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REVIEW

Structural and physico-chemical determinants of the interactions of macrocyclic photosensitizers with cells

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Abstract New therapies have been developed using reactive oxygen species produced by light-activation of photosensitizers (PS). Since the lifetime of these species is extremely short and their diffusion in space is limited, the photo-induced reactions primarily affect the cell organelles labeled by the PS. In addition to the development of molecules with the best optical and photosensitizing properties, considerable research has been done to understand the physico-chemical parameters governing their subcellular localization. In this review, we examine these parameters to establish the structure/efficacy relationships, which allow specific targeting of PS. We examine the effect of subcellular localization on the cellular response to photosensitization processes. We discuss the determinants of subcellular localization, including the hydrophobic/hydrophilic balance, the specific charge effects and the dynamics of PS' transfer through membranes. Specific targeting can also be achieved with molecular structures able to recognize cellular or intracellular receptors, and this is also dealt with in this paper.

Abbreviations

5-ALA	5-Aminolevulinic acid
$AlPcS_n$	Sulfonated aluminum phthalocyanine
ATP	Adenosine-5′-triphosphate
Bcl-2	Antiapoptotic protein identified on B-cell lymphoma 2
Bid	BH3 interacting domain death agonist
BPD	Benzoporphyrin derivative
BPD-MA	Benzoporphyrin derivative monoacid ring A
Ce6	Chlorin e6
DP	Deuteroporphyrin
HP	Hematoporphyrin
HpD	Hematoporphyrin derivative
LCP	Lysyl chlorin p6
m-THPC	Meso-tetrahydroxyphenylchlorin
PBR	Benzodiazepine receptor
PCI	Photochemical internalization
PDT	Photodynamic therapy
Ppe	Pyropheophorbide-a
PpIX	Protoporphyrin IX
PS	Photosensitizer
ROS	Reactive oxygen species
SnET2	Etiopurpurin
$TPPS_n$	Sulfonated tetraphenylporphyrin
ZnPc	Zinc phthalocyanine

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Introduction

The recent development of diode lasers and optical fibers (van den Bergh 1998) has facilitated the use of light-sensitive drugs, in various therapeutic, and experimental applications. These techniques are based on the light irradiation of photosensitizers (PS), which generates active molecular species, such as free radicals, and singlet oxygen. These short-lived species are highly toxic for biological environment (Spikes 1982). Moreover certain PS accumulate selectively in proliferating tissues. This specific quality is used

in the treatment of several oncologic and ophthalmic diseases (Ackroyd et al. 2001; Levy and Obochi 1996; Miller et al. 1999). The difference in the PS concentration between normal and neoplastic tissues and the possibility to focus the light irradiation on the target zone are the key advantages of this application, currently known as photodynamic therapy (PDT) (Dougherty 1985; Dougherty et al. 1998). A new technique, the photochemical internalization (PCI), has been found to improve the therapeutic efficiency of macromolecules such as proteins or nucleic acids (Berg et al. 2003, 1999; Selbo et al. 2001). These macromolecules enter cells by bulk endocytosis, but stay trapped in the endo/lysosomal compartment and cannot reach their cytosolic or nucleic targets. By employing PS that are localized in the membranes of endosomes, PCI permits a light-activated destabilization of the lipid bilayer and liberation of endocytosed macromolecules prior to their destruction by lysosomal enzymes.

Photosensitized reactions

Photosensitization processes are illustrated in Fig. 1. The photosensitizer is excited by absorption of a photon of appropriate wavelength. Following an internal conversion from upper states, the PS reaches the first excited singlet state ^{1}PS. The deactivation of ^{1}PS via an intersystem crossing leads to the excited triplet state ^{3}PS. The lifetime of the triplet state is relatively long (~100 μs) in the absence of quenching. Consequently, the ^{3}PS has enough time to encounter other molecules leading to two types of photosensitized reactions. Firstly, it can react directly with a substrate to form free radicals by transfer of a proton or of an electron (type-1 reactions). These radicals can further combine with oxygen to form oxyradicals. Alternatively, in type-2 reactions, the triplet transfers its energy to molecular oxygen, to form excited singlet oxygen $^1O_2$. A common term used for all these species is the reactive oxygen species (ROS). Both type-1 and type-2 reactions occur, but the singlet oxygen production is considered to be a key factor in the efficacy of photo-induced damage (Weishaupt et al. 1976).

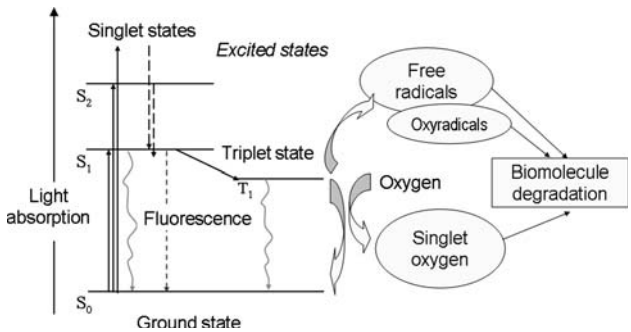

Fig. 1 Simplified scheme of photophysical processes and photosensitized reactions

Photosensitizers

Most of the PS used both clinically and experimentally, are derived from the tetrapyrrole aromatic ring. Porphyrins with a fully conjugated macrocycle represent the cardinal group of PS. Hematoporphyrin (Hp) was the first photosensitizer used in PDT. Hematoporphyrin derivative (HpD) forms the basis of the first commercial preparation Photofrin®. Deuteroporphyrin (DP) (Fig. 2) can be considered as the archetype of dicarboxylic porphyrins. All of these molecules are derived from heme, a natural compound that is the prosthetic group of hemoproteins. Chemical modifications of these molecules may lead to a significant improvement of their properties. Benzoporphyrins for instance, are particularly efficient in absorbing light in the red spectral region where tissues are the most transparent. The benzoporphyrin derivative monoacid ring A, BPD-MA (Richter et al. 1992), is the active component of Visudine® currently used for the treatment of age-related macular degeneration. Other PS derived from chlorophyll or bacteriochlorophyll have been developed. These compounds, called "second generation" PS, are characterized by a reduction of one (chlorins) or two (bacteriochlorins) double bonds of the tetrapyrrole ring, respectively, leading to increased light absorption in the far-red region (Bonnett 1995). Chlorin e6 (Ce6), Etiopurpurin (SnET2), pyropheophorbide-a (Ppe), and bacteriopyropheophorbide (TOOKAD) (Koudinova et al. 2003) belong to this class. Meso-tetrahydroxyphenylchlorin (m-THPC) (see Fig. 2) is a second-generation photosensitizer clinically used under the trade name of Foscan® (Bonnett et al. 1989).

Alongside the aforementioned developments, important work has been carried out in the synthesis of analogs of natural PS. The easily synthesized meso-tetraphenylporphyrin is one of the most important compounds of these synthetic molecules. It can be substituted as in the case of sulfonated meso-tetraphenylporphyrins (TPPS$_n$). Other synthetic macrocycles with their structures being similar to porphyrins (such as phthalocyanines and naphthalocyanines) have been subjects of numerous studies (Bonnett 1995). Aluminum phthalocyanine (AlPc), its sulfonated derivatives (AlPcS$_n$) (Brasseur et al. 1988) (Fig. 2) as well as zinc phthalocyanine (ZnPc) (Reddi et al. 1990) were found to be particularly interesting compounds, because of their photophysical properties.

In contrast to photosensitization by exogenous drugs as cited above, photosensitization can also be achieved by endogenous synthesis of protoporphyrin IX (PpIX) following

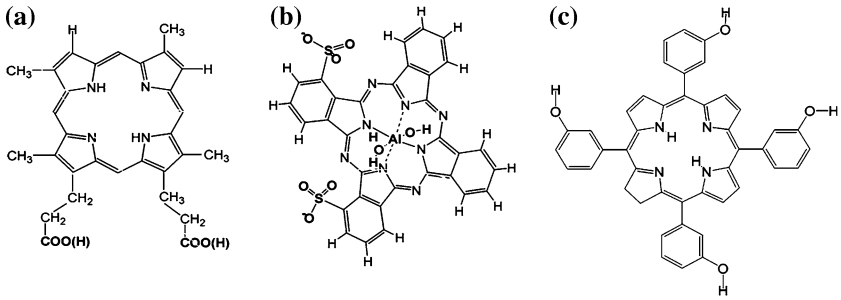

Fig. 2 Structures of photosensitizers: **a** Deuteroporphyrin (*DP*), **b** Disulfonated Aluminum Phthalocyanine ($AlPcS_{2a}$), **c** meso-Tetrahydroxyphenyl-chlorin (*m-THPC*)

the administration of 5-aminolevulinic acid (5-ALA) (Kennedy et al. 1990).

The photo-physical properties of the PS are mainly determined by the tetrapyrrole macrocycle, while its physico-chemical and biological interactions are governed by the nature of its lateral chains. For all groups of PS, extensive research is being carried out into designing the ideal molecule: one with a maximum absorption in the far-red spectral region; low dark toxicity; good photostability; a high singlet oxygen quantum yield; a high specific affinity for the targeted tissue and an appropriate subcellular localization.

Mechanisms of cell death as a function of subcellular localization

The lifetime of photo-induced transient species is extremely short, their diffusion in space is limited and the oxidative reactions primarily affect cell organelles labeled by the PS (Dougherty 1985). Consequently, subcellular localization of the drugs is one of the crucial parameters influencing the efficiency and extent of cell damage, as well as cell response and the mechanism of the resultant cell death.

PDT can induce apoptosis, necrosis or a combination of the two. The ability of PDT-exposed cells to initiate the apoptotic process differs depending on the cell line, the overall light dose, the incubation protocol, the type and concentration of the photosensitizer, as well as other conditions (Dellinger 1996; Kessel and Luo 1998; Kessel et al. 1997; Matroule et al. 1999, 2001; Oleinick et al. 2002). Apoptosis is a complex biochemical process characterized by different stages, specific hallmarks and timing. The morphological features specific to apoptotic cells are cell shrinkage, condensation of nuclear chromatin, formation of apoptotic bodies and DNA fragmentation. In contrast, necrosis results from high levels of cell damage, in which the plasma membrane integrity is lost and the cell is lysed (Almeida et al. 2004; Oleinick et al. 2002).

The subcellular localization and the early responses to light activation indicate that mitochondria play a major role in PS-mediated cell death. Effects on the bioenergetics, (Atlante et al. 1989; Salet and Moreno 1981, 1990) primarily on the mitochondrial permeability transition pore (Salet et al. 1997) have been investigated in detail (Dummin et al. 1997; Morgan and Oseroff 2001; Plaetzer et al. 2002). PDT may induce permeabilization of both mitochondrial membranes, dissipation of the transmembrane potential, and a release of apoptosis-related proteins (such as cytochrome c, apoptosis inducing factor, second mitochondria derived activator of caspases and certain procaspases) into the intermembrane space (Almeida et al. 2004; Oleinick et al. 2002).

On the other hand, it is well known that mitochondria are critical sites for initiating both necrotic and apoptotic cell death. Plaetzer proposes that the mode of cell death is related directly to the changes in the ATP pool. Assays of procaspace-3 activation and nuclear fragmentation on human epidermoid carcinoma cell line A431 have shown that while impairment of mitochondrial functions and rapid decrease of ATP are symptomatic of necrotic cell death, the preservation of high ATP levels allows the execution of the apoptotic program (Plaetzer et al. 2002). The photosensitized damages on lysosomes are generally less lethal than that sustained by mitochondria (Berg and Moan 1994). The release of lysosomal enzymes can nevertheless influence the apoptotic response. Cleavage of Bid by lysosomal proteases can precipitate cytochrome c release from mitochondria and subsequent caspase activation (Matroule et al. 2001; Stoka et al. 2001). On the other hand, Kessel and Luo showed that the cathepsins released from the damaged organelles cleave procaspase-3 and stunt the apoptotic pathway (Kessel and Luo 2001; Kessel et al. 1997). Thus PDT damage to lysosomes can induce apoptosis as well as inhibit it.

It is well known, that with increasing incubation time, PS can migrate from the plasma membrane to more sensitive stores within the cell. Indeed, a short incubation time induces more plasma membrane alterations, while a long incubation time results in more subcellular damage (Kessel 1986; Moan et al. 1984). This photosensitizer redistribution can change the mechanism of cell death. Supporting these findings, Dellinger observes morphological changes typical for the apoptosis of CV-1 cells after prolonged incubation with Photofrin, while the necrosis is predominant after a

short incubation. The microscopy observations suggest that in the latter case the cause of cell necrosis is cell membrane damage leading to degradation and lysis (Dellinger 1996).

Light doses also play a role in determining the cell death mechanism. Lou and Kessel reported that the apoptosis of murine leukemia P388 or L1210 cells was observed at lower PDT doses and that this was associated with mitochondrial and lysosomal photodamage (Luo and Kessel 1997). At high PDT doses, however, necrosis was induced by preferential plasma membrane photodamage. Several studies of cell response to PDT have revealed that photodamage involving lysosomes and mitochondria leads to rapid apoptosis, whereas plasma membrane photodamages delay or prevent the apoptotic response (Dellinger 1996; Kessel et al. 1997; Luo et al. 1996; Luo and Kessel 1997). In conclusion, it appears that apoptosis prevails over necrosis when the photosensitizer accumulates in mitochondria and lysosomes.

All of the above-mentioned results outline the importance of the subcellular localization of the PS. However, the loss of integrity of intracelullar organelles might result in a redistribution of PS during or after irradiation. PS which are initially localized in lysosomes may be released upon the damage of these and can cause a photodamage to more sensitive organelles such as mitochondria, plasma membrane, nucleus or nucleoli (Ruck et al. 1992; Wood et al. 1997). Photodamage-induced redistribution of PS from mitochondria to cytoplasm has also been observed (Morgan et al. 2000).

Determinants of the subcellular localization of photosensitizers

Since the majority of PS exhibit also some fluorescence, the development of confocal fluorescence imaging techniques and a variety of organelle-specific markers permit to determine the subcellular distribution of PS with high sensitivity and good spatial resolution. As detailed in the following paragraphs, mitochondria and lysosomes are major PS localization sites. The localization of PS in the Golgi apparatus and endoplasmic reticulum is less frequent and more likely related to a non-specific solubilization of the photosensitizer in the membranes of intracellular organelles. This type of distribution has been observed for example, for m-THPC in MCF-7 human adenocarcinoma cell line (Teiten et al. 2003). Nevertheless, m-THPC was observed to accumulate in mitochondria of myeloid leukemia cells (Chen et al. 2000). Photosensitizer localization in the plasma membrane has also been observed. Kessel describes lethal photo-damage of leukemia cells, induced by a plasma membrane labeling by mono- and di-acid BDP (Kessel 1989). Using the total internal reflection microspectrofluorimetric technique, Sailer et al. have proved a localization of $TPPS_3$ and $TPPS_4$ in the vicinity of the plasma membrane of endothelial cells (Sailer et al. 2000). It should be noted, that the subcellular localization depends also on the cell type used for the experiments. The 5-ALA induced PpIX was found to be localized in cellular membranes of U373 MG glioblastoma and OV2774 ovarian cells, whereas granular distribution was observed in T47D breast cancer cells (Sailer et al. 2007).

Structural parameters determining subcellular localization

Among the factors governing the interactions between PS and cells, the PS' degree of hydrophobicity, charge and structural asymmetry are of paramount importance.

Hydrophobic/hydrophilic balance

Hydrophobic/hydrophilic balance is modulated by the presence of lipophilic and charged side-chains around the macrocycle. The general findings have shown that hydrophobic PS bearing none or few negative charges can diffuse across the plasma membrane and redistribute between the membranes of cellular organelles, while hydrophilic PS with many positive or negative charges are too polar to cross the plasma membrane and are rather internalized by endocytosis (Berg et al. 1990; Bonneau et al. 2004b; Woodburn et al. 1991). A rational basis for this behavior is discussed in more detail within the paragraph describing the dynamics of the interaction between PS and membranes.

The degree of hydrophobicity is generally given by Log P, defined as the logarithm of the partition coefficient of the molecule between an organic solvent (for instance octanol) and water (Gudzinowicz et al. 1984). Simple pharmacokinetic models based on alternating aqueous and lipid compartments predict a parabolic relation between Log P and the logarithm of the drug concentration at the target site (Aarons et al. 1982; Cooper et al. 1981). Studies on a series of PS differing by their hydrophobicity or charge have been carried out on cells or tumor models in order to investigate this relationship with more realistic biological systems.

Four $TPPS_n$ bearing 1 to 4 sulfonate groups were examined by Sailer et al. (2000) and Berg et al. (1990). The more hydrophobic, $TPPS_1$ and $TPPS_{2a}$ showed diffuse distribution in the cytoplasm and granular structures in BKEz-7 endothelial cells and human cervix carcinoma cells. Microscopy studies indicated that $TPPS_4$ was internalized probably by endocytosis and located in lysosomes. However, some extralysosomal partition of the dye was deduced from energy transfer measurement using acridine orange as an energy acceptor (Berg et al. 1990). This extralysosomal localization and plasma membrane labeling by hydrophilic compounds were confirmed by using the total internal

reflection fluorescence spectroscopy technique (Sailer et al. 2000).

Soukos et al. (1997) have synthesized polycationic, polyanionic, and neutral poly-L-lysine conjugates of Ce6. They have compared their cellular uptake, location and phototoxicity on the human endothelial hybrid cell line EA.hy926 and the human epidermoid squamous carcinoma line A431. Cell viability after irradiation was determined by a (MTT)-micro culture tetrazolium assay. The cationic conjugate was taken up in larger amounts but, due to formation of large aggregates, remained on the surface of the plasma membrane. It is noteworthy that this localization limits the photo-oxidation efficiency of the drug. The anionic and neutral conjugates were internalized by endocytosis and were located in discrete punctuate organelles, probably endo/lysosomes.

Ricchelli et al. (2005) carried out an interesting study on a series of meso-substituted tetra-cationic porphyrins whose degree of lipophilicity was varied through substitution by alkyl chain of various chain lengths. The cell photoinactivation efficiency was directly correlated with both, hydrophobicity and the porphyrin accumulation.

In vivo studies on an animal model have been carried out on a congeneric series of pyropheophorbide PS in order to derive quantitative structure-activity relationship (QSAR) (Henderson et al. 1997). The hydrophobicity of the compounds was modified by the length and the type of the ether side chain. The photosensitizing efficiency increased with increasing the degree of hydrophobicity, with the maximum in the range of Log P 5.5–6.5. PDT activity declined gradually with higher log P even though the degree of incorporation progressively increased with lipophilicity.

Although high hydrophobicity ensures high levels of photosensitizer in target cells, this is not sufficient for optimal PDT efficiency. Probably pharmacodynamic factors, such as differences in intercellular localization or changes in the physical state of the photosensitizer such as aggregation, may account for the differences in PDT activity.

Specific charge effects

Due to their transmembrane potential and negatively charged internal membrane, mitochondria are the sites of accumulation of cationic PS along the membrane potential gradient (Dummin et al. 1997; Morgan and Oseroff 2001). This accumulation also depends on the degree of lipophilicity and the charge delocalization of the PS (Chen 1988). Positively charged tryarylmethane, rhodamine, and cyanine dyes were reported to be efficient mitochondria localizers (Beckman Jr et al. 1987; Kandela et al. 2002; Oseroff et al. 1986). Selective photosensitization of mitochondria in HeLa cells was achieved by cationic Zn(II)Pc. The addition of lipophilic side chains to cationic Zn(II)Pc increases their specific accumulation in the inner mitochondrial membranes, which enhances the phototoxic effect compared to anionic ZnPcS localized in the cytoplasm (Dummin et al. 1997). However, not all cationic PS localize in the mitochondria as demonstrated by the work of Ricchelli et al., which have shown a lysosomal localization of meso-substituted tetra-cationic porphyrins (Ricchelli et al. 2005).

The influence of the symmetry of charge distribution can be illustrated by the work of Kessel et al. (2003). This study concerned two meso-tetraphenylporphyrin derivatives bearing two cationic trimethylamonium groups in adjacent or opposite positions. The compound with adjacent cationic residues labeled the mitochondria, whereas the symmetric conjugate localized in the lysosomes of murine leukemia cells.

It must be pointed out that neutral or anionic PS also localize in mitochondria (Chen et al. 2000) illustrating the complexity of the binding processes. Some of them are being elucidated as discussed later.

Aggregation state

The hydrophobic/hydrophilic balance also governs the self-association of PS in aqueous environment. Association is favored for amphiphilic molecules with polar or charged chains located only on one side of the macrocycle (Dairou et al. 2002). The fluorescence and most frequently the singlet oxygen quantum yields of aggregated species are lower than those of monomeric form, reducing their photodynamic efficacy (Krasnovsky Jr et al. 1990), although this rule is not unequivocal (Dairou et al. 2002; Moan 1984). The formation of large-scale aggregates upon injection of quite hydrophobic PS may considerably modify their pharmacokinetics as compared to monomer forms. Aggregates of the second-generation photosensitizer m-THPC (Foscan) have been identified. Binding to plasma proteins leading to monomerization of aggregates was found to be rather slow (Sasnouski et al. 2005).

Another important question in this domain is the cellular internalization of aggregates. The uptake and the aggregation/disaggregation behavior of PS in tumor cells were studied by time-resolved fluorescence coupled to laser scanning microscopy (Kelbauskas and Dietel 2002). Three derivatives of Ppe and Ce6 bearing alkyl side chains of different lengths (C6–C9) were used in this study. The results showed that the A431 cells took up the aggregates of all compounds but only the less lipophilic molecules disaggregated in the cell. The aggregates were internalized most probably by endocytose pathway and they might partially monomerize in endocytotic vesicles. Intracellular disaggregation of amphiphilic PS was proposed as an explanation for their higher photodynamic efficacy compared to more lipophilic derivatives for which the tumor

response was considerably reduced (Kelbauskas and Dietel 2002).

Cellular uptake and subcellular localization of a series of Ppe ethers bearing C3–C12 acyl chains were studied on human pharyngeal squamous cell carcinoma (FaDu) and radiation-induced fibrosarcoma (RIF) cells in the work of MacDonald et al. (1999). The results revealed a relationship between the lateral chain length, the aggregation state, the subcellular localization and the phototoxicity of these compounds. The authors hypothesize that depending on the aggregation state, there are two internalization pathways. The highly aggregated forms are internalized by endocytose and remain sequestered in the lysosomes, while monomers, mainly localized in the mitochondria, would be internalized by another pathway. Monomers apparently were the active fraction of these compounds because equalizing the extracellular monomer concentration, produced equivalent intracellular concentrations, phototoxicity, and localization patterns. On the other hand, aggregates localized in the lysosomes where they were less active (MacDonald et al. 1999). These results suggest the importance of the parameters governing the aggregation state of PS by an indirect effect on their pharmacokinetics and on their intracellular distribution (Kessel et al. 1987; MacDonald et al. 1999).

Delivery systems

Numerous PS are amphiphilic or hydrophobic compounds that have a tendency to self-associate in physiologic aqueous environment. Hence, different delivery systems such as liposomes, Cremophor EL, cyclodextrin, or lipoproteins have been evaluated in order to facilitate the transport of water-insoluble PS (Derycke and de Witte 2004; Kessel et al. 1991; Richter et al. 1993; Young et al. 1996; Zhou et al. 1988). The use of specific delivery systems can modulate PS pharmacokinetics and cellular uptake. The administration of porphyrins incorporated into DPPC liposomes enhances the accumulation and prolongs the retention of PS by cultured cells and experimental tumors (Cozzani et al. 1985; Jori et al. 1983; Reddi et al. 1990). Hp injected in aqueous solution induces mainly vascular damage, whereas transport by liposomes or LDL enhances the concentration of drug accumulated by tumor tissue (Jori et al. 1986). The vectorization by LDL (Bonneau et al. 2002) influences both, the global cellular uptake and the subcellular localization of $AlPcS_{2a}$ (Bonneau et al. 2004b).

It was reported that LDL-bound PS are at least partly internalized via LDL specific endocytose (Bonneau et al. 2004b; Candide et al. 1986; Schmidt-Erfurth et al. 1994). Moreover, due to their enhanced requirements of cholesterol, several tumor cells over-express the Apo B receptors (Gal et al. 1981; Ho et al. 1978). Consequently, the LDL mediated transport was suggested to enhance the selective accumulation of some PS in the tumor tissue (Jori et al. 1984; Kessel 1986; Reyftmann et al. 1984). It was also pointed out that the pH-dependant exchange of PS between albumin and LDL could play a role in the selective retention of some of these molecules (Mojzisova et al. 2007).

Dynamics of photosensitizer transfer through membranes and subcellular localization

The passive cellular uptake of PS and its partition among subcellular structures is most likely dependent on the dynamics of its permeation through membranes. The quite complex structure of PS renders difficult usual predictions based on octanol/water partition (Gudzinowicz et al. 1984). Correlation was found only for series of compounds sharing a similar structure (Kepczynski et al. 2002). Two factors may account for this difficulty. Firstly, owing to the large size of its macrocycle, PS has a strong tendency to dimerize and aggregate (Ben-Dror et al. 2006). Secondly, asymmetric distribution of side chains around the macrocycle can lead to specific interactions with the membrane bilayer.

Membrane model systems

In order to get a better insight into the dynamics of these processes our group has developed methods based on membrane models consisting in unilamellar vesicles and kinetic analysis (Kuzelova and Brault 1994; Vever-Bizet and Brault 1993). Figure 3 represents the schema of the passive transport of a molecule through a lipid bilayer. For simplicity, it is assumed in this schema that the molecule goes from the aqueous compartment at the bottom to the one at the top. The lower compartment can be seen here as the extracellular medium, but obviously, the same conclusion will apply in the case of the passage through the inner membranes that delineate subcellular structures. The passage through the membrane is governed by three rate constants characterizing the entrance (k_{on}), the diffusion through the lipid phase or flip-flop (k_t), and the exit (k_{off}) steps. We have been interested in amphiphilic PS with polar or charged chains distributed on only one side of the macrocycle (Kuzelova and Brault 1994; Maman et al. 1999). Owing to this asymmetrical structure, it can be postulated that the polar or charged side chains most likely interact with the polar heads of the phospholipids and the macrocycle core is buried in the lipidic phase. As a matter of fact, the fluorescence spectrum of such molecules was indicative of a lipid environment (Brault et al. 1986). Kinetic analysis of these systems predicted that if the photosensitizer transfers through the bilayer, a biexponential

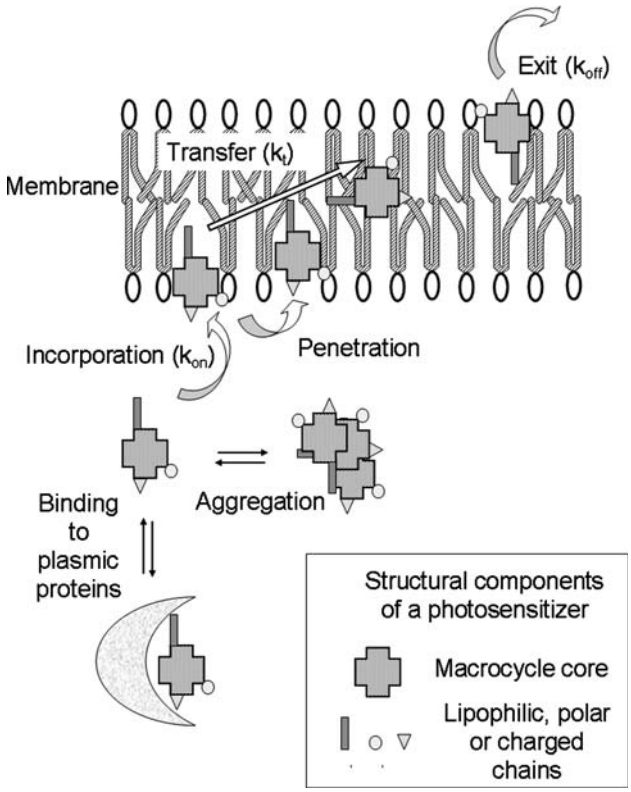

Fig. 3 Simplified scheme of photosensitizer interactions with plasma carriers and cells. The equilibrium between the binding to plasma proteins, aggregation and interaction with the lipid membranes is illustrated. The process of the PS diffusion through the lipid bilayer is characterized by the entrance k_{on}, transfer k_t, and exit k_{off} rate constants

signal should be observed on mixing aqueous solutions of the photosensitizer with aqueous suspensions of vesicles. This was actually observed in stopped-flow experiments for selected concentrations of vesicles. This biphasic signal allowed (Kuzelova and Brault 1994) the calculation of the rate constants k_{on}, k_{off}, and k_t. In fact, for amphiphilic molecules with sufficient water solubility to make them present as monomers, the rate of entrance was found to be extremely fast reaching the limit of diffusion of vesicles and PS in solution. A more sophisticated method involved mixing albumin with vesicles pre-loaded with the photosensitizer that populates both hemileaflets at equilibrium. Albumin possesses high affinity binding site(s) for tetrapyrrole macrocycles. On mixing, albumin extracts the photosensitizer remaining free in solution and displaces the equilibriums governing the interaction of the macrocycle with vesicles. As detailed elsewhere (Kuzelova and Brault 1994) this method allowed clean determination of the rate constants k_t and k_{off}. Combining the two methods, we have been able to characterize the dynamics of interaction of various PS with phospholipidic vesicles differing in lipid composition or for different pH (Bonneau et al. 2004a; Kuzelova and Brault 1995; Maman and Brault 1998; Maman et al. 1999). The rate constants k_{off} and k_t were strongly affected by the physical state of the lipid bilayer, which was modulated by the presence of cholesterol and temperature as detailed elsewhere (Kuzelova and Brault 1994). The membrane thickness also has an important effect on the k_t value (Maman and Brault 1998). Through the control of the ionization state of PS bearing carboxylic chains, pH had a drastic effect on the two rate constants (Bonneau et al. 2004a; Kuzelova and Brault 1995; Maman and Brault 1998). The exit of anionic forms (deprotonated chains) was accelerated while the reverse effect was observed for their flip-flop. As a consequence, for a dicarboxylic porphyrin, it can be predicted that a difference of 1 pH unit between two aqueous compartments separated by a phospholipid bilayer accelerates the transfer from the hemileaflet facing the acidic phase to the other hemileaflet by a factor of about 100 (Bonneau et al. 2004a; Kuzelova and Brault 1995; Maman and Brault 1998). On the other hand, macrocycles bearing non-neutralizable side chains, such as sulfonate, were found to not permeate model membranes over hours (Maman et al. 1999).

Subcellular targeting of photosensitizers

For both PDT and PCI applications subcellular targeting of PS appears as a main challenge. Different specific and non-specific mechanisms of photosensitizer targeting are discussed in this part.

Physico-chemical targeting based on the dynamics of interactions of photosensitizers with membranes

The methods to characterize pertinent dynamic parameters by using membrane models have been detailed above. Bonneau et al. have demonstrated the correlation between the intracellular distribution of deuteroporphyrin (DP) and disulfonated aluminum phthalocyanine ($AlPcS_{2a}$) and the dynamics of their interaction with model lipid membranes (Bonneau et al. 2004b). DP, bearing two carboxyl groups crosses the lipid bilayer within seconds whereas the transfer through the membrane is extremely slow in the case of $AlPcS_{2a}$ bearing two negatively charged sulfonate groups. In agreement with the results obtained using the model system, the fluorescence microscopy on human fibroblasts HS68 showed diffuse and punctuate labeling for DP and $AlPcS_{2a}$, respectively. The authors concluded that the porphyrin molecules quickly enter the cells by passive diffusion and relocate between other intracellular membrane structures. In contrast, permanently charged sulfonate groups anchor $AlPcS_{2a}$ to the membrane surface and restrain its diffusion across the membrane. Consequently $AlPcS_{2a}$ is internalized mostly by bulk endocytose. These

results are in agreement with the endo/lysosomal localization of a series on sulfonated porphyrins and phthalocyanines and the diffuse cytoplasmic localization described for non-sulfonated phthalocyanines (Berg et al. 1990; Malham et al. 1996).

The possibility to control the subcellular localization by the photosensitizer design can be extremely useful for the optimization of these drugs for different applications. For example the diffuse cytoplasmic distribution is generally related to the good photodynamic efficiency, while the endo/lysosomal localization is crucial for non-lethal PCI.

Molecular targeting: benzodiazepine receptor

Recent studies suggest that some PS may exhibit a specific affinity for certain components of cellular organelles, i.e., lipids or proteins. The peripheral benzodiazepine receptor (PBR) is a suitable example to illustrate this specific interaction. PBR is a mitochondrial-binding site for naturally occurring porphyrins such as PpIX and heme (Verma et al. 1987). It is localized on the outer mitochondrial membrane and is involved in numerous functions including mitochondrial respiration, intramitochondrial cholesterol transport involved in steroid biosynthesis (Hauet et al. 2005; Liu et al. 2006), heme biosynthesis, cell proliferation and calcium channel modulation (Kozikowski et al. 1997). Its prominent physiological functions make PBR a very vulnerable PDT target. Hence, many studies of photosensitizer binding to PBR receptor were carried out. Several dicarboxylic porphyrins were reported to be potent inhibitors of the binding of benzodiazepines to peripheral drug receptors in mitochondria (Kessel 1988). The direct correlation between photo-therapeutic potencies of PS, measured by clonogenic assays, and their affinities for mitochondrial benzodiazepine receptors was established for porphyrins (Verma et al. 1998). However, this correlation was not confirmed for series of Ppe. Kessel proposes that the relationship between PDT efficacy and PBR affinity may hold only for sensitizers with the PpIX configuration (Kessel et al. 2001). Morris et al. observed the presence on PBR of low-affinity binding sites for phthalocyanines in rat kidney mitochondria (RKM) and intact Chinese hamster ovary (CHO) cells. The binding to PBR was found to be less relevant to the phthalocyanine-mediated cell photocytotoxicity than other mitochondrial events, such as photodamage to the antiapoptotic protein Bcl-2 (Morris et al. 2002).

It should be noted, that up-regulation of PBR has been observed in certain tumors (Batra and Iosif 1998; Venturini et al. 1998). Hence, the specific affinity of PS for PBR could be exploited to selectively target some tumor tissues.

Targeting with photosensitizer conjugates

The covalent association of a photosensitizer with a molecular structure able to recognize cellular or intracellular receptors is expected to further improve the selectivity of PDT. These conjugates named third generation PS are being the subject of extensive studies that have been reviewed recently (Sharman et al. 2004). Photosensitizer conjugates with ligands selective to various cellular receptors, mostly over-expressed by tumor cells, have been prepared. Receptor to LDL (Schmidt-Erfurth et al. 1997), folic acid (Schneider et al. 2005), sugar (Laville et al. 2003), and polyamines (Garcia et al. 2006) were considered. Interestingly, the possibility to target subcellular structures, in particular the nucleus has been demonstrated (Akhlynina et al. 1997; El-Akra et al. 2006). Such approaches open a large field of applications. However, it should be kept in mind that the global biological efficacy of these conjugates might be further governed by the dynamics of their intracellular distribution before they reach their target.

Conclusion

The subcellular localization of PS is one of crucial factors determining the global biological consequences of photo-induced reactions. The understanding of the mechanisms, governing the intracellular distribution of tetrapyrrole drugs, might lead to development of new PS with optimized interactions with biological structures and enhanced selectivity for target tissues.

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