Tetrapyrrole photosensitisers, determinants of subcellular localisation and mechanisms of photodynamic processes in therapeutic approaches

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Background: Tetrapyrrole photosensitisers are the main drugs used in the various photodynamic therapies. They are based on light-induced reactive oxygen species whose lifetimes are very short, limiting their diffusion in space. The photoinduced alterations primarily affect the labelled structures, at the systemic and subcellular levels. Thus, in parallel to the improvement of the photophysical and photochemical properties of photosensitisers, focus has been given to the parameters governing their localisation.

Objectives and Methods: We analyse the parameters that support the efficiency of photosensitisers, paying particular attention to their structural and physicochemical characteristics, especially the dynamics of photosensitisers towards membranes.

Conclusion: The determinants of systemic and subcellular targeting are discussed with regard to the induced response.

Keywords: kinetics, low-density lipoproteins, photochemical internalisation (PCI), photodynamic therapy (PDT), targeting, tetrapyrrole photosensitisers

1. Introduction

Photosensitisers (PS) are non-toxic dyes that can be activated by harmless light irradiation. The preferential retention of certain photosensitisers by tumours, as compared to normal surrounding tissues, has been recognised for many years [1,2]. This capability, and the ability of these molecules to generate toxic species on light illumination, is the basis of a selective therapeutic approach, the photodynamic therapy (PDT). The administration of the non-toxic PS systemically, locally or topically to the patient is followed, after a time-delay, by the irradiation of the lesion with light, usually from the long-wavelength, red scale of the spectrum. The irradiation leads to the generation of cytotoxic species and subsequently to cell death. One of the most attractive characteristics of PDT, then, is the concept of dual selectivity, as a result both of the selectivity of the PS to its target and of the possibility of restricting the illumination to the diseased area. Originally developed as a tumour treatment [3-5], PDT has recently been successfully applied to non-malignant diseases, such as age-related macular degeneration [6-8].

2. Photosensitisers, photodynamic reactions and therapeutic uses

2.1 Photosensitised reactions
The photocytotoxic effects are initiated by the absorption of light by a photosensitiser. A photosensitiser induces, under the light-irradiation effect, the
Tetrapyrrole photosensitisers, determinants of subcellular localisation and mechanisms of photodynamic processes in therapeutic approaches

![Jablonski diagram](image)

Figure 1. Photodynamic processes. The photophysics of the photosensitisers and the two photochemical reaction pathways from the triplet state of a photosensitizer. On this Jablonski diagram showing the electronic states following absorption of light by a photosensitiser, the arrows represent the different processes (Abs., absorption; Fluo., fluorescence; Phospho., phosphorescence; V.R., vibrational relaxation), the excited singlet states of the photosensitizer are $S_1$, $S_2$, ... and the triplet state ($T_1$) is obtained after intersystem crossing (I.S.C.).

55 alteration of another molecule. When a molecule absorbs a light quantum, its electronic state changes from the ground state to one of many vibrational levels in excited electronic states (Figure 1). The higher excited states are dissipated very rapidly (picoseconds) down to the first excited singlet state with a lifetime of the order of nanoseconds. This excited state can be deactivated by non-radioactive decay or by emission of fluorescence (utilised for cancer diagnosis, see below), or can undergo intersystem crossing (ISC), which allows population of the triplet state. Although ISC is usually forbidden, it is permitted to a large extent for macrocyclic molecules with conjugated double bond systems ($\pi$-electron system). In the long-lived triplet state, the photosensitiser can then react with surrounding molecules. Two reaction types are involved: a type I mechanism involving electron or hydrogen atom transfer from one molecule to the other, and a type II mechanism involving energy transfer to molecular oxygen leading to singlet oxygen formation. Here, it should be noted that, in most cases, direct and indirect evidence exists for the importance of $^{1}\text{O}_2$ in photodynamic processes of PDT [9].

59 For therapeutic use, a good photosensitiser should thus absorb photons efficiently, have a high quantum yield of triplet formation and have a triplet state that is long-lived enough to react with surrounding target molecules. Most of such compounds have tricyclic, heterocyclic or porphyrin-like ring structures with conjugated double bonds [10,11].

75 Whereas both the selective localisation of porphyrins in tumours and tumour regression after light exposure were reported in the 1950s [13,14], the contemporary explosion of interest in the method dates from the discovery of haematoporphyrin derivative (HPD), followed by both chemical and clinical studies [15-18]. HPD has been used to localise malignant tumours [19]. To improve its tumour selectivity, the preparation and purification methods have been developed and patented [20-22]. A semi-purified preparation of HPD — Photofrin® — was the first regulatory approved PS for PDT treatment of various cancers. All these molecules are derived from haem, the natural compound that is the prosthetic group of haemoproteins.

79 However, some disadvantages of the HPD-PDT have been observed: a prolonged skin sensitivity necessitating avoidance of sunlight for many weeks [23], a non-optimal selectivity of the PS for tumour [24,25], a poor penetration of the light excitation wavelength into the tumour and the fact that Photofrin® is a complex mixture of uncertain structures [26]. The development of new PS has therefore become a relevant challenge in recent years. [27,28] In parallel, PDT has found new applications in the treatment of ophthalmic diseases [7,8,29]. Chemical modifications of the porphyrin molecules might lead to a significant improvement of their properties. For instance, benzoporphyrins, which have led to a lot of patents [30-33], are particularly efficient in absorbing light in the red spectral region where tissues are the most transparent. The benzoporphyrin derivative monoacid ring A [34] is the active component of Visudyne®, which is currently used in the treatment of age-related macular degeneration. Photosensitisers derived from chlorophyll or bacteriochlorophyll have also been developed.

83 2.2 Tetrapyrrole photosensitisers

85 Most of the photosensitisers that are used clinically and experimentally are derived from the tetrapyrrole aromatic ring porphyrins [12], with a fully conjugated macrocycle.

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These compounds, called ‘second generation’ photosensitisers, are, respectively, characterised by a reduction of one (chlorin) or two (bacteriochlorin) [35] double bonds in the tetrapyrrole ring, resulting in increased light absorption in the far-red region [30-33,35-37]. For instance, meso-tetrahydroxyphenylchlorin, clinically used under the name of Foscan® [38] and Photosolor®, belong to this class. Others second-generation photosensitisers [39-41] have been developed, for example, the etiopurpurin, pyropheophorbide-a, chlorin e6, and bacteriopyropheophorbide (Tookad®) [42]. In parallel, analogues of natural photosensitisers have been subjects of numerous studies.

The meso-tetraphenylporphin is one of the most important compounds of such synthetic molecules. This macrocycle can be substituted, as in the case of sulfonated meso-tetraphenylporphins. Other synthetic molecules [43-46] with a large macrocycle, such as phthalocyanines or naphthalocyanines, have, for example, been synthesised and studied [47]. The photophysical properties of the aluminium phthalocyanine, its sulfonated derivatives [48] and the zinc phthalocyanine [49] were found to be particularly interesting.

Here, it should be noted that photosensitisation can also be achieved by endogenous synthesis of protoporphyrin IX following the administration of 5-aminolevulinic acid, and not induced by exogenous drugs as cited above [50].

Thus, a great many PS are approved or in trial (Figure 2) and, consequently, the question of the choice of a suitable PS for a particular disease or application is pertinent.

2.3 Therapeutic uses of tetrapyrrole PS

Photodynamic therapy (PDT): PDT is a selective therapeutic approach based, as mentioned above, on the retention of photosensitisers by tumours and the ability of PS molecules to generate short-lived toxic species on light irradiation [1,2]. The light-induced molecular damages result in a targeted cell’s death. Recently, this therapy benefited from the development of laser and optical fibres, and it is now an established procedure for the treatment of some cancers [2,51]. Photofrin®, the first preparation approved since 1995 to treat some cancers, is based on porphyrins. As discussed previously, one of the disadvantages of such first-generation photosensitisers is their absorbance properties in a spectral-range where light is highly attenuated by tissues; a second generation is therefore being developed with improved light absorption in the red region [28].

Owing the very short lifetime of the phototoxic species, the PS is therapeutically active only during the irradiation, and only the labelled tissues are affected. Compared with conventional therapy, an important advantage of the PDT is its dual selectivity due to the preferential retention of the PS in the tumoural tissues and the possibility to restrict the irradiated zone to the diseased area.

Photochemical internalisation (PCI): Recently, with major advances in biotechnology, PS-induced photodynamic effects have found another promising area of application: numerous emerging therapeutic agents are based on polynucleic or polyamino acids. Such drugs possess an enormous potential but their efficacy requires appropriate delivery systems. Indeed, although they can quite easily reach extracellular targets, they have great difficulties in reaching their intracellular targets: after their uptake by endocytosis, they are sequestered in endocytic vesicles and are subsequently degraded in the lysosomes. This is an important limitation for the therapeutic use of such macromolecules with intracellular targets [52-54].

Photochemical internalisation is an interesting new approach to inducing the release of molecules from endocytic vesicles, based on the photodynamic destabilisation of their membrane [55-58]. Indeed, owing to the brief lifetime of the cytotoxic light-induced species, these diffuse < 0.1 µm in a biological environment. The photodynamic processes are thus very localised and, consequently, can damage biomolecules only in the vicinity of the photosensitisser (lipids of the endocytic membranes in the case of PCI-used PS). These alterations of the membrane induce its destabilisation and then the release of its content, including the macromolecule of therapeutic interest, which can then reach its target (Figure 3).

The PCI technology has been found to strongly improve the biological efficiency of various active macromolecules [59]. Recently, the PCI effect has been used in tumour destruction in vivo to treat subcutaneous tumour models in mice [60-62].

Fluorescence diagnosis (called photodynamic diagnosis, PDD): In addition to the curative application of photosensitisers, their fluorescent properties and their selective retention by proliferating tissues can be further exploited for diagnosis [63-65]. The photosensitisers used in PDT accumulate preferentially in tumoural tissues as compared with the normal tissue. The ratio of concentration in target vs. normal tissue is usually about 2 – 4:1 for exogenous photosensitisers, but can be higher [66,67]. For endogenous porphyrins accumulated after treatment with 5-aminolevulinic acid or its ester derivatives, the ratio is more important (around 10:1 [68]). The reasons involved in this preferential accumulation are not fully understood, but imply changes in the activities of the enzymes in the haem synthetic pathway. Nevertheless, this property of both endogenous and exogenous photosensitisers is utilised for diagnosis of different type of cancers [68-70].

3. Pharmacokinetics, tumoural selectivity and tumour destruction

3.1 Pharmacokinetics and biodistribution

Bloodstream injection involves a series of events for the PS. Each step can take very different lengths of time for different PS. First, the PS will be distributed between the various blood components. This equilibrium involves the PS in disaggregating or dissociation from the delivery systems, binding to various serum proteins and lipoproteins and, also, its association to the blood circulating cells. The second step corresponds to the binding of the PS to the blood...
Tetrapyrrole photosensitisers, determinants of subcellular localisation and mechanisms of photodynamic processes in therapeutic approaches

Figure 2. Examples of tetrapyrrole photosensitisers. Dimeric and monomeric compounds of A. Photofrin®, B. Foscan®, C. Vidudyne® used in PDT and D. disulfonated aluminum phthalocyanine efficient for photochemical internalisation (PCI).

vessels wall: the different characteristics of blood vessels in normal and tumoural tissues, as well as the type of vessels in the various organs, will govern this association. Third, the PS will penetrate the wall of the blood vessel. After extravasation, the PS will diffuse throughout the extracellular medium of the tissues or organ to which it has been delivered. If this organ is the liver (or active organ), the PS may be metabolically modified (note that such modification is unlikely for tetrapyrrole PS used clinically). At this moment, the PS may penetrate the tumoural cells by various processes (see later). Finally, the PS will be eliminated from the body, by lymphatic drainage or, if it has been delivered in the liver, generally via fecal elimination. An interesting point is that tumours have little lymphatic drainage, which appears to be one of the reasons for the accumulation of the PS in the cancerous area.
The large temporal variability of these events must be emphasised. Numerous studies have shown that the variation in pharmacokinetics reported for different PS is very large. For example, Photofrin® has been studied in carcinoma patients, and its pharmacokinetics have been described as a triexponential – three-compartment – model, the components having half-lives of 16 h, 7.5 days and 155.5 days. Photofrin® can be detected in the serum for more than 1 year [71]. In tumour-bearing rats, Foscan® has been found to exhibit a triexponential pharmacokinetic model, with half-lives of 0.46, 6.91 and 82.5 h [72], and Tookad® presents a two-compartment kinetics with very rapid half-lives (2 min and 1.3 h [73]).

The overall pattern of PS distribution has been determined in some animal models. In mice, the concentrations were determined to be very high in liver, adrenal gland and urinary bladder, where the concentration peak is greater than in the tumour. In particular, the high-molecular-weight compounds of Photofrin® are fully retained in the liver and the spleen 75 days after injection [74].

In summary, the distribution of PS appears to be complex. Their retention in tumour and their elimination pathways seem to be dependent on the structure of the PS. For instance, the accumulation of sulfonated phthalocyanines in liver is inversely proportional to the degree of sulfonation, and hence their degree of lipophilicity [75].

### 3.2 PS structure, delivery systems and tumoural retention

Since the observation of the localisation of porphyrins and their derivatives in tumour 50 years ago, the phenomenon has been studied intensively but the mechanisms are not yet clearly understood [28,76]. The difficulty is comparing different PS structures, at different times after injection, in different tumour models and at different doses. These diverse conditions explain the variations observed even with the same PS.

As mentioned above, different PS have different pharmacokinetics and distribution. The interval between injection and light irradiation is thus a key to the PDT efficiency. In blood, after injection, the repartition of tetrapyrrole PS helps to define three classes of photosensitiser. First, most of the relatively hydrophilic PS are bound to albumin fractions (e.g., the sulfonated tetraphenylporphyrin derivatives). Second, the asymmetric and amphiphilic PS, such as cis-di(4-sulfonatophenyl)diphenylporphine or the chlorin e6, can be associated with the lipoproteins, inserted into the outer phospholipids or bound to the apoproteins. These compounds are primarily partitioned between albumin and high-density lipoproteins. Third, the hydrophobic PS, which need solubilisation [77,78] (e.g., in liposomes, Tween 80 or cremaphor), can be incorporated in the core of lipoproteins and, in particular, are bound to low-density lipoproteins (LDL). In this case, it should be noted that the solubilisation

![Figure 3. Principle of photochemical internalisation, showing the importance of the subcellular localisation of the photosensitisers. Both the photosensitiser and the active macromolecule are incubated with the cells, endocytosed and co-localise in the endocytic vesicles. Light irradiation induces photochemical reactions, leading to the disruption of vesicular membranes and resulting in cytosolic release of the macromolecules that can then join their intracellular target and induce their activity.](image-url)
Tetapyrrole photosensitisers, determinants of subcellular localisation and mechanisms of photodynamic processes in therapeutic approaches

vehicle can strongly change the behaviour and the localisation of the PS [79].

Most of these compounds are, then, more or less firmly bound to large serum proteins, or are aggregated. Consequently, they are likely to behave like macromolecules in the bloodstream. The possibility of redistribution between the carriers, in particular under the effects of the tumour microenvironment [80,81], has to be taken into consideration, including investigating the tumoural retention processes. Some properties of the proliferating tissue also seem to be important. The accumulation of PS may be favoured by the high number of LDL receptors and/or by the low interstitial pH of targeted tissues [82,83]. Indeed, on the one hand, the increased cholesterol catabolism of proliferating tissues (such as tumour neovessels in macular degeneration) leads to overexpression of LDL receptors [84,85]; and hence LDL, which is a PS natural carrier, could ensure targeting of the tumour cells [86-90]. On the other hand, however, the tumour microenvironment, and in particular the slightly acid pH of the tumour extracellular medium, could play an important role by governing the physicochemical properties of photosensitisers [91-93]. The importance of both these mechanisms is supported by the correlation established between the PS accumulation in tumours and their structure, in particular their lipophilic character, the distribution of their polar and hydrophobic chains around the macrocycle and the electric charges of these chains [28,94]. Moreover, the pH-dependent exchange of photosensitisers between albumin and LDL could play a role in the selective retention of some of these molecules [81]. It has been proposed that, under an acid tumoural environment, a PS redistribution appears in favour of LDL association. The equilibrium shift may allow the PS retention by the tumour even if LDL were not, in blood, the major carrier of the considered photosensitiser.

3.3 Processes of tumour destruction

The three distinct mechanisms involved in the tumour destruction are interrelated. The first mechanism is direct tumour-cell death by apoptosis or necrosis (see below). Cells are killed by the damage caused by the light-generated ROS [95]. However, PDT can also damage the vasculature associated to the tumour, with resulting deprivation of oxygen and nutrients. This leads to tumour infarction.

The last possible reaction under PDT treatment is an immune response against tumour cells. These three mechanisms can influence each other, and their relative contributions in the total response are still to be defined, even if the combination of all three is required for a long-term, optimal cure.

Direct tumour-cell death: In vivo, PDT treatment is able significantly to reduce the number of clonogenic tumour cells in the tumour by direct damage [96]. However, one of the limitations, in vivo, might be the inhomogenous PS distribution within the tumour. Indeed, the PS concentration and the percentage of killed cells appear to decrease with the distance of the tumour cells from the vascular vessels [97].

Another limitation of the cellular effect of PDT is the availability of oxygen within the tumour during the treatment. The PS effects are oxygen dependent, and the cell killing does not occur primarily in hypoxic zones [98]. The rate of $^1\text{O}_2$ generation and subsequent tissue oxygen depletion are affected by the rate of fluence of the irradiation. Low rates of fluence induce higher levels of apoptosis than high rates [99]. Some tumour regions, distant from vessels, appear to be PDT-protected by hypoxia during high-fluence-rate treatments [100]. Fractionating the light irradiation has been proposed as a method to limit the protective effects of PDT-induced hypoxia in the area, [101] allowing re-oxygenation of the region.

Additional photoinduced cell death pathways: A major additional effect in PDT is the result of vascular damage, which appears to contribute to the long-term tumour control [102]. If the photosensitiser is still located in the vessels and is not yet at the cellular level, a microvascular collapse can be observed after PDT [103]. The pharmacokinetics are then of major importance in determining the interval between PS administration and light irradiation. Another key of the treatment is the immune response [104]. The two phenomena involved are the anti-tumour activity of PDT-induced inflammatory cells and the generation of long-term immune response. These effects may be elicited by cytotoxic non-lethal damage, which results in inflammation. This inflammation involves an important invasion by neutrophils, macrophages and other non-specific immune effector cells [105], the effect of which may be decisive to the long-term tumour control. Interestingly, a lysate of tumour cells isolated after PDT has been found to show a strong anti-tumour vaccine activity and protects mice against the development of tumours from the same cell line [106]. Moreover, recent reports have identified autophagy as an alternative cell-death process following PDT [107], although studies on various cells lines are consistent with autophagy serving as a pro-survival response via the recycling of damaged organelles [108]. This mechanism might, then, represent both a potential survival pathway after PDT damage to cellular organelles and a cell-death pathway [109-111].

4. Mechanism of cell death and subcellular localisation

4.1 Modes of cell death and cellular localisation

Although PDT can mediate many different events, its main purpose is to kill cells. The extent of cell death may be influenced by the concentration of PS, its cellular localisation and its physicochemical properties under specific micro-environments, as well as by the oxygen concentration, the wavelength and intensity of irradiation, and the cell type. Because of the interest in mechanisms of cell death, numerous studies have looked at the relative importance of apoptosis and necrosis induced by PDT both in vitro and in vivo [112]. During treatment, the subcellular localisation
of the PS is a crucial parameter in the direct cell-death process. Indeed, the cell killing is due to the molecular damage induced at the cellular level by the transient, photoinduced species that primarily affect the subcellular compartments labelled by the PS [113]. Thus, the subcellular localisation of the photosensitiser at the moment of irradiation influences the efficiency of direct cell death. Moreover, the response and the mechanism of death have also been shown to be governed by this parameter, even if other parameters, such as light dose or PS concentration, have also been shown to influence these processes [114-117].

Apoptosis is a complex biochemical process that has been identified in single cells that are perhaps surrounded by healthy-looking neighbours. Apoptosis is characterised by morphological features, such as the formation of apoptotic bodies, cell shrinkage, condensation of nuclear chromatin and DNA fragmentation [118]. A common feature of a PDT-initiated apoptotic programme is a rapid release of mitochondrial cytochrome c followed by activation of the apoptosome and procaspase 3. By contrast, necrosis results from loss of integrity of the plasma membrane induced by high levels of cell damage.

Another mechanism of cell death has also been described [119]: in vitro, when a cell dies by direct effect, the adjacent cells present lethal cellular damage that is propagated by means of a chain of adjacent cells. The degree of this phenomenon, called the Bystander effect, is higher for cells killed by necrosis than by apoptosis. This effect is thought to be the result of gap-junction communications and diffusion of the diffusible ROS released in the medium [120].

Mitochondria: It has been suggested that mitochondria play a key role in PDT-induced apoptosis [117,121,122]. Indeed, PDT has a major impact on mitochondrial function, in particular on the mitochondrial permeability transition pore and bioenergetics [123,124]. Many components involved in these processes have been identified [125,126]. The permeabilisation of the two mitochondrial membranes should be induced by the photosensitisation, followed by the disappearance of the transmembrane potential and the release of certain components of the apoptotic cascade, such as cytochrome c, second mitochondria-derived activator of caspasmes and certain procaspases [127].

Lysosomes: In general, photodamaging lysosome is less lethal than targeting mitochondria [128]. Nevertheless, the apoptotic cascade can be activated by the release of lysosomal enzymes: Bid can be cleaved by the lysosomal protease to generate a mitochondrion-targeted pro-apoptotic protein [129]. Moreover, the release of cathepsins can be induced by PS located in lysosomes [130]. In the cytosol, these protease cleave procaspase 3, activating the apoptotic pathway [116]. In summary, PDT-induced lysosome damage can induce as well as prevent apoptosis, and often causes slowly induced apoptosis.

Plasma membrane: If the PS is located in the plasma membrane, the balance will switch in favour of necrotic cell death, due to damage to the plasma membrane and rapid depletion of intracellular ATP [131]. It is thought that the mode of cell death is related directly to the changes in the intracellular ATP pool. Another interesting point is that high doses of light might photochemically inactivate enzymes and other elements of the apoptotic cascade, promoting necrosis [132].

In agreement with previous considerations, the apoptosis has been shown to be predominant at low doses of PDT and was associated with mitochondrial and lysosomal photodamage whereas, at high PDT doses, the necrosis was induced by membrane photodamage [133]. In conclusion, it appears that photodamage involving lysosomes and mitochondria may yield rapid apoptosis, whereas membrane photodamage delays or inhibits the apoptotic response. The importance of the subcellular localisation of PS is, then, clearly demonstrated. However, it should be noted that the redistribution of PS during or after irradiation might follow the loss of integrity of intracellular organelles. Thus, PS initially located in lysosomes may be released as a result of lysosome damage and may themselves photodamage more crucial organelles, such as mitochondria [134,135]. The redistribution of PS from mitochondria to cytoplasm can also be photoinduced [122].

4.2 Determinants of the subcellular localisation
Fluorescence microscopy is a sensitive method, and its recent developments with confocal imaging techniques, sensitive detectors and diversification of organelle-specific bright probes allow the determination of intracellular PS localisation with a good resolution. Mitochondria and lysosomes represent the main subcellular targets of PS, whereas localisation in the Golgi apparatus and endoplasmic reticulum mostly correspond to non-specific partition of the PS between the membranes of intracellular organelles. Furthermore, various behaviours can be described for the same PS: some authors have observed a golgian localisation of mesotetrahydroxyporphyrin (Foscan) [136], whereas others have described a mitochondrial localisation [137]. However, it should be noted that the variety of incubation protocols (time delay, chase, medium or buffer of incubation) and cells lines used make comparisons difficult. Aminopyropheophorbide has been observed in cytoplasmic and internal membranes, but not in mitochondria and, for few PS, a plasma membrane localisation has been observed, such as for a mono- and di-acid benzoporphyrin derivatives [139].

Structural parameters: PS–cell interactions and PS subcellular localisation are governed by various factors, among which hydrophobicity/hydrophilicity balance, charge and structural asymmetry are key ones. The hydrophobicity of tetrapyrrole PS is modulated by the presence of lipophilic side chains or charges around the macrocycle; the hydrophobicity level influences intracellular behaviour and the final localisation of the PS in the cell. The relatively hydrophilic PS, bearing...
Tetrapyrrole photosensitisers, determinants of subcellular localisation and mechanisms of photodynamic processes in therapeutic approaches

Polar or charged side chains, are too polar to cross the biological membranes and are usually internalised by endocytosis. By contrast, hydrophobic compounds with no or few polar groups can diffuse across the membranes and may be distributed freely between the membranes of various organelles. For instance, a series of mesotetraphenylporphyrins (mono- to tetrasulfonated, called TPPS1 to TPPS4), in which hydrophobicity has been established using the partition coefficient between organic solvent and water, showed very different localisations [140]. In carcinoma cells, TPPS1 and TPPS2a present a granular distribution, probably corresponding to a lysosomal localisation of TPPS2a [141], whereas TPPS1 localises in the endoplasmic reticulum, Golgi apparatus, nuclear envelope, plasma membrane and small cytosolic vesicles. TPPS3 and TPPS4 localise in the lysosomes, with a secondary localisation in the nucleus and the cytoplasm for the most hydrophilic compound. It should be mentioned here that the behaviour of the asymmetric compounds, such as TPPS2a, is of particular interest and will be discussed below, with particular attention being paid to behavioural dynamics. Moreover, in-vivo studies on an animal model showed that PS lipophilicity was highly predictive for photodynamic activity [94], suggesting that mitochondria are a sensitive subcellular target for PDT [142].

The influence of the charges exerted by the PS lateral groups is likely to be another interesting phenomenon. This specific charge effect is due to the transmembrane potential of mitochondria and to their negatively charged internal membrane. These two characteristics explain why these organelles are the preferential site of accumulation of cationic PS [122]. For example, additional lipophilic side-groups on cationic zinc (II) phthalocyanines increase their preferential accumulation in the inner mitochondria membrane and, consequently, their PDT efficiency [121].

The influence of the symmetricality of charge distribution around the macrocycle has been studied extensively. In addition to TPPS2a, the cellular behaviour of which has already been described, a striking example of this influence is illustrated by two meso-tetraphenylporphyrin derivatives bearing two cationic trimethylammonium groups in adjacent or opposite positions [143]. The symmetrical compound localises in the lysosomes, whereas the asymmetrical compound targets the mitochondria. However, neutral or anionic PS also localise into mitochondria. The complexity of these phenomena may explain the numbers of studies on this subject and the difficulties of predicting subcellular PS behaviour.

Another key point involving structural characteristics of PS is their aggregation state. In general, the singlet oxygen quantum yields and the fluorescence of aggregates are lower than those of monomers, subsequently reducing their PDT efficiency [144]. In an aqueous environment, the aggregation state of the tetrapyrrole PS is governed by the hydrophobic/hydrophilic balance. If the aggregates are taken up by cells, certainly by endocytosis, only the less lipophilic molecules are disaggregated in the intracellular environment [145].

The intracellular aggregates/monomer ratio of amphiphilic PS may explain their high PDT effect as compared to more lipophilic compounds. In agreement, a relationship between lateral chain length, aggregation state and phototoxicity has also been proposed for some PS such as pyropheophorbide-a ethers bearing C3 – C12 acyl chains [142]. The aggregates seem to be endocytosed and sequestered in lysosomes, whereas the monomers are able to localise in mitochondria.

With regard to pharmacokinetics, the use of specific delivery vehicles is likely to modulate the cellular behaviour of PS. It has been shown that the subcellular localisation of haematoporphyrin is not affected by the administration vehicle [146], and the importance of the LDL-mediated delivery in cell cultures is clear in most cases [87,90,147], influencing the global cellular uptake and the subcellular localisation.

Behaviour towards membranes: The behaviour of PS towards the biological membranes, in particular the dynamics of these phenomena, is strongly involved in their cellular uptakes. At the intracellular level, it also influences their partition among the subcellular compartments. In comparison with conventional drugs, this dynamic aspect is of particular importance for PS: as previously explained, their action is confined in space and in time to the structures that have been both labelled and irradiated. This dual selectivity of the photodynamic processes is true at the cellular and at the organ level (Figure 4). Numerous studies have tried to predict PS behaviour towards membranes with regards to the octanol/water partition coefficient. Indeed, for neutral molecules, the membrane permeability is linearly dependent on this coefficient [148]. For PS, correlation has been found only for a series of compounds presenting a similar structure [149]. The importance of the problems encountered can be explained first by the large size of the PS macrocycle, which involves a strong tendency to dimerise and, second, by the asymmetric repartition of side chains around the macrocycle, resulting in specific interaction mechanisms with membrane bilayers. Basically, the passive transport of such molecules across membranes involves three steps (Figure 5), which have been studied on membrane models [93,150-152]. The first step corresponds to the entrance of the photosensitiser in the first hemileaflet. It is characterised by its rate constant and the depth of the PS insertion. It can be postulated that the polar or charged side chains most likely interact with the polar heads of the phospholipids and the macrocycle is buried in the lipidic core of the membrane. Indeed, the fluorescence spectrum of such molecules was indicative of a lipidic environment [153]. The second step corresponds to the transfer of the PS into the second hemileaflet, diffusing through the lipidic core of the membrane. Called ‘flip-flop’, it is defined by its rate constant, which is governed directly by the depth insertion of the PS in the membrane after the first step [154]. The last step is the exit of the PS from the second hemileaflet. For carboxylic compounds, the entrance and exit rate constants
are strongly influenced by the pH, through the control of the ionisation state of PS bearing carboxylic chains, in contrast with compounds bearing unneutralisable chains [93]: at weakly acidic pH, the entrance rate and the depth of PS insertion are increased and, subsequently, the flip-flop is facilitated. Such phenomena can explain the facilitated retention of certain PS by tumoural cells (when the extracellular tumoural microenvironment is acidic). In this hypothesis, the release of the PS in the intracellular medium, at neutral pH, is easier than at the other side of the membrane, where it involves a physicochemical pumping effect. The same kinds of effect could favour a subcellular mitochondrial localisation [87,89]. Indeed, the main impact of the rate of transmembrane crossing has been shown to be highly correlated with the cellular behaviour of asymmetrical amphiphilic tetrapyrrole PS [90]. Moreover, the rate constants of entrance and flip-flop are strongly affected by the physical state of the lipid bilayer; for example, the presence of cholesterol greatly modulates these rates [153].

5. Expert opinion

As discussed above, the cellular response to PDT treatment is closely related to the specific intracellular location of the photoinduced damage. Moreover, the PCI method is based on specific labelling of the endocytosis compartments. For these two approaches, subcellular targeting of PS thus appears as a major challenge.

The dynamics of the behaviour of PS in the lipid bilayer might be a crucial parameter influencing its internalisation pathway and its subsequent subcellular localisation. It is therefore important to define the structural parameters governing these dynamics, with the aim of designing PS, the subcellular localisation of which would be controlled. Our group has demonstrated the correlation between the intracellular localisation of deuteroporphyrin (DP), chlorin e6 (Ce6) and disulfonated aluminum phthalocyanine (AlPcS2a), and the dynamics of their interaction with membranes (Figure 5) [87,89,90]. DP, bearing two carboxyl groups on the same side of the macrocycle, crosses the membranes within seconds. Transfer through the membrane is extremely slow for AlPcS2a, which bears two permanently charged groups in one side of the macrocycle. Ce6, bearing three carboxyl groups on one side, exhibits a median behaviour towards membranes, crossing them with very high degree of difficulty. As expected, labelled cells consequently showed a diffuse labelling pattern for DP; a punctuate one for AlPcS2a and an intermediate pattern for Ce6.
Tetrapyrrole photosensitisers, determinants of subcellular localisation and mechanisms of photodynamic processes in therapeutic approaches

Expert Opin. Ther. Patents (2008) 18(9)

attentions to the eventual influence of LDL on these processes, it has been shown that the distribution of the DP was not modified by its vectorisation by LDL. These results are easily explained by the rapidity of the dynamics of the DP–LDL interaction and the high rate of diffusion of this molecule through membranes. Despite their high affinity for DP, LDL could play only a marginal role in the porphyrin cellular localisation. However, this high affinity confers to LDL an important role as a porphyrin carrier in the blood. The kinetics also suggest a high rate of exchange with other plasma proteins. We could imagine LDL efficiently carrying porphyrins to the tumoural site, where transfer to the cellular membrane occurs rapidly.

On AlPcS2a localisation, the role of LDL was crucial. Whereas LDL vectorisation leads to a lysosomal localisation resulting from an LDL-mediated process of endocytosis, when AlPcS2a is not preloaded on LDL, the efficient incorporation process is bulk endocytosis and the majority of the intracellular vesicles where AlPcS2a is located are not lysosomes. Similar observations have been made regarding sulfonated porphyrins or phthalocyanines for various cell lines [87,156,157] in agreement with their inability to cross the membrane. Indeed, in these cases, the endocytic membranes act as barriers. Compared with the DP case, equilibrium between cells and LDL is then balanced in favour of LDL–PS association. The major cellular incorporation process is thus endocytosis and the LDL influence is enhanced.

LDL had a balanced influence on the localisation of the third compound: Ce6. The plasma membrane labelling was less visible and Ce6 was mainly located in lysosomal compartments. It is important to note that controlling the structural parameters involved in such behaviour offers the possibility of controlling the subcellular localisation of PS, which could be extremely useful in optimising these drugs for different applications. For instance, a diffuse, cytoplasmic distribution is generally related to good photodynamic efficiency, whereas the endosomal/lysosomal localisation is crucial for non-lethal photochemical internalisation.

To conclude, the photophysical properties of PS are determined mainly by the tetrapyrole macrocycle, whereas the physicochemical and biological interactions are governed by the nature of their lateral chains. The characteristics of the ideal PS are still under study and debate: for therapeutic uses, PS should present a low toxicity, a maximum absorption...
in the far-red spectral region, good photostability, a high singlet oxygen quantum yield and a preferential affinity for the targeted tissue, as well as an appropriate subcellular localisation.

The resolution of most of the photophysical and photochemical disagreements of the first generation of PS is one of the main aims of the newer generation. In particular, they present far-red excitations, in the ‘therapeutic window’ of wavelength, and shorter clearances. The targeting of PS is still an important challenge for the photodynamics research community. A high affinity for LDL is certainly desirable for a good PS. At the systemic and subcellular levels, the dynamic aspects of the interaction of PS towards membranes are crucial because they can explain, and then favour, the specificity of the tumoral retention and, for PDT, are key targeting in mitochondria.

We would like to conclude this paper with a particular emphasis on the possibility of redistribution between LDL, other carriers and tumoral cells membranes, in particular under the influence of the tumoral microenvironment. Such phenomena are likely to increase the retention of PS in tumoral cells: the redistribution between carriers has been shown to favour LDL [81], and the acidic extracellular pH involves, as explained above, the physicochemical pumping of the PS in the cells. Particular attention should thus be paid to the dynamic aspects of all processes concerning PS and photodynamic effects.

Declaration of interest

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Tetrapyrrole photosensitisers, determinants of subcellular localisation and mechanisms of photodynamic processes in therapeutic approaches


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