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Photodynamic Antimicrobial Therapy

Mini-Review

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Abstract: Photodynamic antimicrobial therapy (PACT) involves the utilisation of photosensitizers activated by exposure to visible light in order to eradicate microbes (this method has already been applied in photodynamic therapy of tumours). Photodynamic effect of the particular photosensitive substance (PS) is attributed to its ability to penetrate susceptible microorganisms, to absorb the light of certain wavelength, and to generate reactive cytotoxic oxygen products. The target microorganisms for photoinactivation are bacteria, fungi, viruses and protozoa. Photodynamic antimicrobial therapy is proposed as a potentially topical, non-invasive approach suitable for treatment of locally occurring infection. The fact that bacteria are becoming increasingly resistant to antibiotics and antiseptics has lead to an increased interest in the development of new alternative eradication methods, such as PACT. Research and development of photosensitive substances are aimed at finding effective antimicrobial substances, which would have a broad-spectrum potency.

Keywords: *Photodynamic antimicrobial therapy • Photoinactivation • Photosensitizers • Phthalocyanines • Antibacterial effect*

1. Introduction

Photodynamic antimicrobial therapy (PACT) is based on the concept that a non-toxic photosensitiser localized in certain cells can be activated by low doses of visible light of the appropriate wave-length, to generate singlet oxygen and free radicals that are cytotoxic to target cells. This phenomenon was first described by Oscar Raab in 1890 when he noted toxicity of acridine orange, which was dependent on light – against *Paramecium caudatum*. However, studies of this phenomenon and its practical use didn´t appear until the second half of the 20th century. It was first proposed for use in the area of tumour therapy and later their use in antimicrobial therapy were studied [1]. Photodynamic anti-tumour therapy (PDT) is based on the fact that photosensitive substance administered systematically are preferentially incorporated in rapidly proliferating tumorous tissue, and after its irradiation the cell structures are damaged due to the development of reactive cytotoxic products and subsequent apoptosis of tumorous cells. The limiting factor is localisation of the tumour and the possibility of targeted irradiation. The first successful use of PDT was described in skin tumour therapy – by local application of eosin, which accumulates quickly in the proliferating neoplastic cells [1-3]. Nowadays, porphyrin derivatives (Photofrin, Foscan) are used clinically; they were approved for the use in oncologic patients in the USA, Canada, France, the European Union and Japan. They are used for treatment of bladder tumours, lung tumours, gallet and stomach tumours, and for the tumours of neck and head. Although porphyrin substances are effective in PDT, it is known that after parenteral administration there are some undesirable side effects such as prolonged skin photosensitivity [4]. In dermatology, PDT is successfully used for larger inoperable basal cell carcinomas; substances containing the delta-aminolevulinic acid (precursor of photosensitive protoporphyrin IX) are applied. The application of these

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compounds is less widespread in treatment of psoriasis and actinic keratoses. PDT is also used in ophthalmology for age-related macular degeneration therapy.

PDT was first studied in the 1980's and 1990's, PDT for the use in antimicrobial treatment because of the increasing resistance of pathogenic microbes to various types of antibiotics and the subsequent need for alternative therapeutical means. The initial research proved that photosensitive substances, when activated by light, are much more toxic to microbes than to human cells [4-7].

2. Mechanism of photoinactivation

During photoinactivation of microbes or tumour cells, target cells are damaged by the interaction of harmless visible light with a photosensitive substance in the presence of oxygen [8]. The photosensitive substance (PS) itself has no or negligible antibacterial effect but after its irradiation by light of the appropriate wave length (during this process, photon is absorbed by the photosensitive substance), the substance gets from the initial quiescent state to an unstable, excited stage, in which highly reactive cytotoxic products can develop after the reaction with the environment in the presence of oxygen. The result of the reaction are cytotoxic products: hydroxyl radical $OH^{\cdot}$, superoxid radical or singlet oxygen $^1O_2$ (molecular oxygen O_2 in the primary state contains 6 conduction electrons, 2 are unpaired after accepting certain amount of energy; these electrons conjugate and singlet oxygen is generated). Singlet oxygen has a very short half-time period (nanoseconds) and diffusion limited to a distance of up to 100 nm; hence any cytotoxic activity is confined only to its immediate environment. Therefore, the accumulation of the photosensitive substance in the target cell or its close surroundings is required for successful photoinactivation. Lethal cell damage is caused by the destruction of nucleic acids, cell wall or cytoplasmatic membrane while the cellular enzymatic and transport mechanisms are inactivated.

3. Photosensitizers and light sources

The process of photoinactivation of microbes is dependent on the photosensitive substance and light. Especially, important is the type of PS, its concentration, laser type (wave-length of the beam should be as close as possible to the absorption maximum of the PS) and the dose of light applied by irradiation.

Many natural as well as synthetic photosensitive substances are known, typically dyes, usually belonging to aromatic compounds. Photoinactivation of microbes by PACT involves the use of derivates of phenothiazines - toluidine blue O and methylene blue; porphyrins - hematoporphyrin, delta-aminolevulinic acid; xanthenes – rose bengal, chlorins - chlorin(e6), derivates of chlorin(e6) with poly-L-lysine and polyethyleneimine; phthalocyanines - disulphonated phthalocyanine of aluminium, cationic phthalocyanines of zinc, disulphonated phthalocyanine of zinc.

Photodynamic effect of the particular PS is attributed to its ability to penetrate into the sensitive microorganism, to absorb the light of certain wave-length and to generate reactive cytotoxic oxygen products. In this respect, phthalocyanines seem to be a promising group of photosensitive substances [9,10].

Phthalocyanines are heterocyclic adducts composed of a tetrapyrrole nucleus connected by nitrogen bridges. Some of their derivates are used as dyes for printers (blue colour), paints or plastics. They are effective photosensitive substances. After their irradiation, a large quantity of singlet oxygen species are generated (they are able to remain in the excited state for a longer period compared to methylene or toluidine blue). Moreover, they are more resistant to chemical or photochemical degradation. They absorb light at a wave-length between 660-700 nm, which verges on the infra-red end of the spectrum.

Practical use of phthalocyanines was studied in connection with decontamination of blood products. The absorption maximum of phthalocyanines varies from that of erythrocytes and thus the risk of their possible damage is low. Phthalocyanines also showed promising results in photodynamic experimental therapy of cancers. Contrary to the first generation of porphyrins, phthalocyanines were more effective when tested on animals during anti-tumour therapy, and the occurrence of undesirable side effects was lower. Study of antimicrobial characteristics of phthalocyanines in PACT is mainly in the stage of *in vitro* experiments.

Exposure to visible light of a particular wave-length is necessary for the activation of photosensitive substances. Most photosensitive substances are activated by red light in the range of 630–700 nm. This corresponds to the light beam penetrating the tissue up to 0.5–1.5 cm, and, at the same time, represents the limit for therapeutical effect of PDT. Technically, it is possible to use various sources of light. However, lasers and lamps with the possibility of higher light energy seem to be a better option. Lasers are able to generate a monochromatic beam of light and, according to the quantity of light energy, they are divided into high-power and low-power lasers. It is known that while using high-power lasers, lethal antimicrobial effect as a result of

the irradiation itself might occur. A strong antibacterial effect while using the Er:YAG laser for treatment of root canal was described [11], but the necessity of keeping the precise quantity of applied light energy was emphasised. When the subliminal doses were used, there was no bactericidal effect, however, higher doses of energy brought on undesirable complication in the form of structural damage to the root canal. For irradiation of photosensitive substances by PACT, low-power lasers are used (it is important to influence only the photosensitive substance and thus minimize the undesirable damage of the surrounding tissue). Photoinactivation of bacteria is achieved by light doses in the range of mW. Irradiation of bacteria in the absence of PS has no influence on the viability of bacterial cells.

4. Applications of PACT

4.1 Target microorganisms

The target microorganisms for photoinactivation are mainly bacteria. The possibility of bacterial photoinactivation by PACT has been experimentally proved many times – various chemical photosensitive substances have been used to different bacterial strains (Table 1). Significant differences in the effectiveness of PACT was noted with respect to photoinactivation of Gram-positive and Gram-negative bacteria. Gram-positive bacteria are rather sensitive to photoinactivation [12-14]. This antibacterial effect is achieved by photoinactivation mediated by photosensitive substances with different chemical structures (positively and negatively charged substances were effective as well as neutral ones). Gram-negative bacteria are usually resistant to the action of negatively charged or neutral photosensitizers [15-17]. This difference can be explained by the different structures of the bacterial cell wall. There is a permeable outer peptidoglycan layer in Gram-positive bacteria, which enables the penetration of photosensitive substance to the cytoplasmatic membrane, the target of PACT. Thickening of the cell wall of MRSA (methicilin-resistant *Staphylococcus aureus*) or VRE (vancomycin-resistant enterococci) diminishes the penetration of antibiotics, antiseptics and disinfectants, however, the decrease in effectiveness of PACT has not been proved so far [18]. The wall of Gram-

Microorganism	Photosensitive Substance	Reference
Staphylococcus aureus, S. epidermidis	Chlorin (e6) Methylene blue	Gad *et al.* (2004) [38]
Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa	Chlorin (e6)	Tegos *et al.* (2006) [29]
MRSA (methicilin resistant *Staphylococcus aureus*), *Streptococcus sanguinis*	Chlorin (e6)	Embleton *et al.* (2002) [50]
MRSA	Phthalocyanines	Griffiths *et al.* (1997) [28] Soncin *et al.* (2002) [18]
Enterococcus sp., *Pseudomonas aeruginosa, Escherichia coli*	Phthalocyanines	Minnock *et al.* (1996) [30]
Streptococcus mutans, S. sobrinus, Lactobacillus casei, Actinomyces viscosus	Phthalocyanines	Burns *et al.* (1994) [12]
Porphyromonas gingivalis, Bacteroides forsythus, Fusobacterium nucleatum, Campylobacter rectus, Eikenella corrodens, Actinobacillus actinomycetemcomitans, Actinomyces viscosus, Streptococcus sobrinus, S. mitis, S. oralis, S. mutans	Porphyrins	Rovaldi *et al.* (2000) [13]
Porphyromonas gingivalis, Fusobacterium nucleatum, Actinobacillus actinomycetemcomitans	Phthalocyanines	Wilson *et al.* (1993) [14]
Porphyromonas gingivalis	Toluidine blue O	Kömerik *et al.* (2003) [16]
Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae	Phthalocyanines	Wilson *et al.* (2002) [24]
Escherichia coli	Phthalocyanines	Bertoloni *et al.* (1990) [25] Minnock *et al.* (2000) [17] Lacey *et al.* (2001) [23]
Helicobacter pylori	Porphyrins	Hamblin *et al.* (2005) [27]
Candida albicans	Phthalocyanines Toluidine blue O	Chiti *et al.* (2005) [32] Donelly *et al.* (2007) [33]
Aspergillus fumigatus	Porphyrins	Friedberg *et al.* (2001) [34]
Plasmodium falciparum	Phthalocyanines	Lustigman *et al.* (1996) [37]
Acanthamoeba palestinensis	Phthalocyanines	Ferro *et al.* (2006) [36]

Table 1. Summary of substances and microorganisms tested by photosensitisation methods.

negative bacteria is more complex. There is an outer membrane composed of phospholipid double-layer with lipopolysaccharides above the thinner peptidoglycan layer. The wall of Gram-negative bacteria is normally only slightly permeable to large and hydrophobic molecules. Negatively charged photosensitive substances (PSs) are not able to penetrate the lipopolysaccharide but they can be partially effective at higher concentrations when more singlet oxygen is generated in close proximity to the bacterial cells. The sensitivity of Gram-negative cells to photoinactivation is increased by using PSs with polycationic molecular structures – natural or artificially produced by binding of positively charged functional groups (they can promote a tight electrostatic interaction with the negatively charged sites on the outer surface of the bacterial cells). Cationic PSs are able to inactivate wider range of bacteria species than neutral or anionic PSs. In micromolar concentration, they can lower the amount of bacteria by 4-5 logs after incubation up to 5-10 minutes and irradiation of about 50 mW/cm^2 [19]. It is also effective against bacteria resistant to antibiotics and bacteria strains growing in biofilm [20-22]. The sensitivity of Gram-negative bacteria to photoinactivation can also be increased by adding substances such as EDTA (ethylenediaminetetraacetic acid) or polymyxin B, which increase the permeability of the outer wall of Gram-negative bacteria by releasing up to 50% of lipopolysaccharide [23-25]. Nevertheless, some bacteria (e.g. *Burholderia cepacia, Proteus mirabilis*) are already naturally resistant to cationic structures, which may decrease the effect of inactivation mediated by positively charged PS. Moreover, a study published in 1988 describes the isolation of *Salmonella enterica* serovar Typhimurium, showing a low degree of resistance to cationic structures as lipopolysaccharide of this strain had generally lower negative charge when compared to sensitive strains [26]. While studying PACT and different bacterial strains, it was discovered that there is a heterogeneous group of bacteria, which is inactivated only by irradiation by light of certain wave-length, even without the presence of photosensitive substance. Thorough analysis of these bacterial strains proved that their photosensitivity is given by the accumulation of naturally generated porphyrins inside the bacterial body; mainly it concerned precursors generated at synthesis of hematoporphyrins. This group consisting mainly of black-pigmented anaerobes *Porphyromonas* sp. and *Prevotela* sp., in which porphyrins are accumulated, and thus they are photosensitive depending on the outer environment and its conditions. The ability to accumulate porphyrins has been demonstrated in *Propionibacterium acnes* and *Helicobacter pylori* [27]. Our knowledge of naturally produced photoactive porphyrins is still only fragmented and an explanation of the causes of this phenomenon is still unknown.

The available sources show that during PACT wild as well as multi-resistant bacterial strains are killed with equal effectiveness. This has been repeatedly proven with MRSA and *Pseudomonas aeruginosa* [28-30]. The fact is that the resistance to photochemical destruction of microbes mediated by singlet oxygen and other reactive cytotoxic products is highly improbable (it is a natural mechanism of endocellular destruction) and only the theoretical possibility of its occurrence has been discussed. Nevertheless, it was proven *in vitro* that bacteria are able to eject the molecules of porphyrins derivatives by the efflux mechanism and, theoretically, to decrease their sensitivity by lowering the permeability of cell wall for PS [31]. The same mechanism is the basis of bacteria resistance to antiseptics and antibiotics.

Other possible candidates for PACT are pathogenic fungi, especially *Candida albicans* [32,33]. Fungi are more resistant to PACT due to larger cells and the presence of nuclear membrane, which is another obstacle to penetration of PS to the target structure. During experiments, it was necessary to use higher concentrations and longer exposure of the PS, and higher doses of light than necessary for the photoinactivation of bacteria. *Aspergillus fumigatus* has also been tested [34].

Photoinactivation of viruses by PACT has been tested in some countries to eliminate viruses from blood products in special fluorescent boxes. The new indications for PACT include many types of viral skin infections that are caused by the human papilloma virus (different kinds of verrucae) and herpes simplex virus (herpes simplex). In recent years, the possibility to photoinactivate parasitic protozoa has been studied. Photoinactivation of promastigotes and amastigotes of *Leishmania amazonensis* was described in laboratory conditions [35]. Photoinactivation of *Plasmodium falciparum* and *Acanthamoeba palestinensis* was investigated, too [36,37].

4.2 Experimental studies

Photodynamic inactivation has been studied only *in vitro* so far. Laboratory tests are usually carried out on planktonic cultures of microbes, but some authors have tried to confirm bacterial photoinactivation in the presence of blood, saliva, serum or dental plaque. The presence of blood, saliva or serum offers the bacteria certain protection against photoinactivation [38]. It is probably caused by the presence of proteins, which are able to absorb photons of light and thus lower their availability for the interaction with the PS, and therefore lower the number of PS molecules bound to specific

targets in bacterial cell. It has been proven that the bond of the PS to the target cells is five times lower in serum (due to higher content of proteins) than in saliva [39]. Bacteria in biofilm have a different phenotype; they grow slower and are generally more resistant to antimicrobial substances [40]. Wood *et al.* (1999) and Senda *et al.* (2000) [21,41], exposed artificially prepared dental plaque to the cationic derivate of phthalocyanine and proved it not only inactivated bacteria, but they were also able to reduce the thickness of the plaque by half (the examination under the electron microscope showed vacuolisation of cytoplasm and membrane damage). It seems that the damage to the bacterial wall lowered the ability of the cells to bind to each other and to the extracellular matrix. The bactericidal effect was demonstrated only on the surface of the plaque under aerobic conditions; in deeper layers the bactericidal effect was not noted due to lack of oxygen. The lethal effect of photosensitisation is also influenced by pH. Optimal environment is neutral; fluctuation to both sides lowers the effect of photoinactivation [42].

PACT was studied using animal models, as well. The influence of PACT on infected wounds, burns, infection of soft tissues, disorders caused by *Helicobacter pylori*, and brain abscess have been evaluated. Application of PS and local irradiation in mice showed a significant reduction of bacteria infected wounds and burns, better healing of wounds and no sepsis developed [43,44]. In a study on rats, it was possible to eradicate *Porphyromonas gingivalis* in the periodontal pockets surrounding the affected tooth by using PACT (toluidine blue as a photosensitive substance). This therapy led to a significant decrease of bone loss, which is connected to periodontidis - without detected damage of surrounding tissue, which was histologically examined [45]. While studying ferrets, after using PACT with toluidine blue, the mucosal lesions caused by *Helicobacter mustelae* were healed. In immunocompromised mice, after the application of methylene blue and its subsequent activation by light, *C. albicans* was completely eradicated from the mucosal lesion on the surface of tongue [46].

Complications of using PACT include, lower specific toxicity and the possibility of damaging surrounding host cells and commensal microflora. It is fortunate that most *in vitro* studies using tissue culture cells showed that the concentration of the PS and light dose necessary for photoinactivation of bacteria has a minimal influence on the viability of host cells. The methods of precise targeting of PSs on infectious agents continue to be improved, due to the increased lethal effectiveness of PSs and to decrease the concentration and the light dose. Bhatti *et al.* (2000) using monoclonal antibodies against lipopolysaccharide *P. gingivalis* on toluidine blue increased selective photoinactivation of mixed bacterial culture (*Streptococcus sanguinis, P. gingivalis*) [39]. Embleton *et al.* (2005) achieved a similar effect by means of bacteriphages to deliver the photosensitizer to *S. aureus* [47].

4.3 Clinical Use Options

The present knowledge shows that PACT will mainly be used locally with the application of photosensitive substance directly and, at the same time, have open access for the irradiation of a particular light wavelength. This approach has some advantages compared to the standard antimicrobial and antiseptic substances, such as lower risk of damage to physiological microflora and host cells outside the treated areas (so called dual selectivity). The procedure should mainly prevent opportunistic infections. Local administration of the photosensitive substance should avoid the systemic skin photosensitisation, which was a problem of the first generation of porphyrins during photodynamic antitumour therapy [4]. It is assumed that PACT will be used for infected wounds and burns therapy, rapidly expanding infections of soft tissues, abscesses, microbial keratitis, inflammatory processes in ears, nasal sinuses, bladder and stomach [6], further for inflammatory lesions in mouth connected with the presence of dental plaque, *i.e.* for complex treatment of periodontidis and surface tooth decay [48,49]. Previous research has shown that during PACT, wild as well as multi-resistant bacteria strains are killed and thus photodynamic antimicrobial therapy could become an effective mode in treatment of bacterial infections caused by multi-resistant strains, which are a real threat especially in hospitals [5].

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