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Photodynamic antimicrobial chemotherapy (PACT) for the treatment of malaria, leishmaniasis and trypanosomiasis

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Abstract

A photodynamic effect occurs when photosensitiser molecules absorb light and dissipate the absorbed energy by transferring it to biological acceptors (usually oxygen), generating an excess of reactive species that are able to force cells into death pathways. Several tropical diseases present physiopathological aspects that are accessible to the application of a photosensitiser and local illumination. In addition, disease may be transmitted through infected blood donations, and many of the aetiological agents associated with tropical diseases have been shown to be susceptible to the photodynamic approach. However, there has been no systematic investigation of the application of photoantimicrobial agents in the various presentations, whether to human disease or to the disinfection of blood products or even as photo-insecticides. We aim in this review to report the advances in the photoantimicrobial approach that are beneficial to the field of anti-parasite therapy and also have the potential to facilitate the development of low-cost/high-efficiency protocols for underserved populations.

Key words: Tropical diseases; Photodynamic therapy; Singlet oxygen; Photosensitisers; Leishmania; Chagas' disease

Introduction

Tropical diseases (TD) are clinical conditions caused by microbial infections, usually limited geographically to tropical areas. TD can cause exotic symptoms that have been noted by travellers, explorers, and physicians for many years but that were initially not found in western civilization. The tropics present a series of environmental (wet climate, higher temperature, animal reservoirs, insect vectors) and socio-economic factors (water treatment, sanitary conditions) that favour the development of these diseases. Estimates suggest that there may be about 0.5 million of deaths per year due to TD, not including malaria (1).

There is also an enormous related socio-economic burden with millions of people having to deal with diseases that hinder a proper quality of life. In addition, globalisation has increased the potential danger that an exotic microbial disease may be introduced into a new setting where there is no natural resistance. The situation for malaria is somewhat different, being already a threat to millions of travellers from richer economies that require efficient

prophylaxis, besides killing millions of humans annually. Funding of scientific investigation related to tropical medicine has improved markedly in recent years due to industrial as well as government-backed research. However, the complexity of the diseases in terms of the different life forms of the pathogens and their interactions with the host immune system, different vectors etc., present continual challenges in terms of the development of new selective drugs. Finding specific targets in these pathogens has proved to be difficult. An important question still remains: how much more time will be required for effective modern medicines to be supplied for diseases such as trypanosomiasis and leishmaniasis? (2).

One characteristic of the pathogens that cause these diseases is the fact that they live together with the host organisms for many years, having to deal with their immune systems in general and especially with the oxidative artillery of the host. In fact, redox equilibrium is a key issue related to the survival/death balance of the parasite/host

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interaction. It is not surprising, therefore, that the majority of the antiparasitic drugs available in the market work by affecting parasite redox systems (3,4).

One of the most effective ways that is known to cause redox imbalance in cells is the use of photoactivation (5). Based on mechanistic evaluation and available literature data, we will show that photodynamic processes offer a great opportunity for the development of new drugs for controlling TD on several fronts, including reducing parasite infection in the hosts as well as providing alternatives for blood disinfection and vector control.

In terms of healing properties, the application of photosensitisers to TD is likely to be of greatest utility where there is either a skin or a soft tissue manifestation of the disease. Because photodynamic antimicrobial chemotherapy (PACT) is dependent upon exposure to a directed light source, it allows local treatment and control, but consequently cannot affect advanced, disseminated diseases (5,6). Conversely, no microorganism resistance has been reported against PACT, and the low cost of commonly available photosensitisers, coupled with the availability of inexpensive low-power light sources, suggests that this approach is of great potential healthcare benefit in TD (5,6). Although the first published result of photosensitisation applied in biology was an observation made by Raab (7) at the beginning of the last century of the photo-induced killing of *Paramecium caudatum*, the potential use of PACT to kill parasites and control TD has not yet been even moderately exploited. Consequently, the present study is based on the potential application of photoantimicrobial approaches to leishmaniasis, malaria, and African and American trypanosomiasis.

As noted above, the light requirement means that the treatment of disseminated diseases is not feasible at present. However, the application of PACT to blood product disinfection offers considerable potential in blocking TD transmission, and this is also covered in the current study.

Photosensitisers: fundamentals and state of the art

Light absorption by organic molecules is essential to life, allowing the photosynthetic pathway in plants via the photoexcitation of chlorophylls and related pigments (8). Although photosynthesis has an excellent yield of light to chemical energy conversion, side processes compete with normal electron transfer reactions favouring the production of reactive oxygen species (ROS) and nitrogen oxygen species (RNS) (9,10). These species damage biomolecules, causing photoinhibition and photodestruction of the photosynthetic apparatus. In humans, similar photoprocesses are related to premature photoaging and skin cancer, while lower organisms have also evolved the use of photoreactions to protect themselves (4). In plants, photoantimicrobials are used as chemical defence weapons against attack

by herbivorous insects, and the strategy is also used by parasitic fungi to help break plant cell walls (6).

Photodynamic antimicrobial chemotherapy is a therapeutic modality based on the combination of a photosensitiser that is selectively localized in the target tissue and illumination of the area of interest, usually with visible light, resulting in photodamage and cell death (5,6). Exposure of a photosensitiser to light of a specific wavelength will lead to light absorption, the production of excited species and ultimately the transfer of this excitation energy to oxygen to generate ROS (11). The excited species (especially triplet species) transfer energy to oxygen (type II photosensitisation), or undergo direct reaction (electron transfer or other reactions) with the target substrates (type I photosensitisation), usually generating radicals and finally ROS (Figure 1) (11). In addition to causing cell death, photosensitisation reactions in biological tissues can also induce immune stimulatory reactions (12,13), and consequently have the potential to improve the overall host response to infections (3). However, under mild conditions, oxygen does not react with electron pairs of organic molecules in the ground state. Consequently, most organic molecules must be transformed into radical species before they can react with molecular oxygen. A catalyst that allows one-electron reactions is, therefore, necessary to promote the oxidation of biomolecules. The stepwise one-electron reduction of oxygen produces the anion radical superoxide, hydrogen peroxide and the hydroxyl radical, which is one of the most reactive molecules known (11). The reactivity of oxygen can also be dramatically increased by elevating it to the excited state (singlet oxygen), allowing it to add to double bonds of biomolecules. Ideally, photosensitisers should act via both type I and type II mechanisms to increase the range of ROS that can be generated; however, in the presence of biological molecules and interfaces there can be a

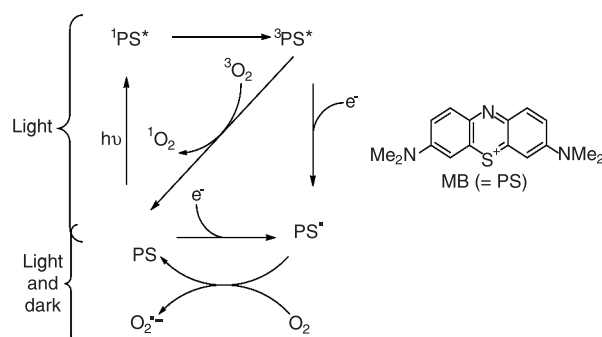


Figure 1. Electron and energy transfer reactions in which methylene blue (MB) is involved in the presence (above) and in the absence (below) of light. PS is photosensitiser in the singlet (1PS) and triplet (3PS) excited states; 3O_2 and 1O_2 are the ground and excited (singlet) states of molecular oxygen (O_2). PS $^-$ is the reduced species of PS, and $O_2^{\cdot-}$ is the anion radical superoxide.

competition between type I and type II mechanisms (14). Methylene blue and other phenothiazinium derivatives are examples of photosensitisers that work both by type I and type II mechanisms (14,15).

Some photosensitisers, including phenothiaziniums and triarylmethanes (e.g., crystal violet, CV) can also be reduced in the dark in the biological environment. The reduction may be directly from reduced co-enzymes, in the case of methylene blue ($E_{1/2} = -0.28$ V), for example, the reduction potential being lower than that of NADH ($E_{1/2} = -0.32$ V) (16). Conversely, it can involve reduction in the electron chain reactions. Reoxidation may involve direct reaction with oxygen, forming the superoxide anion radical. Enzymatic oxidation by enzymes in the electron chain is also possible, in such a way that the photosensitisers work as a bypass in the electron chain reactions. Depending on the quantities involved, this interference in the redox cellular cycle can help cells to survive a possible mitochondrial dysfunction or, at higher concentrations, a redox imbalance may be induced, damaging cells. This explains why molecules such as CV may also be used in disinfection strategies in the dark. In fact, the induction of redox imbalance was used to explain the bacteriostatic and bactericidal activity of CV against *B. subtilis*, *E. coli*, *Str. lactis*, and *S. marcescens* as early as 1933 (17). The authors also reported that ~90% of all Gram-positive strains and only 10% of Gram-negative strains were susceptible. CV is currently frequently employed for this purpose in blood products without photoactivation (18). It is important to mention that the microorganism killing efficiency of CV and other similar molecules in the dark is amplified several times under photoactivation, explaining the promissory strategy of using them as photomicrobicides (5,6,19).

The amount of energy needed to promote an electron from the ground to the excited state (Figure 1) depends on the molecular structure of the photosensitiser. Therefore, the wavelength of light causing the electronic promotion is also highly relevant in the application of the photosensitiser (19). Because of the range of molecular structures of available photosensitisers there is a corresponding array of activation wavelengths, from ultraviolet, through visible, to near infrared. Similarly, there are various biomolecules, which absorb electromagnetic radiation from UV to the visible region, competing with the photosensitisers for the excitation light. Besides light absorption, light scattering is another process that decreases the amount of light penetration in the biological tissues, and the amount of light scattered decreases as the wavelength of light increases (19). Such interferences have been the main drivers towards the development of long-wavelength absorbing photosensitisers absorbing far red or near infrared radiation (5,6,15,19,20).

As noted above, the redox balance of cells infected by parasites is a critical issue in the progression of the parasite infection. In fact several drugs in use today are based on the generation of an intracellular ROS burst that either

kills the parasite or the infected host cell (3). Research in the area of photoactivation reactions in the biological environment has progressed in such a way that certain molecular structures are known to favour photosensitiser localisation in different environments of cells, thus having a significant impact on the mechanism and efficiency of cell death (21,22). Photosensitisers that localize in mitochondria, lysosomes, and nuclei and bind efficiently to membranes have been characterized and a great deal of information concerning structure-activity relationships has been uncovered (15,21-26). The distribution of polar and hydrophobic substituents around the central chemical structure of the photosensitiser, the electronic charge of side chains, and chelation by the central amino groups are all molecular features that seem to play significant roles (23-25). Cationic (positively charged) photosensitisers may be especially effective clinical agents because they can accumulate in negatively charged surfaces including mitochondria (21,23-25), an effect that is driven by the transmembrane potential of the inner mitochondrial membrane (16). Therefore, it is possible to design molecules that could have a significant impact on the host-parasite relationships leading to the cure or control of these diseases, although the rationale for targeting parasite cells inside host cells has not been obtained so far. It should also be emphasized at this point that the major selectivity exhibited by photosensitisers must be that between host and target cells.

Photoantimicrobials

In photodynamic therapy (PDT), the anticancer approach related to PACT, the photosensitisers employed clinically are generally based on the porphyrin nucleus, mainly for historical reasons (27). This situation is changing rapidly since new inexpensive protocols are being developed based on phenothiaziniums, hypericin and triarylmethanes (15). In the case of PACT, the range of molecules considered since its origin is much wider.

The early history of chemotherapy is closely linked with the work of Paul Ehrlich. Indeed, he devised the term "chemotherapy". Ehrlich's research was mainly involved with the differential staining of cells using aniline-derived dyes. A major part of Ehrlich's time was spent examining stains for parasites encountered in TD. His work led to widely used stains such as Trypan red and Trypan blue, and the drugs that were developed from them (5-7).

The development of PACT may be envisaged as an extension of Ehrlich's work, in the sense that several of the photosensitisers in use are, in fact, biological stains. Interestingly, the chemical structure of stains such as methylene blue provided the lead compounds to conventional chemotherapeutic agents, such as aminoquinoline and acrydine antimalarials, e.g., chloroquine and mepacrine, respectively (6).

Photoantimicrobial action, i.e., the effect of the gen-

eration of large amounts of ROS such as singlet oxygen or the hydroxyl radical on microbial species, is extremely destructive for two main reasons: lack of effective microbial defensive mechanisms and multiple sites of attack (5,6). Although cells have several natural defences against ROS, the level of redox imbalance inflicted by PACT is several orders of magnitude larger than the level of protection allowed by enzymatic and molecular antioxidant species in the cell. Besides, antioxidant cellular enzymes such as catalase and superoxide dismutase are inactivated by singlet oxygen (27). It is important to mention that in the photoactivation reaction, different ROS with different mechanisms and different action sites are generated, resulting in multiple sites of attack on the microorganisms. Consequently, an important benefit of PACT is the absence of microbial resistance and, thus, a similar efficacy against conventional drug-sensitive and drug-resistant microbial strains. The multifactorial attack of photoantimicrobial agents has been demonstrated, for example, in both viruses and bacteria (5,6,26,27).

Just as antimicrobial agents have a wide range of chemical forms - β -lactams, tetracyclines, fluoroquinolones, etc., so there is no single chromophoric group, which can be used in photoantimicrobials. Such agents (Figure 2) may belong to the porphyrin, phthalocyanine, azine, triarylmethane, or cyanine families. The effectiveness of a photo-decontamination depends on a series of factors including the type of microorganisms as well as the type of photosensitisers and the reaction medium. It is often relatively straightforward to demonstrate microbial selectivity, given the biological staining heritage of many photoantimicrobial agents (5,6,27-29).

Since photoantimicrobial drug development may be carried out following the same principles as used for conventional drugs - i.e., new compound generation based on a lead structure - although there is a range of structural types, it is not enormous. To use methylene blue as an example, its broad-spectrum activity as a photobactericidal agent has been accredited to the cationic nature of the chromophore, with other, anionic or neutral photosensitisers being essentially inactive against Gram-negative bacteria (29). However, where cell penetration is required, the hydrophilic nature of methylene blue is disadvantageous, and more lipophilic molecules may be more successful - for example in intracellular virus inactivation in red blood cells (30). Among several physical and chemical properties tested, the affinity of a specific photosensitiser for viral nucleic acid seems to best correlate with its virus photokilling efficiency (31).

Considerations in the control of tropical diseases

In terms of developing effective treatments against a specific TD in endemic areas it is important to think about all strategies that would favour a decrease in the pool of infected patients (32). Developing an efficient clinical protocol

that would cure/control the disease would not only favour the patient, but would also decrease the chance that this infection would be transmitted to others by the usual vectors or via blood transfusion. The following sections cover the potential for use of PACT in the treatment of malaria, leishmaniasis and trypanosomiasis. However, it is also important to consider strategies in which photoactivated compounds can be employed in blood product disinfection and in the direct control of vector dissemination.

Blood product disinfection

Whole blood is a mixture of three main fractions: plasma, platelets and red blood cells. There are also therapeutic proteins, such as clotting factors (e.g., Factor VIII). Each of these, and whole blood itself, finds use in haematological replacement - whether in volume, due to blood loss, or in the treatment of a deficiency syndrome, such as haemophilia.

Since the establishment of the germ theory of infectious disease, it has been appreciated that the spread of illness within an individual is closely tied to the circulatory system. By extension, then, the use of blood in transfusion or of blood products in therapy must be subject to very strict controls in order to avoid the transmission of infective agents between donor and recipient. This is further complicated by the practice of "pooling" of blood fractions from multiple donors in order to facilitate supply.

Clearly, the examination, or screening, of donated blood is necessary in order to minimise transfusion-transmission events. However, such screening has significant cost impli-

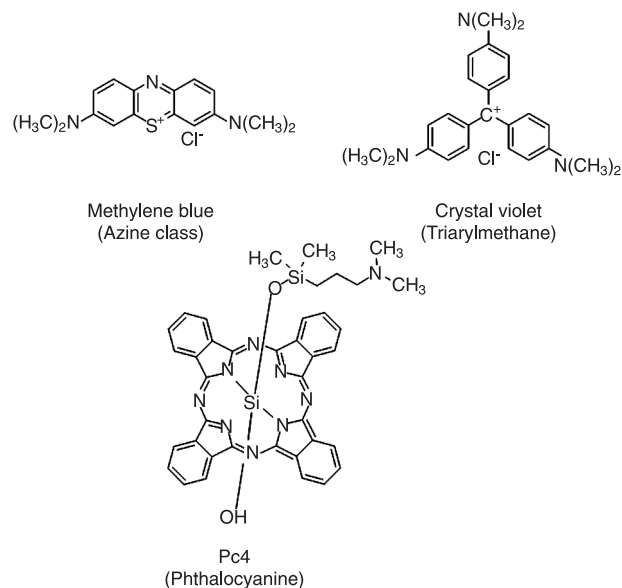


Figure 2. Chemical structures of the principal photosensitisers relevant to tropical diseases.

cations and is thus not carried out universally. In addition, practical screening does not exist for all common infective agents, and there is the additional problem of emerging pathogens/diseases.

In order to guarantee the freedom of blood products from pathogenic microbes, various eradicated treatments have been proposed, including solvent-detergent treatment and ultraviolet irradiation. Most treatments appear to have associated collateral effects. The use of photosensitisers to this end represents a logical step, since blood fractions may be illuminated "in the bag" on a light table or similar apparatus. This is possibly the most important aspect of PACT in relation to TD transmission, as will be understood from the following sections, since there are important TD, which are systemic in the main and thus not, at present, susceptible to the photodynamic approach (33).

The main challenge in blood disinfection is to kill or inactivate the target microorganism without harming blood cells or serum proteins. Reinfusion of damaged blood products, besides not being effective in the patient, can actually be harmful and cause an immune response. Conventional methods of disinfection include the use of chemotherapeutic agents, some of which have the potential to be used as photosensitisers. As mentioned above, CV is one example.

The main advantage of PACT is its multi-target nature, being potentially efficient against viruses, bacteria, fungi, and parasites. As with any other strategy of blood disinfection, there is the potential host toxicity of the photosensitisers. Either all of the photosensitiser must be removed from the treated material prior to transfusion or it must be non-toxic to the recipient. In fact, the majority of the photosensitisers that have been used for blood disinfection are non-toxic (methylene blue, crystal violet, riboflavin). Strategies based on PACT to promote pathogen inactivation in stem cells from cord blood have been proposed (6,34). Even so, strategies aiming to separate photosensitisers from the treated blood, for instance using magnetic nanoparticles, or strategies that would allow the photosensitisation reaction to occur without the direct contact of the photosensitiser with blood (for example, photosensitising surfaces) would possibly allow new and more efficient methods of blood disinfection.

Methylene blue has been used widely as a photosensitiser for blood product disinfection. Using dry pills of methylene blue, plasma containing the agent (1 μ M) is prepared and illuminated at 590 nm. Methylene blue is then separated from the blood using a special depletion filter through which the treated blood product is passed into storage bags. Methylene blue and similar compounds that bind preferentially to nucleic acids or to specific intracellular organelles, in principle, could permit the elimination of nucleate cells (of infecting agents) or viruses. In reality the widespread use of PACT to treat whole blood is awaiting strategies that would allow greater selectivity for infecting

agents, saving red blood cells from being damaged in the photoactivation process (6,33).

Although in use for almost 20 years as a plasma disinfectant agent, methylene blue does not seem to be an ideal photosensitiser for this application due to collateral damage, typically to clotting agents. This damage is likely to be due to the tendency of the agent to damage lipids. Similar photosensitisers, which are either more or less polar than methylene blue, seem to be better suited as blood photodisinfection agents. Thionin, the demethylated derivative of methylene blue, has been shown to be as efficient as the latter as a photokilling agent, without damaging red blood cells and platelets (27,29,33).

One of the protocols used in the pathogenic inactivation of blood products involves irradiation using UV light. UV is by itself a potent microbicidal agent; however, it is also damaging to several blood components. Addition of riboflavin prevents the side effects of UV, like red cell haemolysis and oxidation of haemoglobin to the methaemoglobin, while maintaining the photokilling of microbial organisms. In addition to being a vitamin (vitamin B2), riboflavin is a potent photosensitiser, acting both as a type I and type II mechanism. Because it does not have a formal charge on its structure, it does not interact strongly with proteins or charged membranes and biomolecules, which probably explains the low level of biomolecular damage. Riboflavin is also inherently non-toxic and does not need to be removed after the decontamination process. Therefore, riboflavin shows great promise as a decontamination agent (34,35).

Malaria

Mosquito-borne diseases are transmitted via the blood of bitten individuals. In the case of malaria, the infective agent, *Plasmodium* spp, is injected through the mouthparts of female anopheline mosquitoes and has a life cycle in humans, which includes both bloodstream and tissue phases. There are several infective members of the *Plasmodium* genus. The most serious is *P. falciparum*, with less serious disease caused by *P. ovale*, *P. vivax* and *P. malariae*. Relapsing fevers are often encountered with infection by *P. knowlesi*.

The discovery of the links between mosquitoes, plasmodia and malaria was made over a century ago. Confirmation of the parasite in the bloodstream was made by staining patient blood smears. The stains used were cationic dyes such as methylene blue and the demethylated azures. The selectivity exhibited in this way led one of the founders of antimicrobial chemotherapy, Paul Ehrlich, to use methylene blue as a treatment for the disease, curing two sailors of malaria in 1891 (36).

The fact that stains such as methylene blue are taken up by plasmodial cells inside erythrocytes relative to uninfected erythrocytes demonstrates the damage caused to

the red cell membrane by the parasite, since hydrophilic cations are normally excluded (37). A similar argument is made for the concentration of aminoquinoline antimalarial drugs by infected red blood cells.

PACT may be seen as an application of Ehrlich's approach - several of the photosensitisers proposed for use are, in fact, biological stains, thus exhibiting selectivity for parasites in the first instance. Indeed, methylene blue provided the lead compound from which the aminoquinoline and acrydine antimalarials - chloroquine, mepacrine, etc. - were developed (6,7,27,29,33). In recent years, methylene blue has undergone re-evaluation as a clinical antimalarial (27), this representing use as a traditional, not a photodynamic, drug. However, the acceptance of an intensely coloured, systemic therapeutic agent has been reportedly high despite the obvious sequelae of coloured urine and stools.

Guttman and Ehrlich's successful demonstration of the antimalarial nature of methylene blue was followed by its use as lead structure in many early areas of drug research, not merely tropical medicine (38). However, several close analogues have been shown to be active against the malarial parasite (39), and several of these have also been examined for blood product decontamination protocols (40). Such work is given special significance by the increasing problem of malarial chloroquine resistance.

As discussed above, the use of photosensitisers as antimicrobial agents relies on the production of ROS inside or in close proximity to the target cell. Plainly the demonstrable selectivity of the phenothiazinium stains for plasmodia coupled with established photosensitising activity suggests the use of such compounds against parasitic infection. However, given the location of the malarial parasite in the human body - i.e., the blood stream or the liver - it is difficult to see, at present, how a direct treatment protocol could be achieved. Theoretically, at least, it should be possible to attack the liver stage via implanted optical fibres, but this would be considered to be relatively high risk. Attacking the parasite in the bloodstream is likewise problematic from the point of view of effective illumination of the blood circulation. However, the transmission of malaria via donated blood may be addressed using currently available technology and approaches.

Other photosensitisers examined in terms of pathogen inactivation for use in blood products include the cationic silicon phthalocyanine mentioned above (Figure 2). This has been shown to photoinactivate *P. falciparum* (41,42).

Cutaneous leishmaniasis

Leishmaniasis is an important disease in public health with about 600,000 new cases per year. Worldwide there are about of 12 million people infected with *Leishmania* spp parasites (43). The parasite is spread by the bite of an infected sand fly and can cause disfiguring and sometimes

fatal diseases. The parasite has different life forms in the human host and in sand fly vectors, i.e., amastigotes, which are small and oval and promastigotes, which are longer flagellated forms of the parasite. Domestic dogs are also infected by *Leishmania* parasites and these animals are a reservoir for human leishmaniasis (44).

There are many different diseases caused by *Leishmania* spp. When the parasites do not spread beyond the site of the vector's bite, this may result in cutaneous leishmaniasis. In other instances the amastigotes may spread to the liver or spleen, resulting in visceral leishmaniasis, or to the mucous membranes of the mouth and nose, resulting in mucocutaneous leishmaniasis. Left untreated, these latter diseases result in high rates of mortality, although the use of antimony-containing compounds usually gives good results. The various types of leishmaniasis are confined primarily, but not exclusively, to Central and South America, central Africa, and parts of southern and central Asia (45).

When *Leishmania* parasites first enter the human body, promastigotes are engulfed by macrophages but are resistant to proteolysis and degradation in the phagosome. Once inside the macrophage, the parasite is named an amastigote. By continuing to live inside the macrophage, *Leishmania* amastigotes effectively avoid the humoral branch of the immune system. The parasite invasion into the macrophages is an extremely sophisticated process, in which the parasite presents in its exterior phosphatidylserine, simulating the condition of apoptosis and inducing phagocytosis by the macrophage (46). After being engulfed, the *Leishmania* parasites must endure harsh conditions inside the phagosome. For example, the macrophage uses an oxidative burst (ROS, superoxide and hydroxyl radicals) and RNS, i.e., nitric oxide to destroy foreign material in the phagosome (47). The host-parasite relation for *Leishmania* spp involves three important characteristics: suppression/protection of the parasite from the ROS and RNS cell defences, control of the host apoptosis process and protection against the host immune response. All of these aspects can be neutralised by PACT.

The defence mechanism of the intracellular parasite against oxidative stress involves efficient production of reducing agents. This is true for *Leishmania* spp and also for other parasites such as *P. falciparum*. In both cases, the main treatment involves the uses of drugs that cause an imbalance in the cell redox state to the oxidation side, preventing parasite multiplication (48). For instance, the potentiation of ROS and RNS formation by sodium stibogluconate (Pentostam), one of the drugs used to treat leishmaniasis, was recently described (49). The mechanism by which these reactive radical species produced by the macrophages are neutralised involves glutathione (GSH) or trypanothione (the GSH analogue in the *Leishmania* spp thiol recycling system), using NADPH as reductant, formed in the pentose shunt (3). Therefore, the inhibition of

enzymes related to the control of the redox states in the cells, including glutathione reductase and glucose-6-phosphate dehydrogenase, offers a potential mode of attack against the intracellular parasite.

Current treatment of leishmaniasis is limited to pentavalent antimony derivatives, amphotericin B, and paromomycin, which can cause serious side effects. Other serious drawbacks associated with these medications are the complexity of the treatment protocols and their high cost, making them unsuitable for the majority of underserved populations afflicted by such disease. In addition, the limited number of medications for a widespread population has also created drug resistance; in fact, 60% of leishmaniasis cases have been reported to be resistant to first-line drugs (49).

Due to the clinical efficacy of PDT in localised cutaneous disease and the growing evidence of photoantimicrobial activity, researchers have begun limited clinical trials of PDT to treat cutaneous leishmaniasis and have observed interesting results. 5-Aminolaevulinic acid (ALA) is currently a popular PDT prodrug, being metabolised by both mammalian and microbial cells to the photosensitiser protoporphyrin IX as part of the heme biosynthetic pathway (50). ALA and subsequent red light illumination have been used to treat patients in the Middle East and Europe with old world cutaneous leishmaniasis (*L. major*). These patients achieved a greater than 90% cure rate without evidence of recurrence (51). However, the cost of ALA remains prohibitive and largely inaccessible to the populations in need. Corroborating the clinical studies, Kosaka et al. (52) showed a 25-fold decrease in parasite load in infected mice after one PDT treatment. Importantly, they concluded that the parasitocidal effect of PDT observed was probably mediated via the indirect killing of infected host cells and not through direct parasite killing. Protoporphyrin IX accumulates within the parasite, probably through an endocytic internalization pathway - although this mechanism has not yet been studied - but in amounts insufficient for phototoxicity.

Thus, it is imperative to study the efficacy of PDT against leishmaniasis using photosensitisers that are directly effective against the parasite. In fact, Kosaka and co-workers (52) have provided evidence that positively charged photosensitisers are extremely effective in this respect, as have Bristow et al. (53) using a series of positively charged porphyrins. However, remembering the financial implications of treatment programmes in impoverished regions, a low-cost PDT protocol, based on the administration of methylene blue plus irradiation with a non-coherent light source, has been shown to be effective against *L. amazonensis* parasites *in vitro* and *in vivo*. Given the absence of methylene blue toxicity in humans, the local application of the photosensitiser and illumination of cutaneous lesions of leishmaniasis should be effective against the parasites, including those strains against which conventional therapies are ineffective.

Trypanosomiasis

After malaria, African trypanosomiasis was the principal disease studied by Paul Ehrlich with his application of dyes to stain infected cells. As noted above, this led to the development of the drug suramin from the azoic dyes trypan red, trypan blue, etc., such dyes being effective against rodent models of the disease but not in humans.

Human African trypanosomiasis, caused by the introduction of *Trypanosoma brucei gambiense* or *T. brucei rhodesiense* during biting by the tsetse fly, is usually a disease of the nervous system, sleeping sickness. Conversely, American trypanosomiasis, or Chagas' disease, is normally caused by infection with *T. cruzi*. Neither disease has a significant dermatological aspect (except the puncture wound caused by the biting insect disease vector), but infected persons carry the protozoa in the bloodstream. Thus, although neither the African nor the American form has a locally approachable focus, the causative organisms for both should be targeted as part of blood disinfection protocols. *T. cruzi* has been included in proposed photodisinfection protocols, and the photokilling of the organism has again been demonstrated with the silicon phthalocyanine Pc4 (Figure 2), and thiazole orange (54). As mentioned above, crystal violet is already employed for the inactivation of *T. cruzi* in donated blood, and this also offers a strong potential lead for conventional drug discovery against *T. cruzi*. Earlier experimentation by Do Campo and co-authors (18) using *T. brucei* in citrated guinea pig blood demonstrated that the parasites could be immobilised by a 1% solution of methylene blue or by a 0.001% solution in conjunction with white light illumination from a 100 W source, the immobilised organisms causing no subsequent disease in mice.

Additionally, the redox dyes brilliant cresyl blue, a phenoxazine derivative, and the phenothiazinium new methylene blue have been shown to be inherently trypanocidal - i.e., without light activation - in infected macrophages. This reflects the influence of the redox properties of both dyes on the parasite. Dyes must pass into the interior of the macrophage before killing the parasite. The capacity that some molecules may have of entering host cells without damaging them, and targeting microbial parasitic cells inside the host cell, provides a reasonable hypothesis on which to model blood cell disinfection. As discussed above, in blood disinfection strategies it is important to cause specific damage to parasites without affecting host cells (55). The increased efficiency of singlet oxygen production by new methylene blue, in comparison to that of methylene blue itself is another promising factor in the search for effective photoantimicrobial agents (56).

It should also be noted that the ultraviolet-absorbing Amotosalen (4'-aminomethyl-4,5',8-trimethylpsoralen) has been reported to give complete inactivation of the infective form of *T. cruzi* both in fresh-frozen plasma and in platelet concentrates (57). Rationally, given the severity of disease

associated with trypanosome infection, it is important that such agents are removed from the blood supply. However, most blood industry concerns did not screen for the various trypanosomes until relatively recently. Indeed, there was no standard test available until 2007.

Vector control

The dissemination of TD is strongly influenced by insects since these are the main vectors transmitting the pathogens from one individual to another. Due to deforestation, insect vectors invade cities and are thus considered responsible for establishing new endemic regions of disease. Governments around the world spend significant resources on the control of insect vectors, usually entailing the use of chemical insecticides, which are known to cause side effects in the population and are harmful to the environment. Other strategies have been devised including biological control and photoinsecticides.

Photoactivated insecticides were shown to be an interesting alternative to chemical insecticides. Usually they can be used at low concentration, because they use light energy to "catalytically" generate toxicity. Besides being used at lower concentrations, photoactivated insecticides may be intrinsically less toxic to the environment. First of all they are non-toxic in the dark and second they are less prone to accumulate, because they are usually bleached and further degraded by light. The concept of using photosensitisers to control pest populations was realized as early as in the 1930's using xanthene derivatives against *Anopheles* and *Aedes* larvae (58). The developments in this field have been reviewed by Ben Amor and Jori (59). Porphyrins are particularly interesting as photoinsecticides because they exhibit several absorption bands in the UV/visible spectrum, being therefore efficiently excited by the solar spectrum. The photoactivity efficiency seems to depend both on the octanol/water partition coefficient of the photosensitisers and on specific structural features such as the amphiphilic character (60). In general terms, the efficiency of binding to the membranes seems to be a key factor to explain efficiency of photo-activity. Efficiency of membrane binding can be related to both the value of

the octanol/water partition coefficient and to the optimised structural matches that allow their incorporation within the membrane (23,60).

Conclusions

Major TD such as malaria, leishmaniasis and trypanosomiasis constitute significant morbidity and mortality, mainly in the developing world. Given the size of the problem, with many millions of infected people, the task of providing effective healthcare is often unachievable, both on grounds of the funding available and also the absence of useful therapeutic regimens. However, recent developments in photoantimicrobials offer considerable potential for use in TD where local application of drug and light are feasible, e.g., in presentations such as cutaneous leishmaniasis. This is particularly appealing where relatively inexpensive photosensitisers, such as methylene blue and crystal violet, have been shown to be effective.

The application of photoantimicrobials to blood product disinfection also offers considerable potential in infection control. As crystal violet is already employed in the (non-illuminated) inactivation of *T. cruzi* by various blood collection agencies, the move to photodecontamination would not constitute a quantum leap. In addition, the use of other photosensitisers, such as methylene blue, is already widespread in Europe, thus providing evidence of efficacy and available technology.

The topical/local approach to tropical disease and pathogen inactivation in the blood supply are not claimed to be an answer to the problem of TD. However, both constitute possible additions to the current therapeutic arsenal, with added value where conventional drug resistance is encountered.

As in the case of any therapeutic modality, better knowledge of the mechanisms of action can allow improvements in terms of sensitivity and selectivity. We believe that the development of more robust photosensitisers based on better understanding of specific action mechanisms could allow an increased role of PACT in the treatment and control of TD.

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