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Photodynamic Therapy in Microbial Infections

Terapia fotodynamiczna w zakażeniach drobnoustrojowych

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Abstract

While photodynamic therapy (PDT) is a well-established modality for the treatment of a variety of malignant tumors, several pathways are being explored to probe its potential for the treatment of a variety of non-cancerous conditions. A rapidly emerging application is the use of PDT against infectious diseases of microbial origin. In particular, oral candidiasis, periodontal diseases, and infected wounds can be considered as most suitable for PDT treatment. The association of the photosensitizer with the outer wall and/or the cytoplasmic membrane is a prerequisite for an efficient inactivation process. The main issue which needs to be addressed in order to make antimicrobial PDT a widely used modality is that of host toxicity. In fact, essentially all photosensitizers which are active against microbial cells are also phototoxic to human cells and tissues (*Adv Clin Exp Med* 2006, 15, 3, 421–426).

Key words: photodynamic therapy, microbia, photosensitizers.

Streszczenie

Terapia fotodynamiczna (PDT) jest dobrze znaną metodą leczenia różnych złośliwych nowotworów, istnieje jednak wiele badań mających określić jej zastosowanie w chorobach nienowotworowych. Użycie PDT w chorobach zakaźnych jest coraz powszechniejsze. W szczególności należy brać pod uwagę kandydiazę jamy ustnej, choroby przyzębia i gojenie się zakażonych ran. Związanie się fotouczulacza ze ścianą zewnętrzną i/lub błoną cytoplazmatyczną jest warunkiem wstępnym skutecznego działania. Główny obszar, który wymaga jeszcze zbadania w ramach antydnoboustrojowej PDT to toksyczność w komórkach gospodarza. Zasadniczo, wszystkie preparaty, które są aktywne w stosunku do komórek drobnoustrojów są także fototoksyczne dla komórek i tkanek ludzkich (*Adv Clin Exp Med* 2006, 15, 3, 421–426).

Słowa kluczowe: terapia fotodynamiczna, drobnoustroje, fotouczulacze.

While photodynamic therapy (PDT) is a well-established modality for the treatment of a variety of malignant tumors, several pathways are being explored to probe its potential for the treatment of a variety of non-cancerous conditions [1]. The recent advent of ALA (5-amino-levulinic acid)-PDT is contributing significantly to such novel developments [2]. A rapidly emerging application is the use of PDT against infectious diseases of microbial origin [3]. Indeed, PDT appears to be endowed with some favorable features which could make this technique quite useful in a field which is increasingly challenged by the onset of multidrug-resistant pathogens. Thus, the development of alternatives to antibiotic treatment becomes more and more imperative. PDT seems to be particularly suitable for the treatment of localized infections,

including those which become chronic after prolonged chemotherapy [3]. Photodynamic processes usually act on a multiplicity of targets, hence their mode of action is markedly different from that typical of most antibiotic drugs. Moreover, the photosensitivity of microorganisms is generally independent of their antibiotic-resistance spectra. A careful choice of the irradiation protocol leads to an extensive decrease in the population of pathogens with minimal damaging effects on the host tissues [3, 4] and no adverse consequences on the normal “friendly” flora. Thus, even though PDT is still in its infancy as regards microbiological applications, it is likely to become a mainstream therapeutic option in the near future, at least for specific indications.

Indications

Antimicrobial PDT is still at the dawn of its development. However, the rapid advances of our knowledge as regards the mode of action of photosensitizers on the cells of pathogenic agents and their potential hosts as well as the involvement of an increasing number of research/medical centers in pre-clinical investigations allows one to predict that PDT will be more and more frequently used for the eradication of localized infections. In particular, the following indications can be considered as most suitable for PDT treatment.

Oral Candidiasis

Candida albicans, the causative agent of oral thrush, is readily susceptible to photodynamic inactivation [5]. Topical application of the photosensitizer would allow for photodamage to be confined within the lesion, thus sparing the microflora at other sites. This approach would be of particular importance to HIV-infected patients [6], where *Candida* infections are quite frequent, since a local phototherapy is not expected to cause an increased burden on the immune system or the undesired collateral effects associated with conventional antifungal agents.

Periodontal Diseases

These diseases are consequent to chronic infections caused by a mixture of Gram-positive and Gram-negative bacteria growing as a biofilm to generate so-called subgingival plaque. While current treatment protocols for chronic periodontitis involve the mechanical removal of the biofilm by laborious and often unpleasant procedures, PDT has been proposed as a viable alternative [7]. Several oral pathogens (e.g. *Streptococcus* sp. and *Staphylococcus* sp.) are efficiently eradicated by photodynamic treatment, both in aqueous suspension and as a biofilm [7, 8]. The protocol would involve the deposition of the photosensitizer in the dental pocket followed by irradiation with light delivered via optical fibers. The procedure can usually be completed in a few minutes. This feature would give PDT a significant advantage over treatment with antiseptics and antibiotics, which are difficult to maintain in appreciable concentrations within the periodontal pocket for prolonged periods of time.

Infected Wounds

Indolent and chronic wounds are most frequently contaminated by bacteria, and this contamina-

tion normally causes delayed healing and prolonged hospitalisation. Wound infections are commonly treated with antibiotics or various types of topical products (e.g. polymyxin B, mupirocin, silver nitrate or silver sulfadiazene); however, the emergence of antibiotic-resistant bacterial strains and the toxic effects of silver compounds calls for alternative and more powerful therapeutic approaches. PDT may represent a very useful tool to treat bacterial contaminants of wounds because of the broad specificity of action of adequately selected photosensitizing agents against both wild and antibiotic-resistant strains [3, 4]. Moreover, it has been demonstrated [9] that PDT, especially if applied at low light doses, can up-regulate the expression of growth factors, thereby stimulating wound healing.

Photosensitized Inactivation of Microbial Cells: General Aspects

Microbial cells display a truly large variety in size, subcellular architecture, biochemical composition, and susceptibility to photosensitized processes. However, a generally valid mechanism can be proposed for describing the action of photodynamic processes on such cells, especially since in most cases the cytoplasmic membrane represents the main target. Thus the association of the photosensitizer with the outer wall and/or the cytoplasmic membrane represents a prerequisite for an efficient inactivation process [3]. In Gram-positive bacteria, the outer wall (thickness about 40–80 nm, with a fairly homogeneous biochemical composition) is readily crossed by a variety of compounds, especially those of hydrophilic nature, photosensitizing molecules, even of relatively high molecular weight, can reach the cytoplasmic membrane in significant amounts. The binding process occurs at a very fast rate and the concentration of the photosensitizer in membranous districts reaches maximum values within 1–5 min. of incubation. For Gram-negative bacteria, which contain a highly organized additional layer in the cell wall acting as an effective permeation barrier, no appreciable photosensitivity is observed unless the tight three-dimensional architecture of the outer membrane is preliminarily disrupted so that the photosensitizer can translocate to the plasma membrane [3]. This goal can be achieved either by pre-treatment with agents such as EDTA, which remove the divalent cations (e.g. Ca^{2+} ions), neutralizing the negative charges present on the outer wall [10], or by incubation with cationic compounds, including the nonapeptide polymyxin B [11].

A variety of cationic photosensitisers, including phenothiazines, porphyrins, and phthalocyanines, were successfully tested as photoinactivating agents for Gram-positive and Gram-negative bacteria. The cationic dyes establish an ionic linkage with the anionic groups at the cell surface and, upon photoexcitation, induce an oxidative modification of the outer-wall constituents present in their microenvironment. The formation of oxidized species leads to a perturbation in the native three-dimensional structure, thus allowing the influx of photosensitizer molecules to the cytoplasmic membrane.

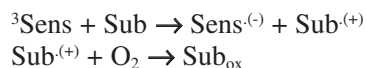
Yeasts, such as *Candida albicans* and *Saccharomyces cerevisiae*, are eukaryotic cells, but they are surrounded by an outer membrane (even though the properties of such membrane are substantially different from those typical of Gram-negative bacteria). As a result, yeast photoinactivation also requires the pre-binding of the photosensitizer with the external wall. However, in this case, even anionic photosensitizers such as haematoporphyrin, are effective [12, 13]. The scheme outlined above does not apply to one class of bacteria, i.e. mollicutes. These bacteria, some of which are also named mycoplasmas, are genetically deficient in cell wall structures; therefore, the cytoplasmic membrane is directly accessible to externally added photosensitizing agents and these cells are very susceptible to photodynamic inactivation mediated by either positively or negatively charged as well as by neutral dyes [14]. The degree of photosensitivity of mollicutes is correlated with their content in cholesterol, which they can readily accumulate from the medium. In particular, cholesterol imparts rigidity to the cytoplasmic membrane and acts as a scavenger of reactive oxygen species: thus, the cholesterol-rich *Mycoplasma hominis* is markedly less photosensitive than the cholesterol-deprived *Acholeplasma laidlawii* [14].

In all cases, the rate and extent of photosensitized inactivation of microbial cells appear to be very similar for wild strains compared with methicillin- or vancomycin-resistant strains [12].

Photosensitized Inactivation of Microbial Cells: Photobiological Aspects

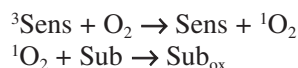
As is well known [3], photodynamic processes proceed by two competitive mechanisms, both of which require the participation of the long-lived photo-excited triplet state of the photosensitizer ($^3\text{Sens}$) as the reactive intermediate. The type I process involves an electron transfer step between the

triplet photosensitizer and a nearby substrate (Sub) with generation of radical species; the latter are then intercepted by oxygen, yielding oxidized products. A typical scheme for a type I photosensitization mechanism can be outlined as follows:



The direction of the electron transfer event between the photosensitizer and the substrate is controlled by their relative redox potential.

Alternatively, a type II mechanism involves an energy transfer step from the triplet photosensitizer to a suitable acceptor, most frequently oxygen. The latter compound is converted to a highly reactive derivative, named singlet oxygen ($^1\text{O}_2$), which in turn attacks photosensitive targets in its surroundings:



It is generally assumed [12] that singlet oxygen represents the main cytotoxic agent responsible for the photosensitized inactivation of microbial cells. Indeed it has been observed that the cultivation of *Staphylococci* in the presence of singlet oxygen scavengers, such as methionine or histidine, provides a high level of protection to the photosensitized cultures [15]. At the same time, some authors demonstrated that singlet oxygen, generated by a photosensitizer deposited on an inert matrix and physically separated from the microbial cell culture, can diffuse through an air-equilibrated medium and cause irreversible damage to *Streptococcus faecium* and *E. coli* [16]. However, the possibility of a contribution to the overall photoprocess from radical-involving mechanisms cannot be ruled out, since the close spatial relationship between a cell-associated photosensitizer and photosensitive cell constituents could favor a direct interaction.

In general, all the photosensitizers which have been found to be very active as photoantimicrobial agents preferentially localize in the cytoplasmic membrane. One important exception is represented by acridines, which largely intercalate with DNA bases [17]. The main alterations of cell functions and morphology caused by photodynamic inactivation are typical of damaged membranous domains (see Table 1). No involvement of the genetic material is generally observed until the late stages of the overall photoprocess, which indicates that such photodamage is not correlated with cell death [3]. This pattern of photo-induced subcellular damage is in agreement with the lack of mutagenic effects as well as the lack of selection of photo-resistant microbial strains even after several photosensitization treatments.

Table 1. Main functional and morphological effects of photosensitized processes in microbial cells**Tabela 1.** Główne czynnościowe i morfologiczne skutki procesów fotouczulania w komórkach drobnoustrojów

Type of photodamage (Rodzaj fotouszkodzeń)	Specific example (Przykład)	Comments (Uwagi)
Functional damage		
Loss of enzymic activities	inactivation of NADH/lactic dehydrogenase	drop in activity in membrane enzymes well correlates with drop in survival
Protein-protein cross-links	many membrane and cytoplasmic proteins	cytoplasmic proteins involved upon Sens rediffusion from the membrane
Inhibition of metabolic processes	inhibition of DNA synthesis and glucose transport	RNA and protein synthesis are consequently blocked
Morphological damage		
Alteration of the mesosome structure	increased volume and frequency of appearance	reflects a disturbed synthesis of membrane and cell wall
Alteration of chromatin	appearance of electron- transparent areas with highly packed nucleic acid	late stage in the overall photoprocess

Conclusions and Perspectives

Several factors appear to promote the use of PDT as an alternative therapeutic approach for those infectious diseases of microbial origin that recur or become chronic after antibiotic treatment. At present, the most promising applications involve local application of the photosensitizer both in terms of the likely speed of treatment and reduced risk of undesired side effects, including the low probability of injury to indigenous bacteria remote from the site of the phototreatment which are often affected during systemic antibiotic therapy. In fact, the major advances which have characterized the research in the field of antimicrobial PDT during the past few years allowed the definition of specific favorable features, including:

1) The short time of the whole phototreatment owing to the generally very fast uptake of the photosensitizing agent by microbial cells followed by the relatively small light doses which are required to obtain a substantial reduction in the population of microbial pathogens;

2) Confinement of the overall photo-damaging process within a restricted spatial range as a consequence of the short lifetime of the photochemically produced cytotoxic species; thus it has been estimated that endocellularly generated singlet oxygen cannot migrate beyond 10 nm from its generation site [3];

3) The broad spectrum of activity of photosensitizers, such as phenothiazines or cationic porphyrins, which have been shown to efficiently photosensitize the inactivation of Gram-positive and Gram-negative bacteria, mycoplasmas, yeasts, and parasites. These photosensitizers exhibit no

detectable toxic action against prokaryotic and eukaryotic cells at photobiologically active doses in the absence of photoactivation;

4) Independence of the degree of antimicrobial photoactivity from the antibiotic-resistance spectra of microbial cells. This property is particularly useful for developing combined therapeutic protocols in the treatment of poorly responding infectious diseases;

5) No significant development of resistance to PDT in microbial cells which have been only partially inactivated and are exposed to repeated sessions of the photosensitizer + visible light treatment;

6) Lack of onset of mutagenic effects in photosensitized cells. This feature underlines a very important difference between PDT and UV light-treatment, which typically causes the development and selection of mutants with unknown properties;

7) Possibility to activate the photosensitizer *in situ* by non-coherent visible light sources, which are typically of low cost, require minor protective measures for the operators and patients, and are powered by inexpensive technology.

One open question in this field is the choice of the photosensitizer(s) to be employed in a given application. A truly large variety of photosensitizing drugs have been tested so far (see [6] and [30] for an exhaustive review), including compounds of natural origin such as hypericin, psoralens, and thiophenes. At present, the attention of investigators is being more and more centered on porphyrins and their analogs, such as phthalocyanines. Moreover, porphyrins typically absorb essentially all the wavelengths in the visible light spectrum. This would allow one to select the irradiation wavelength depending on the thickness of the lesion to be treated. As is known [18], the penetration depth

of light into human tissues is strongly dependent on the wavelength and ranges from a few millimeters in the near-UV/blue region (380–450 nm) to 1.5–2 cm in the red spectral region (650–800 nm). This aspect obviously enhances the selectivity of PDT treatment. Lastly, the chemical structure of porphyrins and their derivatives can be modulated by the introduction of different functions into the molecule, including the coordination of metal ions with the pyrrole nitrogens, the addition of axial ligands to the metal ion, and the attachment of substituents in the peripheral positions of the tetrapyrrolic macrocycle [19]. In this way, the physicochemical properties (e.g. the degree of lipophilicity or hydrophilicity) as well as the photobiological properties of the photosensitizer can be tailored to the biochemical and physiological characteristics of any given microbial pathogen. This would broaden the number of photosensitizing agents which can be used for the phototherapeutic treatment of infectious diseases.

The main area which needs to be addressed in order to make antimicrobial PDT a widely used modality is that of host toxicity. In fact, essentially all those photosensitizers which are active against microbial cells are also phototoxic to human cells and tissues. In order to increase the selectivity of phototreatment, Berthiaume et al. [20] deve-

loped a specific antibacterial photosensitizer immunoconjugate and tested it in mice whose dorsal skin had been infected with *Pseudomonas aeruginosa*, a 75% decrease in the number of viable bacteria was observed in the photo-treated site, whereas little inhibition of bacterial growth occurred in animals that had received a non-specific conjugate. While antibody-targeted photolysis can certainly represent a selective and useful tool for treating specific infections, its widespread use is questionable based on both the cost of the procedure and the intrinsic selectivity associated with the local deposition of the photosensitizer. Several reports point out that careful control of the individual parameters involved in the PDT protocol allows one to achieve a very high differential phototoxicity between microbial and human cells, as shown by experiments with methicillin-resistant *S. aureus* against fibroblasts and keratinocytes as well as with *Helicobacter pylori* against rat gastric mucosa [21]. In general, a 4–5 log reduction in the microbial population can be achieved by using much shorter incubation times prior to irradiation and lower light doses compared with those required to inactivate human cells. An outline of the optimal values which appear to induce an extensive drop in the survival of microbial cells with minimal damage to host tissues is given in Table 2.

Table 2. PDT protocol yielding an efficient and selective phototoxic action on microbial pathogens with minimal damage to host tissues

Tabela 2. Protokół PDT dotyczący skutecznego i selektywnego oddziaływania metody na drobnoustroje patogenne przy minimalnych uszkodzeniach tkanek gospodarza

Parameter (Wskaźnik)	Optimal values (Optymalne wartości)
Cell-photosensitizer incubation time prior to irradiation	1–5 min.
Photosensitizer dose	0.1–1 μM
Delivery system	either free or in combination with dextran, polypeptides and albumin; not effective against Gram(–) if associated with liposomes
Ionic strength	efficiency is independent of the salt concentration
Fluence rate	lower than 50 mW/cm ²
Total light fluence (irradiation time)	lower than 3 J/cm ² (10 min.)

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