Targeted intracellular delivery of photosensitizers

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Abbreviations: BSA, bovine serum albumin; conA, concanavalin A; cdk, cyclin dependent kinase; ER, endoplasmic reticulum; HDL, high density lipoproteins; LDL, low density lipoprotein; MAb, monoclonal antibodies; MSH, \(\alpha\)-melanocyte-stimulating hormone; NES, nuclear export sequences; NLS, nuclear localization sequence; NPC, nuclear pore complex; nup, nucleoporin; PDT, photodynamic therapy; PS, photosensitizer; TPPS, tetraphenylporphyrin sulfonate.

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1. Introduction

Photodynamic therapy (PDT) is a novel treatment, used mainly for anti-cancer therapy, that depends on the retention of photosensitizers (PSs) in tumor cells and irradiation of the tumor with visible light. Upon activation, PSs generate reactive oxygen species (singlet oxygen, $^{1}\text{O}_2$, and free radicals, such as $\text{OH}$, $\text{HO}_2$ and $\cdot\text{O}_2$) which are able to damage membranes, DNA and other cellular structures, meaning that PDT can be a particularly useful alternative treatment for drug-resistant tumors (Canti et al., 1995) and radio-resistant cells (Lofgren et al., 1995). Normal cells, however, are also able to accumulate PSs, so that prolonged skin photosensitization as well as other side effects are some of the limitations of PDT. One of the reasons that large doses of PSs are normally required for efficient tumor-cell killing is that free, unconjugated PSs localize in the cytoplasm, usually in plasma membrane and within mitochondria and in some cases lysosomes, where the PSs exert their photodynamic action. In contrast to cell membranes and other cytoplasmic organelles, the cell nucleus (Wiseman and Hallywell, 1996; Akhlynina et al., 1997, 1999) is known to be a very sensitive target for reactive oxygen species. In order to reduce the dose of PSs administered to patients and hence minimize harmful side effects of PDT, a new approach has been taken to increase the effectiveness of tumor-cell killing through targeted delivery of PS to hypersensitive subcellular sites which is the focus of this review.

2. Some photophysical and photochemical properties of PSs

Molecules of organic PSs are almost always in the singlet ground state ($1S_0$), possessing no unpaired electron spins in the absence of photoactivation. Upon absorption of photons, the PS
molecule is excited to a short-living (~1–100 ns) excited singlet state ($^1S^*$) without a change in its electron spin. This excited state can either decay directly to the ground state ($^1S^0$) or, via intersystem crossing, yield the first excited triplet state ($^3S^*$) with two unpaired electron spins. PS molecules in this state have much longer lifetimes ($\geq$500 ns) and, thus, can undergo many collisions with other molecules. This long lifetime is considered as a precondition for efficient photosensitized reactions. The PS in $^3S^*$ state can react either with its neighbour molecules by an electron or hydrogen transfer process (type I reactions) or by an energy transfer reaction with oxygen (type II reactions) which normally exists in its triplet ground state, $^3O_2$, or $^3\Sigma^-O_2$.

2.1. Type I reactions

The triplet state collision complex formed between an excited triplet state PS, $^3S^*$, and its neighbour singlet state acceptor molecule, $^1A$, can yield radical ions ($^2S^+$, $^2A^-$ and also $^2S^-$ and $^2A^+$, see Darwent et al., 1982; Ochsner, 1997):

$$
\begin{align*}
\uparrow \uparrow ^3S^* + ^1A & \rightarrow \left[ \uparrow \uparrow ^3S^*; ^1A \right]^* \\
& \rightarrow ^2S^+ + ^2A^-
\end{align*}
$$

as well as $^2S$ and $^2A$ radicals (or $S^+$, $A^-$, $S^-$, $A^+$, $S$ and $A$, respectively). Probability of type I reactions is expected to be increased if noncovalent complexes between PS and acceptor molecules are formed before illumination. The majority of radicals produced in these reactions interact with molecular oxygen giving rise to a mixture of so-called reactive oxygen species such as OH, and HO$_2$ and also O$_2^-$ and H$_2$O$_2$, all of which and especially OH, can readily oxidize a very wide variety of biomolecules. The diffusion distances of these radicals in yeast cells were estimated to be not more than 3 nm (Hutchinson, 1957). Recent determinations indicate OH radical diffusion distances of 2.1–14 nm in isolated Ehrlich ascites tumor cell nuclei (Li et al., 1999) and 6.1 nm in HL-60 cells (Elgohary et al., 1998). These very short distances of free diffusion of these radicals are accounted for by their very high reactivity towards constituents of living cells.

The type I reactions are most efficient at low oxygen and high substrate concentrations. For example, experiments with tetrasulfonated chloro-gallium (III)-phthalocyanine showed that at low oxygen concentrations, type I reactions may account for photo-oxidation of membrane components and amino acids (Ferraudi et al., 1988). Deviations in product distributions can be considered as a reliable indicator of type I reactions (Ochsner, 1997).

2.2. Type II reactions

Ground state oxygen, $^3\Sigma^-O_2$, can react with an excited triplet state PS, $^3S^*$, in an energy
transfer reaction yielding singlet oxygen, \( ^1\Delta_g O_2 \) (excitation energy, 7900 cm\(^{-1}\), or 94.4 kJ mol\(^{-1}\)) or \( ^1\Sigma_g^+ O_2 \) (13,200 cm\(^{-1}\), or 157 kJ mol\(^{-1}\)) which also constitute reactive oxygen species.

Highly excited \( ^1\Sigma_g^+ O_2 \) has a very short lifetime in condensed media — e.g., 20 ps in methanol (Schmidt and Bodesheim, 1994) — and is rapidly quenched to produce \( ^1\Delta_g O_2 \). Thus, the energy of the first excited triplet state of a PS usually should not be less than 7900 cm\(^{-1}\) in order to generate singlet oxygen, and should not exceed 18,000 cm\(^{-1}\) (so that unfavourable factors which inhibit its generation may be avoided) (Ochsner, 1997).

Basing on estimated intracellular lifetime of \( ^1\Delta_g O_2 \) (\( \tau_A = 250 \) ns; Baker and Kanofsky, 1992a) and its diffusion coefficient \( (D \approx 1.4 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}; \) Moan and Boye, 1981), the diffusion distance of \( ^1\Delta_g O_2 \) can be theoretically predicted \( (< 6 \tau_A \cdot D) \) to be less than 45 nm within the cell. It was estimated to be 10–20 nm in cells (Moan and Berg, 1991; Takemura et al., 1989) or 11.5 ± 2 nm in thin water films (Becker, 1976, p. 326), and confirmed experimentally by Moan (1990), who showed that the diffusion distance of \( ^1\Delta_g O_2 \) must be less than 25–50 nm because \( ^1\Delta_g O_2 \) generated outside the cell wall of \textit{Escherichia coli} could not induce DNA strand breaks inside the cell.

2.3. Impact of type I and type II reactions

There are several experimental approaches which enable the determination of the type of photosensitized reaction. For example, it is known (Kulig and Smith, 1973; Korytowski et al., 1992; Geiger et al., 1995) that 3β-hydroxy-5α-hydroperoxy-cholest-6-ene, 3β-hydroxy-6α-hydroperoxy-cholest-4-ene, and 3β-hydroxy-6β-hydroperoxy-cholest-4-ene are characteristic of cholesterol reaction with \( ^1\Delta_g O_2 \). In contrast, the corresponding 7α- and 7β-hydroperoxides, in a complex mixture of other products not containing 6α- and 6β-hydroperoxides, are formed during radical oxidation (type I reactions) of cholesterol. The involvement of \( ^1\Delta_g O_2 \) in a reaction can be assessed by the activation of the reaction of PSs by D\(_2\)O where the half-life of \( ^1\Delta_g O_2 \) is 10 times greater than in H\(_2\)O. Generation of \( ^1\Delta_g O_2 \) can be directly estimated by its luminescence at \( \lambda = 1270 \) nm, as has been shown in L1210 cells sensitized with 5-(N-hexadecanoyl)amino eosin (Baker and Kanofsky, 1992b). 1,3-Diphenylisobenzofuran is often used as a relatively specific \( ^1\Delta_g O_2 \) scavenger (Ma et al., 1994). Other approaches are described in special reviews (e.g., Becker, 1976; Ochsner, 1997).

It should be mentioned that type I reactions are favoured in polar media because of the stabilization therein of formed radical pairs and inhibition of reverse electron transfer reaction (Hopf and Whitten, 1978; Foote, 1987), whereas type II reactions are favoured in hydrophobic media where lifetime and solubility of \( ^1\Delta_g O_2 \) are higher (Tomio et al., 1980).

The level of oxygenation of tumours or tumour cells plays a crucial role in PS-mediated reaction, in many of which molecular oxygen is consumed. For Photofrin, the O\(_2\) consumption rate was estimated to be about 6–9 \( \mu \text{M s}^{-1} \) at a light intensity of 50 mW cm\(^{-2}\) (Foster et al., 1991). Such consumption rates can significantly decrease cell oxygen tension and even the number of oxygenated cells in tumours (Foster et al., 1991; Baker and Kanofsky, 1992b; Henning et al., 1995).

Experimental investigations usually revealed both types of reactions in living cells and tissues. Importantly, the mechanism of action of a PS may change from a type II to a type I
reaction as a result of changes in the oxygenation of tumour tissue during photodynamic treatment because of induced tissue hypoxia (Ochsner, 1997).

2.4. Biomolecules undergoing sensitized photoeffects in vitro

It is not possible to indicate a special type of biomacromolecule that possesses a marked selectivity towards sensitized photoeffects, although some of their constituents can clearly be more readily photooxidized than others. Thus, almost all proteins undergo sensitized photooxidation, although only five of the 20 amino acids occurring in natural proteins — Cys, Met, His, Tyr, Trp — are very susceptible to this oxidation. Photooxidation of these specific residues usually leads to the loss or changed functions of the photodamaged proteins (Straight and Spikes, 1985).

Purines are photooxidized more readily than pyrimidines, guanine being the most sensitive under physiological conditions. As a result, guanine residues in nucleic acids are more susceptible to sensitized photooxidation than other bases. Single- and double-strand breaks have been demonstrated in nucleic acids following the photodestruction of guanine residues (Straight and Spikes, 1985).

Lipid molecules containing unsaturated fatty acids as well as steroids, tocopherols and free unsaturated fatty acids, readily undergo sensitized photooxidation. Many other biologically important molecules e.g., ascorbic acid, biotin, folic acids, glutathione, are efficiently photooxidized in the presence of PSs (Straight and Spikes, 1985).

In summary, PSs inflict photodamage on many types of biomolecules without a distinct specificity, their action being mediated largely via reactive oxygen species, no one of which is able to cover distances more than several tens of nanometers. Keeping in mind that cell dimensions are micrometers or tens of micrometers, there seems little doubt that the intracellular action of PSs is principally restricted to their specific subcellular localization, together with the surrounding radius of not more than 40 nm.

3. Subcellular distribution of photosensitizers (PSs)

Since, as already mentioned, most cells in the organism span tens of micrometers, it is clear that PS efficiency will depend not only on the relative distribution of PS between tumor and surrounding tissues and between malignant and normal cells, but also by the subcellular distribution of PSs. Furthermore, the interior of the eukaryotic cell is divided by membranes into compartments differing in their sensitivity to reactions induced by PS-generated reactive oxygen species, in the possibility and rate of replacement of damaged molecules therein, and in the extent to which such damage affects the cell viability, and in the first instance, its ability to divide. It is noteworthy that preferential PS accumulation in tumors is not itself a guarantee of selective photoinduced tumor damage and successful PDT. In experiments on Fischer 344 rats with gliosarcoma 9L, Chopp et al. (1996) found that, despite the 13-fold higher accumulation of Photofrin in the tumor compared to the surrounding healthy tissue, the latter was found to be more sensitive to photodynamic injury at any exposure. It is therefore clear that not only the distribution of PS in various types of tissue, not only their ‘affinity’ for certain cell types,
but also their penetration into various cell compartments and accumulation therein are essential to the mechanism of PS action in situ in the whole organism.

The distribution of a PS within the cell must depend on the route by which it enters, as well as on its physicochemical properties: hydrophobicity/hydrophilicity; type, number, and arrangement of charged groups; number of rings; presence of a central atom in the tetrapyrrrole structure, etc. In an in vivo situation where a PS is introduced into the bloodstream, the picture is still more complicated. Many PSs interact with blood proteins and lipoproteins (see section 3.2.1), whereupon their penetration into particular cells is determined not so much by the PS proper as by the PS-carrying blood component. Thus, PSs may be redistributed between the liposomes, emulsions, or complexes administered to the organism and the blood components (Polo et al., 1992; Richter et al., 1993; Schmidt-Erfurth et al., 1995; Kuzelova and Brault, 1995; Larroque et al., 1996). For this reason, the subcellular distribution of PS observed in vitro may not always reflect the in vivo pattern. Thus, whilst PS hydrophobicity correlated nicely with in vitro cytotoxicity and penetration into L1210 murine leukemia cells in serum-free medium, it did not correlate with photodynamic efficiency in vivo (Kessel et al., 1991).

3.1. Subcellular distribution of PSs in vitro

3.1.1. Ways of PS entry into the cell in vitro

Different PSs, depending on their properties, enter the cell in vitro by different ways, which determines their subsequent subcellular localization and distribution and thereby the efficacy of PDT in vitro. PS molecules may permeate the cell by diffusion both across and along the membranes, by nonspecific endocytosis, or even by pinocytosis which clearly is a highly inefficient mode of selective PS penetration in the cell. Large aggregates or PS-carrying particles may be internalized via phagocytosis (see Fig. 1).

Diffusion-controlled passive transfer into the cell has been shown for hematoporphyrin derivatives (Scourides et al., 1987), hydroxyethyldeuteroporphyrin (Dellinger et al., 1986), benzoporphyrin derivative monoacids (Richter et al., 1996), zinc phthalocyanine (Berg and Moan, 1997). Passive diffusion also partly accounts for the entry into cells in vitro of protoporphyrin IX (Van Graft and Boot, 1996) and 9-acetoxy-2,7,12,17-tetrakis-(beta-methoxyethyl)-porphycene (Szeimies et al., 1996). Partial inhibition of the uptake of these PSs upon lowering the temperature is indicative of the involvement of endocytosis. Nonspecific sorptive endocytosis is operative in the case of N-monoaspartyl chlorin e6 (Robert and Berns, 1989), lysyl chlorin p6 (Leach et al., 1993), and sulfonated aluminum phthalocyanines (Scully et al., 1998), whilst phagocytic uptake of aggregated PSs has been reported for hematoporphyrin diesters (Berg et al., 1993).

3.1.2. Subcellular localization of PSs upon incubation in vitro

In the process of interacting with the cell, PSs firstly come into contact with the plasma membranes, and only then, with differing degrees of efficiency find their way into the cell. The preferential localization of a PS on the cell surface or in its interior can determine the efficiency of photodynamic action and the type of cell death (Dellinger, 1996). As exemplified by experiments using meso-tetraphenylporphines sulfonated to different degrees and NHIK 3025
human carcinoma cells (Berg et al., 1990), the PS penetrated into the cell possessed greater photocytotoxic efficacy than PS bound to the cell surface membrane. It is, therefore, not at all surprising especially in view of the highly localized mode of PS action, that the distribution among intracellular compartments is of great importance even for PSs such as meso-tetraphenylporphine tetrasulfonate and tetrasulfonated aluminum phthalocyanines which are known to preferentially localize at the plasma membrane (Berg et al., 1990; Hubmer et al., 1996).

The localization of PS in the cell will alter according to its mode of cellular uptake and subsequent redistribution. Aluminum phthalocyanine mono- and disulfonates, for example, were found to be diffusely distributed over the cytoplasm of cultured LOX human melanoma cells, whereas the corresponding tri- and tetrasulfonates accumulated in granular lysosomal structures at the cell periphery (Peng et al., 1991a,b). In cultured human meningioma cells, aluminum phthalocyanine di- and tetrasulfonates accumulated in the lysosomes, while aluminum phthalocyanine itself was diffusely distributed over the cytoplasm (Malham et al., 1996). Hydrophobic tetradiocetyamine Zn(II) phthalocyanine and 5,10,15,20-tetra(m-hydroxyphenyl) chlorin showed diffuse localization within the cytoplasm of cultured cells (Ball et al., 1999; Melnikova et al., 1999). Comparison of the distribution of meso-tetraphenylporphyrin sulfonates (TPPSn) in NHIK 3025 cervical carcinoma cells showed TPPS4

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Fig. 1. Schematic depiction of the main pathways by which photosensitizers can be delivered to target cells: liposomal delivery (A), delivery by microspheres (B), antibody conjugates including antibody-linked liposomes (C) (see section 4), and receptor-mediated endocytosis (D) (see section 5.1).
and TPPS\textsubscript{2} (both isomers) to be in the lysosomal/endosomal structures (Berg et al., 1991). A similar distribution was found for TPPS\textsubscript{4} in CT26 murine colon carcinoma cells, whereas TPPS\textsubscript{1} concentrated in small perinuclear vesicles, most probably trans-Golgi (Malik et al., 1997). The lysosomes of EMT6 murine mammary sarcoma cells also accumulated lutetium texaphyrin (PCI-0123, Woodburn et al., 1997).

Intracellular tracking of Photofrin and N-monoaspartyl chlorin \textsubscript{e}\textsubscript{6} revealed that the former enters the cell by diffusion and localizes in mitochondria, while the latter is endocyted and then localized in lysosomes (Roberts and Berns, 1989). Incubation of bladder carcinoma cells with chlorin \textsubscript{e}\textsubscript{6} led to localization of the latter in the plasma membrane and other peripheral cell membranes (Bachor et al., 1991b). Lysyl chlorin \textsubscript{p}\textsubscript{6} was largely found in endosomes (Leach et al., 1993). Lysyl chlorin \textsubscript{p}\textsubscript{6} diester and its triester analog also accumulated in the lysosomes of L1210 murine leukemia cells, whereas lysyl chlorin \textsubscript{e}\textsubscript{6} imide tended to localize in mitochondria and plasma membranes (Kessel et al., 1995). As shown with several cell lines (SCL1 and SCL2, squamous cell carcinoma; HaCaT keratinocytes; N1 fibroblasts), lysosomes are also the destination of 9-acetoxy-2,7,12,17-tetakis-(beta-methoxyethyl)-porphycene (Fickweiler et al., 1999). Benzoporpurin derivative monoacid ring A is accumulated in trans-Golgi (Berg and Moan, 1997), and zwitterions such as Nile Blue and some its derivatives (Georgakoudi and Foster, 1998) tend to localize in lysosomes and trans-Golgi (Lin et al., 1993; Lin and Shulok, 1994).

Mitochondria quite specifically accumulate sulfonated aluminum chlorophthalocyanine (Muller et al., 1997), boronated porphyrin (Hill et al., 1982), the lipophilic cationic porphyrin derivative 5,10,15,20-tetrakis(1-decylpyridinium-4-yl)-21H.23H-porphine tetrabromide (Cernay and Zimmermann, 1996), and lipophilic cationic phthalocyanines, of which the most effective is 2,9,16,23-tetrakis(hexyldimethylammonio) zinc(II) phthalocyanine tetramethylsulfate (Dummin et al., 1997). Protoporphyrin IX, formed subsequent to the introduction of 5-aminolevulinic acid into the cell localizes both in the perinuclear space and in mitochondria (Malik et al., 1996; Liang et al., 1998), whereas exogenously added protoporphyrin IX is found in lysosomes/endosomes (aggregated PS) or in the outer membrane (monomeric form; see Malik et al., 1996). Protoporphyrin IX synthesized in the cell interacts with the mitochondrial (peripheral) benzodiazepine receptor, which is a high-affinity binding site for dicarboxylic porphyrins (Ratcliffe and Matthews, 1995), which is a determinant of phototoxic efficacy (Ratcliffe and Matthews, 1995).

Charged anionic and cationic PSs are usually associated with membrane components, while neutral water-soluble PSs are more diffusely distributed over the whole cell. Consistent with this, Wood et al. (1997) showed that pyridinium Zn(II) phthalocyanine and tetrasulfonated Zn(II) phthalocyanine, carrying four positive and four negative charges respectively, localized in lysosomes, whereas the neutral tetradiethanolamine Zn(II) phthalocyanine was diffusely localized through diverse intracellular membranes, with some preference for the Golgi complex.

Hence, to generalize according to Oleinick and Evans (1998), “the photosensitizers of greatest interest in PDT bind to various cytoplasmic membranes but are not found in the nucleus, do not bind to DNA” (p. S146).

3.1.3. Subcellular redistribution of PSs caused by irradiation in vitro

Even considering the simpler in vitro situation, it should be constantly borne in mind that
not only the photophysical properties of PSs, but also their subcellular distribution in the cell determines the ultimate photocytotoxic effect (Kessel et al., 1997). As mentioned above, many PSs translocate to various membrane-delimited subcellular compartments, subsequent to cell entry. The photochemical reactions induced by irradiation give rise to lipid peroxidation, which may impair membrane integrity and thereby cause PSs release from their primary loci and redistribution (Berg et al., 1991; Ruck et al., 1992; Moan et al., 1994; Strauss et al., 1995; Wood et al., 1997; Woodburn et al., 1997; Malik et al., 1997; Georgakoudi and Foster, 1998; Scully et al., 1998; Ball et al., 1999). Both continuous and fractionated irradiation can result in the damage of secondary targets by PSs in the cell.

Anionic meso-tetraphenylporphyrin di- and tetrasulfonates (TPPS$_{2o}$, TPPS$_{2a}$, and TPPS$_{4}$, respectively), initially accumulating in lysosomes, change their localization upon cell irradiation (Berg et al., 1991): TPPS$_{2o}$ and TPPS$_{2a}$ spread diffusely over the cytoplasm, while TPPS$_{4}$ concentrates in the nuclear region. Interestingly, the irradiation-induced redistribution of PSs appears also to depend on the state of the cells; photoirradiation led to the redistribution, for example, of TPPS$_{4}$ from lysosomes to nuclei in growing but not in stationary cells (Strauss et al., 1995).

Cationic pyridinium zinc phthalocyanine, initially localizing in the lysosomes of RIF-1 cells but diffuses over the cytoplasm as well as selectively staining the nucleoli, upon photoirradiation (Wood et al., 1997; Ball et al., 1999). Anionic tetrasulfonated Zn(II) phthalocyanine and tetraglycine Zn(II) phthalocyanine have been shown to migrate from lysosomes mainly to the region of the nucleus, whereas hydrophobic tetradioctylamine Zn(II) phthalocyanine and 5,10,15,20-tetra($m$-hydroxyphenyl) chlorin as well as amphiphilic polyhematoporphyrin do not change their location (Ball et al., 1999). Tetradiethanolamine Zn(II) phthalocyanine similarly does not change its subcellular distribution, remaining localized largely within the vicinity of the Golgi complex (Wood et al., 1997). The redistribution of PSs from lysosomal compartments can be very rapid, taking minutes or even seconds, as found for polysubstituted Zn(II) phthalocyanines (Wood et al., 1997) and sulfonated aluminum phthalocyanines (Scully et al., 1998).

3.2. Subcellular distribution of PSs administered in vivo

3.2.1. PS complexes with blood proteins

Once administered in vivo (e.g., by systemic injection), PSs form complexes with the protein components of the blood, and only a minor portion thereof may exist in free state (see Table 1). The assertion of Jori (1989) concerning the presence of some free PS in the blood, based on gel-chromatographic separation of blood samples from PS-treated patients and detection of PS at 400 nm, has been disputed by other authors (Maziere et al., 1991; Kongshaug, 1992).

Experiments on incubation of various PSs with serum in vitro demonstrated that practically all PS is bound to blood proteins, exhibiting particular preference (see Table 2).

These data clearly imply that the intracellular localization of PSs in vivo is strongly influenced by their complexation with blood proteins, and redistribution among them and possibly other factors.
3.2.2. Subcellular localization of PSs upon administration in vivo

The data available thus far on the in vivo subcellular distribution of PSs are much more fragmentary than those in vitro, because of the understandable experimental difficulties in performing such studies. It should also be said that, on account of the indirect action of PDT on the tumor vascular epithelium, the concept of the PDT target cell itself may change in some cases (for review, see Dougherty et al., 1998).

An event essential to PS distribution in the blood, and uptake by tumor and normal tissues, is its binding to serum components, including lipoproteins and albumin. Albumin-bound PSs can enter epithelial cells by liquid-phase as well as receptor-mediated endocytosis (Vorbrodt and Trowbridge, 1991; Hastings et al., 1994; Cui et al., 1996), then localize in lysosomes or, to a lesser extent, traverse the cell by transcytosis (Vorbrodt and Trowbridge, 1991). Another type of albumin-accepting cells are macrophages (Faustmann et al., 1991), which trap radical-damaged albumin particularly rapidly (Grant et al., 1992). Albumin has also been shown to carry PSs to the stromal elements of tumor tissue (Kessel et al., 1987).

Low density lipoprotein (LDL)-bound PSs can enter the target cells via receptor-mediated endocytosis (Schmidt et al., 1992; Versluis et al., 1996; for review, see Maziere et al., 1991; Jori and Reddi, 1993; Hamblin and Newman, 1994a,b; Reddi, 1997). In the case of chlorin e₆ covalently bound to LDL, the PS turns up in enzymatically active lysosomes (Schmidt et al., 1992). Subcellular fractionation of rat liver after intraperitoneal injection of hematoporphyrin revealed the latter’s presence in the mitochondrial and the plasma membrane fractions (Cozzani et al., 1981).

In several cases, the subcellular distribution of PS in vitro has been shown to coincide either with that in vivo, or with the lesions caused by irradiation after in vivo administration (for review, see Peng et al., 1996). Boronated porphyrin localized in the mitochondria of glioma cells, for example, both in vivo and in vitro (Hill et al., 1982). On the other hand, in contrast to its pronounced subcellular accumulation in vitro, Photofrin II, meso-tetraphenylporphine disulfonate and Al(III) phthalocyanine trisulfonate mainly stained the plasma membranes of grafted human melanoma LOX cells in nude mice in vivo. Similarly, hydrophilic PSs such as meso-tetraphenylporphine tri- and tetrasulfonates and Al(III) phthalocyanine tetrasulfonate did

<table>
<thead>
<tr>
<th>Photosensitizer</th>
<th>Percentage of injected photosensitizer bound to:</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipoproteins</td>
<td>Heavy proteinsᵃ</td>
</tr>
<tr>
<td>Hematoporphyrin</td>
<td>21</td>
<td>75</td>
</tr>
<tr>
<td>Hematoporphyrin derivative</td>
<td>23</td>
<td>60</td>
</tr>
<tr>
<td>Tetraphenylporphine tetrasulfonate</td>
<td>5</td>
<td>94</td>
</tr>
<tr>
<td>N-Aspartyl chlorin e₆</td>
<td>35ᵈ</td>
<td>65</td>
</tr>
</tbody>
</table>

ᵃ Nonlipoprotein proteins including serum albumin.
ᵇ Presumably aggregated forms (Jori, 1989).
ᵈ High density and low density (1–2%) lipoproteins.
Table 2
In vitro distribution of photosensitizers among human serum proteins (assessed by ultracentrifugation)

<table>
<thead>
<tr>
<th>Photosensitizer</th>
<th>Percentage of photosensitizer bound to human plasma components:</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDL</td>
<td>HDL</td>
</tr>
<tr>
<td>Hematoporphyrin</td>
<td>15</td>
<td>82</td>
</tr>
<tr>
<td>Hematoporphyrin derivative</td>
<td>20</td>
<td>59</td>
</tr>
<tr>
<td>Chlorin e₆</td>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td>N-Aspartyl chlorin e₆</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Al(III) phthalocyanine disulfonate</td>
<td>13</td>
<td>39</td>
</tr>
<tr>
<td>Tetraphenylporphine tetralsulfonate</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Tetraphenylporphine trisulfonate</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>m-Tetrahydroxy-phenyl chlorin</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

a Concentrations were in the range of 5–70 μg per ml plasma.
b Low density lipoproteins.
c High density lipoproteins.
d Nonlipoprotein proteins including serum albumin.
e LDL (5%) and very low density lipoproteins (7%).
not permeate into the cells in vivo, remaining bound to the tumor stroma (Peng et al., 1991a,b).

The redistribution upon irradiation shown in vitro for many PSs of lysosomal localization has been confirmed in experiments in situ using animals. In vivo redistribution of Al(III) phthalocyanine tetrasulfonate, but not zinc phthalocyanine, was observed in rat bladder tumor cells RR 1022 and correlated with the in vitro results (Ruck et al., 1996).

It should be pointed out, however, that for many PSs no clear correlation could yet be established between the binding with corresponding serum proteins and the PDT efficiency in vivo.

4. Systems of PS delivery into the cells

Approaches to enhance the PDT efficacy and selectivity have proceeded in several directions. First, as already mentioned, numerous researchers have synthesized a vast number of new PSs, hoping that they would prove capable of accumulating in desired cellular compartments (Boyle and Dolphin, 1996). The second approach has been made to use various carriers — e.g., polymers, microspheres, liposomes, emulsions — in order to “retarget” the PS in the cell, as shown schematically in Fig. 1. The second strategy will be dealt with in some details below.

4.1. Liposomes

Liposomes are a standard means of delivering drugs into cells. In many cases (though not always, see Biolo et al., 1996) incorporation of PSs into liposomes appreciably enhances its photocytotoxicity (Jori, 1996; Reddi, 1997). However, PSs injected into the bloodstream in liposomes actually reach cells as a component of blood lipoproteins, whereto it is redistributed upon contact of liposomes with serum proteins (Kongshaug, 1992; Richter et al., 1993; Jori and Reddi, 1993; Schmidt-Erfurth et al., 1995; Kuzelova and Brault, 1995; Polo et al., 1995; Larroque et al., 1996). The same happens with the PSs administered into the organism as emulsions (Cremophor EL) (Kongshaug et al., 1995; Soncin et al., 1995a,b; Ometto et al., 1996; Fabris et al., 1997).

4.2. Microspheres

The use of vehicles such as microspheres (Bachor et al., 1991a,b) or nanoparticles (Allemann et al., 1995, 1996) enables intracellular delivery of PSs to phagolysosomes (see Fig. 1B). Covalent attachment of chlorin e6 to 1-μm polystyrene microspheres resulted in the entry of the PS into MGH-U1 human transitional cell bladder carcinoma via phagocytosis (Bachor et al., 1991a). Fluorescence of microspheres and free chlorin e6 was detected respectively in phagolysosomes and on cell membranes (plasma membrane, mitochondrial and nuclear), but no free chlorin e6 was found in the cytoplasm or within the nucleus or lysosomes. The photodynamic activity of chlorin e6 microsphere conjugates in MGH-U1 cells proved to be higher than that of free PS, though the singlet oxygen-generating capacity of chlorin e6 in the free state was nine-fold greater than in microspheres. 0.43 μM free chlorin e6 was not at all
toxic for the cells, whereas the microspheres with the equivalent concentration of PS caused total death of the MGH-U1 cells as determined in a colony-formation test (Bachor et al., 1991a).

4.3. Antibody conjugates

The specificity of the photodynamic action can be improved by conjugating the PS with a ligand or antibody specific for receptors or antigens on the tumor cell surface. PS conjugates with monoclonal antibodies (MAb) against specific tumor cell receptors, for example, have been generated (Oseroff et al., 1986; Yemul et al., 1990; Goff et al., 1991, 1996; Jiang et al., 1991, 1993; Goff et al., 1994; Hemming et al., 1993; Hu et al., 1995; Martsev et al., 1995; Wolford et al., 1996; Berki and Nemeth, 1998; Vrouenraets et al., 1999), to provide enhanced selectivity of surface binding and PS delivery in order to increase photodynamic action. Chlorin e6 conjugates with the MAb OC125 accumulated to two- or three-fold higher levels than free chlorin e6 in murine ovarian tumor cells NIH:OVCAR3 (Goff et al., 1994). Immunoconjugates of chlorin e6 monoethylene diamine monoamide with the MAb IG12 specific for uveal melanoma cells produced four times greater damage in the latter than the free PS at the same concentration, whereas there was no enhancement using conjugates with nonspecific antibodies on various types of cells (Hu et al., 1995).

It is of significance that the main target of most PS–antibody conjugates is the plasma membrane. It has been clearly demonstrated, for example, that, upon incubation of melanoma cells with MAb conjugates of Sn-chlorin e6, photoinduced cell lysis is caused by point lesions of the plasma membrane (Thorpe et al., 1995). On the other hand, it is known that, in contrast to the plasma membrane, lysosomes and especially nuclei are extremely susceptible targets for reactive oxygen species (Akhlynina et al., 1995; Wiseman and Hallywell, 1996). It thus seems desirable to create a selective transport form of PS that can penetrate into the cell interior where it may exert its photodynamic action. One of the ways to this goal may be to modify the MAb–PS conjugates with additional components that would confer on them an ability to be endocytosed by the cell. Attachment of polylysine to MAb OC125 against ovarian cancer cells enhanced the antibody uptake up to 17 times (Hamblin et al., 1996). Internalization of a complex of meso-chlorin e6 mono(N-2-aminoethylamide) with MAb OV-TL16 by ovarian carcinoma cells OVCAR-3 can be achieved by conjugating this complex with N-(2-hydroxypropyl)methacrylamide (Omelyanenko et al., 1998). There appears to be much promise in tumor cell-specific internalizable antibodies. It has been shown recently that m-tetrahydroxyphenylchlorin attached to internalizable MAb 425 specific for head and neck squamous cell carcinoma is more effective than the conjugate with noninternalizable antibodies U36 (Vrouenraets et al., 1999).

The use of immunoliposomes (see Fig. 1C) also improved the efficacy of antibody-conjugated PS, owing to permeation into the target cells. Comparison of the antibody conjugates and monolayer immunoliposomes with sulfonated aluminum phthalocyanine revealed that the photodynamic activity of immunoliposomes on bladder carcinoma cells in vitro was 13 times higher than that of the PS–antibody conjugates alone (Morgan et al., 1994). Pheophorbide a in immunoliposomes with antibodies to T-24 cells (tumor bladder cell line) proved to be more efficient in vitro on these cells than on MGH-U1 human bladder cell line. In this case the PS
was found to accumulate selectively in the lysosomes of the target cells (Bergstrom et al., 1994). It is not clear how practicable this approach may be, however, in view of the in vivo redistribution of PSs from liposomes over various components of the blood, such as lipoproteins.

5. Targeted intracellular delivery of PSs

The fact that a PS introduced into the organism forms complexes with and can be transported by blood proteins provides two possibilities to solve the problem of addressed intracellular delivery: (i) synthesizing and using PSs which bind with high affinity to the desired blood proteins; and (ii) creating protein carriers with preset properties which, among other things, would ensure binding to the desired target cell and subsequent directed transport to the necessary intracellular compartment. The latter approach appears more promising, as in this case the researcher is not restrained by the existing set of serum proteins or the particular features of their interaction or lack thereof with tissues and particular cell types, but is free to design diverse carriers for PSs. This approach is facilitated by the numerous data obtained in the last decade and a half on signals for subcellular localization and the cellular machinery responsible for recognizing such signals and delivering the corresponding molecules to their destinations.

5.1. Internalizable ligand–PS complexes and their subcellular redistribution

As already mentioned, many PSs are readily incorporated into lipoproteins, mainly LDL and high density lipoproteins (HDL). Sufficiently hydrophobic PSs can be incorporated into LDL (Jori et al., 1984; Allison et al., 1991, 1994; Schmidt-Erfurth et al., 1996, 1997), the receptors for which are more abundant in cancerous cells than in surrounding normal tissues, as shown for tumors such as murine lung carcinoma and melanoma and human acute myeloid leukemia and meningioma (Lombardi et al., 1989; Rudling et al., 1990a,b; Vitols, 1991; Versluis et al., 1996). Experiments with PSs pre-incorporated into various lipoproteins have shown that only incorporation into LDL provides for effective photodynamic action (Barel et al., 1986; Allison et al., 1990, 1991). The \( \alpha \)-macroglobulin/LDL receptors are widely found in cells of various types, with LDL uptake most pronounced in adrenals, liver, and spleen (Versluis et al., 1996). Proliferating vascular epithelial cells are also rich in \( \alpha \)-macroglobulin receptors, which may be the cause of preferential PS accumulation there in neovascularized tissues (Schmidt et al., 1992). Significantly, PDT-resistant variants of fibrosarcoma cells express much fewer LDL receptors than the original line (Luna et al., 1995).

The use of lipoproteins as ‘intermediary’ transporters for PS delivery to target cells imposes certain limitations connected with agent redistribution in the blood, depending on the properties of the PS and the blood components which may have not yet been defined (Hopkinson et al., 1999). Furthermore, such a mode of delivery predetermines to a large degree the subsequent subcellular distribution of the PS and thereby its sites of action. Another, increasingly popular approach has made use of conjugates comprising a PS and an addressed internalizable ligand (see Fig. 1D).
By attaching a PS to a macromolecular internalizable ligand, one can explicitly set not only the specificity of its uptake by the cells but sometimes even its subcellular location. Experiments with cultured cells have shown that such manipulation can greatly potentiate the PS effect (Akhlynina et al., 1990, 1993, 1995, 1997; Sobolev et al., 1992). Thus conjugation of chlorin e6 with concanavalin A (conA) increased the photodynamic damage of fibroblasts (Akhlynina et al., 1990), whereas an excess of nonconjugated conA competitively inhibited it. Suppression of internalization of the conA–chlorin e6 conjugate decreased the photodynamic damage, indicating the importance of its uptake by the cells. However, not all internalizable ligands can be easily conjugated with PSs without the loss of receptor-binding ability. To overcome this, both the ligand and the PS can be attached to an additional carrier. Again, the intracellular fate of the ligand after internalization is an important factor in the photodynamic effect achieved. The ligands entering the cell by receptor-mediated endocytosis may then be dispatched either back to the cell surface (recycling) or to other cell compartments by the cell sorting systems.

We have shown a PS delivery system comprising insulin as an internalizable ligand a proportion of which can reach the nucleus (Soler et al., 1989; Burwen and Jones, 1987; Smith and Jarett, 1990; Rosenkranz et al., 1992), and chlorin e6 covalently linked to bovine serum albumin (BSA), to be highly efficient in cultured human hepatoma PLC/PRF/5 cells (Sobolev et al., 1992; Akhlynina et al., 1993, 1995). The conjugate had high affinity for the insulin receptor (half-maximal displacement of [125I]insulin at 1 nM) and was internalized by the PLC/PRF/5 cells. Fluorescence video-intensified microscopy revealed the FITC-labeled conjugate mainly in the perinuclear space and to a lesser extent within the nucleus (Akhlynina et al., 1995). The same localization was observed with the 2′,7′-dichlorofluorescin diacetate assay for the reactive oxygen species generated upon cell irradiation (Akhlynina et al., 1995). 2′,7′-Dichlorofluorescein (or 2′,7′-dichlorodihydrofluorescein) diacetate penetrates into living cells and is deacetylated by intracellular esterases. Reactive oxygen species react with 2′,7′-dichlorofluorescein to yield fluorescent 2′,7′-dichlorofluorescein, which can be used to visualize the sites of intracellular production of reactive oxygen species and sites of PS subcellular location, and also as an intracellular indicator of photodynamic activity in situ (Suematsu et al., 1991; Akhlynina et al., 1995, 1997, 1999; Sarvazian, 1996). The insulin–BSA–(chlorin e6) conjugate at low concentrations suppressed the propagation of hepatoma cells (half-maximal effect at about 1 nM).

Other ligands which have been used in internalizable PS conjugates include transferrin (Hamblin and Newman, 1994a,b), epidermal growth factor (Gijsens and de Witte, 1998), and maleylated BSA, a ligand for ‘scavenger’ receptors (Nagae et al., 1998; Brasseur et al., 1999).

5.2. Complexes of PSs with internalizable ligands and subcellular localization signals

Clearly, internalization of a ligand or its conjugate by the target cell is necessary but not sufficient for damage to be effected in the most vulnerable part of the cell. To this end, it is expedient to take advantage of the mechanism of directed intracellular transport that ensures the delivery of biomolecules to certain intracellular compartments.

This section shall largely concentrate on the signals and pathways by which proteins are targeted to the nucleus, both because this subcellular compartment has proved to be the most
susceptible to damage by reactive oxygen species (Wiseman and Halliwell, 1996), and because it is the compartment to which PSs have been successfully delivered (see section 5.2.2).

5.2.1. Signals mediating targeting to subcellular compartments

The eukaryotic cell is a highly organized system in which specific cellular functions are separated from one another in distinct membrane-bound organelles such as mitochondria, the endoplasmic reticulum (ER), the Golgi apparatus, and the nucleus. Division of the cell into these specialized compartments requires the organized distribution of proteins, as well as other molecules, involving tightly regulated intracellular trafficking/targeting of molecules from their site of synthesis to their site of action. Translation of soluble and integral membrane proteins occurs on ribosomes which coat the cytosolic surface of the ER (Rapoport et al., 1996); from the cytosol, about half of these newly translated proteins are directed to other compartments, generally conferred by targeting signals (Schatz and Dobberstein, 1996).

5.2.1.1. Targeting signals to distinct membrane compartments. Signal sequences are generally recognized in the cytosol by factors that direct the protein to a gated transmembrane channel, which then translocates the protein across the target-organelle membrane in an energy-dependent fashion. Signals for mitochondrial targeting are usually between 20 and 35 amino acids in length, with a profusion of hydroxylated and hydrophobic amino acids as well as basic amino acids, and an absence of acidic ones (e.g., MLSLRQSIRFFKPATRTLCSRYLL, single letter amino acid code, from cytochrome c oxidase polypeptide IV from *Saccharomyces cerevisiae*).

They are able to fold into an amphiphilic α-helix or α-sheet which appears to be essential for function. Composite signals of two targeting sequences in tandem permit the passage of proteins into the mitochondrial intermembrane space; the amino terminal mitochondrial targeting signal is combined with a strongly hydrophobic sequence so that after cleavage of the amino terminal signal, the latter can mediate transfer through the inner mitochondrial membrane (see Schatz and Dobberstein, 1996).

ER import signals (e.g., MMSFVSLLLLGVILFHatQAEQLTKCEVFQ from goat lactose synthase B protein) are strongly hydrophobic, with hydroxylated and negatively charged amino acid residues. The C-terminal sequence KDEL confers ER-retention in nascently expressed proteins, but does not confer ER import per se, and hence only has relevance to proteins expressed intracellularly (see Schatz and Dobberstein, 1996).

Targeting to peroxisomes is mediated by either a C-terminal SKL motif (or variant thereof — e.g., human catalase possesses the KANL sequence — Purdue and Lazarow, 1996) conferring recognition by the PTS1 receptor (Pex5p — Terlecky et al., 1995), or the N-terminal consensus motif R-L-X-H/Q-L, where X is any amino acid (e.g., from 3-ketoacyl-CoA thiolase) which confers interaction with the PTS2 receptor (Pex7p — Marzioch et al., 1994). Whilst the PTS1 sequence is retained in the targeted protein, the PTS2 targeting signal is removed as the protein enters the peroxisomal matrix (Subramani, 1993) in similar fashion to imported mitochondrial/chloroplast/ER proteins.

Targeting to the Golgi apparatus can be effected by the N-terminal 81 amino acids of human β1,4-galactosyltransferase (Yamaguchi and Fukuda, 1995). In terms of intracellular targeting to lysosomes, mannose-6-phosphate modification, involving oligosaccharide chain
biosynthesis and phosphorylation in the trans-region of the Golgi, ensures correct targeting through vesicular transport and recognition by the lysosomal mannose-6-phosphate receptor.

Simple plasma membrane targeting can be effected by signals for the addition of fatty acid sequences such as the N-terminal myristoylation signal of the protooncogene Src (MGSSKSKPK, where G is myristoylated) (Buss et al., 1988), and the 20 amino acid C-terminal farnesylation sequence (KLNPDESQPGCMSCKCVLS, where the underlined C is modified) of c-Ha-Ras (Hancock et al., 1991).

5.2.1.2. Targeting proteins to the nucleus

Gating by the nuclear pore complex (NPC). All passive and active transport into and out of the nucleus occurs through the NPC (Jans and Huebner, 1996; Agutter and Prochnow, 1993; Nigg, 1997). The NPC is a multi-subunit, complex structure, possessing the form of a cylinder within the nuclear envelope, with attached fibrils or filaments on the cytoplasmic side and a basket-like structure on the nucleoplasmic side (Jans and Huebner, 1996; Nigg, 1997). As its name implies, the NPC has a pore/passive channel or molecular sieve function, whereby molecules smaller than 40–45 kDa can diffuse freely into and out of the nucleus. Proteins larger than 45 kDa require specific targeting signals in order to pass through the NPC. In the case of nuclear protein import, a nuclear localization sequence (NLS — Jans and Huebner, 1996; Nigg, 1997; Kalderon et al., 1984a,b; Lanford and Butel, 1984; Hall et al., 1984) or analogous sequence is generally required. Nuclear protein import does not involve cleavage of targeting signals after transport because, in contrast to signals for targeting to the ER, mitochondria etc., nuclear targeting may be required to function several times, and through a number of cell divisions which in the case of most eukaryotes involve dissolution of the nuclear envelope (Nigg, 1997). Proteins are also able to be translocated to the nucleus in their native conformation, again in contrast to targeting to mitochondria/chloroplasts etc., where, subsequent to signal sequence removal on the trans-side of the membrane, completion of protein folding is effected by the action of molecular chaperones/folding enzymes.

NLS-dependent nuclear protein import is energy- and temperature-dependent (Newmeyer and Forbes, 1988; Richardson et al., 1988), and can be inhibited by antibodies specific to the soluble NPC components, the nucleoporins (Featherstone et al., 1988; Dabauvalle et al., 1988; Yokoyama et al., 1995) which make up 5–10% of the NPC mass. The nucleoporins (nups) are believed to represent docking sites for components of the nuclear import machinery during transport through the NPC. Certain nucleoporins are able to bind the cytosolic factors mediating association with and transport through the NPC; RanBP2 (nup358) (Yokoyama et al., 1995; Wu et al., 1995) and Nup2p (Dingwall et al., 1995), for example, are able to interact with the monomeric GTP-binding protein Ran (in its GTP bound form) which plays a key role in translocation through the NPC in both directions, whilst nup62 binds nuclear transport factor NTF2 which interacts with Ran (Paschal and Gerace, 1995). RanBP2, as well as nup116, are able to bind importin β which mediates the docking of signal-containing transport substrates at the NPC (Moroiianu et al., 1995b). Nucleoporins, and in particular their FG repeats, thus serve as the assembly sites at which substrates and transport factors are brought into close vicinity, and can synergise to ferry substrates from one side of the pore to the other.
Signal-mediated nuclear protein import. NLSs are the short modular peptide sequences which are necessary and sufficient for nuclear localization of their respective proteins (Kalderon et al., 1984a,b; Lanford and Butel, 1984; Hall et al., 1984), based on the fact (1) that their mutation or deletion leads to cytoplasmic localization of the protein in question, and (2) that they are active in nuclear targeting of a normally cytoplasmically localized carrier protein, either as a peptide covalently coupled to the carrier, or when encoded in the sequence of a fusion protein. The basis of NLS/targeting sequence-dependence of nuclear protein import is ligand-receptor-like interaction with specific binding proteins (the importins/karyopherins), rather than through binding to DNA or histones binding (Cowie et al., 1986; Hall et al., 1990; Kleinschmidt and Seiter, 1988; Lyons et al., 1987). That conventional NLSs are entry rather than retention signals has been demonstrated by the fact that NLS-deficient carrier proteins (lacking specific signals for nuclear export) microinjected into the nucleus remain nuclear (Lyons et al., 1987; Schmidt-Zachmann et al., 1993), whilst the fact that nuclear accumulated NLS-containing proteins are highly laterally mobile (Rihs and Peters, 1989) implies that binding in the nucleus is not the prime mechanism by which NLSs mediate nuclear localization (Nigg, 1997; see Agutter and Prochnow, 1994; Schmidt-Zachmann et al., 1993).

The process of NLS-mediated transport into the nucleus (see Fig. 2 top) involves two basic steps (Newmeyer and Forbes, 1988; Richardson et al., 1988), the first of which involves energy-independent recognition of the targeting signal of the transport substrate and docking at the NPC, whilst the second is energy-dependent and involves translocation through the pore and into the nucleus. The first step is mediated by the ‘NLS receptor’, the heterodimeric complex of the specific NLS-binding protein importin α (Srp1/Rch1/Kap60/karyopherin α), and nucleoporin-binding NPC docking protein importin β (PTAC97/Kap95/karyopherin β1) (Moroianu et al., 1995a,b; Radu et al., 1995; Goerlich et al., 1995; Imamoto et al., 1995; Enenkel et al., 1995; Rexach and Blobel, 1995), which is also required for high affinity binding to the NLS by importin α (Rexach and Blobel, 1995; Goerlich et al., 1996; Weis et al., 1996a,b).

The subsequent step of energy-dependent translocation of the importin/NLS-containing protein complex through the NPC to the nucleoplasmic side requires Ran in its GDP-bound form (Goerlich et al., 1996; Weis et al., 1996a,b; Moore and Blobel, 1993; Melchior et al., 1993), and modifying factors such as NTF2, the soluble Ran binding protein RanBP1, and RanGAP1 (Ran GTPase activating protein 1) (Moore and Blobel, 1994; Paschal and Gerace, 1995; Coutavas et al., 1993; Bischoff et al., 1994; Bischoff and Ponstingl, 1991). RanBP1 both enables Ran in its GDP form (generated by GTP hydrolysis on the part of RanGAP1) to interact with importin β, as well as acting in concert with NTF2 to maintain transport substrate-importin complex stability and thereby prevent its dissociation during transit through the NPC. Once the complex has reached the nucleoplasm (about a 100–120 nm translocation), Ran-GTP, at high concentration in the nucleus through the action of the guanine nucleotide exchanger RCC1, effects dissociation of the complex such that the NLS-containing protein and importin α are released into the nucleus whilst importin β remains at the NPC (Moroianu et al., 1995a,b; Goerlich et al., 1995). Directionality of the transport process is purported to be provided through compartmentalization of RanGAP1 and RCC1, which are predominantly cytoplasmic and nuclear respectively, which ensure that Ran in the cytoplasm is predominantly
in the GDP-bound form, and that Ran in the nucleus is in the GTP-bound form (Bischoff and Ponstingl, 1991; Izaurralde et al., 1997).

Signals mediating nuclear protein transport. There are a number of different types of nuclear targeting sequences conferring transport between the cytoplasm and nucleus, including conventional or ‘directional’ NLSs that mediate nuclear protein import exclusively, nuclear export

Fig. 2. Better understood pathways of protein import into the nucleus. Nuclear protein import mediated by conventional NLSs (top), importin β1-recognized nuclear targeting sequences (middle), and the M9 shuttle sequence are shown in schematic form, with the specific signal recognizing proteins importin α/β, importin β1 and importin β2 (transportin) respectively, and GDP/GTP state of Ran/TC4 indicated. The compartmentalisation of RanGAP1 and RCC1, the basis of the proposed predominance of RanGDP in the cytoplasm and RanGTP in the nucleus, is also shown. NPC, nuclear pore complex.
sequences (NESs) that mediate protein transport in the reverse direction, and what are best termed 'shuttle signals', that can mediate both nuclear import and export of proteins carrying them.

Conventional NLSs fall into at least three classes, two of which are predominantly basic in nature. They are those resembling the NLS of the SV40 large tumor antigen (T-ag — PKKKRK132) (Kalderon et al., 1984a,b; Lanford and Butel, 1984), comprising a short stretch of basic amino acids, and bipartite NLSs, which consist of two stretches of basic amino acids separated by a spacer of 10–12 amino acids (Robbins et al., 1991). The third less well characterized class of NLS is made up of NLSs resembling those of the yeast homeodomain containing protein Matα2 (Hall et al., 1984, 1990), where charged/polar residues are interspersed with non-polar residues. All three classes of NLS are held to be recognized specifically by the α/β-importin heterodimer during the first step of nuclear transport, as has been shown directly for the importins from several species (Efthymiadis et al., 1997; Huebner et al., 1999; Briggs et al., 1998; Hicks et al., 1996; Smith et al., 1997; Hu and Jans, 1999).

A complicating factor in this context is that higher eukaryotes possess more than one importin α form, with humans and mouse possessing at least five forms, all of which are believed to interact with importin β (Huebner et al., 1999; Hu and Jans, 1999). Despite the lack of quantitative data, it seems clear that the different importin α subunits have distinct NLS-substrate binding properties (Nadler et al., 1997; Sekimoto et al., 1997; Miyamoto et al., 1997; Prieve et al., 1998).

Certain apparently conventional NLSs have been demonstrated to be recognized specifically not by the α/β-importin heterodimer, but by importin β (importin β1) alone, including the T-cell protein tyrosine phosphatase (Tiganis et al., 1997), human immunodeficiency virus (HIV-1) Rev protein (Henderson and Percipalle, 1997), the yeast transcriptional activator GAL4 (Chan et al., 1998) and the parathyroid hormone related protein (PTHrP) (Lam et al., 1999). These proteins appear to be able to be transported to the nucleus in the absence of importin α (Lam et al., 1999), importin β thus fulfilling both the nuclear targeting signal recognition role of importin α, as well as its established NPC docking and Ran-binding roles in the transport process (see Fig. 2 middle). The nuclear targeting signals recognized by importin β as opposed to importin α have been purported to be distinctively Arg-rich (Truant and Cullen, 1999), but the minimal PTHrP sequence recognized by importin β (YLTQETNKVETYKEQPLKTGKKKKGBP94) is clearly T-ag-like, indicating that the situation is more complicated.

It has recently become clear that there are a number of different importin β homologs in eukaryote cells (see Wozniak et al., 1998), with apparently specific transport roles for particular classes of proteins. Importin β4 (Kap123p/Yrb4p), mediates the import of ribosomal proteins into the nucleus, for example, as can β3 (Kap121p/Pse1p/Imp5-RanBP5); importin β2 (Kap104p/transportin) mediates the nuclear import of mRNA binding proteins. Interestingly, a region from ribosomal protein rpL23a (the BIB domain: VHSKKKKTIRSPTFTTPKTLRRQPKYPRKSAPRRNKLHY74) has been shown to be able to be recognized specifically by any of four distinct importin β homologs, including β1-3, each of which can mediate nuclear import (Jaekel and Goerlich, 1998). rpL5 and rpS7 can similarly be transported to the nucleus by all of these importin β homologs, in addition to importin α/β1, although to varying degrees of efficiency. Analogously to the BIB domain,
amino acids 1–41 of rpL25 (MAPSAKATAAKKAVKGTNGKKALKVRTSATFLPKTLKLAR) can be recognized by either importin β3 or β4 (Schlenstedt et al., 1997).

Shuttle sequences are targeting sequences sufficient and necessary to confer nuclear import and export on the proteins carrying them, and hence can mediate transport in both directions through the NPC. The best defined is the 38 residue M9 sequence (NQSSNFPMKGNGFGRGSPPYGGQQYFAKPRQNYGGY) of the human mRNA-binding protein hnRNP A1 (Michael et al., 1995a,b), which is largely hydrophobic, in contrast to conventional NLSs. M9-dependent nuclear import requires importin β2 and Ran (see Bonifaci et al., 1997; Nakielny et al., 1996). Apart from the fact that no correlate of importin α is involved, the sequence of events in terms of M9 recognition and Ran-mediated passage through the NPC in the import direction is comparable to that for nuclear import mediated by the α/β-importin heterodimer and conventional NLSs (Fig. 2, bottom). All importin βs have a Ran requirement for transport function, and would appear to mediate transport pathways similar to that of transportin or importin β1 (see previously).

In the export direction, the importin β homolog exportin (Crm1p/Xpo1p/Kap124p) mediates the export from the nucleus of leucine-rich NES-containing proteins, whilst CAS (Cse1p/Kap109p) (Kutay et al., 1997) specifically transports importin α from the nucleus to the cytoplasm to enable it to carry out further cycles of nuclear import of conventional NLS-containing proteins. The correlates of conventional NLSs which mediate nuclear transport in the export direction are NESs, the largely hydrophobic, leucine-rich sequences resembling that of HIV-1 Rev (LQLPPLERLTL) that are sufficient and necessary for nuclear export of their respective proteins. NES-mediated nuclear protein export is a rapid, active process (Bogerd et al., 1995; Fischer et al., 1995), mediated by the importin β homolog exportin and requiring Ran-GTP (Stade et al., 1997; Fornerod et al., 1997; Engelmeier et al., 1999).

Regulation of nuclear targeting. One means by which NLS-dependent nuclear protein import can be regulated in either an enhancing or inhibitory fashion is through phosphorylation in the vicinity of the NLS (Jans and Huebner, 1996; Jans et al., 1998). The modular sequences conferring phosphorylation-regulated nuclear protein import — phosphorylation-regulated NLSs (prNLSs) (Jans and Huebner, 1996; Jans et al., 1998) — which are able to confer phosphorylation-regulated nuclear protein import on heterologous proteins, have been identified for a number of transcription factors and other proteins.

The best characterized example of a prNLS in which phosphorylation enhances NLS-dependent nuclear protein import is that of T-ag, where synergistic action of the protein kinase CK2 (Ser111Ser112) and double-stranded DNA-dependent protein kinase (dsDNA-PK) sites, 13 and 4 amino acids N-terminal to the NLS (amino acids 126–132) respectively, which increases the rate of nuclear import of T-ag by about 50-fold (Rihs et al., 1991; Jans et al., 1991; Jans and Jans, 1994; Jans, 1995). The mechanism of facilitation of nuclear import is through the CK2 and dsDNA-PK sites enhancing recognition of the T-ag NLS by importin α/β (Huebner et al., 1997; Xiao et al., 1996, 1998).

In terms of prNLSs where phosphorylation inhibits NLS-dependent nuclear protein import, phosphorylation by the cyclin dependent kinase (cdk) cdc2 at Thr124 adjacent to the T-ag NLS inhibits T-ag nuclear import, specifically reducing the maximal level of nuclear accumulation (Jans et al., 1991). T-ag thus possesses a specialized sort of prNLS, comprising both enhancing and inhibitory phosphorylation sites. The CK2/dsDNA-PK sites increase the rate of NLS-
dependent nuclear import by enhancing recognition by importin \( \alpha/\beta \), whilst phosphorylation at the \( \text{cdc2} \) site adjacent to the NLS inhibits transport, markedly reducing the level of maximal nuclear accumulation (Jans et al., 1991). Whereas the CK2 and dsDNA-PK sites synergise at the level of both phosphorylation and importin interaction, the CK2 and \( \text{cdc2} \) sites function completely independently of one another in terms of both regulating T-ag nuclear transport, and influencing phosphorylation at the other site (Jans et al., 1991); that is, CK2-phosphorylation does not enhance nuclear import by reducing phosphorylation at the inhibitory \( \text{cdc2} \) site, nor does Thr\(^{124} \) phosphorylation inhibit nuclear transport by impairing Ser\(^{112} \) phosphorylation.

Despite their diverse origins, the prNLSs of the viral protein T-ag (Rihs et al., 1991; Jans et al., 1991; Jans and Jans, 1994; Jans, 1995), the yeast transcription factor SWI5 (Jans et al., 1995) and the \textit{Drosophila} morphogen Dorsal (Briggs et al., 1998) are all functional in higher mammalian cells with respect to both NLS activity, and regulation thereof by phosphorylation. The functionality of the T-ag NLS has also been demonstrated in yeast and plant cells (Shiozaki and Yanagida, 1992). Since prNLSs thus appear to function in different cell types and species, it can be concluded that phosphorylation and prNLSs as a mechanism of regulating signal-dependent nuclear protein import are conserved across eukaryotes, from yeast to flies to higher mammals. This is important in terms of using these sequences for targeting in a clinical setting.

Substituting the kinase site within prNLSs enables novel prNLSs to be engineered where nuclear targeting can be effected in response to different cellular signals. It has proved possible to substitute one kinase site with that for another in the context of the T-ag prNLS, and show an alteration in the cellular signals regulating nuclear import, according to the specific phosphorylation site introduced (Xiao et al., 1996; Xiao and Jans, 1998). In particular, the CK2 site of the T-ag prNLS was substituted with consensus sites for either PK-A or PK-C, and the resultant proteins shown to be able to render nuclear import uniquely responsive to cAMP and PK-A activation (Xiao et al., 1996), or phorbol esters/PK-C activation (Xiao and Jans, 1998), respectively. This demonstrates clearly that the kinase sites confer the specificity in terms of the cellular signals that can induce NLS-dependent nuclear import or otherwise through prNLSs.

Substitution of the negatively charged amino acids Asp or Glu in place of phosphorylation site serine/threonine residues within prNLSs can create an engineered constitutive prNLS which does not require phosphorylation in response to cellular signals to enhance or inhibit NLS function. This has been shown for T-ag, including the CK2 site (Jans et al., 1991; Jans and Jans, 1994; Xiao et al., 1997, 1998), and Dorsal/rel (Mosialos et al., 1991; Briggs et al., 1998). That the effects on nuclear transport of phosphorylation can thus be simulated by Asp/Glu indicates that it is negative charge at the respective sites that is mechanistically important in regulating nuclear import.

Apart from the mechanisms discussed above with respect to prNLSs where phosphorylation can either enhance NLS recognition by importin \( \alpha/\beta \) or inhibit NLS accessibility through negative charge near the NLS, several other specific mechanisms exist to regulate signal-dependent nuclear protein import. These include intermolecular/intramolecular NLS masking and cytoplasmic retention (Jans et al., 1998).
5.2.2. Modular vehicles for targeted PS delivery

For cell-specific targeted nuclear delivery of chlorin e₆, Akhlynina et al. (1997, 1999) prepared conjugates containing insulin as a model internalizable ligand and modified versions of the T-ag NLS. Conjugates were constructed using either BSA or β-galactosidase/T-ag-β-galactosidase fusion protein (P10) as carriers to which chlorin e₆, insulin, and in the case of the BSA-containing conjugates, NLS-containing peptides, were successively linked. The various constructs thus made are shown schematically, together with the sequences of the peptides used, in Table 3.

Akhlynina et al. (1997, 1999) set out to compare the photodynamic activity of constructs without and with the T-ag NLS (amino acids 126–132) together with the T-ag N-terminal flanking sequence (amino acids 111–125 — see Table 3) which have been shown to enhance T-ag nuclear transport. As mentioned in part (c) of section 5.2.1.2.3, the CK2 phosphorylation site (Ser¹¹²) within this flanking region increases the rate of T-ag nuclear import by about 50-fold, whilst phosphorylation by the cdk cdc2 at another site (Thr¹²⁴) reduces the maximal level of transport. This enabled the authors to design an NLS optimised for efficient nuclear targeting (P101Lys) spanning T-ag amino acids 111–132, including the CKII site, but lacking the cdk site (see Table 3).

Photosensitising activity was measured using the insulin receptor-bearing PLC/PRF/5 human hepatoma and C6 rat glioma cell lines (Table 4).

The construct using the T-ag-β-galactosidase fusion protein P10, containing T-ag amino acids 111–135 with a non-phosphorylatable alanine residue at the cdk site threonine, comparable to the P101Lys peptide, showed the highest photodynamic activity. The EC₅₀ was

Table 3
Outline of PS transporting modular constructs together with the amino acid sequences of the NLS-peptides/sequences used (Akhlynina et al., 1997, 1999)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Carrier</th>
<th>NLS Peptide/protein sequenceᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-P101Thr-(chlorin e₆)-insulin BSAᵇ</td>
<td>P101Thr: NH₂-Cys-Gly-Pro-Gly-<strong>Ser</strong>¹¹²-Asp-Asp-Glu-Ala-Ala-Asp-Ala-Gln-His-Ala-Ala¹²⁴-Pro-Pro-Lys-Thr¹²⁸-Lys-Arg-Lys-Val¹³²-Gly-Tyr-COOH</td>
<td></td>
</tr>
<tr>
<td>β-Gal-(chlorin e₆)-insulin β-Gal</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Numbers refer to the T-ag amino acid sequence, with the NLS (amino acids 126–132) underlined. Ser¹¹² (bold type) is the site of CK2 phosphorylation which enhances T-ag nuclear import, and Thr¹²⁴ (substituted by Ala — shown in bold type — in all cases) is the site of phosphorylation by cdc2 which inhibits T-ag nuclear import. Substitution of Lys¹²⁸ (bold type) by Thr abolishes NLS activity (see part (c) of section 5.2.1.2.3).
ᵇ Abbreviations: BSA, bovine serum albumin; β-Gal, β-galactosidase.
ᶜ T-ag-β-galactosidase fusion protein containing a variant of T-ag amino acids 111–135 fused N-terminal to E. coli β-galactosidase amino acids 9–1023.
0.13 nM on PLC/PRF/5 cells, while the β-galactosidase construct, completely lacking an NLS, exhibited an EC50 value of 2.0 nM; free chlorin e6 had an EC50 value 2500-fold higher (Table 4).

As shown in experiments using confocal laser fluorescence microscopy and video-intensified microscopy, BSA-P101Lys-(chlorin e6)-insulin accumulated both within the nucleus and the cytoplasm while BSA-P101Thr-(chlorin e6)-insulin accumulated mainly within the cytoplasm, this differential localization presumably representing the basis of the difference between the EC50s of the two constructs (see Table 3). P10-(chlorin e6)-insulin and β-galactosidase-(chlorin e6)-insulin exhibited qualitatively the same differences in subcellular localization within PLC/PRF/5 cells as BSA-P101Lys-(chlorin e6)-insulin and BSA-P101Thr-(chlorin e6)-insulin, respectively. Chlorin e6 localized in the cytoplasm (see also section 2.1.1.2).

That the results with respect to intracellular localization of NLS-containing constructs are not explicable in terms of photodynamic action after irradiation damaging the nuclear envelope leading to subsequently enhanced nuclear transport is unlikely, based both on kinetic considerations, and the fact that, since the T-ag NLS is exclusively a nuclear entry signal like other NLSs and is not able to mediate nuclear accumulation in the absence of an intact nuclear envelope (see Efthymiadis et al., 1997, 1998).

These data (Akhlynina et al., 1997) show that nuclear targeting of photosensitizing constructs through the inclusion of NLSs results in enhanced photodynamic activity, maximally about 2500-fold that of free chlorin e6. Bisland et al. (1998) have more recently confirmed these results.

It is known that only a small proportion of internalized insulin is transported to the nucleus under normal conditions (Smith and Jarett, 1990). This is due in part to the fact that insulin is unable to escape from endosomes subsequent to its internalization. It is known that human adenovirus disrupts the membrane of endocytotic vesicles during receptor-mediated

---

### Table 4
The photodynamic activity of NLS-containing chlorin e6 constructs (Akhlynina et al., 1997)

<table>
<thead>
<tr>
<th>Constructa</th>
<th>EC50 ± SD (nM)b</th>
<th>PLC/PRF/5 cells</th>
<th>C6 glioma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorin e6</td>
<td>350 ± 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P10-(chlorin e6)-insulin(1:5:7)</td>
<td>7.5 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-galactosidase-(chlorin e6)-insulin(1:5:7)</td>
<td>38 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA-P101Lys-(chlorin e6)-insulin(1:4:5:3)</td>
<td>23.0 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA-P101Thr-(chlorin e6)-insulin(1:4:7:3)</td>
<td>&gt; 150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorin e6</td>
<td>320 ± 2</td>
<td>&gt; 1000</td>
<td></td>
</tr>
<tr>
<td>P10-(chlorin e6)-insulin(1:3:8)</td>
<td>0.13 ± 0.06</td>
<td>ndc</td>
<td></td>
</tr>
<tr>
<td>β-galactosidase-(chlorin e6)-insulin(1:2:8)</td>
<td>2.0 ± 0.4</td>
<td>ndc</td>
<td></td>
</tr>
<tr>
<td>BSA-P101Lys-(chlorin e6)-insulin(1:1:11:2)</td>
<td>50 ± 8</td>
<td>28 ± 3</td>
<td></td>
</tr>
<tr>
<td>BSA-P101Thr-(chlorin e6)-insulin(1:1:8:2)</td>
<td>104 ± 21</td>
<td>74 ± 29</td>
<td></td>
</tr>
</tbody>
</table>

- a The ratio of components in the constructs is indicated in parentheses.
- b Determined from colony-formation test.
- c nd, not determined.
endocytosis, enabling access to the cytosol and delivery of its DNA into the nucleus (Greber et al., 1993). Adenoviruses have been used to increase the delivery of a variety of proteins into cells (Fitzgerald et al., 1983; Seth, 1994), while the incorporation of adenovirus particles into gene transfer complexes has been shown to enhance gene delivery, through effecting the release of endocytosed DNA into the cytoplasm (Sobolev et al., 1998). Akhlynina et al. (1999) used attenuated Ad5 adenovirus strain dl312 (deleted for the tumorigenic E1A region) in combination with the most potent photosensitising NLS-containing construct — P10-(chlorin e₆)-insulin — and showed that the nuclear accumulation of the construct and its efficiency were significantly increased (see Fig. 3). Hence, even though the constructs and adenovirus are internalised through distinct cellular receptors, their occurrence within the same endocytic vesicles presumably results in escape of the construct into the cytosol through adenovirus-mediated disruption of the endosomal membrane. The NLSs of the constructs in the cytoplasm

Fig. 3. Fluorescence micrographs of photoactivation in situ in living PLC/PRF/5 human hepatoma cells incubated with chlorin e₆-insulin-containing conjugates either lacking an NLS (B) or including the T-ag NLS in the presence of attenuated adenovirus (Ad5 dl312) (A) (see section 5.2.2). Cells were incubated with conjugates for 18 h at 37°C, washed, incubated with dichlorofluorescein diacetate (DCFD — see section 5.1) for 5 min at 37°C, and then washed and irradiated. Fluorescence due to the production of 2',7'-dichlorofluorescein from DCFD through reaction with photosensitizer-generated, reactive oxygen species was visualized using video-enhanced microscopy, with quantitation using a line plot (positions indicated) presented in C and D. Comparison of panels A/C and B/D indicates the enhancement of NLS-mediated nuclear delivery by the endosomolytic action of the adenovirus (see also section 5.2.2; Akhlynina et al., 1999). Scale bar (panel B), 10 µm.
are subsequently recognised by the cellular nuclear import machinery (see section 5.2.1.2) to mediate delivery of the constructs into the nucleus. Enhanced endosomal exit ultimately results in increased photodynamic activity in the nucleus (Fig. 3).

The data above clearly indicate that NLS-mediated nuclear localization of PS enhances its activity, confirming the nucleus to be a hypersensitive site for photodynamic damage (Wiseman and Halliwell, 1996; Akhlynina et al., 1997). The precise mechanism of nuclear photodamage-induced cell death, and in particular how this may relate to processes such as the destruction of tumor vasculature, is unclear at this stage.

The data summarized here indicate that it is possible to design highly efficient molecular constructs (e.g., of the type depicted schematically in Fig. 4) that possess specific and distinct sequence modules conferring cell-specific targeting, internalization, intracellular vesicle escape,

Fig. 4. Schematic depiction of the route of cellular uptake of a modular conjugate to deliver photosensitizers to the nucleus of specific cells subsequent to receptor-mediated endocytosis. The various sequence modules for cell surface binding and uptake (a ligand), an endosomolytic sequence, and a nuclear targeting sequence (NLS), as well as the photosensitizer, are linked to the carrier molecule. The conjugate is thus able to bind to a specific receptor of the target cell (1), enter the cell by receptor-mediated endocytosis (2), escape from the endosome to the cytoplasm through action of the endosomolytic component (3), and localize efficiently in the nucleus through the NLS conferring interaction with the cellular nuclear import machinery (4) (see also Section 5.2.2). Subsequent photo-irradiation (5) results in oxidative damage to the nucleus, demonstrated to be a hypersensitive subcellular site for photosensitizer action (Akhlynina et al., 1997, 1999).
and targeting to the nucleus. Individual sequence components/modules can retain their activities, and contribute to the overall goal of achieving cell-specific, efficient PDT of tumor or other conditions. Although insulin was used as a model ligand able to confer both cell-type specificity and receptor-mediated endocytosis on a photosensitizing construct, the approach is clearly applicable to a wide variety of ligands and cancer cell types, e.g., ligands such as insulin-like growth factors (neuroblastomas and osteosarcomas), nerve growth factor (neuroblastomas or glaucomas), or melanocyte-stimulating hormone (melanoma), could clearly be used conjugated to NLS-BSA-chlorin e₆ or P10-chlorin e₆ to target chlorin e₆ to the nucleus of requisite tumor cell types (see also below).

6. Unsolved problems and prospects

Though addressed intracellular delivery systems have proved to be highly efficient in vitro, it must be admitted that these are only the very first steps toward successful therapy relying on this principle. Based on the advances discussed above (e.g., Akhlynina et al., 1997, 1999; Bisland et al., 1998), an idealized scheme for a modular delivery system is depicted in Fig. 4, which incorporates the three basic components of (1) an internalizable ligand conferring cell-specific delivery, (2) a sequence with endosomolytic activity, and (3) a nuclear targeting signal, in addition to the photosensitizer. Future prospects with respect to each of these modular components are discussed below, as well as the nature of the conjugate itself.

To date, internalizable peptide ligands for which receptors are more abundant on tumor cells than on the surrounding normal cells include α-melanocyte-stimulating hormone (MSH), specific for a number of melanomas (Morandini et al., 1994; Siegrist et al., 1994; Jiang et al., 1996; Akhlynina et al., 1999), a variant of epidermal growth factor (Huang et al., 1997), and somatostatin (De Jong et al., 1998a,b). Depending on the tissue (e.g., brain), growth factors such as those for insulin or insulin-related growth factor can be placed in the same category. Tumor cell-specific internalizable antibodies may be a better alternative for PS delivery inside the cell. An interesting prospect in this context is the possibility of using internalizable polypeptide ligands, such as acidic and basic fibroblast and platelet-derived growth factors and interleukins-1, -2, and -5 (see Burwen and Jones, 1987; Jans, 1994; Jans and Hassan, 1998), as cell-specific targeting agents, which themselves are nuclear localizing through possession of functional NLSs. These ligands intrinsically thus may confer not only cell-specific delivery, but also efficient nuclear translocation of PSs.

There are options to ‘improve’ intracellular transport, in particular, by introducing a component mediating the release from endosomes, such as amphipathic oligopeptides that have already been shown to appreciably promote liberation of macromolecules from the endocytotic pathway compartments (Plank et al., 1994). Specific examples (see Plank et al., 1994) include fusogenic peptides from influenza virus hemagglutinin (Wagner et al., 1992; Midoux et al., 1993), and acidic synthetic derivatives such as the peptide GALA (Parente et al., 1988; Anderson et al., 1993).

In terms of nuclear targeting itself, despite the success in using modifications of the T-ag prNLS (Akhlynina et al., 1997; 1999), alternative targeting signals may be employed, including the more recently characterized modular sequences such as M9 or the BIB domain (see part (c)
of section 5.2.1.2.3). The latter, as well as the amino terminus of rpL25, are able to be recognized by more than one nuclear import receptor, meaning that these signals may be even more efficient than the optimised T-ag NLS in targeting PSs to the nucleus. Direct comparison of relative nuclear import efficiencies should be performed in the near future to enable the optimal nuclear import pathway(s) to be exploited, to enhance PDT nuclear drug delivery in general.

An important aspect is also the technological feasibility of creating PS carriers. It seems unlikely that the multicomponent carriers presently produced in a laborious and expensive fashion through covalent linkage of different peptide modules via bifunctional cross-linking reagents, even if they prove highly efficient in vivo, will find broad clinical application in the near future. Therefore we believe that special attention should be paid to developing recombinant chimeric vehicles for PS that would include modules for addressed delivery both to the target cells and into the most vulnerable compartments thereof.

In this context we have succeeded in producing bacterially expressed, modular recombinant polypeptide vehicles for PSs comprising (i) MSH as the internalizable ligand, (ii) the optimized nuclear targeting signal of T-ag, (iii) the *E. coli* hemoglobin-like protein HMP as a carrier, and (iv) an endosomolytic amphipathic peptide. These vehicles delivered the PS into the nuclei and perinuclear space of murine melanoma M3 cells and provided for a much greater photodynamic effect than nonmodified PS. Analysis of the M3 cell survival vs the irradiation dosage (at equimolar chlorin e₆ concentrations) demonstrated that the D₀ (67 kJ) for the PS-vehicle complex is tenfold less than for free PS (D₀ = 620 kJ), i.e., that the melanoma cell nuclei are ten times more sensitive to photodynamic injury than the sites affected by free PS. These results are also indicative of the prospects of using recombinant chimeric multicomponent vehicles for PSs (Smirnova, Rosenkranz, Baeva, Lunin, Sergienko, Voronina, Jans and Sobolev, 1999, manuscript in preparation).

There may be a problem, of course, in the potential antigenicity of the addressed delivery constructs in the organism. The use of human proteins as ligands and carriers may alleviate this problem; again, there already are ways and means of substantially attenuating the immunogenicity of macromolecules (Allemann et al., 1995; Saito et al., 1996).

### 7. General conclusions

PSs are substances possessing a local action within target cells which is extended not more than to tens of nanometers around sites of their subcellular localization due to the short diffusion distances of reactive oxygen species and free radicals produced upon illumination of PSs. PSs used for PDT localize in various cytoplasmic membranous structures but are not found in the most vulnerable intracellular sites for reactive oxygen species such as the cell nucleus.

The data summarized here show that it is possible to design highly efficient molecular constructs — PS carriers — with specific modules conferring cell-specific targeting, internalization, intracellular vesicle escape, and targeting to the most vulnerable intracellular compartments such as the nucleus. Nuclear targeting of these PS-carrying constructs resulted in enhanced photodynamic activity, maximally about 2500-fold that of free PS. Future work
should optimise this approach, hopefully to the point at which tumor cells can be killed rapidly and efficiently, with a minimum of normal cell and tissue damage.

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