18,19].

In terms of antiviral agents, the positive charge of phenothiazinium derivatives allows efficient binding to viral nucleic acid – thus the considerable use of methylene blue and its congeners as nucleic acid probes [20]. In the disinfection of blood products this is given extra importance since such products do not contain viable nucleic acid

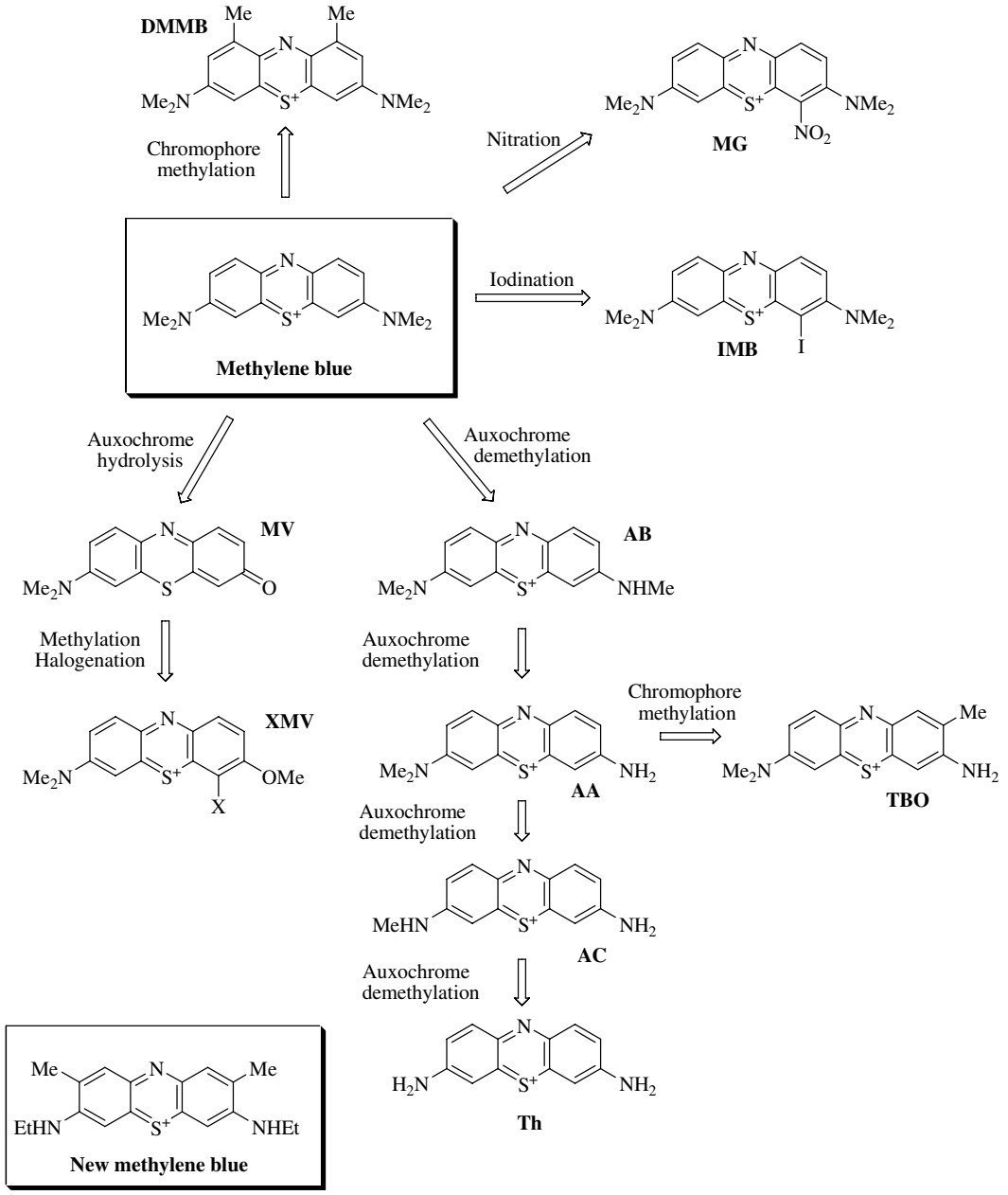


Fig. 1. Methylene blue and other phenothiazinium derivatives. Arrows do not necessarily infer synthetic routes. Key: MB – methylene blue; DMMB – dimethyl methylene blue; MG – methylene green; IMB – iodomethylene blue; MV – methylene violet; XMV – halogenated methylene violet; AB – azure B; AA – azure A; AC – azure C; TBO – toluidine blue O; Th – thionin; NMB – new methylene blue. For MG and IMB the auxochrome adjacent to $-NO_2$ or –I may be NHMe.

and would thus not be damaged in this way. Conversely, although some anionic photosensitisers have been reported to have antiviral capabilities, these occur against different viral targets (e.g., capsid [21]) which have some commonality with host cell structure, allowing a greater potential for collateral damage.

As photosensitisers proposed for PACT should be broad-spectrum in activity, antifungal capability is also important. Here there is less difference between the classes of photosensitiser, both anionic and cationic types exhibiting efficient phototoxicity against, e.g., *Candida albicans* [22,23].

The requirement for blood product photodecontamination procedures lies in the presence in the blood supply of pathogenic microorganisms. The identity of such pathogens can vary depending on the geographic location of the donor, and thus, for example, decontamination procedures must also cover the inactivation of protozoal species where these occur, as well as viruses, bacteria and yeasts. Again, methylene blue and its derivatives are known to be active against such protozoa as *Plasmodium falciparum* and *Trypanosoma cruzi* [24,25], although little has been reported on this activity in terms of modern blood decontamination work.

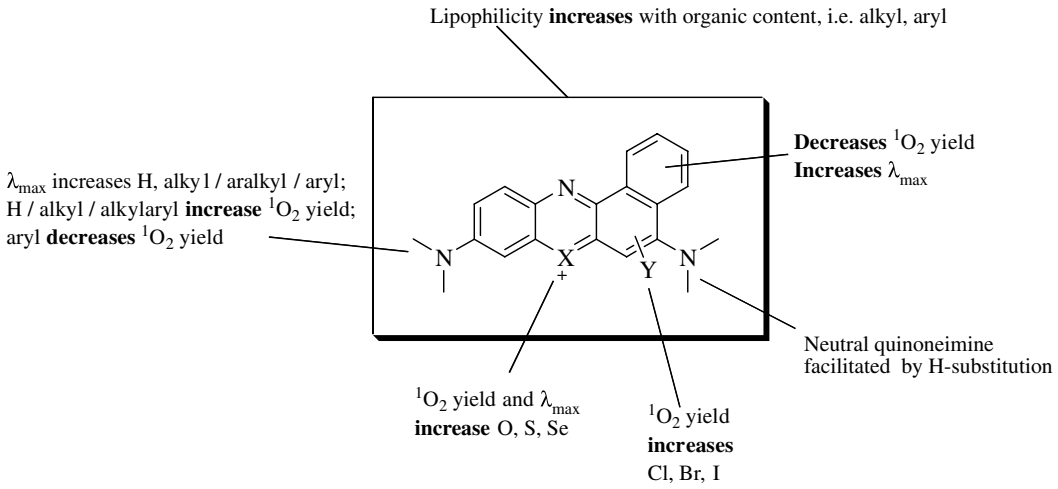


Fig. 2. Structure-function relationships for phenothiazinium and benzo[*a*]phenothiazinium photosensitisers.

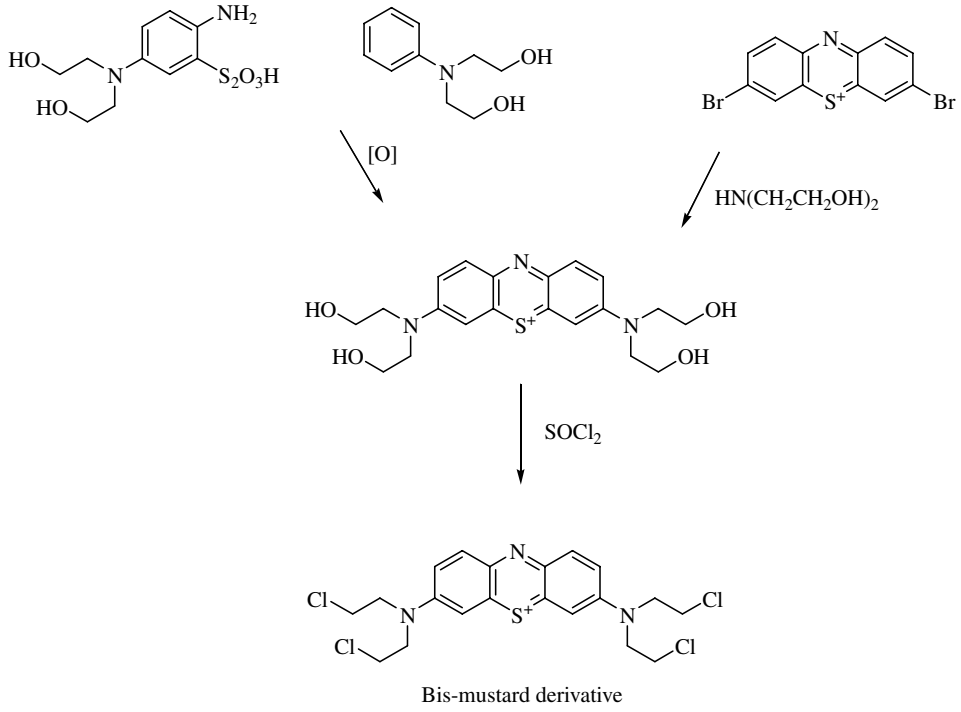


Fig. 3. Derivatisation of a phenothiazinium photosensitiser, post-chromophore synthesis.

Thus there is considerable historical precedent showing that cationic photosensitisers based on the phenothiazine system are very well placed as suitable agents for use in PACT, whether in the decontamination of blood products, the treatment of the diseased state or carrier disinfection. However, the number of compounds examined here remains low, and little has been reported concerning either structure-function relationships, for use in basic photosensitiser design, or structure-activity relationships, to inform future efforts. The following sections therefore examine lead compounds and their derivatives to provide such information, with special regard to the use of such compounds in blood disinfection protocols.

1.2. Basic design – what makes an effective photosensitiser molecule?

The general properties associated with a successful photoantimicrobial agent are outlined in Table 1 and are discussed below.

1.2.1. Compound purity

In standard anti-infective research, potential compounds for testing are expected to be discrete entities with respect to molecular purity. The concept of pure dyes in the textile industry is not usually encountered, as this is not essential for efficient dyeing – indeed in some cases

Table 1
Ideal characteristics for a photoantimicrobial agent

Essential
Pure compound, not a mixture
Significant yield of singlet oxygen
High selectivity for microorganisms
Low toxicity/non-mutagenic in mammalian cells
Non-specific in terms of biomolecular targeting
Preferable
Water soluble for ease of application
Light absorption outside that of relevant biomolecules, e.g., haem in red blood cell decontamination
Photostable during application

dyestuffs containing impurities are more effective in the dyeing process. Clearly in the present context photosensitising compounds must be subject to the same considerations as standard drugs. Chemical and isomeric purity are essential.

1.2.2. Singlet oxygen generation

The various electronic transitions possible in photosensitiser action are well known and are shown in Fig. 4. Generally, effective photosensitiser molecules are able to populate the excited triplet state ($^*T_1$) significantly, and it is possible to design such molecules based on the enhancement of factors supporting this.

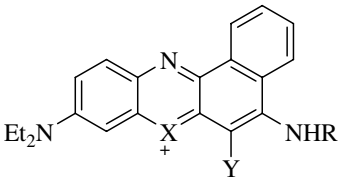
For the triplet excited state to be sufficiently populated, a relatively long-lived excited singlet and efficient conversion of $^*S_1 \rightarrow {}^*T_1$ is required. The $^*S_1$ state is deactivated both radiatively (fluorescence) and non-radiatively. Facile deactivation is also possible via structural flexibility in donor-acceptor chromophoric systems, such as those currently described, the acceptor being the cationic nucleus and the donors the auxochromic groups. In terms of the particular features common to the phenothiazinium-based photosensitisers and their benzologues, the formation of a twisted intramolecular charge transfer (TICT) complex between the cationic chromophore and an amino auxochrome has been proposed as a possible relaxation route [26]. A TICT mechanism allowing charge separation and rapid non-radiative relaxation reportedly occurs most strongly with *N*-aryl and *N*,*N*-dialkyl auxochromes.

The $^*T_1$ state can itself be stabilised by the inclusion of atoms of a high atomic number due to their large spin-orbital coupling constants (the "heavy atom" effect). The peripheral inclusion of bromine or iodine in the chromophore, or (usually) of sulphur or selenium as ring heteroatoms is standard practice in the maximisation of singlet oxygen production (Table 2) [27].

1.2.3. Selectivity

Unlike the situation with standard antimicrobial drugs the concept of selectivity in PACT refers to that solely between human and microbial cells. The great advantage of photosensitisers such as methylene blue is that they are non-selective with respect to the site of action within the microbial target. For example, post-illumination damage has been reported at the cell wall, the cytoplasmic membrane, ribosomes and nucleic acid of bacteria [28]. Nucleic acid targeting may be seen as advantageous in the inactiva-

Table 2
Changes in physicochemical properties of Nile Blue analogues with halogenation [27]



X	Y	R	λ_{max} (nm)	$^1O_2$ quantum yield	log *P*	pK_a	Ref.
O	H	H	623	0.005	2.25	10.0	[20]
O	Br	H	643	0.007	–	8.0	[20]
O	I	H	642	0.025	2.55	10.0	[20]
S	I	H	660	0.23	3.70	6.5	[20]
O	H	Et	632	0.005	2.69	–	[19]
S	H	Et	652	0.025	2.76	–	[19]
Se	H	Et	659	0.650	2.10	–	[19]

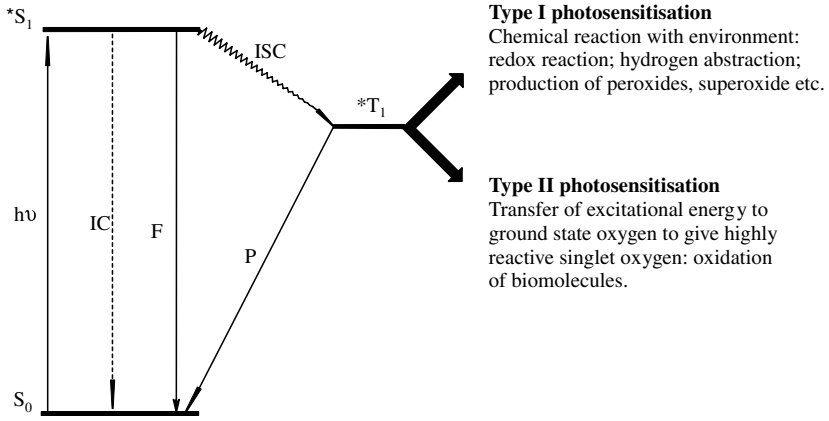


Fig. 4. Adapted Jablonski diagram indicating excitation/relaxation pathways involved in photosensitisation. Key: S_0 – singlet electronic ground state of photosensitiser; $^*S_1$ – singlet excited state; $^*T_1$ – triplet excited state; h – light absorption; IC – internal conversion (including TICT); F – fluorescence; P – phosphorescence; ISC – inter-system crossing.

tion of viruses in blood products, e.g., plasma, but this is not essential. Indeed although the DNA-intercalative nature of methylene blue is well-established, the use of this photosensitiser with HIV-1 produced post illumination damage to enzymes such as reverse transcriptase and the viral capsid as well as nucleic acid [29].

1.2.4. Absorption wavelength

Photosensitisers used for biological application must absorb light outside that of the operative environment. For the present case, the most important example is given by haem absorption in red blood cells. Realistically here, photosensitisers must exhibit significant light absorption >630 nm in order to be effective. The requirement in platelet concentrates and plasma, having little visible absorption, is less stringent.

For a given photosensitiser, both benzannelation and the inclusion of heavy atoms usually increase the maximum wavelength of absorption considerably. The modification/elaboration of auxochromic side chains has a lesser effect (Fig. 2).

1.2.5. Metabolism/cellular breakdown

It is well established that photosensitisers based on azine chromophores are reduced, at varying rates, by ubiquitous cellular enzymes, to colourless leuco forms (Fig. 5), which are inactive as photosensitisers [28]. It is possible to prevent this process, or at least to inhibit it significantly, by informed drug design [30]. However, this may again have a pharmacological impact. In addition, the reduction of such azine cations may aid in uptake at the target site, thus increasing activity if intracellular reoxidation is possible. Such activity has been reported for methylene blue and the malarial parasite *P. falciparum* [5,6].

1.2.6. Holistic consideration

In designing photosensitisers for biological purposes it is important to investigate the effects of functionalisation, as discussed above, on the molecule as a whole. A normal effect either of ring annelation/expansion or of heavy atom substitution is that of increased lipophilicity, – the new molecule exhibits increased organic partitioning, e.g., into lipids in biological media (or, *in vitro*, in the standard aqueous buffer-octanol system).

In terms of biological behaviour, such increased lipophilicity (hydrophobicity) is often reflected in greater lipoprotein binding, which may lower the bioavailability of the photosensitiser, depending on the end use. Given intracellular microbial uptake, the new molecules may also exhibit altered organelle targeting – e.g., ribosomes instead of cell wall, etc. Moreover, the gross selectivity between microbial and host cells may be affected. Such alteration of behaviour may or may not be detrimental to activity, but it should be considered at the drug design stage nevertheless.

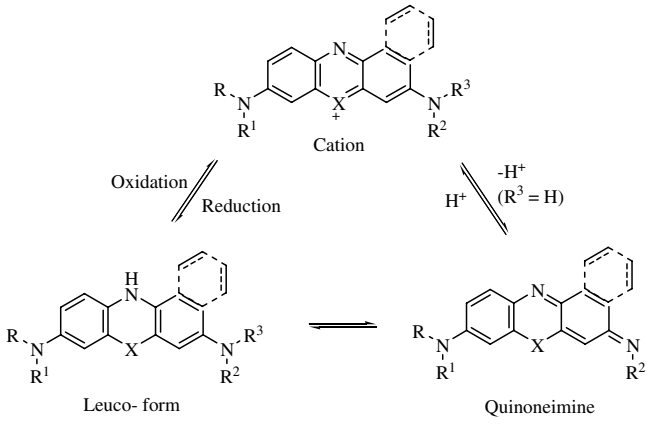


Fig. 5. Cationic, reduced (leuco) and quinoneimine forms of the phenothiazinium and benzo[*a*]phenothiazinium chromophore. R groups = alkyl, aryl or H; X = O, S, Se.

2. Blood products

Blood transfusion and the use of products derived from whole blood are essential in modern medicine. However, the presence of pathogens in donated blood can lead to infection in the recipient (transfusion-transmitted infection, TTI), as has been seen with haemophiliacs who received HIV-contaminated clotting factor preparations. Blood donations are screened stringently, both prior to donation in the form of complex questionnaires and post-donation in the microbial testing of the blood.

2.1. Blood fractions

The major fractions of blood are red blood cell concentrate, platelet concentrate and plasma, while products such as clotting factors are isolated from these.

Plasma is the aqueous fraction of the blood and contains the proteins and factors necessary for function – e.g., serum lipoproteins and clotting factors. Plasma is often frozen promptly and thawed before use. Fresh frozen plasma (FFP) stored at −30 °C has a shelf life of 5 years.

Platelets are required in the treatment of bleeding disorders – e.g., in thrombocytopenia as a result of the chemotherapy of leukaemia or of cirrhosis. They are obtained from donors by plateletpheresis or concentrated from blood donations. Plateletpheresis-derived platelet concentrates (PCs) are single donor products; "random donor" PCs are pool-prepared from 4 to 6 donations. The product may be stored at 20–24 °C for up to 5 days.

Red blood cell concentrates (RBCCs) are utilised in the improvement of oxygen-carrying capability due to blood loss either after trauma or surgery. Red blood cell concentrates can be stored in red cell preservation solutions at 2–6 °C for 42–49 days.

Transfusion-transmission illnesses may arise from viral, bacterial, fungal or protozoal contamination, depending on the medical history, and often the geographical location, of the donor, and to this list must now be added prion disease.

The screening of donated blood is not infallible. "Window" periods of infection occur, normally straight after

colonisation, in which the microbe is undetectable by serological testing [31]. Blood donation during such a period could obviously lead to transfusion-transmission of disease. In addition, the large-scale manufacture of blood products utilizing the principle of ''pooling'', i.e., combining many blood fractions from different donors, has a concomitant increase in the risk of resultant infection. In terms of the elimination of the disease threat, there are problems in selectivity, the degree of complexity depending on the blood fraction concerned.

2.2. Pathogen inactivation

Current approaches to pathogenic inactivation in plasma include heating (e.g., pasteurization), UV treatment or nanofiltration. Unfortunately, none of these is completely effective, nor without the disadvantage of collateral damage, e.g., to proteins.

Pathogen reduction procedures for human blood components are in routine (clinical) use for human plasma and in clinical trials for platelet concentrates.

Table 3 gives an overview on existing decontamination procedures for stable products, which includes mostly plasma factor concentrates used in batches of several hundred litres of plasma.

The targets for the labile product, human fresh-frozen plasma, are mostly viruses, but also leucocytes are given in Table 4. Bacteria and other microbes in human plasma and plasma proteins are of lesser importance, since some plasma proteins allow sterile filtration. Furthermore, plasma protein solutions are usually frozen and stored at −30 to −40 °C and only thawed before clinical use.

Recently, due to the prion problems in transfusion medicine, emphasis is on small volume single donor inactivation procedures for human plasma and platelets.

The main difference between plasma and platelet or red cell fractions is the cellular nature of the latter two. This nature makes pathogen deactivation much more complex: physical methods, e.g., filtration or washing, can remove extracellular pathogens but not intracellular. The detergent effects so successful against viral contaminants in plasma are also potentially damaging to the cellular membranes of platelets and erythrocytes.

Pathogenic microbial species in platelet or red blood cell concentrates may be cell-associated, extra- or intracellular or a combination of these, generally depending on the pathogen. For example, yeasts and bacteria are usually, though not always, extracellular pathogens while protozoans (e.g., the malarial parasite *P. falciparum*) or viruses such as HIV may be present in the extracellular medium or inside the cell. Due to its storage at 22 °C, the threat to platelets comes mainly from bacteria, since their growth is not inhibited at this temperature [32]. Conversely, since most bacterial species exhibit poor growth characteristics at 4 °C (*Yersinia enterocolitica* and *Pseudomonas fluorescens* are the main exceptions), the main problem to be addressed in erythrocyte pathogen inactivation lies in the treatment of intracellular pathogens such as HIV. Given the problems of selectivity and collateral damage associated with the regimens above, intracellular disinfection in blood fractions must be carried out more along the lines of standard antimicrobial chemotherapy. An added complication here is the requirement for leucocyte reduction.

By definition, targeting intracellular pathogens requires transport of the toxic agent into the cell interior. Molecules having the ability to do this usually satisfy certain gross physicochemical criteria, such as cationic ionisation and positive $\log P$ (hydrophilic–lipophilic balance). Thus classical chemotherapeutic molecules such as chloroquine are concentrated by erythrocytes, whereas methylene blue

Table 3
Decontamination of blood products: stable products

Procedure	Product	Target	Introduction
Pasteurisation	Albumin, clotting factors	Envel. viruses	1944, 1981
Dry + wet heat	Fibrin glue, immuno-globulins	Some non-envel. viruses	1985
Solvent/deterg.	All plasma proteins	Envel. viruses	1985
pH 4	Immunoglobulins	Envel. viruses	1991
Sulfonation	Immunoglobulins	Envel. viruses	1989
β-Propiolactone	Immunoglobulins	Envel. viruses	1992
Nanofiltration	Factor VIII, immuno-gobulins	Envel., non-envel. viruses	1994

Modified according to [40].

Table 4
Decontamination of blood products: labile products

Procedure	Product	Target	Introduction
Solvent/deterg.	Fresh-frozen plasma	Envel. viruses	1992
Methylene blue/light	Fresh-frozen plasma	Envel. viruses, some non-envel. viruses	1991
Leukocyte depletion	Platelet concentrates red blood cell conc. fresh-frozen plasma	Leukocytes leukocytes leukocytes	Since 1957
UVB irradiation	Platelet concentrates	Leukocytes	1992
Gamma irradiation	Platelet concentrates	Leukocytes	1985

Modified according to [40].

(cationic/log $P < 0$) is not. Typical anionic photosensitiser molecules such as porphyrin carboxylates and phthalocyanine sulfonates (anionic/log $P > 0$) are excluded from erythrocytes (Fig. 6).

Once inside the blood cell, the agent must locate the pathogen. In terms of antiviral treatment the ability to intercalate into nucleic acid is advantageous, but not essential since there are other targets such as proteins and enzymes, which do not require intercalation. In addition, intercalative ability may be undesirable if the compound cannot be removed completely from the blood sample post-treatment, since this has the potential to cause post-transfusion mutagenesis.

2.3. Photodynamic inactivation

The photodynamic approach to the problem of blood production disinfection/pathogen eradication offers selectivity with more control over collateral damage. Long wavelength visible light ($\geqslant$630 nm) can be used to illuminate all fractions of whole blood and – unlike ultraviolet – has no inherent effect on cellular or proteinaceous material. Clearly the historical use of vital stains such as methylene blue and the azures provides considerable foundation for the investigation of improved photosensitisers based on the phenothiazinium and derivative chromophores. Thorough knowledge of the chemistry entailed in phenothiazinium design and synthesis allows a more considered, logical approach to the problem of selectivity and, thus, to the minimization of collateral damage.

There are presently three photodynamic procedures in routine clinical use or which have been cleared for clinical trials, using single donor human plasma units for therapeutic use:

(a) The methylene blue procedure using visible light [33].
(b) The amotosalen procedure using UVA-Light [34]. This is in reality a photochemical, rather than a photodynamic approach, relying as it does on UV activation of a psoralen derivative in the presence of nucleic acid to form adducts where the psoralen nucleus is covalently bonded to one or two nucleotide bases.

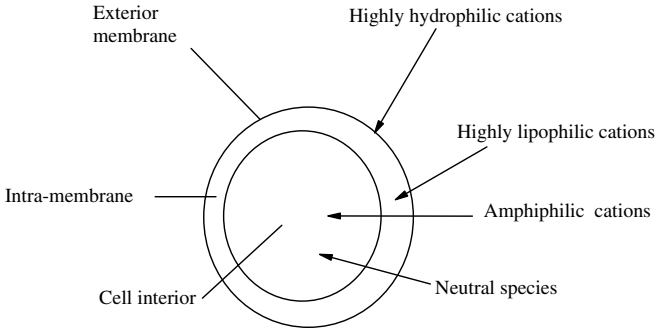


Fig. 6. Gross classification of erythrocyte localisation.

(c) The Riboflavin Procedure using UVB-Light [35]. Similar in operation to MB, riboflavin (vitamin B2) produces singlet oxygen on illumination. The basis for its use lies in the low toxicity of this essential nutrient. However, like psoralens, it is activated by long-wavelength UV and is thus unlikely to be of use in red blood cell concentrates.

As already mentioned, the mechanism of action of methylene blue (MB) can work in two ways. By excitation of MB with light, it can go into a singlet and triplet stage and transfer the energy through either electron (Type I mechanism) or energy transfer (Type II mechanism).

On intercalation with DNA or RNA, the Type I mechanism can lead through oxidized species like hydroxyl radicals and the Type II mechanism through singlet oxygen. The results are breakages of nucleic acids, mostly at the guanosine site. Therefore in contrast to the Amotosalen procedure there is no covalent adduct formed. Riboflavin can act in a similar way to methylene blue.

The methylene blue Procedure for human plasma was developed by Mohr et al. in 1991 [33] and further improved by MacoPharma [36]. The single donor plasma is connected to a closed plastic blood bag system, which includes a 0.65 μm membrane plasma filter to eliminate cell contaminations. The pack contains 85 μg of methylene blue hydrochloride, which leads, using 266 ml of plasma to a final concentration of 1 μM. The unit is illuminated with 180 J cm^{-2}, using sodium lamps at 590 nm for about 20 min, using a validated PC-controlled machine. The photodynamic step is followed by a filtration step, which removes more than 90% of the residual MB and its photoproducts (mostly azures A, B, C and Thionin) [37]. The procedure has been validated for a volume variation from 200–315 ml of plasma.

As the illumination machine uses standard high pressure sodium lamps it has an integrated cooling and agitation system and allows monitoring of the light intensity through the illumination step.

This procedure is efficient to inactivate at least 5 logs of enveloped viruses relevant to transfusion medicine, e.g., HIV and HCV, but is less efficient for non lipid-enveloped viruses [36]. This procedure has some influence on the efficacy of plasma coagulation proteins. Fibrinogen and Factor VIII activity is decreased by around 25% using the photodynamic MB procedure. Other factors, including inhibitors are not reduced. This is confirmed by proteomics analysis [38]. Significant efforts have been undertaken to prove that there is enough safety margin with respect to the pharmacology and toxicology of the MB-treated plasma, to allow the use of such plasma in specific clinical applications [39]. Thus MB-treated plasma is considered equivalent to standard human fresh-frozen plasma.

The Theraflex MB-plasma system uses a MB-quality described in the European Pharmacopeia. It is a procedure, which fits into the routine operation of a blood bank and therefore has found wide acceptance in most European

countries. More than four million units of MB-treated plasma have been used clinically to date.

2.4. Thionin/light + low-dose UVB for the decontamination of platelet concentrates

Despite its documented efficacy as a photoantimicrobial, methylene blue is not suitable for photodynamic pathogen inactivation of platelet concentrates (PC) for two reasons:

(a) Bacteria and residual leucocytes are not inactivated.
(b) Platelets are heavily damaged by illumination in the presence of MB.

It was, however, found that the fully demethylated derivative of MB, thionin (Th) was similarly effective in inactivating free viruses while leaving platelet functions almost fully intact [41]. A possible explanation is that Th is more hydrophilic than MB and may therefore exhibit less binding to platelet and other cell exteriors. As a consequence, Th/light, even more than MB/light, is ineffective in the inactivation of leukocytes and bacteria in the presence of plasma. This is due to the fact that plasma is known to contain compounds that quench photodynamic type II-reactions involving singlet oxygen. Significant quenchers in plasma occur in both the lipoprotein-fraction, and in the aqueous phase, e.g., urea, tocopherols, carotenoids, ascorbic acid and bilirubin [42]. Nevertheless, the lipid-enveloped viruses tested were inactivated by Th/light treatment. In this respect, Th was at least as effective as MB. Obviously Th, similar to MB, has a high affinity for viral structures including the viral genome. It is also remarkable that in contrast to most non-enveloped viruses tested, namely the animal parvoviruses, the human parvovirus B19 was found to be highly sensitive to Th/light treatment [41].

In plasma-free suspensions, thionin at a concentration between 1 and 5 μM was effective in inactivating a number of Gram-positive and also some Gram-negative bacteria. This was not unexpected because the established photobactericidal activity of other phenothiazinium dyes, particularly methylene blue and toluidine blue (TBO) [43,44]. For example, Wilson and his coworkers used the two photosensitisers to inactivate oral bacteria in multispecies biofilms [45,46]. Recent publications related to the photodynamic inactivation of bacteria in blood components in the presence of phenothiazinium derivatives have included studies of the photobactericidal activity of the compounds against *Y. enterocolitica* [47]. Here it was found that dimethyl-methylene blue (DMMB) was more active than TBO and MB, and that new methylene blue (NMB) was more active than DMMB [48]. Another study revealed that DMMB and NMB were also more potent than TBO and MB in photoinactivating different strains of *Staphylococcus aureus* [49]. However, reports suggesting that the photoactivity of TBO was unaffected by the presence of blood and serum [50,51] are not supported by the results of the investigations on the phototreatment of PC with thionin: it should be remembered that plasma is necessary to maintain the storage stability of the platelets in PC [52,53].

There is an additional requirement that residual leucocytes in PC should be inactivated because they may contain cell-associated viruses and may cause alloimmunization and refractoriness to further platelet transfusions in the recipients of PC-transfusions [54,55]. However, like MB, Th is not effective in photoinactivating leucocytes. As mentioned, this might be due to the fact that both compounds are hydrophilic and therefore unable to penetrate cellular membranes: According to Wainwright the cytotoxicity of phenothiazine dyes is increased with increasing hydrophobicity, i.e., the more hydrophilic compounds are not so cytotoxic [28,49]. This assumption is supported by data published by Skripchenko and Wagner indicating that white blood cells were inactivated by illumination in the presence of DMMB, whereas the less hydrophobic MB did not cause significant changes in leukocyte viability [56].

3. Phenothiazine derivatives

3.1. Phenothiazinium photosensitisers

As mentioned above, methylene blue (MB) and toluidine blue (TBO) have been examined as lead compounds in both photochemotherapeutic and conventional approaches. There are also several closely related congeners which are commercially available and which are often employed to provide data in short series research. These congeners are the demethylated MB analogues, azure A, B and C and thionin (previous section). In addition there are derivatives having peripheral substitution (Fig. 2), such as new methylene blue (2,7 dimethyl), dimethyl methylene blue (Taylor's blue, 1,9-dimethyl) and methylene green (4-nitro). Toluidine blue itself has a methyl group at C-2 of the chromophore (Fig. 2). Indicative data for these compounds as suitable photosensitisers are given in Table 5.

A considerable number of MB analogues was produced in work by Taylor aimed at compounds useful in biological staining, and from which the stain Taylor's Blue evolved (Table 6) [57]. The absorption maxima for this set show that auxochromic variation is of little use in providing a significant bathochromic shift. In addition, chromophore alkylation was shown to have a slightly negative effect here [9,30].

3.1.1. Use in pathogen eradication in blood products

Several phenothiazinium derivatives have been used in routine blood staining since the early 20th century, e.g., new methylene blue, toluidine blue, azure B, etc. [58]. The utility of these phenothiaziniums in blood staining arises from the interaction between the planar cationic structure and sialylated structures (i.e., derivatised with sialic acid) in the red blood cell membrane.

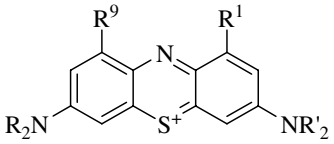
The higher percentage of sialylation in reticulocytes (immature red cells) means that phenothiaziniums such as

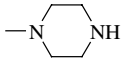
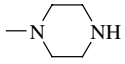
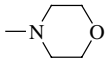
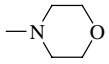
Table 5
Photoproperties and log *P* values for phenothiazine-based photosensitisers

Photosensitiser	λ_{max} (H_2O, nm)	Relative $^1O_2$ yield[a]	log *P*
Methylene blue (MB)	656	1.00	−0.10
Azure A (AA)	623	0.86	+0.70
Azure B (AB)	648	0.41	−0.09
Azure C (AC)	616	0.71	−0.40
Thionin (Th)	595	1.16	−0.35
Methylene green (MG)	654	0.50	−0.28
Toluidine blue O (TBO)	625	0.86	−0.21
New methylene blue (NMB)	630	1.35	+1.20
Dimethyl methylene blue (DMMB)	648	1.21	+1.01
Iodomethylene blue (IMB)	??	–	–
Methylene violet (MV)	580	0.73	+0.11
Halomethylene violet (XMV [68])	573 (X = Br), 575 (X = I)	0.91, 1.46	–

For compound structures, see Fig. 1.
[a] Spectrophotometric measurement of the oxidation of 1,3-diphenylisobenzofuran, $^1O_2$ yield relative to that of MB.

Table 6
Absorption data for methylene blue derivatives in ethanol [57]



NR_2	NR'_2	R^1	R^9	λ_{max} (nm)
NMe_2	NMe_2	Me	H	655
NMe_2	NMe_2	Me	Me	655
NEt_2	NEt_2	Me	Me	650
NMe_2	NMe_2	H	H	668
NEt_2	NEt_2	H	H	672
NPr^n_2	NPr^n_2	H	H	678
		H	H	670
		H	H	665

MB and the azures are useful stains both in the demonstration of erythropoiesis [59,60], and in the decay of blood cells due to ageing [61].

The combination of cationic/hydrophilic character of most of the commercially available phenothiaziniums aids in membrane targeting, i.e., at hydrophilic (sialylated) regions of the glycocalyx membrane structure [62,63]. Those compounds with the ability also to dissolve in lipids, either due to more positive lipophilicity (e.g., dimethyl methylene blue [57]) or to the formation of small amounts of a neutral quinoneimine species, e.g., toluidine blue O or azure B (Fig. 5) [64] exhibit greater intracellular distribution – DMMB stains erythrocytes blue-green [57]. Proven ability to localise inside blood cells is obviously important in terms of putative of cellular photoantivirals.

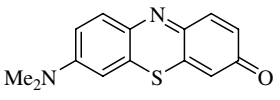
It should be noted that methylene blue is under constant examination in terms of its ability to inactivate new or emerging pathogens which threaten the blood supply. For example, as well as its efficacy against HIV-1, HBV and HCV, MB has recently been reported to be highly effective, under white light illumination, in the inactivation of West Nile virus, and to inhibit completely its infectivity in an inoculated animal model [65].

3.2. Phenothiazones

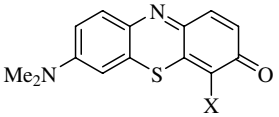
Methylene violet (MV) is simply produced via the alkaline hydrolysis of methylene blue, which leaves a double-bonded oxygen in place of one of the auxochromic dimethylamino moieties. MV is thus a neutral species [66] and, relative to methylene blue, provides a suitable demonstration of the improved cellular uptake of neutral species. The neutral analogue is considerably more effective against intracellular viruses in red blood cells than is cationic/hydrophilic MB, the latter being mainly excluded from the cell interior [67]. However, improved uptake is balanced by the inhibition of action of MV (and its halogenated analogues) in the presence of plasma proteins [68]. This is presumably due to the high levels of lipoprotein binding expected of a neutral lipophilic material [67]. In addition, the neutral character of MV means that it has low aqueous solubility, which complicates its administration. Greater aqueous solubility was attained on the conversion of the neutral oxo (>C=O) function to alkoxy, with concomitant regeneration of a phenothiazinium salt (Fig. 7). Halogenated versions (XMV, Fig. 1) exhibited similar singlet oxygen yields to MB in combination with lowered protein binding compared with the parent MV [68].

3.2.1. Use in pathogen eradication

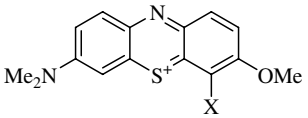
Both toluidine blue O [69] and methylene violet are also known to cause nucleic acid photodamage, although the latter acts in part via direct electron transfer to the nucleotide bases, causing "nicks" and also by photoadduct formation [70]. Thus, whereas TBO acts via a Type II photosensitisation mechanism, MV exhibits a mixture of Type I in conjunction with photochemical (psoralen-type) attack.



Methylene violet



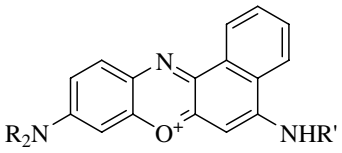
Neutral



Cationic

Fig. 7. Neutral and cationic methylene violet (MV) analogues.

Table 7
Absorption data for Nile blue derivatives in ethanol [74,75]



	R	R′	λ_{max} (nm)
(Nile Blue A)	Et	H	628
	Me	Benzyl	635
	Et	Benzyl	642
	n-Pr	Benzyl	646
	n-Bu	Benzyl	647
	n-Amyl	Benzyl	646
	n-Hex	Benzyl	647
	Me	Phenyl	648
	Et	Phenyl	660
	n-Pr	Phenyl	660
	n-Bu	Phenyl	661
	n-Pe	Phenyl	662
	n-Hex	Phenyl	663

3.3. Benzo[a]phenothiazinium derivatives

The use of Nile blue as a stain in haematology is well established – for example, Nile blue stains intraerythrocytic *Plasmodium berghei* [71]. In addition, Nile blue was employed as a lead compound in photosensitiser development for cancer PDT by Foley et al. [26]. The absence of any singlet oxygen production by the lead compound was remedied both by halogen atom inclusion and replacement of the oxygen of the central ring by the heavier sulfur or selenium [72]. Both of these approaches yielded useful photosensitisers with significant singlet oxygen producing potential, and demonstrating admirably the heavy atom effect. In addition levels of mammalian toxicity were sufficiently low for further development to be considered [73].

There exists a sound understanding concerning important structure-light absorption relationships in the benzannelated derivatives mainly due to work published by Crossley et al. in the 1950s, in an investigation of the series as conventional chemotherapeutics for tuberculosis [74–78]. The effect of both increasing alkyl chain length in the auxochromic moiety and of heteroatom substitution on absorption wavelength were shown to follow the usual patterns as discussed above (Table 7). Interestingly, the use of *N*-aryl groups at position 5- of the benzo[*a*]phenoxazine nucleus provided a number of highly active antimycobacterial compounds, having equal or greater activity against *Mycobacterium bovis* when compared to streptomycin [75]. For the current work this is unfortunate, since it has

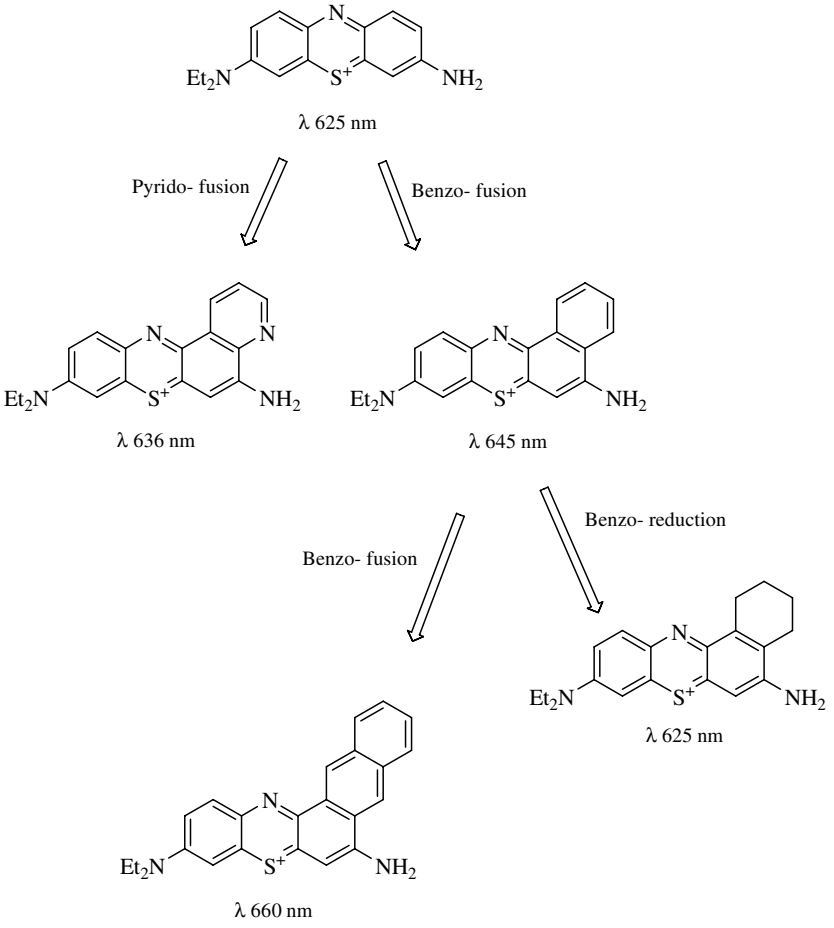


Fig. 8. Variation in wavelength maxima with aromatic character.

also been shown that bis(*N*-arylamino)phenothiazinium derivatives do not produce measurable quantities of singlet oxygen [79]. However, the use of *N*-benzyl derivatives in Crossley's study also realised highly active antitubercular activity [74]. Although little mammalian toxicological data is available on any of these compounds they retain considerable potential in terms of blood product use.

3.4. Other annelated derivatives

Recent work by one of the authors (MW) has also shown that other aryl fusions of the phenothiazinium nucleus are possible, furnishing derivatives of similar type, in terms of photoproperties, but with the potential for future chromophore modification. These compounds include naphtho [2,3 *a*]- and pyrido[2,3 *a*]-fusions (Fig. 8) [80].

4. Conclusion

The current use of MB in plasma photodecontamination encouraged the testing of similar compounds for platelet concentrates and this has resulted in the present proposal of thionin/UV to this end. The situation with red cell decontamination is still some way from being resolved, largely due to problems with haemolysis. However, a more rational approach to photosensitiser design, taking into account red cell membrane binding should provide dividends.

The synthetic chemistry associated with methylene blue and its congeners is, by now, well understood, if not widely appreciated. The preparation of close MB analogues is thus relatively straightforward, while that of more complex molecular structures involving extended chromophores is at least achievable, if not yet in high yield. Consequently, the provision of full ranges of compounds for screening in the search for, for example, potential red cell photoantimicrobials is feasible.

5. Abbreviations

CFU	colony-forming units
DMMB	dimethyl methylene blue
DNA	deoxyribonucleic acid
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
MB	methylene blue
MV	methylene violet
NMB	new methylene blue
PACT	photoantimicrobial chemotherapy
PC	Platelet concentrate
PDT	photodynamic therapy
RNA	ribonucleic acid
TBO	toluidine blue O
Th	thionin
TICT	twisted intramolecular charge transfer
UV	ultraviolet

References

[1] B. Zeina, J. Greenman, W.M. Purcell, B. Das, Killing of cutaneous microbial species by photodynamic therapy, Br. J. Dermatol. 144 (2001) 274–278.
[2] F. DeEds, Antiseptic value of phenothiazines in urinary tract infections, J. Pharmacol. 65 (1939) 353–371.
[3] L.M. DeWitt, Preliminary report of experiments in the vital staining of tubercles, J. Infect. Dis. 12 (1913) 68–92.
[4] M. Wainwright, K.B. Crossley, Methylene Blue – a therapeutic dye for all seasons? J. Chemother. 14 (2002) 431–443.
[5] J.L. Vennerstrom, M.T. Makler, C.K. Angerhofer, J.A. Williams, Antimalarial dyes revisited: xanthenes, azines, oxazines, and thiazines, Antimicrob. Agents Chemother. 39 (1995) 2671–2677.
[6] M. Wainwright, L. Amaral, The phenothiazinium chromophore and the evolution of antimalarial drugs, Trop. Med. Int. Health 10 (2005) 501–511.
[7] R.H. Schirmer, B. Coulibaly, A. Stich, et al., Methylene blue as an antimalarial agent, Redox Rep. 8 (2003) 272–276.
[8] G. Csaba, J. Korosi, A new antitumour agent: phenazathionium-mustard salt, Neoplasma 15 (1968) 443–445.
[9] M. Wainwright, Phenothiazinium photosensitisers V. Photobactericidal activities of chromophore-methylated phenothiazinium salts, Dyes Pigments 73 (2006) 7–12.
[10] S.J. Wagner, A. Skripchenko, D. Robinette, J.W. Foley, L. Cincotta, Factors affecting virus photoinactivation by a series of phenothiazine dyes, Photochem. Photobiol. 67 (1998) 343–349.
[11] M. Wainwright, R.M. Giddens, Phenothiazinium photosensitisers: choices in synthesis and application, Dyes Pigments 57 (2003) 245–257.
[12] J.W. Foley, L. Cincotta, A.H. Cincotta, Structure and properties of novel benzo[*a*]phenoxazinium photochemotherapeutic agents, Proc. SPIE 847 (1987) 90–95.
[13] M. Wainwright, Photodynamic antimicrobial chemotherapy (PACT), J. Antimicrob. Chemother. 42 (1998) 13–28.
[14] M. Wainwright, D.A. Phoenix, J. Marland, D.R.A. Wareing, F.J. Bolton, A study of photobactericidal activity in the phenothiazinium series, FEMS Immunol. Med. Microbiol. 19 (1997) 75–80.
[15] M. Wainwright, The use of dyes in modern biomedicine, Biotech. Histochem. 78 (2003) 147–155.
[16] J. O'Neill, M. Wilson, M. Wainwright, Comparative antistreptococcal activity of a range of photobactericidal agents, J. Chemother. 15 (2003) 329–334.
[17] A. Segalla, C.D. Borsarelli, S.E. Braslavsky, et al., Photophysical, photochemical and antibacterial photosensitizing properties of a novel octationic Zn(II)-phtalocyanine, Photochem. Photobiol. Sci. 1 (2002) 641–648.
[18] A. Minnock, D.I. Vernon, J. Schofield, J. Griffiths, J.H. Parish, S.B. Brown, Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both Gram-negative and Gram-positive bacteria, J. Photochem. Photobiol., B: Biol. 32 (1996) 159–164.
[19] N.S. Soukos, L.A. Ximenez-Fyvie, M.R. Hamblin, S.S. Socransky, T. Hasan, Targeted antibacterial photochemotherapy, Antimicrob. Agents Chemother. 42 (1998) 2595–2601.
[20] E.M. Tuite, J.M. Kelly, New trends in photobiology: photochemical interactions of methylene blue and analogues with DNA and other biological substrates, J. Photochem. Photobiol., B: Biol. 21 (1993) 103–124.
[21] L. Corash, Virus inactivation in cellular components, Vox Sang. 70 (1996) 9–16.
[22] M. Wilson, N. Mia, Effect of environmental factors on the lethal photosensitization of *Candida albicans in vitro*, Lasers Med. Sci. 9 (1994) 105–109.
[23] S.C. de Souza, J.C. Junqueira, I. Balducci, C.Y. Koga-Ito, E. Munin, A.O.C. Jorge, Photosensitization of different *Candida* species by low power laser light, J. Photochem. Photobiol., B: Biol. 83 (2006) 34–38.

[24] J.P. Thurston, The chemotherapy of *Plasmodium berghei*. I. Resistance to drugs, Parasitology 43 (1953) 246–252.
[25] C. Boda, B. Enanga, B. Courtioux, J.C. Breton, B. Bouteille, Trypanocidal activity of methylene blue evidence for *in vitro* efficacy and *in vivo* failure, Chemotherapy 52 (2006) 16–19.
[26] L. Cincotta, J.W. Foley, A.H. Cincotta, Novel phenothiazinium photosensitizers for photodynamic therapy, SPIE Adv. Photochemother. 997 (1988) 145–153.
[27] L. Cincotta, J.W. Foley, A.H. Cincotta, Novel red absorbing benzo[*a*]phenoxazinium and benzo[*a*]phenothiazinium photosensitizers: *in vitro* evaluation, Photochem. Photobiol. 46 (1987) 751–758.
[28] M. Wainwright, Methylene blue derivatives – suitable photoantimicrobials for blood product disinfection? Int. J. Antimicrob. Agents 16 (2000) 381–394.
[29] B. Bachmann, J. Knuver-Hopf, B. Lambrecht, H. Mohr, Target structures for HIV-1 inactivation by methylene blue and light, J. Med. Virol. 47 (1995) 172–178.
[30] M. Wainwright, D.A. Phoenix, L. Rice, S.M. Burrow, J.J. Waring, Increased cytotoxicity and phototoxicity in the methylene blue series via chromophore methylation, J. Photochem. Photobiol., B: Biol. 40 (1997) 233–239.
[31] R.Y. Dodd, The risk of transfusion-transmitted infection, New Engl. J. Med. 327 (1992) 419–421.
[32] A.J. Blajchman, M. Goldman, F. Baeza, Improving the bacteriological safety of platelet transfusions, Tranfusion Med. Rev. 18 (2004) 11–24.
[33] B. Lambrecht, H. Mohr, J. Knuever-Hopf, H. Schmitt, Photoinactivation of viruses in human fresh plasma by phenothiazine dyes in combination with visible light, Vox. Sang. 60 (1991) 207–213.
[34] E. Snyder, J. McCullough, S.J. Slichter, R.G. Strauss, I. Lopez-Plaza, J.-S. Lin, L. Corash, M.G. Conlan, Clinical safety of platelets photochemically treated with amotosalen HCl and ultraviolet A light for pathogen inactivation: the SPRINT trial, Transfusion 45 (2005) 1864–1875.
[35] P.H. Ruane, R. Edrich, D. Gampp, S.D. Keil, R.L. Leonard, R.P. Goodrich, Photochemical inactivation of selected viruses and bacteria in platelet concentrates using riboflavin and light, Transfusion 44 (2004) 877–885.
[36] L.M. Williamson, R.A. Cardigan, C.V. Prowse, Methylene blue-treated fresh-frozen plasma: what is its contribution to blood safety? Transfusion 43 (2003) 1322–1329.
[37] T. Verpoort, S. Chollet, C. Heron, et al., Filtration of Methylene Blue and photoproducts after photodynamic treatment of plasma using Blueflex, ISBT-Congress, Istanbul, 2003; Abstr. P246.
[38] J.D. Tissot, D.F. Hochstrasser, B. Schneider, J.J. Morgenthaler, P. Schneider, No evidence for protein modification in fresh-frozen plasma after photochemical treatment: an analysis by high-resolution two-dimensional electropheresis, Brit. J. Hematol. 86 (1994) 143–149.
[39] P. Pohler, W.H. Walker, S. Reichenberg, H. Mohr, U. Gravemann, T.H. Müller, Methylene blue treated plasma: pharmacokinetic and toxicological profile of MB and photoproducts, Vox. Sang. 87 (2004) 93. Abstr. A 10.2.
[40] Pathogen inactivation of Labile Blood Products. Council of Europe Publishing, February 2001; ISBN: 92-871-4560-1, Strasbourg, France.
[41] H. Mohr, A. Redecker-Klein, Inactivation of pathogens in platelet concentrates by using a two-step procedure, Vox Sang. 84 (2002) 96–104.
[42] J.R. Karnofsky, Quenching of singlet oxygen by human plasma, Photochem. Photobiol. 51 (1990) 299–303.
[43] T. T'ung, Photodynamic action of methylene blue on bacteria, Proc. Soc. Exp. Biol. Med. 33 (1935) 328–330.
[44] T. T'ung, S.H. Zia, Photodynamic action of various dyes on bacteria, Proc. Soc. Exp. Biol. Med. 36 (1937) 326–330.
[45] M. Wilson, J. Dobson, W. Harvey, Sensitization of oral bacteria to killing by low-power laser radiation, Curr. Microbiol. 25 (1992) 77–81.
[46] S. Sarkar, M. Wilson, Lethal photosensitization of bacteria in subgingival plaque from patient with chronic perodontitis, J. Periodont. Res. 28 (1993) 204–210.
[47] M. Wainwright, D.A. Phoenix, D.R.A. Wareing, T.E. Smillie, Photobactericidal activity of phenothiaziniums against *Yersinia enterocolitica*, J. Chemother. 13 (2001) 503–509.
[48] M. Wainwright, The emerging chemistry of blood disinfection, Chem. Soc. Rev. 31 (2002) 126–136.
[49] M. Wainwright, D.A. Phoenix, S.L. Laycock, D.R.A. Wareing, P.A. Wright, Photobacterial activity of phenothiazinum dyes against methicillin-resistant strains of *Staphylococcus aureus*, FEMS Microbiol. Lett. 160 (1998) 177–181.
[50] M. Wilson, S. Sarkar, J.S. Bulman, Effect of blood on lethal photosensitization of bacteria in subgingival plaque from patients with chronic periodontitis, Lasers Med. Sci. 8 (1993) 297–303.
[51] M. Wilson, J. Pratten, Lethal photosensitization of *Staphylococcus aureus in vitro*: effect of growth phase, serum and pre-irradiation time, Lasers Surg. Med. 16 (1995) 272–276.
[52] H. Gulliksson, S. Larsson, G. Kumlien, A. Shanwell, Storage of platelets in additive solutions: effects of phosphate, Vox Sang. 78 (2000) 176–184.
[53] S. Murphy, The efficacy of synthetic media in the storage of human platelets for transfusion, Transfus. Med. Rev. 13 (1999) 153–163.
[54] W. Dzik, Use of leukodepletion filters for the removal of bacteria, Immunol. Invest. 24 (1995) 95–115.
[55] J.G. Eernisse, A. Brand, Prevention of platelet refractoriness due to HLA antibodies by administration of leukocyte-poor blood components, Exp. Hematol. 9 (1981) 77–83.
[56] A.A. Skripchenko, S.J. Wagner, Inactivation of WBCs in RBC suspensions by photoactive phenothiazine dyes: comparison of dimethylmethylene blue and MB, Transfusion 40 (2000) 968–975.
[57] K.B. Taylor, G.M. Jeffree, A new basic metachromatic dye, 1:9-dimethyl methylene blue, Histochem. J. 1 (1969) 199–204.
[58] M. Wainwright, Pathogen inactivation in blood products, Curr. Med. Chem. 9 (2002) 127–143.
[59] B. Rudensky, Comparison of a semi-automated new Coulter methylene blue method with fluorescence flow cytometry in reticulocyte counting, Scand. J. Clin. Lab. Invest. 57 (1997) 291–296.
[60] T. Inoue, N. Tatsumi, Evaluation of erythropoiesis by new methylene blue staining to establish reticulocyte maturity in bone marrow aspirates and peripheral blood, Acta. Cytol. 35 (1991) 479–480.
[61] T.J. Greenwalt, E.A. Steane, F.O. Lau, K. Sweeney-Hammond, Aging of the human erythrocyte, Prog. Clin. Biol. Res. 43 (1980) 195–212.
[62] M. Gliesing, K.J. Halbhuber, Topo-optical studies of gradually disintegrated erythrocyte membrane derivates: different kinds of ghosts, Acta. Histochem. 86 (1989) 117–121.
[63] J. Makovitzky, S. Bozsoky, E. Laszlo, Topo-optical reactions of the human blood platelet membrane, Histochemistry 79 (1983) 281–287.
[64] P.N. Marshall, S.A. Bentley, S.M. Lewis, Purified azure B as a reticulocyte stain, J. Clin. Pathol. 29 (1976) 1060–1063.
[65] J.F. Papin, R.A. Floyd, D.P. Dittmer, Methylene blue photoinactivation abolishes West Nile virus infectivity *in vivo*, Antiviral Res. 68 (2005) 84–87.
[66] L. Adamcikova, K. Paylikova, P. Sevcik, The decay of methylene blue in alkaline solution, React. Kin. Catal. Lett. 69 (2000) 91–94.
[67] A. Skripchenko, D. Robinette, S.J. Wagner, Comparison of methylene blue and methylene violet for photoinactivation of intracellular and extracellular virus in red cell suspensions, Photochem. Photobiol. 65 (1997) 451–455.
[68] M.A. Houghtaling, R. Perera, K.E. Owen, S. Wagner, R.J. Kuhn, H. Morrison, Photobiological properties of positively charged methylene violet analogs, Photochem. Photobiol. 71 (2000) 20–28.
[69] D. Smelt, P. Repanovici, A. Pascaru, R. Portocala, Photodynamic effect of toluidine blue on MM virus, Rev. Roum. Med. Virol. 27 (1976) 203–207.
[70] H. Morrison, T. Mohammad, R. Kurukulasuriya, Photobiological properties of methylene violet, Photochem. Photobiol. 66 (1997) 245–252.

[71] L.K. Eveland, E.G. Allen, Nile blue stain: *Plasmodium berghei* and uninfected erythrocytes, Trans. Roy. Soc. Trop. Med. Hyg. 66 (1972) 512–513.
[72] Photo-inactivation of cancer cells, US Patent 4962197, 09.10.90.
[73] C.W. Lin, J.R. Shulok, S.D. Kirley, et al., Photodynamic destruction of lysosomes mediated by Nile blue photosensitizers, Photochem. Photobiol. 58 (1993) 81–91.
[74] M.L. Crossley, P.F. Dreisbach, C.M. Hofmann, R.P. Parker, Chemotherapeutic dyes. I. 5-Aralkylamino-9-alkylaminobenzo[*a*]phenoxazines, J. Am. Chem. Soc. 74 (1952) 573–578.
[75] M.L. Crossley, R.J. Turner, C.M. Hofmann, P.F. Dreisbach, R.P. Parker, Chemotherapeutic dyes. II. 5-Arylamino-9-dialkylaminobenzo[*a*]phenoxazines, J. Am. Chem. Soc. 74 (1952) 578–584.
[76] M.L. Crossley, C.M. Hofmann, P.F. Dreisbach, Chemotherapeutic dyes. III. 5-Heterocyclicamino-9-dialkylaminobenzo[*a*]phenoxazines, J. Am. Chem. Soc. 74 (1952) 584–586.
[77] R.C. Clapp, J.H. Clark, J.P. English, C.E. Fellows, R.E. Grotz, R.G. Shepherd, Chemotherapeutic dyes. IV. Phenoxazines and benzo[*a*]phenoxazines, J. Am. Chem. Soc. 74 (1952) 1989–1993.
[78] R.C. Clapp, J.P. English, C.E. Fellows, J. Forsythe, R.E. Grotz, R.G. Shepherd, Chemotherapeutic dyes. V. Benzo[*a*]phenothiazines and benzo[*a*]phenazines, J. Am. Chem. Soc. 74 (1952) 1994–1996.
[79] M. Wainwright, N.J. Grice, L.E.C. Pye, Phenothiazine Photosensitisers. Part II. 3,7-Bis(arylamino)phenothiazines, Dyes Pigments 42 (1999) 45–51.
[80] M. Wainwright, Publication in preparation.