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Photodynamic Therapy: Current Status and Future Directions

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Key Words
Photodynamic therapy · Photosensitizer · Porphyrin · Singlet oxygen · Hydroxyl radical · Superoxide

Abstract
Photodynamic therapy (PDT) is a minimally invasive therapeutic modality used for the management of a variety of cancers and benign diseases. The destruction of unwanted cells and tissues in PDT is achieved by the use of visible or near-infrared radiation to activate a light-absorbing compound (a photosensitizer, PS), which, in the presence of molecular oxygen, leads to the production of singlet oxygen and other reactive oxygen species. These cytotoxic species damage and kill target cells. The development of new PSs with properties optimized for PDT applications is crucial for the improvement of the therapeutic outcome. This review outlines the principles of PDT and discusses the relationship between the structure and physicochemical properties of a PS, its cellular uptake and subcellular localization, and its effect on PDT outcome and efficacy.

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Introduction

Photodynamic therapy (PDT) is a noninvasive, highly selective method for the destruction of unwanted cells and tissues. It was clinically approved more than a quarter of a century ago for the treatment of a small number of selected tumors [1] and has expanded tremendously to include areas of application as diverse as cardiology [2, 3], urology [4], immunology [5], ophthalmology [6, 7], dentistry [8, 9], dermatology [10, 11] and cosmetics [12, 13]. Antimicrobial/antiviral PDT has been successfully used for the treatment of viral infections [14, 15], against antibiotic-resistant bacterial [16, 17] and fungal strains [18–20], for the inactivation of pathogens in blood products [21], for water sterilization [22, 23] and for disinfection and sanitation of surfaces [24, 25]. The photodynamic process is successfully used for drug delivery and the release of endocytosed macromolecules in the cytosol [26, 27].

Among the main advantages of PDT is its high selectivity. It is based on the requirement for the simultaneous presence of three components, photosensitizer (PS), molecular oxygen, and visible or near-infrared (NIR) light, none of which is toxic or cell/tissue damaging by itself. Ideally, the PS is taken up and accumulates preferentially in the targeted cells. Because the PS is harmless in the absence

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of light, generalized toxicity in PDT is avoided by illuminating only the desired area. A PS absorbs light energy and transfers it to other nonabsorbing molecules. Such energy or electron transfer generates highly reactive species, which kill the targeted cells, damage tumor-associated vasculature and activate an antitumor immune response.

Mechanism of the Photodynamic Action

PSs are substances with a particular arrangement of electrons in their molecular orbitals. Like almost all molecules, at ground (singlet) state, PSs have couples of electrons with opposite spins in low-energy molecular orbitals. The absorption of light with the appropriate wavelength lifts an electron to a high-energy orbital without changing its spin. This is a short-lived (nanoseconds) excited singlet (S_1) state and the PS can lose its energy and return to the ground state by emitting light (fluorescence) or heat. Alternatively, the S_1 state can undergo a process known as intersystem crossing, where the spin of the excited electron is inverted. This inversion of the electron spin is the reason for the relatively long life (microseconds) of the excited triplet (T_1) state. Radiative triplet-to-singlet transition is forbidden because it requires a change of the electron spin, which is a slow process [28]. From the T_1 state, the PS can relax back to the ground state by emitting light (phosphorescence) or by transferring energy to another molecule. It can also lose energy through internal conversion or radiationless transitions during collisions with other molecules. The longer the life of the PS in the T_1 state, the higher are the chances that it will encounter a collision with another molecule, ending up with the production of chemically reactive species.

Type I and Type II Processes

While in a T_1 state, a PS can be involved in two types of processes (fig. 1). In a type I process, the PS in a T_1 state abstracts an electron from a reducing molecule in its vicinity. Among such electron-donating molecules are reduced NADPH, guanine in nucleic acids, and tryptophan and tyrosine in proteins. As a result, a pair of radical anion ($PS^{-}\cdot$) and radical cations ($biomolecule^{+}\cdot$) are formed. In an aerobic environment, the PS radical anion donates its extra electron to O_2, producing a superoxide anion radical ($O_2^{-}\cdot$) and restoring the PS [29].

Superoxide can act as either a univalent oxidant or a reductant. It can oxidize small molecules such as sulfite, tetrahydroflavins, leukoflavins, catecholamines, the enediolate tautomers of sugars, and other good reductants

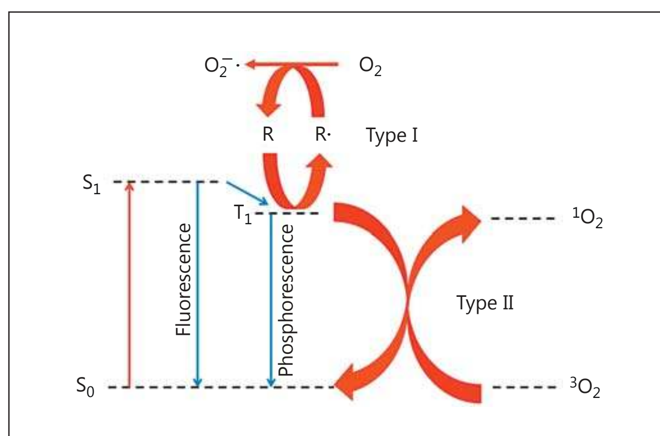
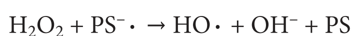


Fig. 1. Photoexcitation and participation of a PS in type I and type II processes.

[30], but it does not react directly with nucleic acids, lipids or carbohydrates [31]. The superoxide radical, however, can react at almost diffusion-limited rates ($k \geq 10^9$ M^{-1} s^{-1}) with other biologically relevant radicals, releasing potentially toxic cell-damaging products including organic hydroperoxides and quinones [32]. Among such radicals are nitric oxide ($NO\cdot$) and the phenoxyl radicals generated by one-electron oxidation of phenols. In biosystems, the most abundant phenol is the amino acid tyrosine. The tyrosyl radical reacts with superoxide with a rate constant of 1.5×10^9 M^{-1} s^{-1} [33]. It has been suggested that hydrogen bonding to an amine group increases the electrophilicity of $O_2^{-}\cdot$ and the reduction potential of the $O_2/O_2^{-}\cdot$ couple, which results in a dramatic increase of $O_2^{-}\cdot$ reactivity [34]. Another biologically important radical reacting with superoxide at an almost diffusion-limited rate ($k \sim 3 \times 10^9$ M^{-1} s^{-1}) is the guanine neutral radical [G(–H)·]. Reactions of $O_2^{-}\cdot$ with G(–H)· formed in polynucleotides ($k = 4.7 \times 10^8$ M^{-1} s^{-1}) results in the accumulation of oxidatively modified guanine bases [35]. Reaction of superoxide with nitric oxide ($NO\cdot$) ($k \sim 4–6 \times 10^9$ M^{-1} s^{-1}) produces a strong oxidant, peroxynitrite ($ONOO^-$) [36, 37]. Peroxynitrite in turn can react with CO_2 and bicarbonates ($k \sim 3 \times 10^4$ M^{-1} s^{-1}) [38] to yield nitrosoperoxycarbonate, a precursor of the carbonate radical anion ($CO_3^{-}\cdot$) [39]. The carbonate radical anion is a one-electron oxidant capable of abstracting electrons from tyrosine and tryptophan [40]. In addition to reacting with other free radicals, superoxide is capable of oxidizing [4Fe-4S] clusters of proteins [41]. Such proteins are mainly dehydratases and Krebs cycle enzymes, and destruction of their [4Fe-4S] clusters has deleterious consequences.

First, it inactivates these enzymes, which impairs the Krebs cycle and hence aerobic energy production and biosynthetic pathways that depend on such enzymes. Second, the iron that is released from the [4Fe-4S] clusters acts as a catalyst of H_2O_2 decomposition in the Fenton reaction, generating the powerful oxidant hydroxyl radical ($HO\cdot$) [42, 43]. The Fe^{2+} released from the [4Fe-4S] clusters would bind to anionic molecules including proteins, nucleic acids, lipids and other cell membrane components and would be kept in a reduced Fe^{2+} state by cellular reductants. Hydrogen peroxide, which is relatively stable and can diffuse across membranes, would reach Fe^{2+} bound to biomolecules, and $HO\cdot$ would be generated at the spot [44]. In addition to the Fenton reaction, $HO\cdot$ can be photogenerated in a reaction where H_2O_2 is reduced by the PS radical anion [29]:



$$H_2O_2 + PS^{-}\cdot \rightarrow HO\cdot + OH^{-} + PS$$

Due to its extremely high reactivity, $HO\cdot$ would damage targets it encounters at the site of its formation [45].

In type II photoreaction, the T_1 state of the PS transfers its energy directly to molecular oxygen, itself a triplet in the ground state ($^3O_2$). This energy not only promotes one of the two unpaired electrons of $^3O_2$ to a high-energy orbital, but also inverts its spin, converting ground state molecular oxygen into singlet oxygen ($^1O_2$). Energy transfer to yield $^1O_2$ (type II) competes with electron transfer (type I), and it is believed that most PSs generate both $^1O_2$ and radicals. Because energy transfer to O_2 occurs at a higher rate ($k \approx 1\text{–}3 \times 10^9$ M^{-1} s^{-1}) than electron transfer (e.g. to give $O_2^{-}\cdot$, estimated as $k \leq 1 \times 10^7$ M^{-1} s^{-1}) [46], and $^1O_2$ is more reactive than $O_2^{-}\cdot$, singlet oxygen is considered the primary damaging species in PDT. In addition, biological systems are enzymatically protected against superoxide, but antioxidant enzymes that eliminate $^1O_2$ have not evolved, presumably because of its short lifetime.

Molecular oxygen has two S_1 states, denoted as $^1\Delta_g$ and $^1\Sigma_g$. These two states differ by the structure of the π-antibonding orbitals and by their energetic levels – 95 kJ/mol (22.5 kcal/mol) versus 158 kJ/mol (37.5 kcal mol) above the ground state, respectively [45] (fig. 2). The higher energy excited state of $^1O_2$ ($^1\Sigma_g$) is very short-lived due to a spin-allowed transition to the $^1\Delta_g$ state. The lifetime of the $^1\Delta_g$ state is determined by two processes: physical quenching, where the excess energy of $^1O_2$ is transferred to another molecule without the formation of products and the consumption of O_2, and chemical quenching, which is a chemical reaction between $^1O_2$ and another molecule, generating identifiable products [47].

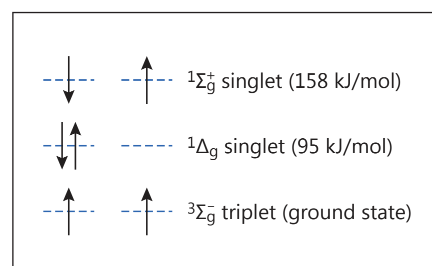


Fig. 2. Schematic representation of the electron configuration and energetic levels of ground and excited molecular oxygen states.

The presence of electrons with opposite spins, which removes the spin restriction typical for triplet oxygen [45], makes singlet oxygen highly reactive. It is an electrophile, oxidizing substrates that are not reacting with triplet oxygen. Singlet oxygen reacts rapidly with unsaturated carbon-carbon bonds and neutral nucleophiles such as sulfides and amines, as well as with anions [47], producing peroxides as initial products [28]. The decomposition of peroxides in turn generates radicals that can initiate a variety of chemical reactions, ultimately generating biologically active products.

In a biological environment, the lifetime of $^1O_2$ is limited by both physical and chemical quenching. As a result of physical quenching, the lifespan of $^1O_2$ in pure water is only about 4 μs [48], but in cells it is shorter due to the contribution of chemical quenching [49]. Even if chemical quenching does not occur, the maximal length $^1O_2$ can travel would not exceed 150 nm [49]. This is an extremely short distance. It is much smaller than the size of an average mammalian cell (10–30 μm) and even than the size of most cellular organelles. The short lifetime of $^1O_2$ makes localization of the PS a key factor in determining which cellular structures will be damaged and, as a consequence, the outcome of the PDT treatment.

Photodynamic Targets at the Molecular Level

Cells contain a variety of biomolecules that are potential substrates for oxidation by $^1O_2$ and other reactive species generated by PDT. In principle, the probability of such species reacting with a potential target depends on the distance between the PS and the target, the abundance of the target and the specific reaction rate constants. Singlet oxygen is considered the main damaging factor in PDT, but PSs operating mainly by the type I mechanism can also be highly effective [50]. PSs rarely

participate only in type I or type II processes; therefore, photodynamic activation usually generates a mixture of $^1O_2$ and radicals [51]. Recent studies suggest that high PDT efficacy results from a synergistic action of species generated by type I and type II reactions ($O_2^{-}\cdot$, $HO\cdot$ and $^1O_2$) [52–54].

Proteins

Due to their abundance and relatively high rate constants for reactions with $^1O_2$ and other reactive oxygen species (ROS), proteins are regarded as a main photodynamic target. Amino acid residues differ dramatically with respect to rate constants for reaction with $^1O_2$. This results in selective damage to particular amino acid side chains. Under physiological conditions, cysteine, methionine, tyrosine, histidine and tryptophan react with $^1O_2$ at rates $>10^7$ M^{-1} s^{-1} [46]. These amino acid residues are primary sites of oxidative modification of proteins. The reaction mechanisms involved are complex and lead to a number of final products [55]. Cysteine and methionine are oxidized mainly to sulfoxides, histidine yields a thermally unstable endoperoxide, tryptophan produces N-formylkynurenine, and tyrosine can undergo phenolic oxidative coupling. In the pH range of 7.0–8.5, protein sulfhydryl groups are the most sensitive to photooxidation, followed by histidine, tryptophan and tyrosine. Abstraction of a hydrogen atom from cysteine residues produces a thiyl radical that will cross-link to a second thiyl radical and form disulfide bridges.

The hydroxyl radical, in contrast, reacts with most amino acids at diffusion-controlled rates [56]. Therefore, protein damage by $HO\cdot$ depends on the concentration of the target and the reactivity of initially formed products. For example, oxidation of tyrosine by the hydroxyl radical generates a tyrosyl radical [57]. As mentioned earlier, the tyrosyl radical reacts with $O_2^{-}\cdot$ at an almost diffusion-limited rate [33]. Such reaction leads to either regeneration of tyrosine or the addition of $O_2^{-}\cdot$ to form tyrosine hydroperoxide [58, 59]. In proteins, the formation of tyrosine hydroperoxide is favored when the tyrosine is at the N-terminus [58]. It has been suggested that hydrogen bonding of $O_2^{-}\cdot$ to an amine group favors the addition reaction, thereby promoting the formation of tyrosine hydroperoxide [34]. Thus, without directly reacting with amino acid residues, $O_2^{-}\cdot$ contributes to protein damage by releasing Fe from [4Fe-4S] clusters, participating in reactions with free-radical intermediates and generating $ONOO^-$. Peroxynitrite reacts directly with sulfur-containing amino acids [60] and is a source of the highly oxidative carbonate radical [38].

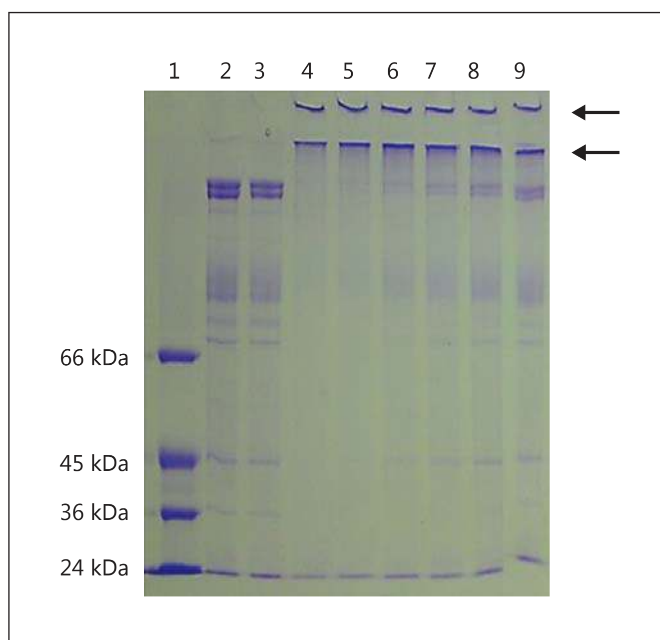


Fig. 3. Photo-induced cross-linking of erythrocyte membrane proteins. Hemoglobin-free erythrocyte membranes were illuminated for 30 min in the presence of Zn(II) *meso*-tetrakis(*N*-butylpyiridinium-2-yl)porphyrin (ZnTnBu-2-PyP^{4+}). Proteins were separated by sodium dodecyl sulfate gel electrophoresis and stained with Coomassie brilliant blue. Lanes: 1 – molecular weight markers; 2 – untreated erythrocyte membranes; 3 – dark control (incubated with 32 μM ZnTnBu-2-PyP^{4+} in the dark); 4–9 – erythrocyte membranes illuminated in the presence of different concentrations of ZnTnBu-2-PyP^{4+} (lanes: 4–32 μM; 5–16 μM; 6–8 μM; 7–4 μM; 8–2 μM; 9–1 μM). Arrows point to protein aggregates.

Besides photooxidation of amino acid residues, other chemical modifications of proteins can take place [61]. They include PS/protein photo-binding and protein cross-linking through the coupling of two tyrosine units. Covalent cross-linking, which is regarded as a secondary reaction between photooxidation products of susceptible amino acid residues and other groups in the protein [62], leads to the formation of molecular aggregates (fig. 3).

Modification of an individual protein depends not only on the content but also on the position of vulnerable amino acid residues in the folded protein. Therefore, proteins differ substantially in their susceptibility to PDT damage. An additional factor, which affects protein damage in PDT, is the proximity of the PS to vulnerable sites [61], which in turn depends on PS localization.

As a consequence of photo-induced modifications, proteins lose their catalytic [63], cell signaling and oth-

er essential functions, which ultimately leads to cell death (reviewed in detail by Pattison et al. [51]).

PDT-Induced Lipid Peroxidation

Lipids are less abundant than proteins, but a high content of unsaturated fatty acids, especially in biomembranes, makes membranous organelles and plasma membrane prime targets for photogenerated $^1O_2$ and other ROS [64]. The rates of reaction of singlet oxygen with unsaturated fatty acids are in the range of 0.74–2.4 × 10^5 M^{-1} s^{-1} and depend on the number of double bonds in the unsaturated fatty acids [65]. An additional factor, which contributes to PDT-induced lipid damage, is the high solubility of oxygen in lipids. Consequently, in its excited state, a PS localized in a lipid environment has a higher chance than in water to encounter O_2 and to produce $^1O_2$ and oxygen-derived radicals. In contrast to radicals, which induce lipid peroxidation via hydrogen abstraction, $^1O_2$ can add directly to unsaturated fatty acids, generating lipid peroxides [66]. In the presence of traces of transition metals, lipid peroxides decompose, giving rise to alkoxyl and peroxyl radicals [67]. These radicals initiate free-radical chain reactions, which destroy the lipid bilayer of biomembranes and generate chemically reactive and biologically active products [68]. Thus, the consequences of lipid peroxidation are not limited to the direct damage of lipids, but include secondary modifications of proteins and polynucleotides [69]. In addition to affecting membrane functions [70], lipid peroxidation can lead to alterations in metabolism and cell signaling [71–74], resulting ultimately in cell death (for a detailed review see Girotti and Kriska [66]).

Photosensitized Modification of Nucleic Acids

Oxidatively generated DNA damage is among the causes of cell death in PDT. It can be mediated by one-electron oxidation, $O_2^{-}\cdot$/HO· and $^1O_2$ [69, 75].

Direct one-electron oxidation occurs when an excited PS in a T_1 state abstracts an electron/hydrogen atom from a DNA base [76]. Among the bases, guanine is particularly vulnerable due to its low ionization potential [77]. A guanine cation radical ($G^{+}\cdot$) is the immediate product. It can be converted to 8-oxo-7,8-dihydro-2′-deoxyguanosine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine by competing one-electron oxidation and reduction reactions [77, 78] or is deprotonated to a highly reactive guanine radical, Gua(–H)·. In an addition reaction with $O_2^{-}\cdot$, Gua(–H)· is converted to 2,2,4-triamino-5(2H)-oxazolone [77, 78]. The guanine cation radical can react with lysine, arginine and serine in proteins in a nucleophilic addition reaction, giving rise to DNA-protein cross-links [75].

The most damaging among the ROS generated by the type I photo process is the hydroxyl radical, which indiscriminately reacts with all DNA constituents at diffusion-limited rates. The main way that HO· causes base modifications is by addition to double bonds [79]. This reaction competes with hydrogen abstraction by HO· from the methyl group of thymine and the 2-amino group of guanine [79]. Hydrogen abstraction from deoxyribose DNA backbone initiates reactions, causing DNA strand breaks [75, 80].

In contrast to HO·, singlet oxygen ($^1\Delta_g$ state) is a highly selective DNA modifier [81]. Its prime target is guanine [79] (k = 5 × 10^6 M^{-1} s^{-1} [82]), which through a series of reactions is converted into 8-oxo-7,8-dihydro-2′-deoxyguanosine [83], a specific $^1O_2$ oxidation product [78] that can be detected after PDT treatment [84]. Since $^1O_2$ does not react with 2-deoxyribose, it cannot cut the DNA backbone, and previously reported formation of DNA nicks can be explained by secondary oxidation of 8-oxo-7,8-dihydro-2′-deoxyguanosine by $^1O_2$ [77]. Details about reactive species, mechanisms of oxidatively generated DNA modifications, reactivity of products and biological consequences can be found elsewhere [85–87].

DNA damage induced by PDT can result in genotoxicity and mutagenicity [88]. Compared with the impairment on DNA induced by other anticancer treatment regimens, however, the extent of nucleic acid damage is considered to be much smaller [89] and there are no reports that PDT causes secondary tumors [90].

Photosensitizers

The majority of PSs currently in use for PDT are cyclic tetrapyrrolic structures: porphyrins and their analogs, chlorins, bacteriochlorins, phthalocyanines, etc. (fig. 4). Their advantages and disadvantages have been reviewed in detail [91]. The first PS clinically approved for PDT treatment was a porphyrin named ‘hematoporphyrin derivative’ (fig. 5a). Its commercial form, Photofrin®, is widely used in clinical PDT [92]. Serious shortcomings of this first PS (reviewed by Nyman and Hynninen [93]) stimulated intensive research into the development of compounds which can fulfill as many as possible of the formulated requirements for a good PS [94]. Significant success has been achieved in several aspects: (1) synthesis of stable PSs absorbing light in the red and NIR region of the spectrum (650–800 nm), which penetrates deeper

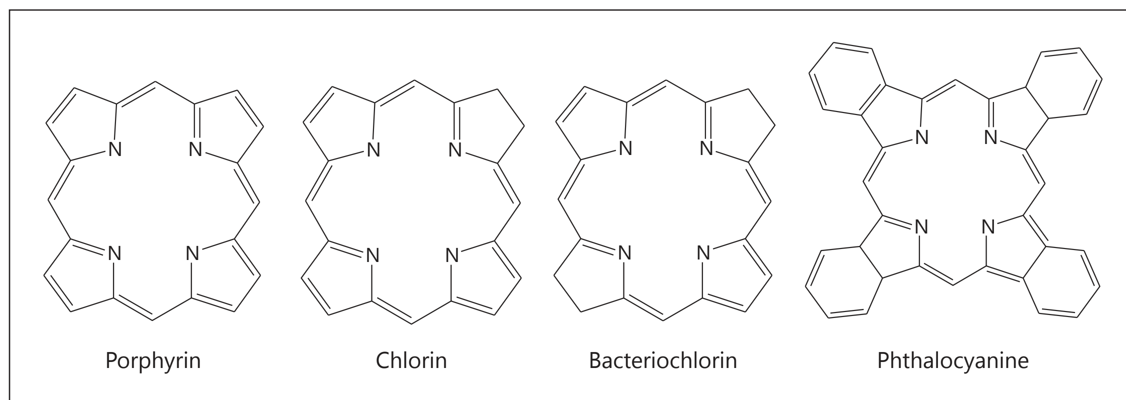


Fig. 4. Main groups of PSs used in PDT.

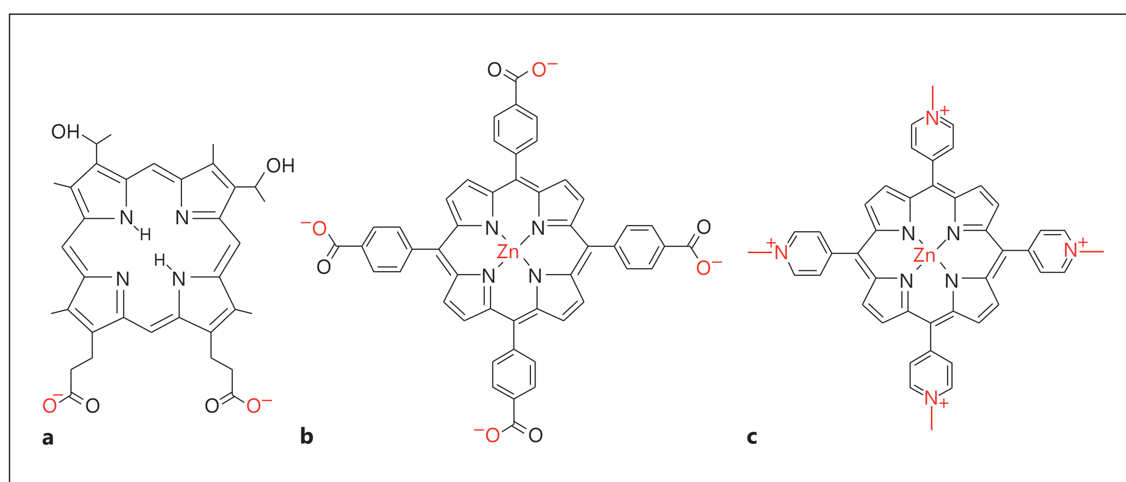


Fig. 5. Structures of anionic and cationic PSs. **a** Hematoporphyrin derivative (monomer). **b** Zn(II) *meso*-tetrakis(4-carboxyphenyl)porphyrin ($ZnTBAP^{4-}$). **c** Zn(II) *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphyrin ($ZnTM\text{-}4\text{-}PyP^{4+}$).

into the tissues [52, 95, 96], (2) improvement of the selectivity (increasing the target/healthy tissue ratio) and (3) accelerating elimination from the body in order to reduce side effects [97–100]. Increased understanding of the mechanisms of the photodynamic action at cellular and molecular levels helped in formulating strategies for further improvement of PSs. Priority was given to the targeting of the PS to particular cellular organelles, increasing PS uptake by targeted cells and tissues, shifting absorbance of light to longer wavelengths, and accelerating PS clearance from the organism.

Illumination is a key element in PDT and optical properties of tissues determine the depth and efficacy of the treatment. Light scattering and light absorption by endogenous chromophores (hemoglobin, melanin, etc.) limit light penetration at wavelengths (λ) <650 nm. At λ >1,300 nm, light penetration in tissues is reduced due to absorption by water molecules. Therefore, maximal penetration of light into tissues can be achieved at the so-called 'phototherapeutic window' [101, 102], which is schematically illustrated in figure 6. Among the natural pigments with strong absorption in the NIR region of the spectrum (720–850 nm) are bacteriochlorins and bacteriopurpurins [103], but they are unstable, easily photobleach, and permit limited chemical modifications [54, 104]. Recently developed new synthetic strategies allowed the synthesis of artificial stable bacteriochlorins, which demonstrated improved pharmacokinetics and high PDT efficacy [52, 54,

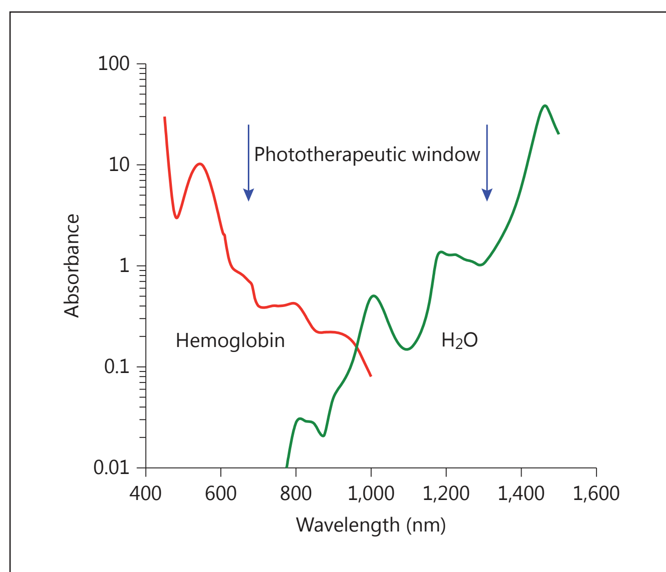


Fig. 6. The optical (phototherapeutic) window, where the absorption and scattering of light by tissues is minimal. For simplicity, only absorption by hemoglobin and water is presented in the logarithmic scale [modified from 102].

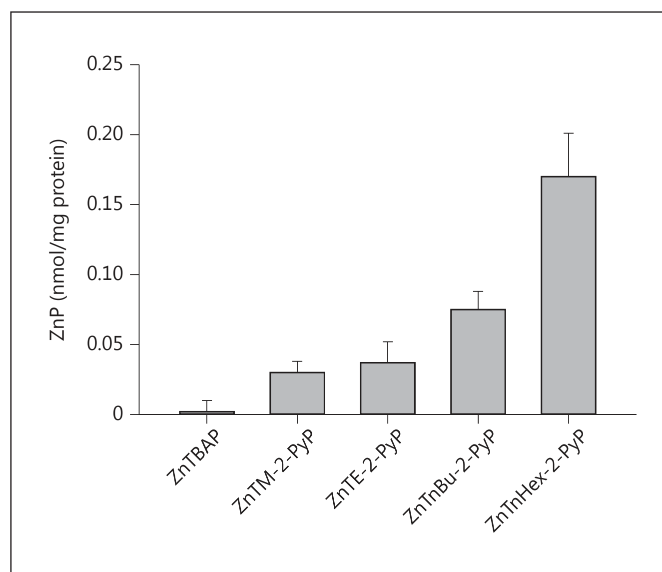


Fig. 7. Uptake of isomeric ZnPs by human colon adenocarcinoma cells (LS174T). After 24 h of incubation with 20 μM ZnPs the cells were washed and lysed. PSs were determined in cell-free extracts by estimating the area under the florescence emission peaks. Mean ± SE is presented (n = 3). ZnTBAP = Zn(II) *meso*-tetrakis (4-carboxyphenyl)porphyrin; ZnTM-2-PyP = Zn(II) *meso*-tetrakis(*N*-methylpyridinium-2-yl)porphyrin; ZnTE-2-PyP = Zn(II) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin; ZnTnBu-2-PyP = Zn(II) *meso*-tetrakis(*N*-butylpyiridinium-2-yl)porphyrin; ZnTnHex-2-PyP = Zn(II) *meso*-tetrakis(*N*-hexylpyiridinium-2-yl)porphyrin.

95, 96, 105]. Light with λ >850 nm, however, does not provide enough energy for excitation of $^3O_2$ to $^1O_2$ [100, 106]. This drawback is eliminated by simultaneous absorption of two photons by the PS, to provide the same energy as a single photon of shorter wavelength [107].

Uptake of PSs

Due to the limited lifespan and, as a consequence, limited migration distance of $^1O_2$ and other photo-generated reactive species in the biological environment, localization of the PS molecule is the main factor determining which structures will be preferentially damaged in PDT. The nature of such targets and the extent of photodamage have a major impact on the physiological reactions and, ultimately, on the PDT outcome.

Three properties of the PS molecule – charge, lipophilicity and three-dimensional structure – are the main determinants of PS uptake and subcellular distribution.

Charge

Investigations on the effect of electrical charges on PS uptake have revealed that negative charges are unfavorable for PS transport across membranes. However, diffusion across the plasma membrane is possible if a PS possesses up to two negative charges, provided their unfavorable effect is compensated with sufficient lipophilicity [102]. More than two negative charges cannot be compensated, which prevents free diffusion of the PS across the plasma membrane [102, 108]. Such PSs are taken up by endocytosis and accumulate mainly in lysosomes [108]. In contrast, positively charged PSs are efficiently taken up by cells and accumulate intracellularly to concentrations higher than in the environment. The two PSs shown in figure 5, Zn(II) *meso*-tetrakis(4-carboxyphenyl)porphyrin (ZnTBAP^{4-}) (fig. 5b) and Zn(II) *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (ZnTM-4-PyP^{4+}) (fig. 5c) have very similar structures but differ mainly by the net electric charge. The uptake of the cationic Zn-porphyrin, however, is much more effective (fig. 7), which is the reason behind its higher PDT efficacy. Charge differences can explain the inferior PDT activity of the anionic hematoporphyrin derivative (fig. 5a) compared to the cationic Zn(II) *N*-alkylpyridylporphyrins [109]. Positively charged PSs are electrostatically at-

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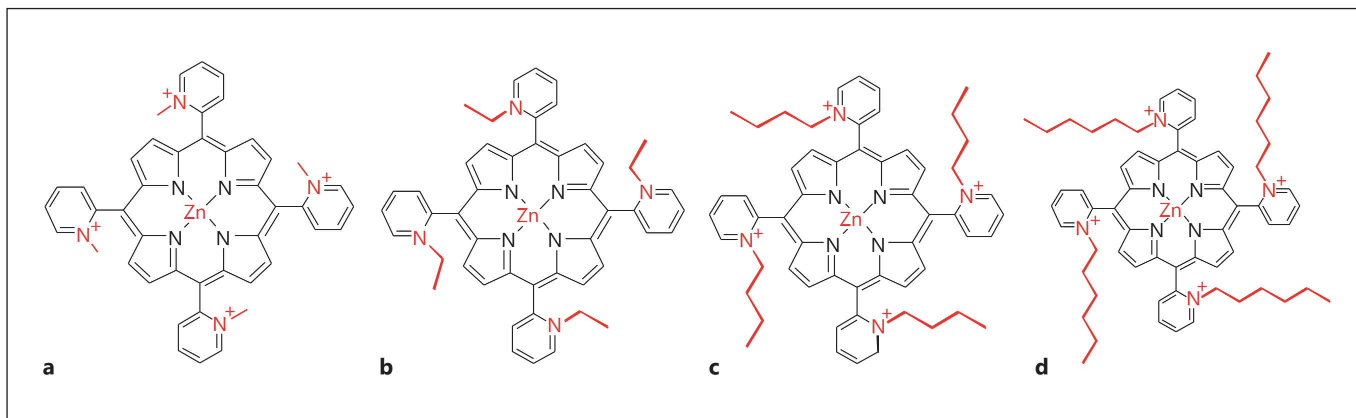

Fig. 8. Structures of cationic Zn(II) *N*-alkylpyridylporphyrins with progressively increasing lipophilicity. For simplicity, only *ortho*-isomers are shown. **a** Zn(II) *meso*-tetrakis(*N*-methylpyridinium-2-yl)porphyrin (ZnTM-2-PyP^{4+}). **b** Zn(II) *meso*-tetrakis(*N*-ethylpyridinium-2-yl)porphyrin (ZnTE-2-PyP^{4+}). **c** Zn(II) *meso*-tetrakis(*N*- butylpyiridinium-2-yl)porphyrin (ZnTnBu-2-PyP^{4+}). **d** Zn(II) *meso*-tetrakis(*N*-hexylpyiridinium-2-yl)porphyrin (ZnTnHex-2-PyP^{4+}).

Fig. 9. Isomers of the hydrophilic methyl and the amphiphilic hexyl Zn-porphyrin derivatives. **a** *Ortho*, Zn(II) *meso*-tetrakis(*N*-methylpyridinium-2-yl)porphyrin (ZnTM-2-PyP^{4+}). **b** *Meta*, Zn(II) *meso*-tetrakis(*N*-methylpyridinium-3-yl)porphyrin (ZnTM-3-PyP^{4+}). **c** *Para*, Zn(II) *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (ZnTM-4-PyP^{4+}). **d** *Ortho*, Zn(II) *meso*-tetrakis(*N*-hexylpyiridinium-2-yl)porphyrin (ZnTnHex-2-PyP^{4+}). **e** *Meta*, Zn(II) *meso*-tetrakis(*N*-hexylpyiridinium-3-yl)porphyrin (ZnTnHex-3-PyP^{4+}). **f** *Para*, Zn(II) *meso* tetrakis(*N*-hexylpyiridinium-3-yl)porphyrin (ZnTnHex-4-PyP^{4+}).

tracted by the predominantly negatively charged components of the plasma and mitochondrial membranes. A critical force driving such positively charged molecules inside cells and mitochondria is the transmembrane potential. Cationic PSs bind to anionic regions on proteins, which probably play an important role in their transport and preferential accumulation in tumor tissue [110]. In addition to the total net charge, distribution (position) of charges in the molecule strongly influences the uptake of PSs [110].

Lipophilicity

Investigations carried out with a series of cationic *meso*-substituted porphyrins, where the balance between hydrophilicity and lipophilicity was varied through an increase in the length of the alkyl chains (fig. 8), showed that the uptake of the PS by cancer cells is strongly influenced by the length of the side chain (fig. 7) [111]. Initially, the positively charged porphyrin is attracted electrostatically, but at the vicinity of the membrane lipid bilayer, hydrophobic forces start to dominate. Uptake increases by increasing the length of the alkyl chain from 1 to 8 carbons [112]. It parallels photodynamic efficacy, which also increases as the polarity decreases, reaching a maximum for amphiphilic porphyrins. An increase in the length of the alkyl chains above certain limits leads to suppression of cellular uptake [99]. It has been proposed that the length of the aliphatic chain affects the binding of molecules to cellular membranes. A chain with optimal length eventually favors the insertion of the PS deeper into the lipid bilayer of biological membranes [99].

Lipophilicity affects not only the uptake of the PSs, but also their subcellular distribution. For cationic metalloporphyrins, an increase in the length of the aliphatic tail from 2 to 6 carbons shifts subcellular localization of the molecules from cytosolic to mitochondrial [111].

Shape, Size and Three-Dimensional Structure of the Molecule

In contrast to the contribution of charges and lipophilicity, much less is known about the effect of shape and size of the PS molecule on cellular uptake, subcellular distribution and PDT activity. In a study investigating how lipophilicity of asymmetric porphyrins affects their incorporation in membranes, Engelmann et al. [113] found that lipophilicity alone was not a sufficient predictor of membrane binding. The spatial structure of the molecule is a factor which determines the depth a PS would penetrate into the lipid bilayer of membranes, and the strength of hydrophobic and hydrophilic interactions. It has been concluded that PDT efficacy depends not only on the amount of bound PS but also on its location [113].

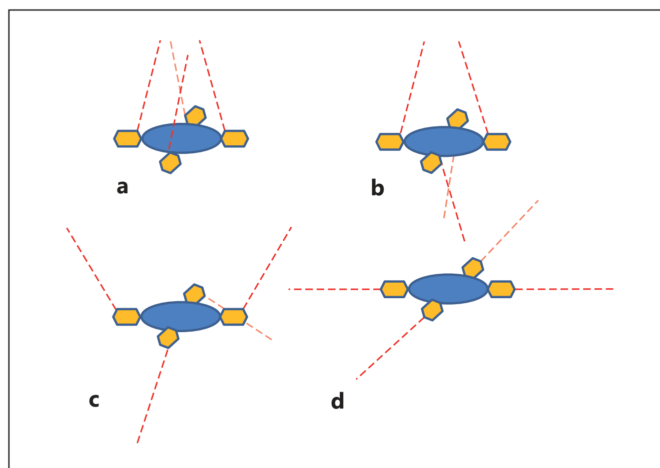

Fig. 10. Schematic representation of the three-dimensional shapes of the *ortho*-, *meta*- and *para*-hexyl isomers. The porphyrin core is represented by a blue ellipse, pyridyl substituents at *meso*-position by yellow hexagons and aliphatic chains by dotted red lines. For simplicity, only αααα and αβαβ atropisomers of *ortho*-isomer are presented. **a** ZnTnHex-2-PyP αααα. **b** ZnTnHex-2-PyP αβαβ. **c** ZnTnHex-3-PyP. **d** ZnTnHex-4-PyP [modified from 111].

Studies on structurally related cationic metalloporphyrins revealed that, together with charge and lipophilicity, shape and bulkiness play an important role in cellular uptake and subcellular distribution of the molecules (for detailed review see Tovmasyan et al. [114]). The *ortho*-isomer ZnTM-2-PyP^{4+} (fig. 9a), of the hydrophilic methyl analog, displays cytoplasmic distribution with preferential lysosomal uptake. The *meta*-methyl isomer ZnTM-3-PyP^{4+} (fig. 9b) is found in both the nucleus and the cytoplasm, and accumulation of its *para*-analog (fig. 9c) is predominantly nuclear. Thus, moving the substituents from *ortho*- to *para*-position shifts the localization of the hydrophilic PS from cytoplasmic to nuclear [111]. For the amphiphilic hexyl derivatives, however, moving the hexyl alkyl chain from *ortho*- to *para*-position (fig. 9d–f) does not lead to the translocation of the PS to the nucleus. In contrast, such change in the spatial structure of the molecule affects its incorporation in the lipid bilayer of the membranes [111]. The reasons for such differences among the structural isomers of the same compound can be understood if the three-dimensional shape of the molecules is taken into consideration. Each structural *ortho*-isomer can have up to 4 atropoisomers, each determining a particular three-dimension-

al shape. The effect of the position of the alkyl chain on the shape and size of the molecule is schematically illustrated in figure 10. The αααα atropo-isomer of the *ortho*-analog is more compact, with charges allocated on one side and aliphatic tails facing the opposite side of the molecule (fig. 10a). The αβαβ atropo-isomer is more symmetrical, with positive charges in the middle and lipophilic tails at both sides of the molecule (fig. 10b). Such conformation hides the positive charges and obstructs electrostatic interactions with neighboring molecules. In the *meta*-analog (fig. 10c), the aliphatic chains are more extended, and charges can be less accessible than in the corresponding *ortho*-isomer. The *para*-analog (fig. 10d), in contrast, is more planar and more flexible, with positive charges encircled by the aliphatic tails [111]. Such differences in size, shape, charge accessibility and orientation of the lipophilic chains determine the strength of electrostatic and hydrophobic interactions of the PS with neighboring molecules, which directs the translocation and the distribution of the PS to cellular structures and organelles.

Relationship between Subcellular Distribution of PSs and PDT Efficacy

Cellular compartments vary substantially by sensitivity toward photo-generated reactive species. As a consequence, the subcellular distribution of PSs is a strong predictor of their PDT efficacy. The localization of photodamage to specific subcellular targets is critical for the activation of particular signaling/regulatory pathways and the magnitude and type of cellular response, as well as for the mode of cell death following PDT [115].

Lysosomes

Initially, lysosomes were considered the main intracellular PDT target, and it was believed that disruption of lysosomes was the main reason behind cell killing. It has been proposed that as a result of photo-induced disruption of lysosomal membranes, the cell is digested by released hydrolytic enzymes. Further studies demonstrated, however, that although PSs localized in lysosomes can lead to cell killing upon illumination, the relative efficacy of lysosome-localized PSs is significantly lower than that seen with PSs localized in mitochondria and other organelles. A possible reason is inactivation of lysosomal enzymes by the PDT treatment and/or inhibition by cytosolic inhibitors [116].

Mitochondria

PSs that localize to mitochondria are reported to be more efficient in killing cells than those that localize at other cellular sites. Mitochondria are among the first organelles to show ultrastructural changes after photosensitization. Mitochondrial targeting is considered particularly important for effective anticancer therapy because inhibition of mitochondrial functions and/or damage to mitochondrial components is very critical for cell survival and may induce rapid apoptotic response [99]. Photodynamic damage to mitochondria is a primary event in a chain of processes that result in disruption of the electron transport chain, dissipation of the mitochondrial membrane potential and mitochondrial swelling.

Biomembranes

Photodynamic efficacy is directly proportional to membrane binding of PSs, indicating that favorable membrane interactions are a key factor for achieving high PDT efficacy [112]. If a PS is anchored within a membrane, the excited triplet species formed after illumination would encounter an elevated concentration of oxygen and more singlet oxygen would be generated. In addition, $^1O_2$ produced within the membrane has higher chances to react with sensitive membrane components, including polyunsaturated fatty acids. In contrast, if the PS remains at the membrane boundary or in the water environment, it will interact with lower aqueous oxygen concentrations and a larger fraction of the generated singlet oxygen will be deactivated before encountering and oxidizing sensitive cellular components [117]. The complexity of biomembranes and their crucial role for cell survival can explain the high PDT activity of membrane-localized PSs. Even mild PDT-induced membrane oxidation can result in loss of membrane barrier function [70], inactivation of membrane-bound protein complexes [61], modifications of cell receptors and interruption of cell signaling cascades [118] (or alterations in any other essential membrane functions), which ultimately can lead to cell death.

Cytoskeleton

Cytoskeletal elements are another attractive target for PDT. PSs with a high affinity for binding to nonpolymerized tubulin can be highly cytotoxic. By specifically binding to tubulin and after illumination, preventing its polymerization, low doses of PSs can act in a manner similar to inhibitors of microtubule function [119, 120]. Photodynamic action targeted at tubulin induces the formation of micronuclei and giant cells and the accumulation of cells in mitosis [116].

The Mode of Cell Death

Various factors govern the type of cell death by PDT (reviewed in detail by Mroz et al. [121]). Among the most important are physicochemical properties of PS, its subcellular localization and local concentration, the concentration of oxygen, and the wavelength and intensity of light used for illumination [88, 122]. All other conditions being equal, PSs that localize to mitochondria induce apoptotic cell death within a certain threshold of oxidative stress. Particularly with mitochondria, apoptotic cell death can ensue not only from oxidative damage induced by primary, photo-degenerated ROS, but also by superoxide anion generated as a secondary product due to photodamage of components of the electron transport chain [123]. In contrast, PSs targeting lysosomes either delay or block the apoptotic program, thus predisposing the cells to necrosis. Mild oxidative damage by PSs localized in the plasma membrane cause apoptosis, but extensive damage leading to loss of plasma membrane integrity brings necrotic cell death [115, 124, 125]. Photosynthesizers targeting the endoplasmic reticulum/Golgi membranes have been reported to mediate necrosis. In summary, the type of death following PDT depends on the quantity, the site and the type of ROS generated by the photodynamic process, which in turn determine the extent of the oxidative damage [126]. The same PS can cause necrosis if illumination is done under normoxic conditions, but induces apoptotic death if illumination is carried out under hypoxic conditions [84]. It has been proposed that photoinactivation of essential enzymes and other components of the apoptotic cascade is the main reason for necrotic death under conditions of high PDT intensity [88]. Sublethal damage signals the cell to die by apoptosis, while severe damage prevents ATP production and execution of the apoptotic pathways and forces the cell to die by necrosis [122, 127].

Results from recent studies suggest that the type of photo-induced cell death has a strong impact on the clinical outcome of PDT. PDT with very low light fluence rates, which is known to cause predominantly apoptotic cell death [128–130], minimizes side effects, improves tumor control and reduces treatment-related morbidity without hampering therapeutic efficacy [131–133].

Adverse Reactions and Current Limitations of PDT

Compared to surgery or radiation therapy, PDT is less invasive and adverse reactions are relatively mild and not long-lasting. Depending on the PS and the therapeutic protocol, adverse events associated with PDT include photosensitivity, erythema, edema, fever, pleural effusion, constipation, anemia and respiratory insufficiency [134]. The principal side effect of PDT is pain, which usually occurs in the early part of irradiation and then gradually decreases over time [134–136]. The mechanism of PDT-induced pain is not well understood, but in most cases it can be well controlled by a combination of opiate, opioid and nonsteroidal anti-inflammatory drugs [137]. Photosensitivity is another common complication, which can last for months. In the majority of cases it is mild-to-moderate and requires no treatment. Photophobia, visual discomfort and dyschromias are also listed among the side effects of PDT [134]. It is important to stress that most side effects can be alleviated by the proper selection of the type of PS and PS dosage, parameters of illumination and other details of the PDT treatment protocol. Standardization of the treatment protocols and prediction of the PDT response, however, are seriously hampered by the lack of established PDT dosimetry [138]. In contrast to ionizing radiation, no agreement has been reached on how the doses of PS and light should be measured, and even no widely accepted definition of dose exists. In addition, the optimum PS and light doses as well as drug-light time interval may vary from patient to patient or lesion to lesion, which prevents the application of standardized protocols and the achievement of highest response rates.

Among the limitations of PSs currently used for clinical PDT are the difficulty in treating large tumor masses and the limited depth of treatment. Visible light can penetrate the tissues not deeper than 5–10 mm, which restricts the application of PDT to mainly superficial lesions. A detailed description of the current state of PDT and its limitations can be found in comprehensive reviews [134, 138].

Strategies for Perfecting PDT

The development of better, more efficient compounds, free of the shortcomings of the first- and second-generation PSs, is among the primary strategies for improving PDT. This includes synthesis of PSs with strong absorption bands in the NIR region of the spectrum [139], which overcomes one of the main limitations of the currently clinically approved PSs – the insufficient depth of treatment. Improvement of PSs can be aided by the latest advances in nanotechnology. Nanoparticles have been used as PSs themselves, for delivery of PSs or as energy trans-

ducers [100]. Targeted delivery of PSs is achieved by conjugation with antibodies, engineered synthesis of molecules with specific structure and even by attachment of PSs to magnetic nanoparticles. In the latter case, an externally applied magnetic field directs the PS to the lesion [100]. Attachment of different modules to PSs, for example DNA- or peptide-based linkers and cancer cell-specific delivery vehicles, is used to improve both target specificity and pharmacological properties [140]. In addition to the efforts in developing better PSs, various strategies driven by the current understanding of photophysics, photochemistry, photobiology and the latest technological advances have been evolved to meet the requirements for effective PDT outcome. Deep treatment, using PSs absorbing in the NIR region of the spectrum, is achieved by two-photon PDT [141]. It is based on the development of laser technology, which allows the application of short (approx. 100 fs) laser pulses with high peak power. Instead of one, two light photons are absorbed and each photon accounts for only half of the excitation energy [142]. Metronomic PDT is based on the application of very low doses of PSs combined with low rates of irradiation lasting for extended periods of time [143]. As mentioned before, the outcome is cell death by apoptosis with minimal tissue necrosis.

Irrespective of the advances in laser technology, synthetic chemistry, nanotechnology and photobiology, PDT, more than a quarter of a century after its first clinical approval, is still not accepted as 'standard' therapy even in areas of medicine where real improvement in outcome using standard therapy has not been achieved [144]. The solution of this problem has been summarized by Moghissi [144], who recommends that, in order to overcome the current challenges and rise to the height of its potential, PDT needs commitment and funds [144].

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