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Review

Photodynamic antimicrobial chemotherapy (PACT)

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Whereas the photodynamic therapy (PDT) of cancer has recently shown rapid clinical acceptance, photodynamic antimicrobial chemotherapy (PACT)—which predates the related cancer regimen—is not widely appreciated. Like PDT, PACT utilizes photosensitizers and visible or ultraviolet light in order to give a phototoxic response, normally via oxidative damage. Currently, the major use of PACT is in the disinfection of blood products, particularly for viral inactivation, although more clinically-based protocols are being developed, e.g. in the treatment of oral infection. The technique has been shown to be effective *in vitro* against bacteria (including drug-resistant strains), yeasts, viruses and parasites. A wide range of photosensitizers, both natural and synthetic, is available with differing physicochemical make-up and light-absorption properties. PACT is proposed as a potential, low-cost approach to the treatment of locally occurring infection.

Introduction

The field of antimicrobial chemotherapy is one of constant challenge, particularly in view of the rapid evolutionary changes and wide variety of pathogens encountered. As it is probable that the time of momentous victories in the war against microbes has now passed, and that the scale of advances in medical science brought about with the advent of agents such as the β-lactam antibiotics will probably not be equalled, the discovery of new drugs and therapies is an imperative. Of particular importance in this area has been the appearance of drug resistance in a wide range of pathogens—leading to increased morbidity from infections which, in the past, had been trivial and easily treated.

In addition, localized infections need not be treated with systemic medication if an efficient alternative is available. In this way, effective systemic agents can be withheld for more life-threatening infections. Such a situation is desirable since (i) the development of microbial resistance to systemic agents would be avoided and (ii) it is important that poorly funded healthcare systems (e.g. in the developing world) are able to conserve expensive systemic drugs.

Although research into synthetic antimicrobials continues, there are few novel, effective compounds available to supplement the physician's armamentarium. Increased targeting of pathogens might be achieved using antibodies, but this is somewhat removed from the principle of the 'magic bullet'—the Holy Grail of the medicinal chemist. Also, antibody-labelled drugs are likely to be relatively expensive.

In the past decade great advances have been made in the alternative treatment of tumours using photosensitizing porphyrin derivatives in conjunction with laser light. The combination of photosensitizing drug and light causes the production of reactive oxygen species in the tumour environment, leading to tumour death. This is known as photodynamic therapy (PDT) and—theoretically at least—has dual selectivity for the target cells in that the porphyrin derivatives are tumour-specific and the laser light is delivered directly to the tumour mass via fibre optics. Any porphyrins in the periphery should thus remain non-illuminated. Phototoxic side-effects due to skin accumulation of porphyrin drugs have, however, been reported widely, and so the search for improved drugs continues.[1,2]

Whereas PDT was developed from the observation of endogenous porphyrin fluorescence during surgery, the use of photosensitizers in microbial eradication can be traced back to before the age of chemotherapy. The idea

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of the 'magic bullet', alluded to above, was introduced at the turn of the century by Paul Ehrlich after decades of experimentation on the staining effects of aniline dyes on animal and microbial cells. Ehrlich thus formulated the principle of selectivity and laid the foundations of modern chemotherapy. It is this work which underlies the principle of photodynamic antimicrobial action—if a live microbe can be demonstrated selectively with a vital stain such as methylene blue, it should be possible to destroy the stained microbe on illumination.

The science of photodynamic antimicrobial chemotherapy (PACT) is still in its infancy, but follows similar principles to that of PDT. Indeed, while PDT is currently used only in the more accessible tumours, the use of PACT may also be limited to localized infection due to the problems of systemic light delivery. However, with the advent of optical fibre technology, deep-seated—if not disseminated—infection should become amenable to the photodynamic approach.

Additionally, it is argued that the widespread systemic use of antibiotics is a cause of multidrug resistance and superinfection via effects on normal 'friendly' flora.[3] Local therapy using photodynamic agents would lessen the risk of such collateral effects.

A very wide selection of light sources is available, ranging from state-of-the-art laser technology to basic tungsten-filament lamps. Indeed, the assumption that a laser is essential for the photodynamic therapy of malignant disease has hindered the growth and acceptance of this discipline considerably. What is important, both in PDT and PACT, is the ability to excite the photosensitizer at its target site with minimal photoeffect on the surrounding tissue. For example, the disinfection of virally-contaminated blood currently carried out in parts of Europe utilizes light boxes containing fluorescent tubes.[4] Examples of the light sources available for PACT are given in Table I, but it should be remembered that PACT, like PDT, uses low-power light rather than the lasers used in ablative therapy: microbial photokilling is attained with milliwatts rather than tens (or hundreds) of watts. The terminology used to describe the quantity of light used in a PACT procedure warrants some explanation. The power density of a light source is normally given in mW/cm^2 whereas the light dose describes the energy received (e.g. by a wound or a Petri dish) and as such can be calculated as the power density multiplied by the illumination time (in seconds). It should thus be noted that the power density, the illumination time or both can be varied to give the same light dose. However, a high power density over a short time period may give different results, in terms of microbial kill, from those of a low power density over a longer time even though the light dose is the same in each case.

The use of directed light against microbial pathogens *in situ* also raises the problem of the possibility of collateral damage. Such effects can be minimized through a thorough knowledge of the light-absorption characteristics of the proposed photosensitizer and of its target environment. In addition, local rather than systemic administration of the photosensitizer should inhibit its dissemination to the periphery leading to the generalized skin photosensitization which has been a problem not only with the first generation porphyrin drugs used in PDT but also with common prescription drugs such as the sulphonamides and chlorpromazine.[5]

Although PACT is gaining increasing acceptance it is not, at present, a mainstream therapeutic option. The burgeoning use of photosensitizers in viral decontamination is perhaps a little too removed from the clinical milieu, but the recent demonstration of the technique against a range of oral pathogens and also against drug-resistant bacteria should encourage its use in a wider arena. In addition, photosensitizers, being readily available and inexpensive, should be attractive in the area of low-cost topical healthcare regimens.

Photosensitizers and photodynamic antimicrobial action

When an aromatic molecule absorbs light of a certain energy it may undergo an electronic transition to the

Table I. A selection of light sources available for PACT

	Wavelength (nm)	Power (W)	Power density (mW/cm^2)
Lasers			
argon	488–514	20	
argon ion pumped dye	585 or 630	3–4	
tuneable dye	400–1000	20	
Wavelength-filtered lamps			
250 W quartz halogen	620–640		40 (16 cm^2 area)
500 W tungsten filament (slide projector)	>600		7–10 (25 cm^2 area)
1–5 W xenon arc	600–700		150 (20 cm^2 area)

singlet excited state (electron spins paired). Depending on its molecular structure and environment, the molecule may then lose its energy by electronic or physical processes, thus returning to the ground state, or it may undergo a transition to the triplet excited state (electron spins unpaired). At this stage the molecule may again undergo electronic decay back to the ground state, it may undergo redox reactions with its environment, or its excitational energy may be transferred to molecular oxygen (also a triplet-state molecule) leading to the formation of the labile singlet oxygen (Figure 1).

The ability of a molecule to instigate redox reactions and/or to form singlet oxygen depends on the production of a sufficient population of triplet state molecules. This in turn depends on the decay rates of both the triplet and initially-formed singlet states. Thus, for example, a highly fluorescent molecule which undergoes significant electronic decay from the excited singlet state would not be expected to form a high proportion of the triplet excited state.

Photosensitizers are usually aromatic molecules which are efficient in the formation of long-lived triplet excited states. In terms of the energy absorbed by the aromatic π-system, this again depends on the molecular structure involved: furocoumarin photosensitizers (psoralens) absorb relatively high energy ultraviolet (UV) light (*c.* 300–350 nm), whereas macrocyclic, heteroaromatic molecules such as the phthalocyanines absorb lower energy, near-infrared light (*c.* 700 nm, Table II).

Studies carried out regarding photodynamic action against microorganisms have been mainly concerned with well-established species such as *Salmonella typhimurium*, as used in the Ames test, or the yeast, *Saccharomyces cerevisiae*. While these may be good models, a more useful method would be to include in testing protocols the pathogenic species against which PACT is proposed to be used. Growing the organisms as part of a biofilm would also increase the relevance of test results.

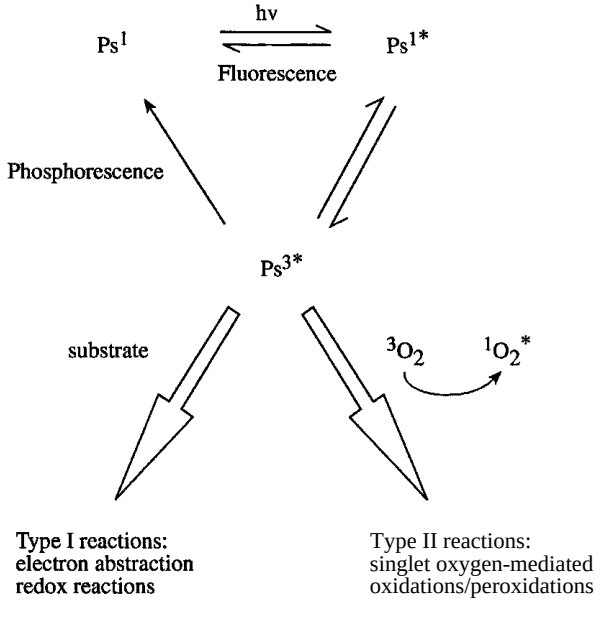


Figure 1. Photosensitization processes.

In carrying out such testing protocols the possibility of multiple sites of photodamage should be remembered—a 'one drug–one site of action' hypothesis is too often assumed. Obviously microbial morphology can vary with species and this will lead to differences in photosensitizer localization. In addition, the time allowed for photosensitizer uptake before illumination may be important—a photosensitizer that is taken up slowly by the microorganism may at first cause only cell wall photodamage whereas different effects, e.g. nucleic acid strand breakage, will be apparent on longer incubation times.

The sites of photosensitizer action and the effects of photodynamic inactivation of microbial species are mentioned under the various classes of photosensitizer which follow. However, to illustrate these points briefly, the related phenothiazinium photosensitizers toluidine blue O (TBO) and methylene blue are considered here. Against *Escherichia coli*, TBO is known to be membrane active, since it causes increased permeability,[6] whereas methylene blue causes strand breaks in this organism's nucleic acid.[7] However, in another Gram-negative organism, *Proteus mirabilis*, methylene blue causes photodamage in the cell envelope, as proved by enhanced cell lysis,[8] as well as DNA photodamage.[9]

The photosensitizing efficacy of the putative PACT agent must also be considered. The standard acridine photosensitizer, acriflavine, is ineffective against *E. coli* if it is excluded from the cell,[10] whereas polymer-immobilized rose bengal causes photoinactivation of *E. coli*,[11] reflecting the far greater singlet oxygen-generating efficiency of the latter agent.

Photodynamic microbial damage at the molecular level is, in many cases, well established. Type I photodamage due to electron or hydrogen abstraction by the photosensitizer, subsequent redox reactions and oxygenation products relies on close proximity of the photosensitizer and the biomolecular target. A type I reaction with water in the microbial milieu can give rise to hydroxyl radicals (HO·) which can also react with biomolecules or combine

Table II. Photosensitizer absorption maxima

Photosensitizer type	λ_{max} range in buffer (nm)
Phenothiazinium	620–660
Phenazine	500–550
Acridine	400–500
Cyanine	500–600
Porphyrin	600–650
Phthalocyanine	660–700
Psoralen	300–380
Perylenequinonoid	600–650

Table III. Photocytotoxic pathways in microbial cells

Site of action	Action	Result	Consequence	Cytotoxic event
Water	Hydrogen abstraction	Formation of hydroxyl radical ($HO^{\cdot}$)	Formation of hydrogen peroxide, superoxide (O_2^{-})	Further oxidative processes
Cell wall/membrane unsaturated lipids/sterols	Peroxidation	Peroxidation	Hydroperoxide formation	Increased ion permeability (Na^+/K^+ leakage)
Peptide	Hydrogen abstraction	Peptide cross-linking	Enzyme inactivation	Loss of repair facility; lysis
Viral protein coat	Oxidation of Tyr/Met/His residues	Protein degradation		Loss of viral infectivity
Respiratory chain	Redox reactions			Inhibition of respiration
Cytoplasmic enzymes/viral enzymes (e.g. reverse transcriptase)	Oxidation or cross-linking (as above)			Inhibition of ribosome assembly; inhibition of replication/infectivity
Nucleic acid residues (typically guanosine)	Oxidation of base or sugar	8-Hydroxyguanosine	Nucleotide degradation; sugar degradation/cleavage	Base substitution; strand cleavage; mutation; inhibition of replication

to give hydrogen peroxide *in situ* with subsequent cytotoxic results.

Typical type I reactions, e.g. at the bacterial cytoplasmic membrane, include the abstraction of allylic hydrogens from unsaturated molecules such as phospholipids (Table III). The radical species thus formed may undergo reaction with oxygen to yield the lipid hydroperoxide. Lipid peroxidation is detrimental to membrane integrity, leading to loss of fluidity and increased ion permeability.[12] Other cell wall/membrane targets include aminolipids and peptides. Thus inactivation of membrane enzymes and receptors is also possible.[13]

If the triplet state photosensitizer transfers its energy to molecular oxygen, the singlet oxygen formed *in situ* then reacts rapidly with its environment—cell wall, nucleic acids, peptides, etc. This is type II photodynamic action (Figure 1). The short half-life of singlet oxygen again ensures a localized response.

Type II processes are generally accepted as the major pathways in photooxidative microbial cell damage. As with the type I pathway discussed above, singlet oxygen will also react with molecules involved in the maintenance and structure of the cell wall/membrane such as phospholipids, peptides and sterols (e.g. in yeasts). However, the products from such reactions may be slightly different. For example, whereas type I reaction with cholesterol may result in the formation of cholesterol-7α or 7β-hydroperoxide, the formation of the 5α-isomer is indicative of type II reaction with singlet oxygen only.[13] Reaction of singlet oxygen with other molecules involved in the cell wall/membrane can also occur. Thus the amino acid, tryptophan, undergoes cycloaddition with singlet oxygen and the reactive intermediate formed degrades to give a formanilide derivative which may react further, e.g. in peptide crosslinking (Table III). Methionine residues also react with singlet oxygen, producing methionine sulphoxide.[1]

Nucleic acids are known to react mainly through guanosine residues. Again there exists a difference in selectivity between type I and type II processes. The former is mediated through hydroxyl radical attack at the sugar moiety whereas the latter is an attack of singlet oxygen at the guanine base[14] (Table III). Type II photooxidation of guanine residues with the acridine dye acriflavine leads to DNA strand-breakage and the photoactivity of the same dye has been shown to cause both nuclear and mitochondrial mutations in yeasts.[15]

While the predominant type of photodynamic action may often be determined by the class of compound, the exact mode of action is also closely governed by the site of action. This, in turn, is a function of the physicochemical make-up of the photosensitizer, just as with any other medicinal compound. This is important in terms of the preliminary in-vitro testing of putative compounds: a promising photosensitizer in chemical assays may not perform well against its microbial target due to meta-

bolism, reduction and other factors or simply because it localizes in a non-vital region of the target cell. The reverse may also be true; for example, the triphenylmethane dye crystal violet (gentian violet) shows no photosensitizing behaviour in chemical tests, yet its inherent bactericidal activity is enhanced by illumination.[16] This is thought to result from intracellular adsorption of the photosensitizer causing rigidity of its structure and inhibition of the rotational energy loss from the singlet excited state. This leads to increases in the triplet-state yield and thus in the photosensitizing efficacy.[17]

Once a lead compound is identified, the normal process of structure optimization can be begun in a similar way to that followed in mainstream medicinal chemistry research. Physicochemical parameters such as lipophilicity (log *P*) and ionization (pK_a) are obviously of importance here, but other, more specialized, factors such as light-absorption characteristics (the maximum wavelength of absorption, λ_{max}, and the intensity of the absorption, ε_{max}) and the efficiency of singlet oxygen production (Φ_Δ) must be included in a putative photoantimicrobial profile. Indeed the five parameters mentioned are all related to the electronic structure of the compound. For instance, while the addition of a halogen such as iodine to a photosensitizer molecule[18] usually yields a compound having a considerably increased singlet oxygen efficiency, the resulting compound is also much more lipophilic (increased log *P*), and may localize in a completely different microbial compartment to the lead compound.

The aim of the current review is to provide some idea of the wide range of photosensitizers—and structural types—available for use against pathogenic organisms and also to outline the versatility of PACT.

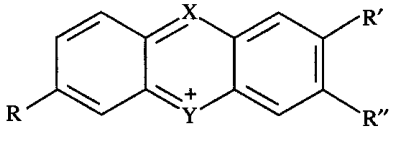
Cationic azine photosensitizers

As can be seen from Figure 2, the commercial azines are very closely related in structure. The simple tricyclic skeleton forms the basis of many dyes and stains and, as mentioned previously, the first synthetic antibacterial compounds were azine derivatives.

Phenothaziniums. These are blue dyes (λ_{max} = 600–660 nm) such as methylene blue and TBO. Methylene blue has been used widely in histology for over a century. The first report of its photodynamic action (against bacteriophages and viruses) appeared as early as 1930.[19] It is also utilized in bacterial testing of milk, microbial reduction of the phenothiazinium chromophore causing decolorization. Both methylene blue and TBO are used in surgical identification at reasonably high concentrations (normally 1% w/v)[20,21] without causing human toxicity. In terms of PACT, it has been shown that the light dose required to kill bacteria treated with TBO is far lower than that causing toxicity in cultured human keratinocytes and fibroblasts.[22]

The size and shape of the phenothiaziniums, in line with other linear tricyclic heteroaromatics, make them ideal nucleic acid intercalators (Figure 2). Methylene blue's photodynamic action against nucleic acids is selective for guanosine residues, leading preferentially to the formation of 8-hydroxyguanosine[23] and strand breaks (low PCR yield).[24] Effective inactivation of HIV-1 has been attained using 1 μM methylene blue and 10.5 mW/cm^2 of red light.[24]

A great deal of work has been carried out on the binding of phenothiazinium dyes to nucleic acids.[25] This led, in many early cases, to the supposition that the mode of action of such dyes, in terms of their photoactivity, must be against DNA. While photosensitizers such as methylene blue, TBO and a congener (Azure B) do bind to the DNA of simpler species such as bacteriophages, this has been shown to have little effect on replication.[26] Indeed, against bacteria the effect of differing photosensitizer structure is more apparent. Thus the site of action of methylene blue against *E. coli* is known to be DNA[7] whereas the closely related TBO is membrane-active.[27] The utility of phenothiazinium photosensitizers against a



	R	R″	R′	X	Y	λ_{max} (nm)
Methylene blue	$(CH_3)_2N$	$N(CH_3)_2$	H	N	S	660
Toluidine blue O	$(CH_3)_2N$	NH_2	CH_3	N	S	625
Neutral red	$(CH_3)_2N$	NH_2	CH_3	N	NH	540
Proflavine	H_2N	NH_2	H	CH	NH	456
Acridine orange	$(CH_3)_2N$	$N(CH_3)_2$	H	CH	NH	492
Aminacrine	H	H	H	C-NH_2	NH	410
Ethacridine	H_2N	H	OC_2H_5	C-NH_2	NH	420

Figure 2. Azine photosensitizers (λ_{max} values in ethanol).

range of bacterial strains has recently been reported, using a low-power white light source (1.75 mW/cm^2 for 1 h).[28] It was noticeable that the phenothiaziniums were more effective against Gram-positive strains than against Gram-negative strains.

Methylene blue has been widely used by several European transfusion services in the photodecontamination of blood plasma and has been shown to be particularly effective in the inactivation of viruses.[29] Generally accepted conditions are a photosensitizer concentration of 1 μM and a red light (600–700 nm) fluence rate of approximately 10 mW/cm^2.[24,29] The technique was first suggested in 1955 but was not used routinely until 1992.[4] Methylene blue is known to cause viral envelope photodamage[30] but in recent investigations on HIV-1, it was shown that it also exerts a photodynamic effect against the viral core proteins, viral RNA and reverse transcriptase.[24] Thus, even if the outer coat of the virus remains intact after treatment its infectivity is destroyed due to the loss of reverse transcriptase activity. Unfortunately, methylene blue is much less active against intracellular viruses and thus would not be of use in the disinfection of red blood cell concentrates.[31]

As far as collateral effects are concerned, methylene blue also mediates photodamage to some plasma proteins and this results in a drop in clotting factor activity.[32] The specificity of methylene blue derivatives for a particular pathogenic target might, however, be increased by coupling the phenothiazinium chromophore via a reactive maleimido or succinimido group to antibodies.[33]

In terms of the non-antiviral potential of the phenothiazinium photosensitizers, a great deal of work has been reported by Wilson *et al.*,[34] mainly concerning photoantimicrobial effects against oral pathogens. Thus, important organisms implicated in dental caries, such as *Streptococcus* spp., are reported to be eradicated using TBO or methylene blue. This work has been carried out *in vitro*, including in the presence of substances from the oral environment, such as demineralized dentine and collagen.[35] The bacteria remain susceptible to PACT even when present as part of a biofilm.[36,37] Interestingly, the photoactivity of the phenothiaziniums is unaffected by the presence of blood.[38] *Candida albicans*, the causative agent in oral thrush, is also susceptible to the TBO/methylene blue photodynamic approach.[39] This may be significant in view of the high incidence of candida infection in HIV-infected patients, since locally administered PACT would not be expected to cause either an increased burden on the immune system or the additional side-effects concomitant with conventional antifungals. The photoeradication of *Helicobacter pylori*, again as part of a biofilm, has also been demonstrated using TBO.[40]

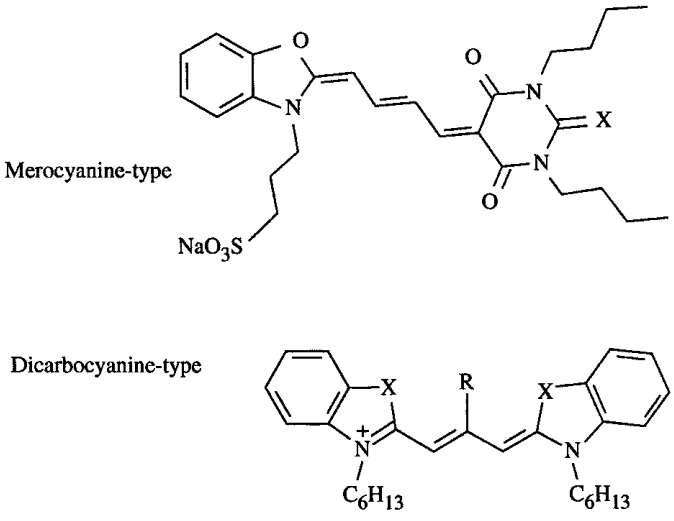
The closely related phenazine photosensitizer neutral red (Figure 2) has been used as an effective photosensitizing antiviral, especially against herpes simplex virus.[41] Clinical trials of its use in herpetic infection of the skin and mucous membranes, particularly against genital herpes lesions, were carried out in the early 1970s[42] but were discontinued as a result of the occurrence, in a small percentage of cases, of post-treatment carcinogenesis.[43] Neutral red is bioisosteric with TBO and, similarly, exerts its photodynamic effect against the viral envelope.

Acridines. The first report of photodynamic action in a biological system was the acridine-mediated photoinactivation of paramecium reported by Raab in 1900.[44] Since then the wide use of aminoacridines as biological stains and, more recently, as molecular probes has resulted in a wealth of literature dealing with the interaction of such compounds with a wide range of microorganisms. The most widely used aminoacridines are the nucleic acid probe, proflavine (Figure 2) and the lysosomal agent, acridine orange (Figure 2). However, other, simpler aminoacridines have also been investigated in terms of their DNA interactions and mutagenic potential for various bacteria and yeasts. The photosensitizing abilities of the aminoacridines are well established and their use in probing cellular photodamage has been widely reported.[15] The testing of obsolete aminoacridine antibacterial drugs such as aminacrine (9-aminoacridine, Figure 2) and ethacridine (7-ethoxy-3,9-acridinediamine, Figure 2) as photodynamic agents is a logical step given the bacterial selectivity of these compounds. The author has shown recently that simple aminoacridines exhibit considerable increases in antibacterial activity on illumination with white light, but less so than proflavine or acridine orange.[45]

Cyanines and merocyanine 540

In common with the phenothiaziniums and acridines, cyanine dyes were tested against microbial targets in the early part of this century. Indeed the cyanines evolved from the use of acridines as wound antibacterials in World War I.[46] It is often forgotten that several cyanines, for example pyrvinium and stilbazium, are still commercially available as anthelmintic preparations.[47] Surprisingly, the combination of visible-light absorption and proven antimicrobial activity has not encouraged a great deal of original research in this area. Indeed, the major part of the work has been carried out on one structure, merocyanine 540 (MC540, Figure 3). MC540 has been used in the purging of leukaemic cells from autologous bone marrow grafts, but its use in the photoinactivation of blood-borne enveloped viruses has also been studied.[48]

The chemical structure of MC540 is appealing to the medicinal chemist as it offers much scope for functionalization. Thus, replacing a ring heteroatom with one of higher atomic number (e.g. oxygen for sulphur, Figure 3) normally leads to increased singlet oxygen yields[49]—the 'heavy atom effect'—though for cyanine dyes these are usually low in comparison with, for example, methylene blue. The use of heavy atoms also stabilizes the polymethine chain to photoisomerization (a major deacti-



X	R	λ_{max} (nm)	$\Phi_{\Delta\ (liposomes)}$	$\Phi_{\Delta(EtOH)}$
Merocyanines	–	–	–	–
O (MC540)	–	535	–	0.001
S	–	555	–	0.002
Se	–	561	–	0.38
Dicarbocyanines	–	–	–	–
S	Et	551	0.01	0.001
Se	H	574	0.08	0.014
O (DHOCI)	H	–	–	–

Figure 3. Cyanine photosensitizers.

vation pathway).[50] As expected, varying the *N*-alkyl side-chains in MC540 facilitates changes in the lipophilicity of the system (Figure 3).[48]

Perhaps the main drawback to the use of merocyanines in PACT is the inactivation of such compounds by plasma and serum components, although recent work has shown that this effect may be inhibited by the replacement of the ring oxygen with sulphur or selenium (Figure 3).[51]

Macrocyclic photosensitizers

Porphyrins. While the use of porphyrins as photodynamic antimicrobial drugs does not have the same rationale as that of the azine derivatives, their use in this area has been reviewed by Malik *et al.*[52] Whereas azine derivatives have a demonstrated selectivity evolved through pathogen staining, the use of porphyrins is an extension of anticancer PDT. Haematoporphyrin derivative (HpD), a closely related mixture of oligomeric photosensitizers derived from blood, is the first preparation given FDA approval for use in clinical PDT and has some activity against both bacteria and viruses.

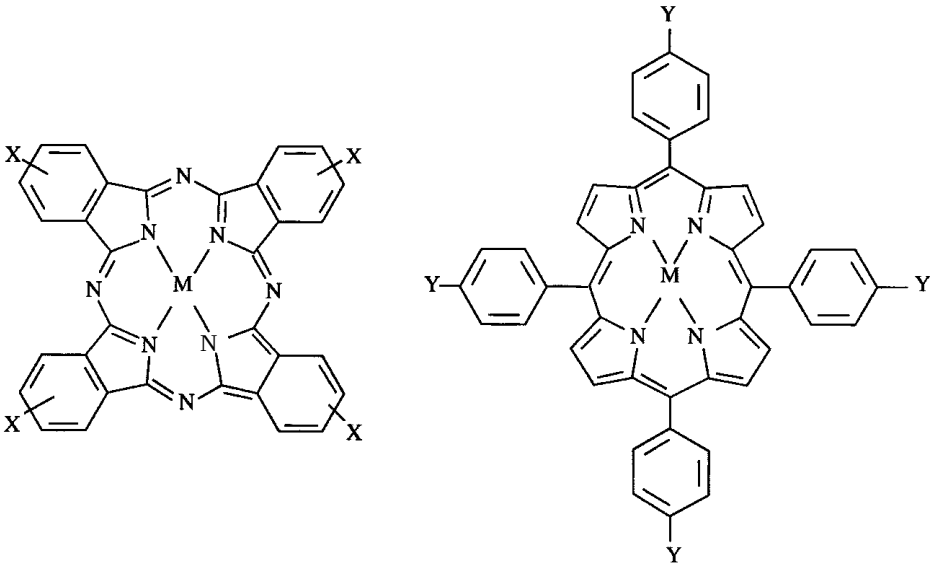
While both naturally-derived and synthetic porphyrins are available, the former class has the obvious disadvantage of having a similar light-absorption profile to that of the endogenous porphyrins in clinical presentation. Their use in, for example, the disinfection of open wounds might, therefore, be problematic on the grounds of endogenous light absorption causing a decrease in photosensitizing efficiency.

The natural-product porphyrins, as a class, are effective against a range of anaerobic bacteria *in vitro*, and indeed haemin was reported to be active without light activation.[53] Binding of the porphyrins to the target cell has been studied and the Gram-negative organisms appear to be the more refractory to PACT, presumably due to their more complex cell wall.[53] However, in an investigation of synthetic *meso*-substituted porphyrins (Figure 4, molecules a and b), the cationic derivatives were generally more photoactive than the anionic photosensitizers.[54] In addition, earlier work by Nitzan *et al.*[55] had shown that HpD and deuteroporphyrin—both anionic porphyrins—were active against Gram-positive bacteria but inactive against Gram-negative species such as *E. coli* or *Pseudomonas aeruginosa*, even at concentrations of 200 mg/mL, unless used in conjunction with a nonapeptide derivative of colistin in order to open channels in the bacterial membrane.

Porphyrins and the closely related chlorins have also been demonstrated as effective virucidal agents *in vitro*[56,57] apparently causing photodamage to the viral envelope.[58] Benzoporphyrin derivative has been tested against HIV in whole blood and is reported to be more selective than methylene blue against intracellular virus, giving complete inactivation at a concentration of 0.5 mg/mL and a light dose of 13 J/cm^2.[58]

Phthalocyanines. Formally, the phthalocyanines are tetrabenzotetraazaporphyrins (Figure 4, molecules d–k). Greatly increased aromatic character explains why the near infrared absorption of these compounds is more intense than that of the parent porphyrin nucleus (Table II). In addition, the relatively straightforward synthetic routes to the phthalocyanines has meant that a wide range of compounds is available in terms of the central metal/semi-metal atom and side-chain functionality. In terms of their photosensitizing potential, phthalocyanines give high yields of singlet oxygen—greater than that of standard photosensitizers such as methylene blue.[59] As for the porphyrins, there is little evidence of historic use of phthalocyanines in either the treatment or staining of microorganisms and, once again, the considerable current interest in this class of photosensitizers must be considered to be a development of cancer PDT work.

The majority of PACT investigation associated with phthalocyanines has been in the area of blood product disinfection. Although, unlike methylene blue, the technique is not yet used clinically, the efficacy of phthalocyanines in the photoinactivation of viruses in various compartments is considerable. Again, enveloped viruses such as HIV, VSV and HSV are generally amenable



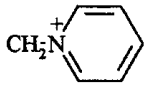
	Substitutions at positions		
Molecule	**M**	**X**	**Y**
a	2H		SO_3H
b	2H		$N(CH_3)_3^+$
c	$HOSiOSiCH_2CH_2N(CH_3)_2$	H	
d,e,f	GaIII / AlIII / ZnII	SO_3H / $C(CH_3)_3$	
g	2H	$C(CH_3)_3$	
	Zn		
h			
j	Zn	$SO_2N(CH_2CH_2OH)_2$	
k	Zn	SO_3H	

Figure 4. Macrocyclic photosensitizers.

to photoinactivation,[60–62] whereas the non-enveloped virus, cephalomyocarditis virus, is not, indicating that the viral envelope is a target for phthalocyanine photosensitization.[63] The use of phthalocyanines against various forms of HIV infection has been reported, and once again paralleling the development of these compounds in cancer PDT, aluminium and silicon phthalocyanines appear to show considerable promise, both groups of compounds exhibiting light absorption in the near infrared and efficient sensitization of singlet oxygen. In terms of chemical make-up, the silicon phthalocyanines used as photodynamic agents are functionalized axially through the silicon atom, rather than in the periphery of the aromatic system and this appears to produce highly active compounds. Thus, for example, silicon phthalocyanine bearing a cationic dialkylaminoalkylsilyloxy-residue on the central silicon (Figure 4, molecule c) was active not only against cell-free HIV but also against the actively replicating virus and latently infected red blood cells (using a photosensitizer concentration of 2 μM and a power density of 25 mW/cm^2).[64] Such an activity profile against viruses is obviously highly desirable although there remains the problem of red blood cell damage which requires the addition of an antioxidant such as vitamin E.[60] It has also been reported that the use of high irradiance (e.g. 80 mW/cm^2) in conjunction with sulphonated aluminium phthalocyanine (1 μM) is less toxic to red blood cells.[65] In addition, the synchronous use of thiol-containing species such as reduced glutathione with phthalocyanines inhibits IgG binding to the treated erythrocytes, thus facilitating subsequent crossmatching.[66]

In terms of structure–activity relationships for the phthalocyanines, there appears little general correlation between the antiviral potency and the central atom of the phthalocyanine, although against vaccinia virus the activity increased in the order Ga(III) < Al(III) < Zn(II).[67] The degree of phthalocyanine sulphonation and butylation (Figure 4, molecules d–f) was also found to affect both

the antiviral activity and the extent of haemolysis.

Although widely publicized, viral contamination is not, of course, the sole factor in disease transmission through donated blood. It has been shown that blood-borne pathogens involved in tropical diseases may be inactivated using PACT. Thus the cationic silicon phthalocyanine mentioned above (Figure 4, molecule c) has been shown to mediate the photoinactivation of *Plasmodium falciparum* and *Trypanosoma cruzi* under conditions similar to those stated above (2 μM photosensitizer, 25 mW/cm^2 illumination).[68,69]

Photobactericidal testing of phthalocyanines has also been carried out *in vitro*. Thus Wilson *et al.* have demonstrated the photokilling of *Streptococcus sanguis* in biofilms[70] and also of methicillin-resistant *Staphylococcus aureus* by aluminium phthalocyanine.[71] Metal-free tetra(*tert*-butyl)phthalocyanine (Figure 4, molecule g) has been incorporated into polymer films as a photobactericidal material which is effective against *S. aureus*.[72] That the bacteria were killed only on illumination suggests that the site of action must be the cell wall, i.e. the photosensitizer was immobilized and so could not enter the bacterial cell, thus singlet oxygen generated at the polymer–cell interface would react immediately on contact with the cell wall. Testing of anionic, cationic and neutral zinc phthalocyanines (Figure 4, molecules h, j and k) against both Gram-positive and Gram-negative bacteria showed that only the positively charged phthalocyanine (a pyridinium salt) was active.[73] This effect is similar to that noted above with cationic *meso*-porphyrins[54] and indicates the presence of a specific site of action for the active species since the neutral phthalocyanine showed similar uptake without activity.

Naturally occurring photosensitizers

Whereas HpD is now widely used photodynamically in the clinic, the major use of haematoporphyrin in nature is not in photosensitization. There are, however, many examples of natural product photosensitizers which have evolved over millions of years—either, in plants, as chemical defence against microbial or herbivorous attack or, in fungi, to facilitate plant parasitization. The isolation and elaboration of such compounds as the furanocoumarins and the perylenequinone pigments represent major advances in this area of research.

Psoralens (furanocoumarins). Psoralen derivatives have been used for millennia in Asia in the treatment of various skin disorders, but more recently the structure–activity relationships and sites of action of the psoralens have been established and synthetic analogues prepared. The field of psoralen photomedicine and, in particular, photopheresis (extracorporeal chemotherapy) in the treatment of lymphoma, has become extremely active in recent years,[74] with a wide range of newly synthesized compounds as well as the in-vitro testing of natural congeners and structural isomers of the furanocoumarin nucleus.

Whereas the concept of photopheresis is based on the selectivity of psoralen derivatives for malignant cells, such as the lymphocytes implicated in cutaneous T-cell lymphoma, the affinity of psoralens for nucleic acid intercalation has also indicated their use in PACT as photodynamic antivirals. Illumination of the viral nucleic acid–psoralen complex with the relevant wavelength of UV light leads to damage via photoadduct formation and thus to viral inactivation (Figure 5). Currently, several groups are investigating the use of psoralens for the disinfection of blood, against both non-associated and intracellular viruses. The use of psoralens differs from the other photosensitizers discussed above in that psoralens intercalate in viral DNA rather than in sites in the viral envelope and also that the furanocoumarin chromophore absorbs UV light (normally UVA, 320–400 nm), i.e. at higher energies. In terms of collateral damage there is, of course, a higher degree of risk to healthy cells (particularly by nucleic acid photodamage) with UV light although this may be ameliorated using specific long wavelengths of UVA.[75] Typical disinfection conditions, e.g. for amino-methyltrimethylpsoralen, are 50 mg/mL of agent and a UVA dose of 38 J/cm^2.[75]

That simple psoralens cause nucleic acid cross-linking is well established.[74] Psoralen functionalization in the pyrone ring (ring C, Figure 6) can yield compounds that are unable to form cycloadducts via this ring due to steric factors. Since methylation in the furanocoumarin nucleus furnishes compounds, e.g. 4,8,5′-trimethylpsoralen or 4,6,4′-trimethylangelicin, which cause DNA cross-linking,[76] the steric factor is obviously important. The 3-ethoxycarbonyl analogue is sufficiently hindered to give rise only to mono-adducts with DNA.[76]

Psoralens can also cause hydroxylation of guanosine in nucleic acids, a mechanism often associated with the intermediacy of singlet oxygen.[77] Thus, although psoralen photodamage to viruses normally results from psoralen photoadducts with nucleic acids,[78] damage can certainly be envisaged as occurring via a photodynamic route. If this is indeed the case, the ongoing efforts in new drug design and synthesis in this area, and particularly those involving increasing the singlet oxygen yield, are well justified.[79] Indeed, the photoactivities of brominated psoralen derivatives against viruses were considerably higher than the parent compounds.[80]

There are two main reasons for the development of analogues of psoralen for PACT: increasing the selectivity for viruses, or increasing the photoactivity at the target site, for example via the heavy atom effect. To this end, many bioisosters and structural isomers of the furanocoumarin nucleus have been isolated or synthesized *de novo*.[81] The replacement of the furan oxygen with sulphur or selenium gives rise to compounds having much

improved photoactivity. In addition, the 8-azapsoralens—i.e. analogues arising from replacement of carbon-8 with nitrogen (Figure 6, X = N)—exhibit lower incidences of DNA cross-link formation.[76]

In common with the other photosensitizers used in the inactivation of viruses in blood products, psoralens have also been investigated in the eradication of other microorganisms. Thus, 4′-aminomethyl-4,5′,8-trimethylpsoralen–UVA treatment has been shown to give complete inactivation of the infective form of *T. cruzi* both in fresh frozen plasma and in platelet concentrates. As with viral photoinactivation, parasite DNA showed

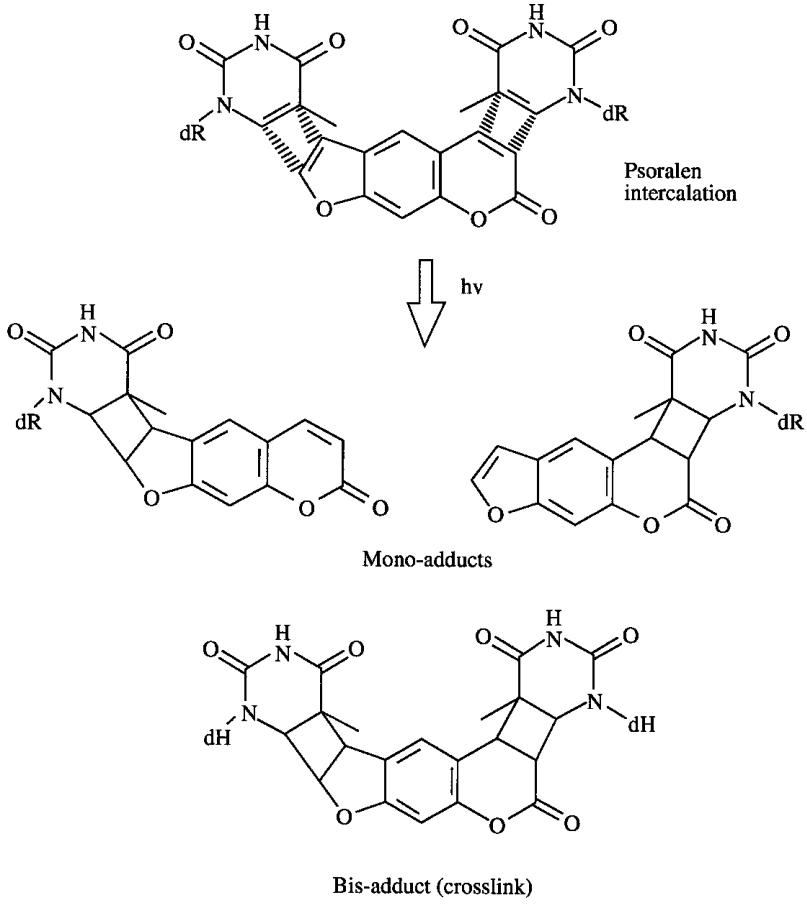
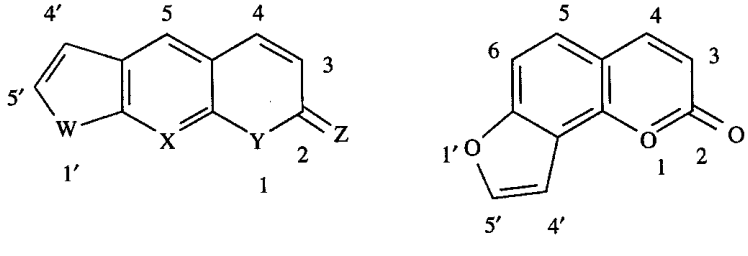


Figure 5. Psoralen–thymidine photoadduct formation.



W	X	Y	Z	
O	CH	O	O	psoralen
S	CH	O	O	thienocoumarin
O	N	O	O	8-azacoumarin
O	CH	O	S	2-thiofuranocoumarin
O	CH	O	Se	2-selenofuranocoumarin

Figure 6. Psoralens, angelicins and bioisosters.

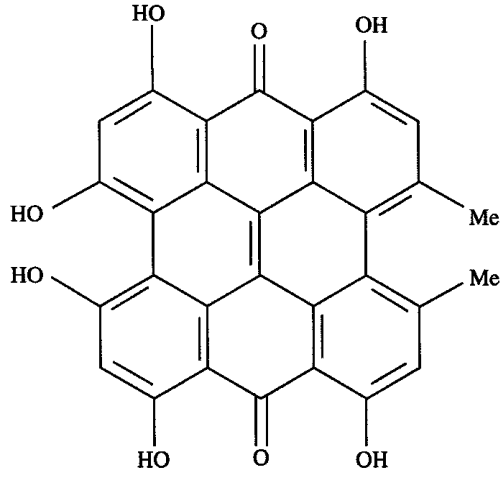
the presence of psoralen photoadduct formation.[69] 8-Methoxypsoralen inactivated bacteria in platelet concentrates, although Gram-positive organisms were far more susceptible than Gram-negative species.[82] Psoralen–UVA has also been implicated in the photokilling of pathogenic *S. aureus* colonizing atopic dermatitis lesions after a 30 mg (oral) dose of 8-methoxypsoralen and subsequent illumination with UVA at 5 J/cm^2.[83]

Perylenequinonoid pigments. Domestic cattle have long been known to suffer from a skin disorder (hypericism) after the ingestion of a photosensitizer contained in the weed St John's wort (*Hypericum perforatum*). Skin photosensitization results from the transport of the perylenequinonoid pigment (PQP) hypericin (Figure 7) in the animal's bloodstream to the epidermal capillaries and subsequent activation by sunlight. Similarly, traditional Chinese medicine has made use of extracts of *Hypocrella bambusae*—containing the photosensitizer hypocrellin—in the treatment of skin disease. The PQPs and the related hypericin implicated here were investigated, and their favourable photoproperties—near infrared absorption and high singlet oxygen efficiencies—have promoted their in-vitro testing both for PDT and PACT.[84,85]

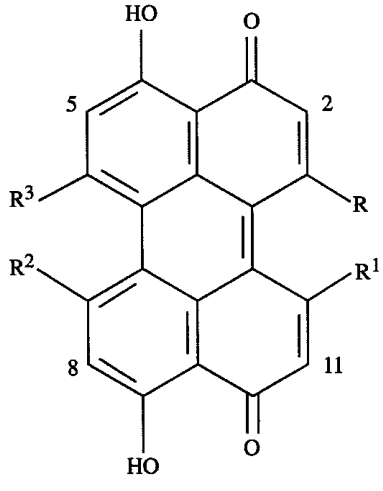
In general the PQPs have high, positive log *P* values (high lipophilicity) coupled with a formal negative charge. Along with the large pseudoplanar area, this makes the PQPs obvious candidates as antivirals—indeed hypericin is currently undergoing clinical trials in AIDS patients.[86] The activity of hypericin against HIV is increased by illumination, thus the PQPs are also under investigation in photodynamic disinfection of blood products.[87] The mode of action involved in the photodamage of HIV by hypericin is thought to be via cross-linking of viral capsid proteins.[88]

Increased interest in hypocrellins (Figure 7) has arisen due to the fact that they have been found to be inhibitors of protein kinase C (PKC), a key target for antiviral and anticancer drugs. Semisynthetic approaches to new hypocrellin photosensitizers, involving their action against PKC, were made, starting from the known PQP cercosporin via conjugate addition of, e.g., thiophenol at positions 5 and 8. Calphostin C (Figure 7) appears to be more active in PKC inhibition than any of the newly tested derivatives, and it is suggested that the increased photoactivity against PKC arises by the addition of cysteine residues in active sites of the protein (through –SH) at positions 5 and 8 of calphostin C. Thus, when these positions are blocked chemically, the photoactivity is decreased.[89] Such studies have demonstrated the potential of the PQPs for functionalization, leading to changes in physicochemical properties and improved characteristics for PACT.[90]

Miscellaneous natural product photosensitizers. The continuous discovery of new natural products provides a source of new and novel photosensitizers. Several of these have been shown to be biologically active, and their photoinactivation potential against viruses has been tested. Such



Hypericin



Perylenequinonoids

R	R^1	R^2	R^3	
R/R^1	$-CH_2C(OH)MeCH(COMe)-$	OMe	OMe	hypocrellin A
R/R^1	$-CH_2CMe=C(COMe)-$	OMe	OMe	hypocrellin B
$-CH_2CHMeCOPh$	$-CH_2CHMeOCO-p-C_6H_4OH$	OMe	OMe	calphostin C

Figure 7. Hypericin and the perylenequinonoid pigments.

compounds include the psoralen-related gilvocarcins,[91,92] the terthiophenes[93–95] and benzophenanthridines such as sanguinarine.[96,97]

Conclusion and future directions

Major advances have been made in photodynamic antimicrobial chemotherapy (PACT) in the past decade. While the early stages of this research have been mainly concerned with the disinfection of whole blood and blood products, the development of spin-off regimens involved with the eradication of localized infection by pathogenic microorganisms—for example in the treatment of oral candidiasis—can be envisaged in the next few years. Similarly, the use of an arginine-haematoporphyrin derivative which is effective against *P. aeruginosa* has been suggested for use in locally infected wounds.[98]

The main area which needs to be addressed is that of host toxicity. The photosensitizers covered here which are derived from vital stains are known to be non-toxic in much higher concentrations than those required for effective pathogen killing. However, the phototoxic effects of these agents on host cells are, in many cases, unclear. Conversely, established photosensitizers such as methylene blue are used in cancer PDT only when application directly to the immediate tumour environment is feasible—for example, by direct instillation into the bladder.[99] This underlines the probable local limitation to PACT, although work on the differential phototoxicity of one photosensitizer in bacterial and human cells *in vitro* has been reported.[22] Similar work on the efficacy of methylene blue and TBO against *H. pylori* on rat gastric mucosa has shown that photokilling of the bacteria occurred at much lower light doses ($\leqslant$200 J/cm^2) than that required to damage the underlying mucosa ($\geqslant$500 J/cm^2).[100]

In order to increase selectivity, the use of specific antibody-linked photosensitizers has been investigated, and was useful in conferring activity on the anionic photosensitizer tin chlorin-e6 against Gram-negative bacteria such as *P. aeruginosa*, as the non-conjugated photosensitizer had no effect on bacterial growth (using 630 mm light with a power density of 100 mW/cm^2 for 1600 s).[101] Varying the photosensitizer–antibody linking group has been implicated in changing the type I/type II mechanism. The use of a dextran carbazate polymer as linker allowed the formation of type I-induced hydroperoxides *in situ* and these increased the efficacy of the photosensitizer conjugate.[102]

That antibody-labelling can mean the difference between bacterial photolysis and unaffected bacterial growth in Gram-negative species as above is obviously important. However, the efficacy of cationic compared with anionic photosensitizers against Gram-negative bacteria is well established without recourse to antibody labelling. In addition, the use of antibodies would be of less import if PACT were to be used in local infections. Antibody conjugation to photosensitizers shows greater promise in the area of anti-cancer PDT.

Several bacterial pathogens are known to synthesize porphyrins. Thus, pigmented microorganisms such as *Prevotella* and *Porphyromonas* spp. may be susceptible to photoinactivation using these endogenous photosensitizers.[103] Although such an approach would obviate the use of synthetic compounds, the natural photosensitizers involved might also cause collateral damage.[104]

Given the small fraction of available photosensitizing compounds (and, within this, the wide variety of structures) that have been investigated for use in PACT, there is obviously great potential for such a technique to be included in the clinic. The search for microbial specificity will undoubtedly continue, but this is more likely to result from properly organised drug development including de-novo photosensitizer synthesis.

Whilst it is not suggested that PACT will replace systemic antimicrobial chemotherapy, improvements may be obtained using the photodynamic approach in the clinical treatment of local infection, both in terms of the likely speed of treatment and in the lowering of treatment cost. Local therapy of infection, where possible, also has the advantage of being less injurious to indigenous bacteria remote from the site of infection which are at risk during systemic antibiotic therapy.

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