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Mechanisms in photodynamic therapy: part one—-photosensitizers, photochemistry and cellular localization

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Summary

The use of non-toxic dyes or photosensitizers (PS) in combination with harmless visible light that is known as photodynamic therapy (PDT) has been known for over a hundred years, but is only now becoming widely used. Originally developed as a tumor therapy, some of its most successful applications are for non-malignant disease. In a series of three reviews we will discuss the mechanisms that operate in the field of PDT. Part one discusses the recent explosion in discovery and chemical synthesis of new PS. Some guidelines on how to choose an ideal PS for a particular application are presented. The photochemistry and photophysics of PS and the two pathways known as Type I (radicals and reactive oxygen species) and Type II (singlet oxygen) photochemical processes are discussed. To carry out PDT effectively in vivo, it is necessary to ensure sufficient light reaches all the diseased tissue. This involves understanding how light travels within various tissues and the relative effects of absorption and scattering. The fact that most of the PS are also fluorescent allows various optical imaging and monitoring strategies to be combined with PDT. The most important factor governing the outcome of PDT is how the PS interacts with cells in the target tissue or tumor, and the key aspect of this interaction is the subcellular localization of the PS. Examples of PS that localize in mitochondria, lysosomes, endoplasmic reticulum, Golgi apparatus and plasma membranes are given. Finally the use of 5-aminolevulinic acid as a natural precursor of the heme biosynthetic pathway, stimulates accumulation of the PS protoporphyrin IX is described.

Keywords

Photodynamic therapy Photosensitizers; Photochemistry; Photophysics; Tissue optics; Subcellular localization

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Introduction

Photodynamic therapy (PDT) can be defined as the administration of a nontoxic drug or dye known as a PS either systemically, locally, or topically to a patient bearing a lesion (frequently but not always cancer), followed after some time by the illumination of the lesion with visible light (usually long wavelength red light), which, in the presence of oxygen, leads to the generation of cytotoxic species and consequently to cell death and tissue destruction. The concept dates from the early days of the twentieth century when workers used dyes such as eosin together with light to treat skin cancer [1]. Hematoporphyrin (HP) was also first used at this time [2] and sporadic reports [3] of both selective localization of porphyrins in tumors and regression after exposure to visible light appeared until the 1960s. The modern explosion of interest in PDT dates from the discovery of hematoporphyrin derivative (HPD) by Lipson and Baldes in 1960 [4], and was fueled by pioneering studies in both basic science and clinical application [5–7] by Dougherty et al. (notable among many groups). A semi-purified preparation of HPD known as Photofrin® (PF) was the first PS to gain regulatory approval for treatment of various cancers in many countries throughout the world, including the United States. After experience of treating tumors with HPD–PDT was accumulated, it was realized that this compound had disadvantages, including prolonged skin sensitivity necessitating avoidance of sunlight for many weeks [8], sub-optimal tumor selectivity [9], poor light penetration into the tumor due to the relatively short wavelength used (630 nm) [10], and the fact that it was a complex mixture of uncertain structure [11]. In recent times much work has been done on developing new PS [12,13], and at the present time there is such a great number of potential PS for PDT that it is diffcult to decide which ones are suitable for which particular disease or application. Some PS can easily be prepared by partial syntheses starting from abundant natural starting materials, such as heme, chlorophyll and bacteriochlorophyll. This route leads to both economical and environmental advantages compared to complicated total chemical synthesis [14].

In parallel with the advances in chemistry there has also been much activity in developing new light sources. These include user-friendly lasers frequently based on solid state laser diodes, as well as inexpensive light-emitting diodes and filtered broad-band lamps [15]. Advances in knowledge of tissue optics has allowed great improvements to be made in treatment planning and predicting how the light is distributed within the target tissue or organ and therefore to optimizing the clinical outcome. The recognition that different tissue types have very different optical properties, and even that the same tissue type or organ can vary markedly between individuals in how light is absorbed and scattered. The fact that most PS are also fluorescent as well as photochemically active, means that imaging and detection strategies can be applied in PDT protocols. These techniques are sometimes known as photo(dynamic) detection or diagnosis. They may be carried out to detect otherwise hidden disease such as dysplasia, to delineate tumor borders, or to visualize disease in inaccessible areas such as the esophagus, bronchus or colon that can, however, be reached endoscopically. Another application of fluorescent imaging and quantification is in its ability to improve PDT dosimetry. For instance, fluorescence measurements can be made to quantify the actual amount of PS in the patient’s lesion before deciding on the appropriate

illumination parameters. Fluorescence measurements can be also be made to measure photobleaching (see later) of the PS in the tissue, which under some circumstances, can be a surrogate marker for optimal completion of the treatment. Although PS are usually selected based on photochemical and pharmacokinetic considerations, in the future there may also be an additional factor to be taken into consideration involving the need for fluorescence imaging.

In order to make rational choices from among the myriad available PS and light sources available, it is necessary to understand some of the mechanistic aspects of how PS behave upon illumination and what happens to the PS when they are put in contact with mammalian cells in tissue culture. In recent years there has been an explosion of knowledge in cell biology about signal transduction pathways, transcription factors and regulation of cell cycle control, inflammation and cell death. Some of the mechanisms involved after PDT of cells in culture will be covered in the second part of this review series. The precise way that PDT influences these cellular pathways, is largely governed by where in the cell the PS is located. This subcellular localization in turn is governed by the chemical nature of the PS (molecular weight, lipophilicity, amphiphilicity, ionic charge and protein binding charactersitics), the concentration of the PS, the incubation time, the serum concentration and the phenotype of the target cell.

One of the chief attractions of PDT as a therapy is the concept of dual selectivity. Collateral damage to normal tissue can be minimized by increasing the selective accumulation of the PS in the tumor or other diseased tissue, and by delivering the light in a spatially confined and focused manner. Nevertheless PDT can have side effects including long-lasting skin photosensitivity, occasional systemic and metabolic disturbances, and excessive tissue destruction at the treated site. It is hoped that advances in mechanistic understanding of PDT will minimize the risk reward ratio and extend the number of disorders both serious and more minor that can be treated.

Photochemistry and photophysics

Photosensitizers

Hematoporphyrin derivative or Photofrin was the first PS to be studied in detail. However, it proved highly frustrating for scientists who attempted to determine its chemical structure and to identify its components [11,16–19]. There was significant variation between batches and attempts to fractionate it into its individual component molecules frequently yielded mixtures as complicated as the starting material [20,21]. Although there is good evidence for the presence of hematoporphyrin oligomers, it is uncertain whether they are predominantly ethers or esters, and the whether the side chains are predominantly vinyl or hydroxy ethyl groups [22]. When these uncertainties were combined with other significant deficiencies of the preparation, enthusiasm for its widespread use was decreased. These deficiencies include a long-lasting skin photosensitivity so that patients may have to avoid sunlight for as long as eight weeks, the lack of a reasonably-sized absorption band > 650 nm, and the fact that its tumor-localizing properties were not as pronounced as first thought. These considerations spurred a large effort amongst organic chemists to develop novel PS that could in theory be

candidates for mediating PDT. The net result is a collection of probably hundreds of compounds and it can be bewildering to try to choose among them.

The characteristics of the ideal PS have been discussed in recent reviews [23,24]. They should have low levels of dark toxicity to both humans and experimental animals and low incidence of administrative toxicity (i.e. hypotension or allergic reaction). They should absorb light in the red or far-red wavelengths in order to penetrate tissue (see later). Absorption bands at shorter wavelengths have less tissue penetration and are more likely to lead to skin photosensitivity (the power in sunlight drops off at $\lambda > 600$ nm). Absorption bands at high wavelengths (> 800 nm) mean that the photons will not have sufficient energy for the PS triplet state to transfer energy to the ground state oxygen molecule to excite it to the singlet state (see later). They should have relatively high absorption bands (>20,000–30,000 M^{-1} cm^{-1}) to minimize the dose of PS needed to achieve the desired effect. Synthesis of the PS should be relatively easy and the starting materials readily available to make large-scale production feasible. The PS should be a pure compound with a constant composition and a stable shelf life, and be ideally water soluble or soluble in a harmless aqueous solvent mixture. It should not aggregate unduly in biological environments as this reduces its photochemical efficiency. The pharmacokinetic elimination from the patient should be rapid, i.e. less than one day to avoid the necessity for post-treatment protection from light exposure and prolonged skin photosensitivity. A short interval between injection and illumination is desirable to facilitate outpatient treatment that is both patient-friendly and cost-effective. Pain on treatment is undesirable, as PDT does not usually require anesthesia or heavy sedation. Although high PDT activity is thought to be a good thing, it is possible to have excessively powerful PS that are unforgiving. With limitations in both PS and light dosimetry, highly active PS may easily permit treatment overdosage. It is at present uncertain whether it is better to have a PS “tailored” to a specific indication and to have families or portfolios of PS for various diseases or patient types, or to seek one PS that works against most diseases. Lastly a desirable feature might be to have an inbuilt method of PS dosimetry monitoring and following response to treatment by measuring in vivo fluorescence and its loss by photobleaching.

The majority of PS used both clinically and experimentally, are derived from the tetrapyrrole aromatic nucleus found in many naturally occurring pigments such as heme chlorophyll and bacteriochlorophyll. Tetrapyrroles usually have a relatively large absorption band in the region of 400 nm known as the Soret band, and a set of progressively smaller absorption bands as the spectrum moves into the red wavelengths known as the Q-bands. Naturally occurring porphyrins are fully conjugated (non-reduced) tetrapyrroles and vary in the number and type of side groups particularly carboxylic acid groups (uroporphyrin has eight, coproporphyrin has four and protoporphyrin has two). Porphyrins have the longest wavelength absorption band in the region of 630-nm and it tends to be small. Chlorins are tetrapyrroles with the double bond in one pyrrole ring reduced. This means that the longest wavelength absorption band shifts to the region of 650–690 nm and increases severalfold in height; both these factors are highly desirable for PDT. Bacteriochlorins have two pyrrole rings with reduced double bonds, and this leads to the absorption band shifting even further into the red, and increasing further in magnitude. Bacteriochlorins may turn out to be even more effective PS than chlorins, but with relatively few candidate molecules and some

questions about the stability of these molecules upon storage this remains to be seen. There are a set of classical chemical derivatives generally obtained from naturally occurring porphyrins and chlorins that include such structures as purpurins, pheophorbides, pyropheophorbides, pheophytins and phorbins some of which have been studied (a few extensively) as PS for PDT.

A second widely studied structural group of PS is the phthalocyanines (PC), and to a lesser extent, their related cousins the naphthalocyanines. Again their longest absorption band is in > 650 nm and usually has a respectable magnitude. As can be imagined the presence of four phenyl groups (or even worse four naphthyl groups) causes solubility and aggregation problems. PCs are frequently prepared with sulfonic acid groups to provide water solubility and with centrally coordinated metal atoms. It was found that the assymetrically substituted disulfonic acids acted as the best PS (compared to mono-, symmetrically di-, tri- and tetra-substituted sulfonic acids) in both the zinc [25] and aluminum [26] series of PC derivatives.

Another broad class of potential PS includes completely synthetic, non-naturally-occurring, conjugated pyrrolic ring systems. These comprise such structures as texaphyrins [23], porphycenes [27], and sapphyrins [28]. A last class of compounds that have been studied as PS are non-tetrapyrrole-derived naturally occurring or synthetic dyes. Examples of the first group are hypericin (from St Johns wort) [29] and from the second group are toluidine blue O [30] and Rose Bengal [31]. As yet these compounds have perhaps been more often studied as agents to mediate antimicrobial photoin-activation [32] rather than as PS designed to kill mammalian cells for applications such as cancer.

Examples of PS from some of these structural groups that have either received regulatory approval or are undergoing clinical or advanced pre-clinical testing are given in Fig. 1. The tetrapyrrole nucleus frequently holds a co-coordinated metal atom, but it has been found that only diamagnetic metals such as (Zn, Pd, In, Sn, Lu) allow the tetrapyrrole to retain its photosensitizing ability, while paramagnetic metals such as (Fe, Cu, Gd) do not [33]. Many of these compounds are lipophilic and some are even insoluble in water. These compounds must either be delivered in an emulsion or else incorporated in liposomes. Although much research has been undertaken in this area, the structural features of the molecule necessary to make the ideal PS, i.e. with both a high selectivity for tumors or other target lesions, and a high level of phototoxicity towards cells and tissue in the illuminated area, are still unknown.

Photophysics

Fig. 2 graphically illustrates the processes of light absorption and energy transfer that are at the heart of PDT. The ground state PS has two electrons with opposite spins (this is known as singlet state) in the low energy molecular orbital. Following the absorption of light (photons), one of these electrons is boosted into a high-energy orbital but keeps its spin (first excited singlet state). This is a short-lived (nanoseconds) species and can lose its energy by emitting light (fluorescence) or by internal conversion into heat. The fact that most PS are fluorescent has led to the development of sensitive assays to quantify the amount of PS in cells or tissues, and allows in vivo fluorescence imaging in living animals or patients to measure the pharmacokinetics and distribution of the PS. The excited singlet state PS may

also undergo the process known as intersystem crossing whereby the spin of the excited electron inverts to form the relatively long-lived (microseconds) excited triplet-state that has electron spins parallel. The long lifetime of the PS triplet-state is explained by the fact that the loss of energy by emission of light (phosphorescence) is a “spin-forbidden” process as the PS would move directly from a triplet to a singlet-state.

Photochemistry

The PS excited triplet can undergo two kinds of reactions (Fig. 2). Firstly, in a Type 1 reaction, it can react directly with a substrate, such as the cell membrane or a molecule, and transfer a proton or an electron to form a radical anion or radical cation, respectively. These radicals may further react with oxygen to produce reactive oxygen species. Alternatively in a Type 2 reaction, the triplet PS can transfer its energy directly to molecular oxygen (itself a triplet in the ground state), to form excited-state singlet oxygen. Both Type 1 and Type 2 reactions can occur simultaneously, and the ratio between these processes depends on the type of PS used, the concentrations of substrate and oxygen.

Type 1 pathways frequently involve initial production of superoxide anion by electron transfer from the triplet PS to molecular oxygen (monovalent reduction) [34,35]. Superoxide is not particularly reactive in biological systems and does not by itself cause much oxidative damage, but can react with itself to produce hydrogen peroxide and oxygen, a reaction known as “dismutation” that can be catalyzed by the enzyme superoxide dismutase (SOD). Hydrogen peroxide is important in biological systems because it can pass readily through cell membranes and cannot be excluded from cells. Hydrogen peroxide is actually necessary for the function of many enzymes, and thus is required (like oxygen itself) for health. Superoxide is also important in the production of the highly reactive hydroxyl radical ($HO^{\bullet}$). In this process, superoxide actually acts as a reducing agent, not as an oxidizing agent. This is because superoxide donates one electron to reduce the metal ions (such as ferric iron or Fe^{3+}) that act as the catalyst to convert hydrogen peroxide (H_2O_2) into the hydroxyl radical ($HO^{\bullet}$). This reaction is called the Fenton reaction, and was discovered over a hundred years ago. It is important in biological systems because most cells have some level of iron, copper, or other metals, which can catalyze this reaction. The reduced metal (ferrous iron or Fe^{2+}) then catalyzes the breaking of the oxygen–oxygen bond of hydrogen peroxide to produce a hydroxyl radical ($HO^{\bullet}$) and a hydroxide ion (HO^{-}). Superoxide can react with the hydroxyl radical ($HO^{\bullet}$) to form singlet oxygen, or with nitric oxide (NO^{-}) (also a radical) to produce peroxynitrite ($OONO^{-}$), another highly reactive oxidizing molecule.

Like H_2O_2, $HO^{\bullet}$ passes easily through membranes and cannot be kept out of cells. Hydroxyl radical damage is “diffusion rate-limited”. This highly reactive radical can add to an organic (carbon-containing) substrate (represented by R below), this could be, for example, a fatty acid which would form a hydroxylated adduct that is itself a radical. The hydroxyl radical can also oxidize the organic substrate by “stealing” or abstracting an electron from it. The resulting oxidized substrate is again itself a radical, and can react with other molecules in a chain reaction. For example, it could react with ground-state oxygen to produce a peroxyl radical ($ROO^{\bullet}$). The peroxyl radical again is highly reactive, and can react with another organic substrate in a chain reaction. This type of chain reaction is common in the oxidative

damage of fatty acids and other lipids, and demonstrates why radicals such as the hydroxyl radical can cause so much more damage than one might have expected.

These ROS, together with singlet oxygen produced via Type 2 pathway, are oxidizing agents that can directly react with many biological molecules. Amino acid residues in proteins are important targets that include cysteine, methionine, tyrosine, histidine, and tryptophan [36,37]. Due to their reactivity, these amino acids are the primary target of an oxidative attack on proteins. The reaction mechanisms are rather complex and as a rule lead to a number of final products. Cysteine and methionine are oxidized mainly to sulfoxides, histidine yields a thermally unstable endoperoxide, tryptophan reacts by a complicated mechanism to give *N*-formylkynurenine, tyrosine can undergo phenolic oxidative coupling. Unsaturated lipids typically undergo enetype reactions to give lipid hydroperoxides (LOOHs derived from phospholipids and cholesterol) [38–41].

DNA can be oxidatively damaged at both the nucleic bases (the individual molecules that make up the genetic code) and at the sugars that link the DNA strands by oxidation of the sugar linkages, or cross-linking of DNA to protein (a form of damage particularly difficult for the cell to repair). Although all cells have some capability of repairing oxidative damage to proteins and DNA, excess damage can cause mutations or cell death. Of the four bases in nucleic acids guanine is the most susceptible to oxidation by $^1O_2$. The reaction mechanism has been extensively studied in connection with oxidative cleavage of DNA [42]. The first step is a [4 + 2] cycloaddition to the C-4 and C-8 carbons of the purine ring leading to an unstable endoperoxide [43]. The subsequent complicated sequence of reactions and the final products depend on whether the guanine moiety is bound in an oligonucleotide or a double-stranded DNA [44].

Because of the high reactivity and short half-life of singlet oxygen and hydroxyl radicals, only molecules and structures that are proximal to the area of its production (areas of PS localization) are directly affected by PDT. The half-life of singlet oxygen in biological systems is < 40 ns, and, therefore, the radius of the action of singlet oxygen is of the order of 20 nm [45].

Light delivery

In PDT it is important to be able to predict the spatial distribution of light in the target tissue. Light is either scattered or absorbed when it enters tissue and the extent of both processes depends on tissue type and light wavelength. Tissue optics involves measuring the spatial/temporal distribution and the size distribution of tissue structures and their absorption and scattering properties. This is rather involved because the biological tissue is inhomogeneous and the presence of microscopic inhomogenities (macromolecules, cell organelles, organized cell structure, interstitial layers, etc.) makes it turbid. Multiple scattering within a turbid medium leads to spreading of a light beam and loss of directionality. Absorption is largely due to endogenous tissue chromophores such as hemoglobin, myoglobin and cytochromes. Complete characterization light transport in tissue is a formidable task; therefore, heuristic approaches with different levels of approximations have been developed to model it. An effort for modeling light transport also requires accurate values for the optical properties of the tissue. Scattering is generally the most important factor in limiting light penetration into

most tissues and is measured by μ_s (which for soft tissues is in the range 100–1000 cm^{-1}). Absorption is usually of lesser importance and measured by μ_a (values in the range of 0.1–5 cm^{-1} for most tissue at green and longer wavelengths). The third parameter necessary to define tissue optical properties is the anisotropy factor that measures the direction of scattering of light. It is possible to use mathematical approaches such as diffusion theory or Monte Carlo modeling to predict how light will travel into target tissue and the illumination parameters (fluence, fluence rate, wavelength, angle of incidence) may then be adjusted to maximize the light dose. The combination of absorption of lower wavelength light by the important tissue chromophores (oxy and deoxyhemoglobin and melanin) together with reduced light scattering at longer wavelengths and the occurrence of water absorption at wavelengths greater than 1300 nm has led to the concept of the “optical window” in tissue (see Fig. 3). In terms of PDT the average effective penetration depth (intensity reduced to 37%) is about 1–3 mm at 630 nm, the wavelength used for clinical treatment with PF, while penetration is approximately twice that at 700–850 nm [46,47].

The increased penetration depth of longer wavelength light is a major incentive for the development of PS absorbing at such wavelengths, and a naphthalocyanine (776 nm) [48] and bacteriochlorin (780 nm) [49] fall into this category. The absorption of light by the PS itself can limit tissue light penetration. This phenomenon has been termed “self-shielding” and is particularly pronounced with PS that absorb very strongly at the treatment wavelength [50]. Many PS are prone to photo-destruction during light exposure; a process called “photobleaching” [51]. This thought to happen when the singlet oxygen or other ROS produced upon illumination reacts with the PS molecule itself in a manner that reduces its efficiency for further photosensitization processes. PS of different chemical structures have widely varying photobleaching rates and in some cases (particularly that of PPIX) the first product of photobleaching is actually a better PS than the starting molecule. Nevertheless photobleaching usually means loss of PDT reactivity but this may still have beneficial effects regarding treatment differential. These are based on the following considerations: there exists a threshold PDT dose to produce tissue necrosis [48]. If photobleaching occurs (which does not have such a threshold) before this threshold is achieved, no tissue damage is incurred. This is desirable for normal tissue exposed to therapeutic light but not for the tumor tissue to be treated. Thus, the net result is that one can achieve greater depth of tumor necrosis while sparing the normal skin.

Subcellular localization

PS uptake by cancer or other cells is crucial for effective PDT. ROS have a short half-life and act close to their site of generation, therefore to a certain degree the type of photodamage that occurs in cells loaded with a PS and illuminated depends on the precise subcellular localization of the PS within the cell. The understanding of PS localization principles is therefore important for choosing the most effective PS for each application. Confocal laser scanning fluorescence microscopy has made the determination of intracellular location of PS much easier, and gives more sensitivity and better spatial resolution than earlier non-confocal techniques. Co-localization of subcellular organelle specific probes with differing fluorescence emission maxima to that of the PS can be used to more closely identify the site of localization [52] and these probes can also be used to

identify sites of damage after illumination [53]. Fluorescence resonance energy transfer (FRET) [54] can also be used to determine intracellular location of PS.

Intracellular distributions in cultured cells have been determined for a range of PS with widely differing structures. The important structural features are (a) the net ionic charge which can range from −4 (anionic) to +4 cationic, (b) the degree of hydrophobicity expressed as the logarithm of the octanol/water partition coefficient, (c) the degree of asymmetry present in the molecule. PS which are hydrophobic and have two or less negative charges can diffuse across the plasma membrane, and then relocate to other intracellular membranes. These PS also tend to have the greatest uptakes into cells in vitro, especially when present in relatively low concentrations in the medium (<1 μM). Those PS which are less hydrophobic and have >2 negative charges tend to be too polar to diffuse across the plasma membrane, and are therefore taken up by endocytosis.

Some PS distribute very broadly in various intracellular membranes. An example is pyropheophorbide—a methyl ester that was reported to be localized in endoplasmic reticulum, Golgi apparatus, lysosomes and mitochondria, in NCI-h446 cells [55].

Lysosomes

In 1993 lysosomes were proposed to be a critical intracellular target for localization of PS [56]. However, succeeding studies [57] have found that although lysosomally localized PS can lead to cell killing upon illumination, the relative efficacy is significantly lower than that seen with PS localized in mitochondria and other organelles [58]. This may be due to the tendency of PS with greater degrees of aggregation to accumulate in lysosomes.

Woodburn et al. [59] studied intracellular localization, in V79 Chinese hamster lung fibroblasts and C6 glioma cells, of a series of porphyrins derived from HP and PPIX with side chains chemically modified to give hydrophobic and anionic or cationic residues at physiological pH. Compounds were selected to represent all combinations of these characteristics and it was found that those with a net cationic character localized in mitochondria, while those with net anionic character localized in lysosomes. As the anionic porphyrins all carried two negative charges, these results are in accord with previous work suggesting that sensitizers with a net charge of −2 or greater accumulate in lysosomes. Nagata et al. [60] showed that the chlorin-based PS, ATX-S10 (Na) had a primary site of accumulation in lysosomes but cells underwent apoptosis upon illumination doses leading to 70% cell death, suggesting that apoptotic pathways may be activated via mitochondrial destabilization following the damage of lysosomes by PDT.

A technique termed photochemical internalization was developed by Berg et al. [61,62]. This procedure relied on co-incubating cells with a macromolecule that needed to be delivered into the cell cytoplasm and a PS such as aluminum phthalocyanine disulfonate (AlPCS2). Both of these molecules were incorporated into lysosomes with the PS localizing in the lysosomal membrane. On delivery of the correct amount of light the lysosome was ruptured by photochemical damage to its membrane thus releasing the intact macromolecule into the cytoplasm. This technology has been used to increase the intracellular delivery of genes [63], viruses [64], peptide nucleic acids [65], and ribosome inactivating proteins [66].

The initial intracellular localization of PS in lysosomes may redistribute due to photodynamic action after only a small amount of light has been delivered. It was found that exposure of cells pre-incubated with anionic porphyrins, to light doses that inactivated 20% of the cells resulted in relocalization of the sensitizers from the lysosomes to the cytoplasm in general, and, more specifically, the nucleus [67,68]. This behavior was attributed to photodynamic permeabilization of the lysosomal membrane, thus allowing small molecules, including the PS to leak out into the cytoplasm.

Many workers have proposed to deliver PS to cells by targeting the PS molecules to a cell-type specific receptor. This is often accomplished by covalently attaching one or more PS molecules to the specific ligand of the receptor. When most of these receptors bind their natural ligand to which is covalently attached the PS, the entire construct is internalized into the cell and follows the endosomal-lysosomal pathway discussed above. In many cases PS delivered by this method gives the most specific lysosomal fluorescence seen in PDT. An example of this is given in Fig. 4A and B where the class A scavenger receptors whose expression is specific to mature macrophages is targeted by a conjugate between maleylated albumin and the PS chlorin (e6) delivered to the J774 mouse macrophage tumor cell line [69]. The maleylated albumin is endocytosed after binding to its receptor and the PS ends up specifically localized in lysosomes. There is good red and green overlap (orange) with the lysosomal marker in Fig. 4A while the separation of red and the green mitochondrial marker is complete in Fig. 4B.

Mitochondria

Mitochondria have been found to be a very important subcellular target for many PS used in PDT [70]. This is related to the tendency of many PS to produce apoptosis by mitochondrial damage after illumination (see Section 5). Benzoporphyrin derivative (BPD) is one of the well-studied mitochondrial-localized PS [71], however, cellular localization depends on cell type and BPD formulation (free BPD, liposomal or encapsulated in polycationic liposomes) used [71,72]. Some endothelial cells (ECV304) preferentially accumulated BPD in perinuclear region, others (HUVEC) in cytoplasm; polycationic liposomal BPD was mostly deposited in the mitochondria while free BPD was also found in perinuclear region [71,72]. Our results with liposomal BPD are shown in Fig. 4C and D. Co-incubation with green Lysotracker (Fig. 4C) showed that BPD may localize in lysosomes to a small extent but the majority was extralysosomal. Co-incubation with Mitotracker (Fig. 4D) showed that a part of the BPD was in mitochondria, but that there was a significant amount of BPD that was in neither lysosomes nor mitochondria and hence probably in the endoplasmic reticulum or Golgi.

Two mesotetraphenylporphyrin derivatives bearing adjacent: 5,10-di[4-*N*-trimethylaminophenyl]-15,20-diphenylporphyrin (DADP-a) or opposite: 5,15-di[4-(*N*-trimethylaminophenyl)-10,20-diphenylporphyrin (DADP-o) cationic-*N*-(CH3)3+ groups on two of the para-phenyl positions were compared in study by Kessel et al. [73]. DADP-a localized in mitochondria, while DADP-o (a much more symmetric molecule) localized in lysosomes, and led to extensive lysosomal photodamage after irradiation.

PS with cationic charges and which are also hydrophobic can localize in mitochondria [74]; this is thought to be due to the influence of the mitochondrial membrane potential as well as the lipid bilayer of the membrane [75]. It is known that carcinoma cell mitochondria preferentially accumulate and retain certain cationic dyes to a much greater extent than most normal cells. Oseroff et al. [76] evaluated 10 rhodamine and cyanine dyes as carcinoma-specific mitochondrial PS in vitro. The most effective, *N*,*N*′-bis(2-ethyl-1,3-dioxolane)kryptocyanine, caused marked, light-dependent killing of human bladder, squamous, and colon carcinoma cell lines after 30-min incubations at 1–0.01 μM but was minimally toxic to human keratinocytes and to normal monkey kidney epithelial cells. Dummin et al. [74] prepared cationic zinc (II) phthalocyanines with lipophilic side-chains and showed they specifically accumulated in the inner mitochondrial membranes. On irradiation of the incubated HeLa cells, the cristae were affected and finally completely destroyed. The respiration stopped and the energy metabolism was shut down.

It was known previously that Pc4 localized in mitochondria and Golgi complexes and ER [77]. At early times (0–1 h) after introduction of Pc 4 to LY-R cells, the dye was found in the mitochondria, lysosomes and Golgi apparatus, as well as other cytoplasmic membranes, but not in the plasma membrane or the nucleus. Over the next 2 h, there was some loss of Pc 4 from the lysosomes but an accumulation in the Golgi apparatus and the mitochondria. The exact binding site of Pc4 was discovered only recently. Pc 4-PDT photodamaged Bcl-2 and Bcl-xL, antiapoptotic proteins interacting with the permeability transition pore complex that forms at contact sites between the inner and outer mitochondrial membranes. These complexes and the inner membrane are unique in containing the phospho-lipid cardiolipin. Nonyl-acridine orange (NAO) is a specific probe of cardiolipin and Morris et al. [54] showed evidence for fluorescence resonance energy transfer from NAO to Pc 4, defining a binding site for the photosensitizer.

Plasma membrane

Compounds that localize in plasma membranes of cultured cells are relatively uncommon in the PDT field. Aveline and Redmond [78] used confocal fluorescence microscopy to show that deuteroporphyrin IX (DP) and its monobromo and dibromo derivatives localized preferentially in the plasma membrane of L1210 cells. PF shows a dynamic distribution in human carcinoma cells: the plasma membranes are the main target sites of PF after a brief (3 h) incubation, while the Golgi complex is affected after prolonged (24 h) incubation [79]. The effects of PDT on cells with plasma membrane-localized PF was found to be a cessation of proliferation post PDT at Photofrin dose less than 7 μg/ml, and at higher dose (28 μg/ml) plasma membrane disruption and cell swelling were observed immediately after PDT. Characteristics of typical apoptosis such as phosphatidylserine externalization and DNA fragmentation were not detected in the cell death process caused by this PDT regime.

Golgi apparatus and endoplasmic reticulum

Teiten et al. [80] studied Foscan subcellular localization in the MCF-7 human adenocarcinoma cell line by means of confocal microscopy and microspectrofluorometry. The fluorescence topographic profiles recorded after cells co-stained with Foscan and organelle-specific fluorescent probes revealed that Foscan presents low localization in

lysosomes and a weak accumulation in mitochondria. However, the Foscan fluorescence topographic profile turned out to co-localize perfectly with that obtained for the endoplasmic reticulum (ER) and the Golgi apparatus. The patterns of fluorescence derived from confocal microscopy studies were consistent with predominant localization of Foscan in these organelles. Furthermore, evaluation of enzymatic activity of selected organelles immediately after laser light irradiation (650 nm) indicated the Golgi apparatus and ER as the primary damaged sites resulting from Foscan-mediated PDT in the MCF-7 cell line.

ALA-induced PPIX

Recently there has been much interest in a different approach to PDT where, instead of the PS being administered in a pre-synthesized form, a metabolic precursor is administered and the PS is synthesized in situ in tumors or cells of the target tissue [81]. The precursor is 5-aminolevulinic acid (ALA) which interacts with the heme biosynthetic pathway as shown in Fig. 5. Almost all types of cells of the human body, with the exception of mature red blood cells, are equipped with this metabolic machinery. In the first step of the pathway ALA is formed from glycine and succinyl CoA. The synthesis of ALA by ALA-synthetase is under feedback regulation by the amount of heme in the cell. The last step in the pathway is incorporation of iron into PPIX catalyzed by the enzyme ferrochelatase and this is rate-limiting. By adding exogenous ALA, the feedback inhibition is bypassed, and PPIX will accumulate because of the limited capacity of ferrochelatase to transform PPIX to heme. PPIX is formed in the mitochondria of cells, but rapidly diffuses to other intracellular membrane sites. Gaullier et al. [82] found early staining in mitochondria but at later time points the plasma membrane showed strong staining, and fluorescent spots (shown to be lysosomes by co-localization experiments with lysosomal probes) were observed within the cytoplasm especially in the perinuclear region. Fluorescence spectra suggested that the PPIX microenvironments were quite different at short and long incubation times.

In vivo the ALA may be administered orally [83], intravenously [84], or topically [85]. The reasons why cancer cells tend to synthesize more PPIX than normal cells, has been much investigated. Hypotheses include greater expression of heme biosynthesis enzymes, porphobilinogen deaminase [86], coproporphyrinogen oxidase [87], or reduced expression of ferrochelatase [88], but increased delivery of ALA to the tumor may play a role especially in topical application [89]. Recent attempts to increase the efficacy of ALA-mediated PDT include the use of iron chelators to decrease the amount of PPIX converted to heme by ferrochelatase by removing the free iron that is the necessary for the enzyme to work [90]. Another approach is to administer ALA as one of various alkyl esters (methyl, pentyl, hexyl or benzyl) in order to increase cellular uptake by making the molecule more lipophilic [91]. Since ALA is frequently applied topically to the skin, the ALA-methyl ester that penetrates through the skin’s natural permeability barrier much better than the polar ALA, recently received approval to treat basal cell skin cancers [92].

Conclusion

The recent explosion in the synthesis and development of novel PS has led to a “logjam” in the investigation of the pros and cons of which compound should be used for which

application. PDT is an inherently complex technology that depends on multiple variables including the chemical and photochemical properties of the PS, the PS dosage and delivery vehicle, the drug–light time interval, the wavelength, energy dose, power density and pulse structure of the light, and the oxygenation state of the tissue. Therefore, it is unreasonable to expect a comprehensive study of all these parameters for each of several hundred candidate molecules. The way around this otherwise insurmountable problem is to carry out rational mechanistic studies that would allow predictions of efficacy and preferred clinical application to be made based on the PS chemical structure and simple spectroscopic and photophysical measurements. There are signs that this is starting to happen. Well-designed structure–function studies on rationally chosen sets of candidate PS molecules may allow more or less general conclusions to be deduced [93,94]. These experiments may have to be carried out both in vitro and in vivo. There have been instances when PS that were highly active against cancer cells in culture, were completely inactive against tumors in animal models. There may be an analogy with the pharmaceutical industry that has begun to develop computer software that can predict drug performance from chemical structure and target identity using such benchmarks as the druggability rules [95] and pharmacophore analysis [96]. It may not be too futuristic to expect similar developments in the PDT field, especially as more clinical approvals are obtained and more money becomes available for this type of research. The growth of non-cancer applications of PDT to include ophthalmology, cardiovascular, immunology and anti-infection.

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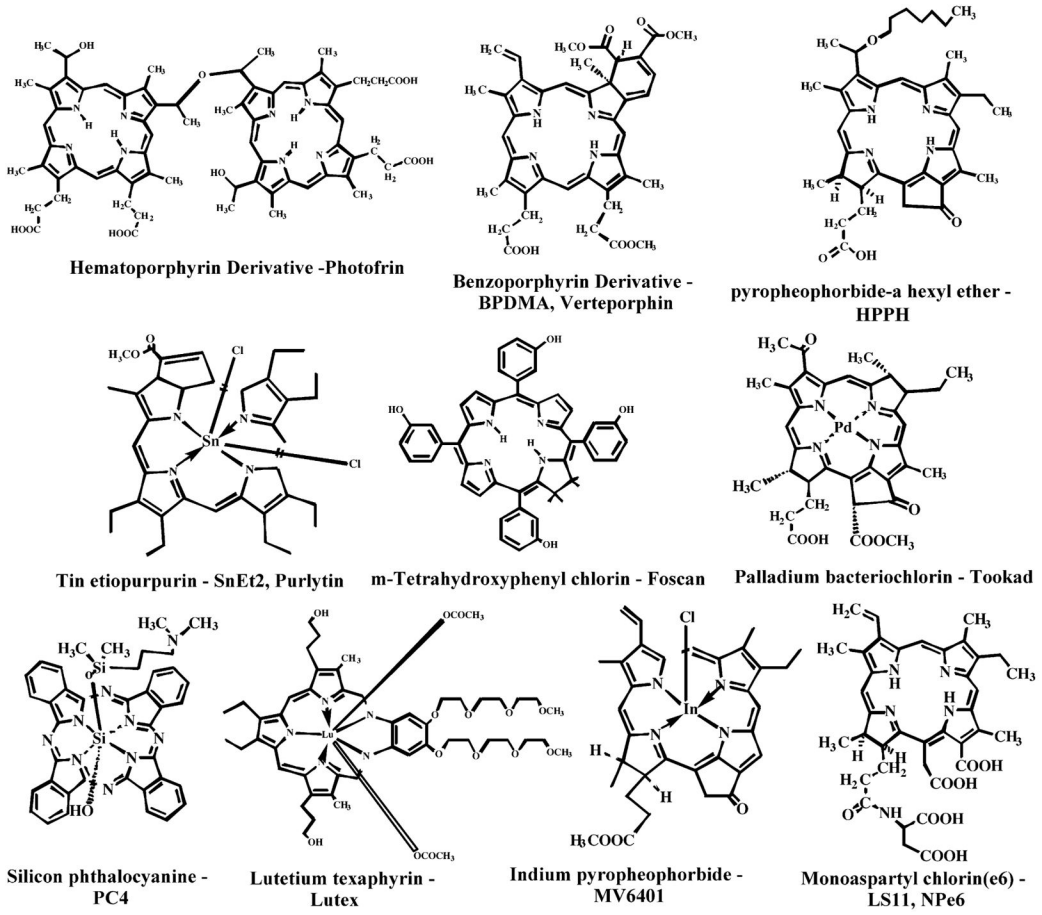

Figure 1.
Structures of PS either clinically approved or in trials.

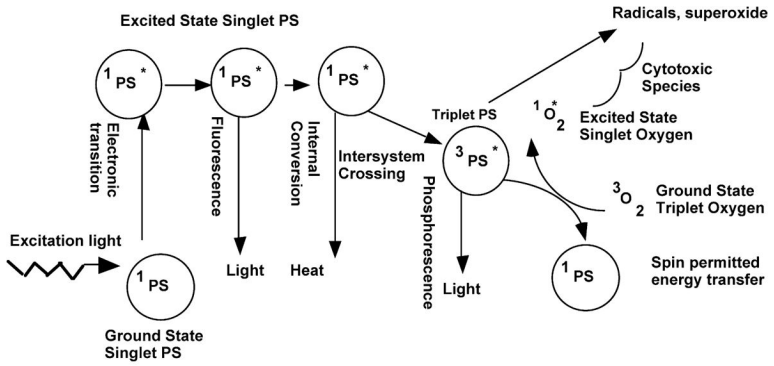

Figure 2.
Graphical illustration of the photophysical and photochemical mechanisms of PDT.

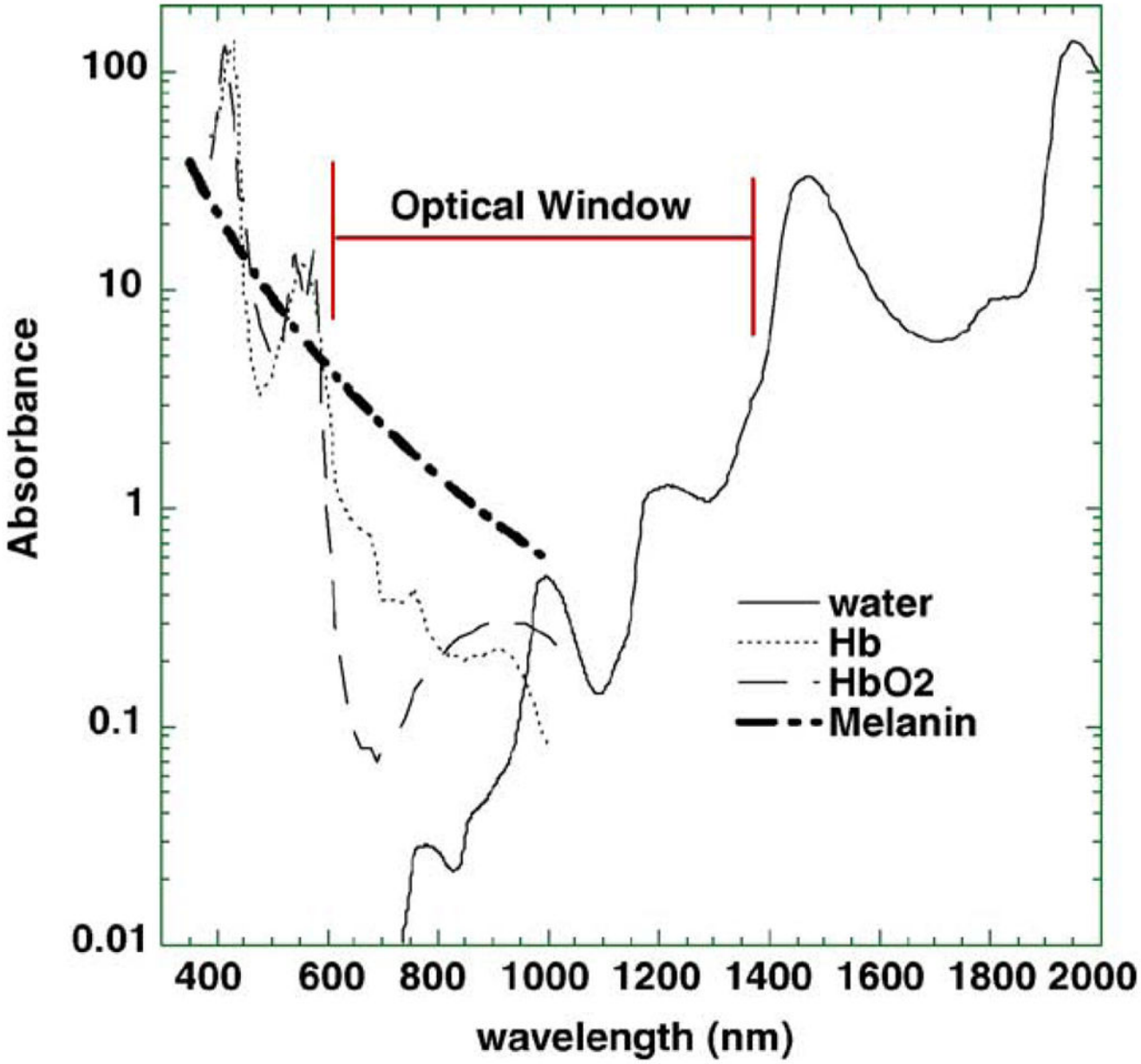

Figure 3.
Optical window in tissue. Absorption spectra of important tissue chromophores such as water, oxy- and deoxyhemoglobin and melanin are plotted on a logarithmic scale.

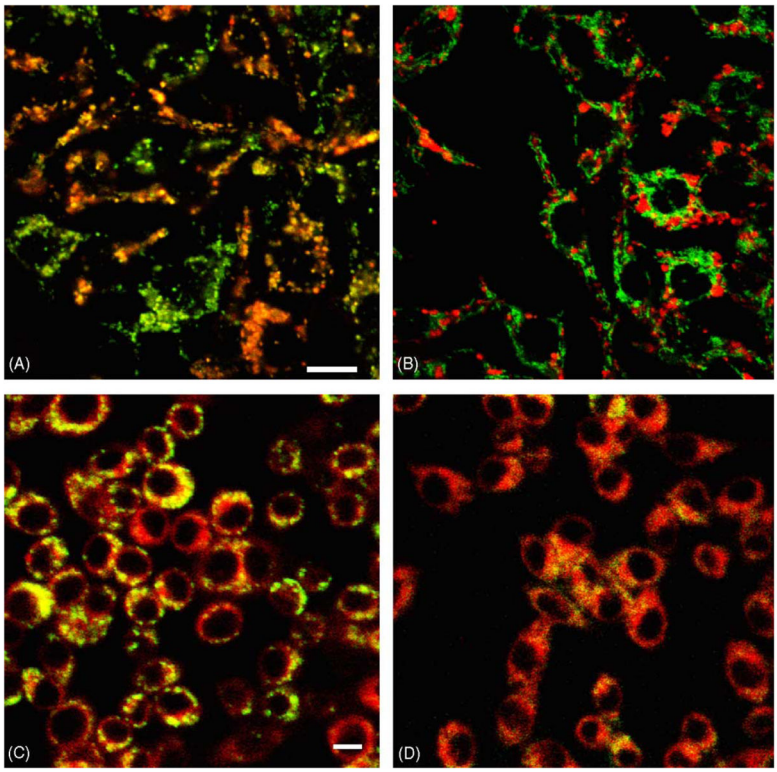

Figure 4.

Scanning laser confocal fluorescence microscope images of cells loaded with PS (red fluorescence) and organelle specific green fluorescent probes. (A and B) J774 macrophage cells that have been incubated with a lysosomal-targeted photosensitizer–protein conjugate with (A) lysotracker green and (B) mitotracker green. (C and D) J774 cells that were incubated with BPD and (C) lysotracker green and (D) mitotracker green. Scale bar is 10 μm.

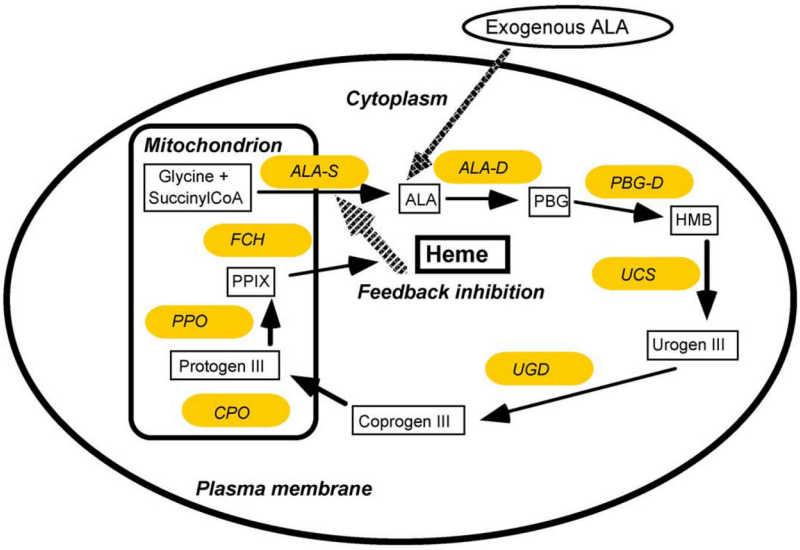

Figure 5.

ALA-induced PPIX. Schematic illustrating the interaction of the heme biosynthesis pathway with exogenous ALA to give intracellular PPIX. Abbreviations are ALA-D: ALA dehydratase; ALA-S: ALA synthetase; Coprogen III: coproporphyrinogen III; CPO: coproporphyrinogen oxidase; FCH: ferrochelatase; HMB: hydroxymethylbilane, PBG-D: porphobilinogren deaminase; protogen III: protoporphyrinogen; PPO: protoporphyrinogen oxidase; Urogen III: uropor-phyrinogen III; UCS: uroporphyrinogen cosynthase, UGD: uroporphyrinogen decarboxylase.