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Review Article

Photoantimicrobials - a PACT against resistance and infection

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CONTENTS

Abstract

The upsurge in multidrug-resistant bacteria, *e.g.,* methicillin-resistant *Staphylococcus aureus* (MRSA), in the healthcare milieu represents a serious clinical problem with associated high morbidity and mortality. Since resistant strains are selected by the overuse of single-site/mode-of-action therapeutics, different approaches to bacterial eradication are required. Similarly, traditional disinfection protocols for donated blood and blood products are inadequate in the face of the AIDS pandemic and other emerging pathogens. Photosensitizing drugs operate via the generation of reactive oxygen species upon illumination *in situ*. The lack of microbial resistance mechanisms, *e.g.,* against singlet oxygen, ensures a wide range of topical/local applications across bacteria, viruses, yeasts and protozoa. This underlines the potential of photoantimicrobial chemotherapy (PACT) in infection control, whether in antisepsis or in the inhibition of transfusion/transmission of disease.

Introduction

In recent years, the incidence of fatal bacterial infections in hospital patients has increased markedly because of drug resistance. At present in England and Wales alone, there are over 5,000 deaths per year from infections arising from methicillin-resistant *Staphylococcus aureus* (MRSA) (1). In many cases, such infections are contracted within the hospital environment. In addition, there is emerging resistance to the drug of last resort, vancomycin (2), and common disinfectant drugs such as mupirocin, used against MRSA colonization, are also becoming less effective (3). This, after several decades of "wonder drugs" and the apparent supremacy of man over microbe, is quite a shocking phenomenon. If we look beyond simple infections, more serious illnesses such as tuberculosis (TB) have been on the increase for over 20 years, even without the added impetus of the AIDS pandemic.

With hindsight, the two scenarios are merely different sides of the same coin. The increases in mortality from nosocomial infection with drug-resistant bacteria (typically MRSA) are due to overprescription and misuse of antibacterial drugs. The rise in cases of TB is mainly due to a lack of policing of patient compliance and follow-up. Similarly, increased resistance is also being encountered to antivirals in immunocompromised patients (4). In each case, microbial species have been able to circumvent the actions of effective drugs, either by increased evolutionary selection (relatively simple for a constantly mutating RNA virus such as HIV-1 [5]), or by partial survival due to noncompletion of therapy.

It is 75 years since Fleming's discovery of the antibacterial activity of penicillin. In the early years of its use, it is scarcely surprising that it was considered to be miraculous in nature. However, logically, the natural penicillins are merely evolutionary chemical defense weapons used by molds against colonizing bacteria, and yet we are surprised that this defense has been reversed, also via evolution. The key here lies in the mode of action of the β-lactams, or rather, in the fact that they have only one mode of action. This makes it relatively straightforward, *i.e.,* a matter of time, for bacterial species exposed to these agents to evolve strains having detoxifying mechanisms, such as the overexpression of penicillinases or penicillin-binding proteins. Similarly, for other drug types, target alteration/overexpression of efflux pumps may be employed to the same effect, as in quinolone resistance.

Beating drug resistance is, of course, a major concern for the pharmaceutical industry. The usual approach is to circumvent resistance mechanisms by targeting novel structures in the bacterial cell, etc. Thus, the much-vaunted success of oxazolidinone drugs such as linezolid (Zyvox) lay in its attack against a ribosomal target (6).

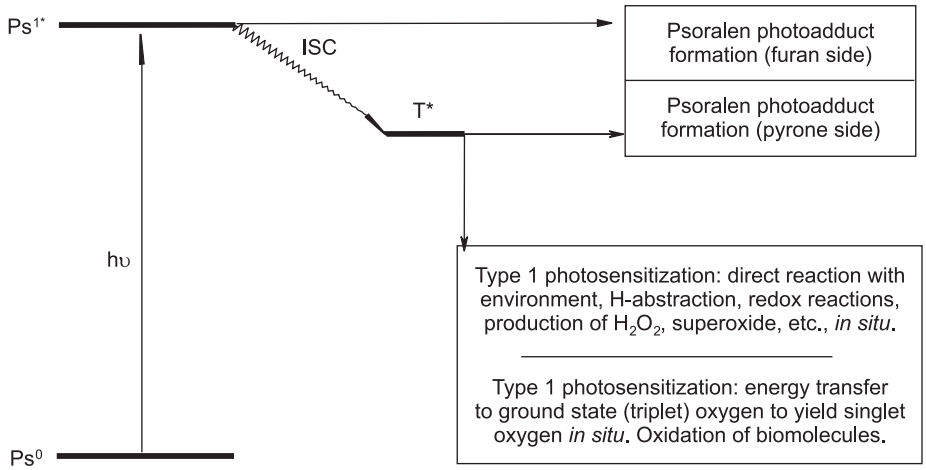

Fig. 1. Photosensitization pathways (relaxation routes omitted for clarity).

However, clinical resistance to linezolid occurred within 6-12 months of introduction (7).

The selection of resistant strains of bacteria is aided by drugs with a single mode of action/site of action and the overuse of such agents, particularly where they are unlikely to be effective, *e.g.,* the use of β-lactam antibiotics against viral disease. While resistance may be slowed by a conservational approach to therapy and the use of drug combinations, this is only a holding strategy and new drugs acting via a single site/mode of action are doomed to short useful lifetimes.

Simplistically then, one answer to the problem of evolved resistance is to develop antibacterial agents where the mode of action is multifactorial, although this is not a straightforward prospect. Whatever the answer, consideration should be given to ideas whether of traditional antibacterial type or not. While it is certain that new pharmaceutical agents will emerge in the fight against bacteria, the availability of these agents is not immediate. As mentioned above, there are now strains of bacteria which are resistant even to the last line of defense, *e.g.,* vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA, respectively) (8, 9). To slow the rise of resistance, we need to change the way in which we employ such valuable drugs, using effective alternatives where possible.

The photodynamic approach

The efficacy of certain types of dye (*e.g.*, methylene blue, acriflavine, crystal violet) against microbial species formed the basis of modern chemotherapy a century ago (10). The selectivity, particularly of cationic (positively charged) dyes, for bacteria over mammalian cells was used by pioneering scientists such as Ehrlich, Browning *et al*. to develop early synthetic antibacterials such as the aminoacridines, although much of the impetus for such work was lost at the inception of the antibiotic era (11).

The recent renaissance in the use of dyes and their derivatives in cancer treatment (photodynamic therapy, PDT) relies on the fact that the dyes employed belong to a class of agents called photosensitizers. These can utilize light energy, either in the promotion of a chemical reaction between the photosensitizer and its environment, or to transfer the energy to oxygen, forming highly toxic singlet oxygen *in situ* (Fig. 1). Thus, given the added bacterial selectivity of the cationic agents (as opposed to the neutral or anionic derivatives used in cancer PDT) and the rapid reactivity of singlet oxygen, a highly effective antibacterial effect is possible, and this is known as photoantimicrobial chemotherapy, or PACT (12). Obviously, the requirement for light activation means that the photobactericidal effect is only experienced in the illuminated region, thus endowing extra selectivity.

In addition, due to its high reactivity, the cell-killing agent singlet oxygen is a nonspecific oxidizing agent. Consequently there is no cellular defense against it; indeed, antioxidant enzymes such as catalase and superoxide dismutase are inactivated by it (13). Also, due to the unique mode of action, there is no significant difference in activity between organisms which are susceptible or resistant to conventional antibiotics (14).

The high reactivity of singlet oxygen has other benefits. While the cellular localization of the photosensitizer is determined by its physicochemical properties, the diffusion of singlet oxygen is sufficient to be able to inactivate different structures, biomolecules, etc., in the immediate environment. Singlet oxygen is thus a multifactorial antimicrobial agent. This can be seen, for example, in the analysis of photoinactivated HIV-1, where damage was observed to viral nucleic acid, the viral capsid and essential enzymes such as reverse transcriptase (15).

Similar use may be made of photosensitizers as an alternative therapy for yeast infection. Several groups have shown that *Candida* spp. are highly susceptible to photosensitizers such as the phenothiazinium toluidine blue (16, 17). Although there is, as yet, scant evidence for clinical azole antifungal resistance, it is possible that the photodynamic approach may be employed as a palliative measure in the treatment of immunocompromised patients. The nature of fungal disease, *i.e.,* in the skin and soft tissues, makes the photoantimicrobial approach attractive, particularly in terms of the likely comparative speed of treatment.

The clinical use of photosensitizing drugs as antimicrobials is limited to topical therapy, at least in the short term, because of the light delivery requirement. However, even with such restriction, there remain many accessible areas of the body which could thus benefit (in addition to the obvious treatment of skin diseases). Indeed, the topical approach to both drug and light application still allows for the treatment of a range of bacterial diseases. Systemic infection is at present inaccessible, but the use of PACT locally would allow the maintenance of conventional drugs for the treatment of life-threatening diseases such as septicemia.

At the present time, photoantimicrobial drugs must exhibit considerable advantages over conventional agents before they are even considered for the clinic. It may be that the problem of drug resistance has to become significantly worse before their introduction occurs, but resistance levels against all commonly used topical drugs are now on the increase, and conventional clinical options are diminishing rapidly.

Additionally, bacterial resistance to PACT is unlikely. Even exclusion of the photosensitizer from the cell is no guarantee of detoxification, since singlet oxygen produced outside the cell still leads to cell death due to oxidative reactions at the cell wall. This has been demonstrated, for example, with polystyrene-immobilized photosensitizers (18).

Photodynamic therapy is reasonably well accepted for the treatment of nonmelanoma skin tumors. Unfortunately for PACT, this clinical acceptance has led to the assumption that the photosensitizers employed in PDT will be effective photoantimicrobials. In most cases this is incorrect, since most anticancer photosensitizers are anionic in nature (usually sulfonic or carboxylic acids; see Fig. 3). Moreover, there is quite adequate literature to indicate the antimicrobial potential of cationic dyes.

It is now reasonably well accepted that cationic photosensitizers are more effective, especially as broad-spectrum antibacterials, than *e.g.,* porphyrin or phthalocyanine congeners having an anionic functionality (19, 20). This can be seen in the markedly greater activities of cationic photosensitizers against Gram-negative bacteria due to the greater complexity of the bacterial cell wall. Even against the more susceptible Gram-positive strains, cationic derivatives appear to be more effective. This has been reported (Fig. 2) for several phenothiazinium derivatives (cationic), compared to the anionic compounds protoporphyrin IX (PPIX) and zinc phthalocyanine tetrasulfonate (ZnTSPc) (Fig. 3) against the Gram-positive bacterium *Streptococcus sanguis* (21). Much improved phthalocyanine derivatives carrying multiple cationic charges have been reported recently (Fig. 3) (22, 23).

Indications

Local infection

In terms of application to the problem of drug resistance in healthcare, photosensitizers have a niche market. Their use is of obvious advantage in burns and wounds, and, used topically (*e.g.*, in wounds), they have

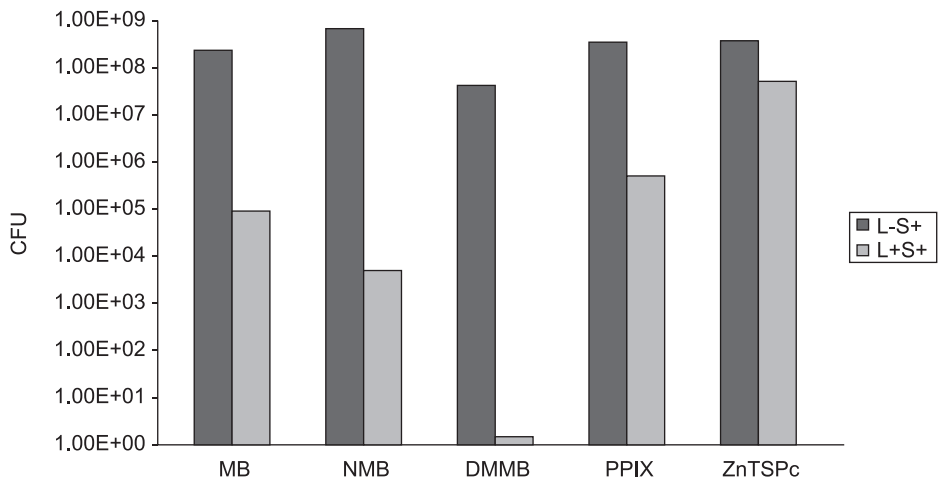

Fig. 2. Bactericidal activity of cationic and anionic photosensitizers against *Streptococcus sanguis* (21). CFU = colony-forming units; L-S+ = dark conditions; L+S+ = light conditions.

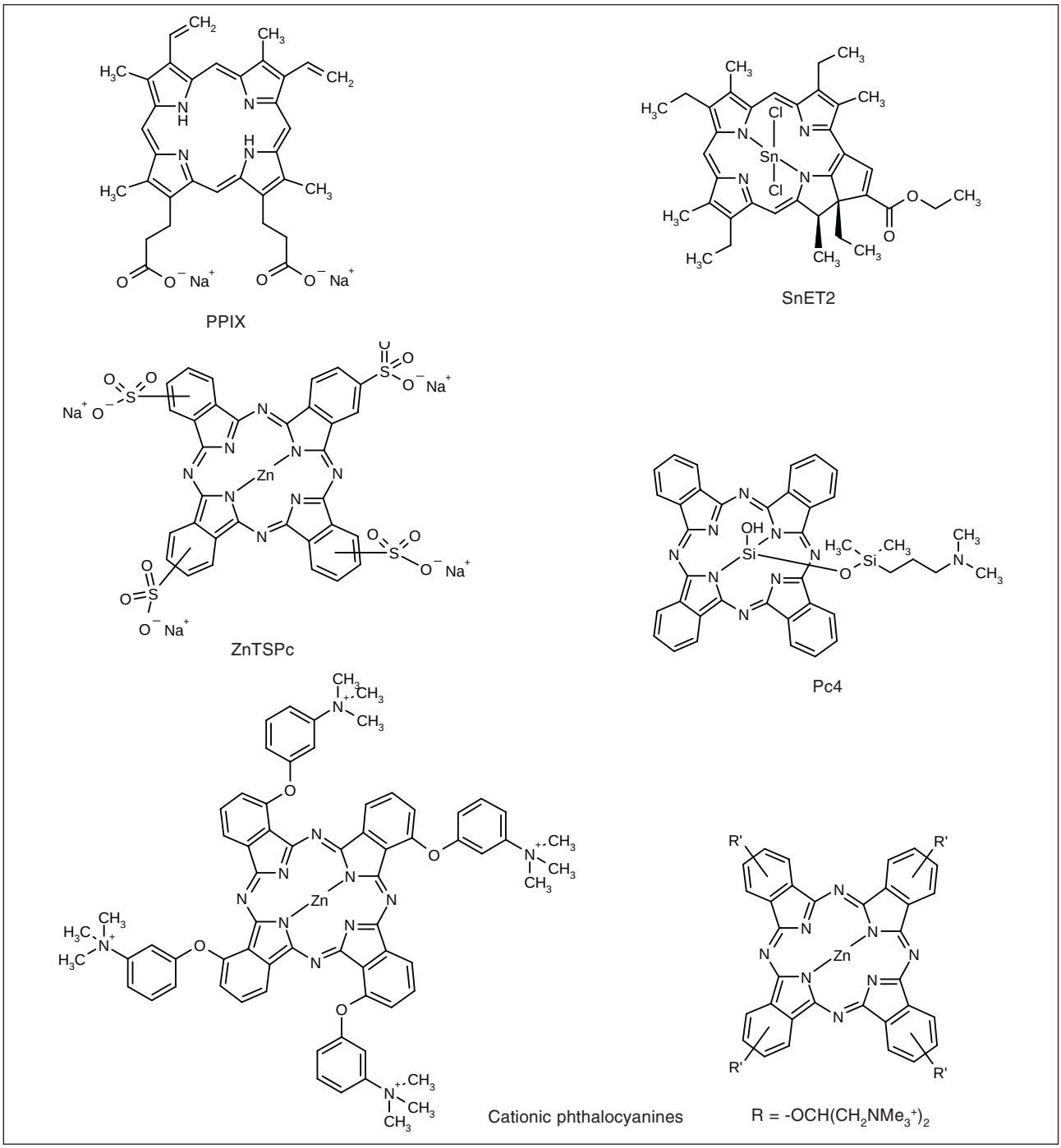

Fig. 3. Porphyrin- and phthalocyanine-based antimicrobials.

the potential to cut drug-resistant infection transmission considerably. Work by the author has shown that cationic photosensitizers of the phenothiazinium series are highly active against MRSA, more so even than vancomycin (14) (Fig. 4).

In addition to infection of the oral cavity as a major site of application, the topical use of PACT in localized infection includes a number of clinical indications which are currently difficult to treat, either due to intractability or length of therapy. For example, *Helicobacter pylori* is a bacterial species implicated in gastric ulceration. The treatment of this organism requires combination ("triple") therapy with acid suppressors and antimicrobials over an extended period of time. However, the organism is known to be susceptible to PACT, *e.g.,* using the phenothiazine derivative toluidine blue (24). Although treatment would

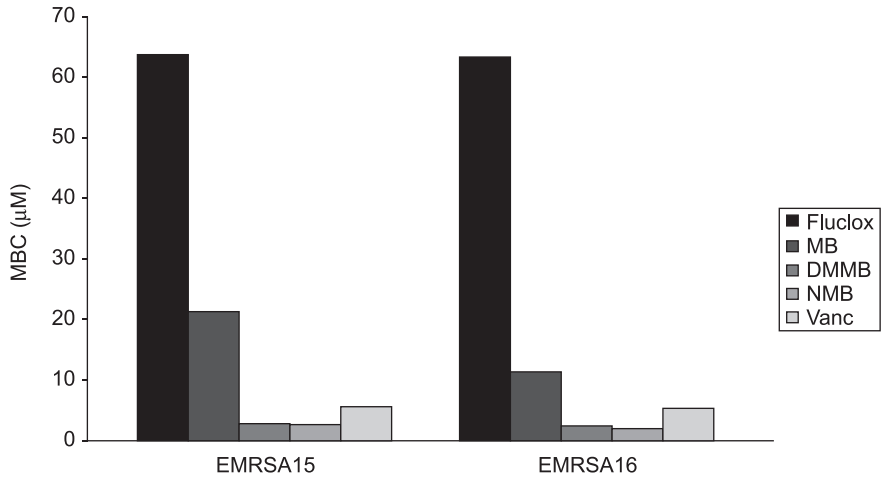

Fig. 4. Phenothiazinium photobactericidal activity against epidemic strains of MRSA (EMRSA) compared to flucloxacillin and vancomycin (14).

require endoscopy, this could be carried out on an outpatient basis, possibly with a single treatment.

Similarly, multidrug-resistant tuberculosis is a massive clinical problem in the developing world, and since the fall of the Iron Curtain, also in the former Soviet Union (25). There, PACT is apparently being employed in the treatment of drug-resistant pulmonary TB (26).

Viral infections, particularly of the skin, are also possible targets for PACT (27). Indeed, a clinical trial of photosensitizers such as proflavine (acridine) and neutral red (phenazine) with white or ultraviolet light for the treatment of genital herpes lesions (herpes simplex virus, HSV-2) was carried out in the 1970s in the United States (28). Although the approach was generally successful in terms of lesion clearing, some patients presented with post-treatment Bowen's disease of the treated area, leading to discontinuation of the trial (29). It is unclear whether carcinogenesis was due to activation by photodamaged virions, to direct mutagenicity by the activated heterocyclic dyes, or to the adverse cellular effects of ultraviolet radiation. The modern approach to such therapy, with the aid of some 20 years' experience in rational photodynamic treatment, would be quite different in terms of both photosensitizer choice and lesion illumination/dosimetry.

Other viral infections are also susceptible to photosensitizers. Human papillomavirus (HPV) is involved in the production of papillomas in, for example, the respiratory tract. This manifestation has been successfully treated in recent years using porphyrin-based preparations such as protoporphyrin IX (PPIX), derived *in situ* from 5-aminolevulinic acid (ALA) (30). The involvement of HPV in genital warts has also attracted research into their photodynamic eradication (31), although results are so far (*e.g.,* for ALA/PPIX treatment) indecisive, often due to pain associated with ALA treatment (32). As with much preclinical photosensitizer work, the establishment of suitable photosensitizers is a key factor and too much reliance has thus far been put on successful anticancer photosensitizers. The acridine derivative proflavine has been shown to be effective in the treatment of warts where there was resistance to the therapeutic of choice, idoxuridine (33).

In cancers with viral etiologies, successful photodynamic treatment may indicate that the viral agent has also been eradicated, particularly in the absence of recurrence for significant follow-up periods. An example of this is given by the treatment of cervical cancer (HPV) with ALA (34). The AIDS-related cancer Kaposi's sarcoma can be treated successfully with the purpurin derivative SnET2 (35) (Fig. 3), but this is now less common in developed countries where AIDS is treated with modern highly active antiretroviral therapy (HAART), since the antiviral effects are sufficient to suppress human herpesvirus type 8 (HHV-8), which is thought to be the etiological agent in KS (36). Whether PACT will be made any more affordable in the developing nations than is HAART remains to be seen.

Blood products

Currently, the main commercial application of photoantimicrobials lies in blood product decontamination, with methylene blue (MB; Fig. 5) now in use by several European blood collection/banking agencies for the disinfection of plasma. The area of pathogen inactivation is full of potential for current and future photosensitizer development. It should be noted that psoralen-based agents, which function by photochemical reaction (Fig. 1) rather than by production of reactive oxygen species, are not included in the present article.

The use of photodecontamination in blood products was proposed as early as 1956, but was not realized until the early 1990s with the impetus given by the possibility

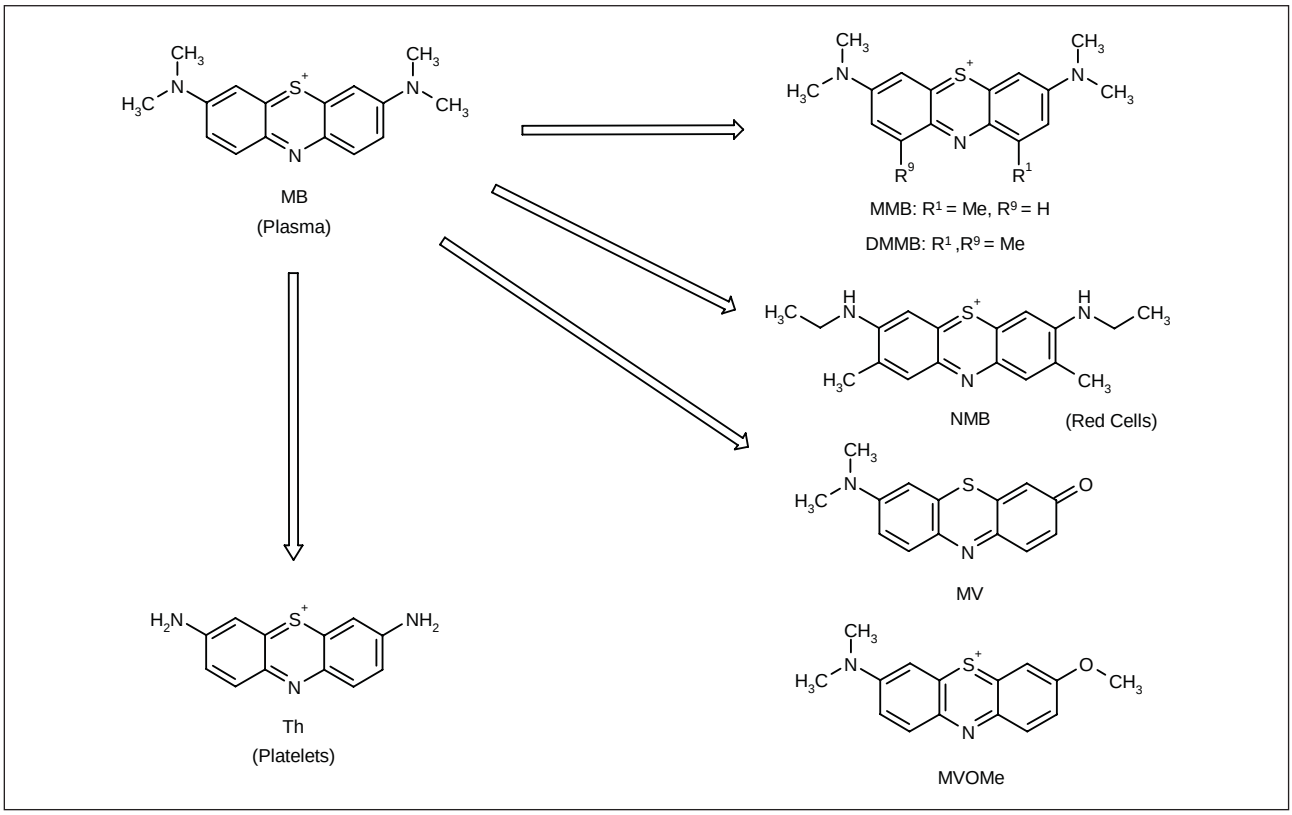

Fig. 5. Development of phenothiazinium-based blood fraction decontaminants.

of HIV transmission. The phenothiazinium photosensitizer methylene blue is now in use by various blood collection/product concerns across Europe in blood plasma decontamination, *e.g.,* the Maco Pharma Blueflex® system (37). Since viruses are not the sole possible contaminants, any photosensitizer used must be effective against other microbial pathogens such as bacteria and yeasts. Activity against emerging diseases, such as West Nile virus, is also important (38).

It is possible that different photosensitizers will be employed for different blood fractions, although it would be more attractive if a single agent were to be found applicable for plasma, platelets and red cells. However, targeting microbial contaminants in the different fractions is not straightforward. As with many drug development projects – and not solely in the field of photosensitizer research – methylene blue has been employed as a lead compound in blood decontamination protocols, but, as already noted, has found commercial use only in plasma decontamination. This is due to the cellular nature of the other fractions. Due to its planar surface area and positive charge, methylene blue can target and inactivate viruses in a protein suspension, but this is considerably more difficult in the presence of cells, since the virus can be extra- or intracellular, as well as in the suspending media. The putative decontaminating agent must therefore be able to enter blood cells, and methylene blue does not do this sufficiently. In addition, it causes membrane damage (39).

This has led to an ongoing search for more suitable photosensitizers for the cellular fractions. A further complication to improved cellular uptake for putative red cell photosensitizers lies in the problem – by definition – of heme absorption in this particular fraction. This means that photosensitizers used here must absorb light strongly beyond 630 nm (methylene blue absorbs light at 660 nm).

Phenothiazine-based photosensitizers have featured significantly in blood decontamination, as shown in Figure 5. Thionin (Th), activated by yellow light, is currently under investigation for use in platelets, possibly in conjunction with a subsequent long-wavelength ultraviolet illumination step (40). However, the low maximum wavelength of absorption for thionin means that this is of little use for red cell work.

Due to the inefficiency of methylene blue in crossing red cell membranes, more lipophilic analogues have been investigated. Increasing alkyl character is a standard method for achieving greater lipophilicity, as demonstrated here using alkylated methylene blue derivatives (Table I). Due to the steric constraints inherent with the dimethylamino moieties in methylene blue, only the 1- and 9-positions are open to substitution with groups other than hydrogen. Thus, the mono- and dimethylated derivatives (MMB and DMMB, respectively; Fig. 5) offer

Table I: Properties of several phenothiazinium photosensitizers (structures are given in Fig. 5); [a]maximum wavelength of absorption in water; [b]yield of singlet oxygen relative to that of methylene blue; [c]lipophilicity (= log_{10} partition coefficient between phosphate buffer and 1-octanol).

	λ_{max} (nm)[a]	RSOE[b]	LogP[c]
Methylene blue (MB)	656	1.00	–0.10
Methyl methylene blue (MMB)	654	1.11	+0.70
Dimethyl methylene blue (DMMB)	648	1.21	+1.01
New methylene blue	630	1.35	+1.20

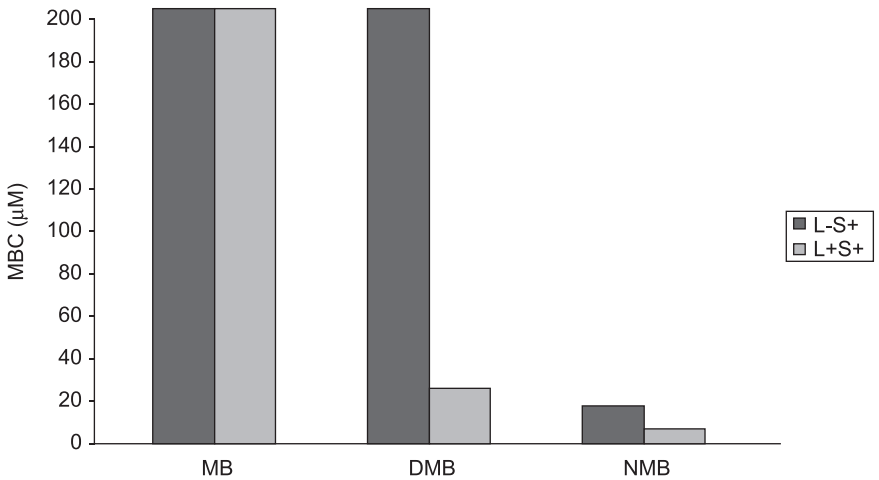

Fig. 6. Activity of phenothiazinium photosensitizers against *Yersinia enterocolitica* in red blood cell concentrate (43). MBC = minimum bactericidal concentration.

both increased lipophilicity and singlet oxygen yield (Table I). This is also the case for new methylene blue, which has a 2,8-dimethylated chromophore but differs in the amino function, having ethylamino in place of dimethylamino groups (Fig. 5).

The increased lipophilicities of the dimethylated derivatives have been demonstrated in recent work by the author, *viz.* in the eradication of *Yersinia enterocolitica* in red blood cell concentrates. This Gram-negative organism can grow at the (relatively) low temperatures at which red blood cells are stored (4 C), thus posing a significant threat of transfusion/transmission (41). Both new methylene blue and dimethyl methylene blue (DMMB) were effective photobactericides, whereas methylene blue (MB) was not, emphasizing the intracellular activity of the derivatives (Fig. 6) (42). Similar intracellular activity against viruses has been reported by Wagner *et al.*, and considerable work has been carried out in developing DMMB for red cell disinfection (43-45).

Another approach to improved cellular uptake is the use of neutral rather than cationic photosensitizers, thus facilitating membrane passage. Methylene violet (MV; Fig. 5), a neutral species having a double-bonded oxygen in place of one of the dimethylamino groups in methylene blue, has been shown to have considerably greater intracellular activity against viruses than methylene blue (46). However, the plasma inhibition and low aqueous solubility of this compound are disadvantageous. The synthesis of new derivatives having alkoxyl groups instead of the oxo function (as in MVOMe; Fig. 5) has circumvented these problems (47). The long-wavelength absorption characteristics (*ca.* 580 nm) of these latter compounds, however, are not optimal for red cell work.

Other photosensitizer types have been examined for the red cell fraction, including phthalocyanines such as the silicon derivative Pc4 (Fig. 3). This compound has excellent photoproperties with regard to absorption wavelength and singlet oxygen production and is able to enter the cell. Activity against a range of pathogens has been reported, including viruses, bacteria and protozoal agents of tropical disease such as *Plasmodium falciparum* (malaria) and *Trypanosoma cruzii* (trypanosomiasis, or Chagas' disease) (48, 49).

Both dimethyl methylene blue and Pc4 have been extensively researched for red cell decontamination purposes. However, in both cases, the longevity of treated cells has been a cause for concern, membrane damage (due to oxidation) leading to potassium leakage, decreased ATP levels, *etc.*, over time (normal requisite shelf life of 42 days). Such damage has been decreased by the use of antioxidant agents such as Trolox® or dipyramidole (50).

Since many of the drawbacks in the development of useful photosensitizers for blood decontamination relate to their possible toxicity in transfused patients, the use of a known nontoxic photosensitizer would be a logical approach. Research using riboflavin (vitamin B2) as a decontaminant has been claimed as successful in each fraction against a variety of pathogens, including *P. falciparum* and West Nile virus (51).

The photodecontamination of blood products represents the major clinical effort involving the use of photosensitizers, both currently and in the near future. Many of the lessons learned here can, and should, inform future research in other areas of PACT.

Conclusions and future prospects

The photoantimicrobial effect has been known for over a century but the use of photosensitizers in the clinical setting has been unnecessary due to the availability of effective systemic antimicrobials. In the age of the hospital superbug, however, there is an urgent requirement for alternative therapies and this can be filled, at least in part, by PACT.

The fact that cationic photosensitizers have been shown to be broad-spectrum antimicrobials – *i.e.,* not merely antibacterials or antivirals – allows for considerable potential use. In addition, the novel mode of action of the photosensitizers (via singlet oxygen) means that they are equally active against conventional drug-susceptible and -resistant strains, and that there is a very low probability for resistance development. However, in the immediate future, clinical trials of photoantimicrobial agents are required, aimed at topical/local application.

The broad-spectrum activity of cationic photosensitizers underpins the application of these agents in both infection treatment and disinfection. In addition, there will undoubtedly continue to be a considerable contribution to blood supply/blood product safety, which will increase considerably if a red cell photodecontamination protocol is realized.

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