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Targeted intracellular delivery of photosensitizers to enhance photodynamic efficiency

ANDREY A ROSENKRANZ, 1,2 DAVID A JANS3 and ALEXANDER S SOBOLEV1,2

1Department of Biophysics, Biological Faculty, Moscow State University, 2Laboratory of Molecular Genetics of Intracellular Transport, Institute of Gene Biology, Russian Academy of Science, Moscow, Russia and 3Nuclear Signaling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra, Australian Capital Territory, Australia

Summary Photodynamic therapy (PDT) is a novel treatment, used mainly for anticancer therapy, that depends on the retention of photosensitizers (PS) in tumour cells and irradiation of the tumour with appropriate wavelength light. Photosensitizers are molecules such as porphyrins and chlorins that, on photoactivation, effect strongly localized oxidative damage within target cells. The PS 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 experimental approaches discussed in the present paper indicate that it is possible to design highly efficient molecular constructs, PS carriers, with specific modules conferring cell-specific targeting, internalization, escape from intracellular vesicles and targeting to the most vulnerable intracellular compartments, such as the nucleus. Nuclear targeting of these PS-carrying constructs results in enhanced photodynamic activity, maximally about 2500-fold that of free PS. Future work is intended to optimize this approach to the point at which tumour cells can be killed rapidly and efficiently, while minimizing normal cell and tissue damage.

Key words: nuclear import, nuclear localization sequence, photodynamic therapy, photosensitizer, receptor-mediated endocytosis, targeted delivery.

Introduction

Photodynamic therapy (PDT) is a relatively new cytotoxic treatment, predominantly used in anticancer approaches, that depends on the retention of photosensitizers (PS) in tumour cells and their activation within the tumour through irradiation with light of the appropriate wavelength. Photovativated PS generate reactive oxygen species (singlet oxygen, \( ^{1}\text{O}_2 \), and free radicals, such as \( \cdot\text{OH}, \cdot\text{HO}^- \) and \( \cdot\text{O}_2^- \)) which are able to damage cellular structures, meaning that PDT is particularly attractive as an alternative means to kill drug- and radioresistant tumour cells.1,2 Normal cells, however, are also able to accumulate PS and be damaged by them, so that prolonged skin photosensitization, light-sensitivity of the eye and other side-effects have proved to be severe limitations of PDT. When photoactivated, PS inflict damage on many types of biomolecules without any specificity, their action being mediated largely via the reactive oxygen species mentioned, none of which travel more than several tens of nanometers before reacting with a biomolecule. Keeping in mind that cell dimensions are of the order of micrometres or tens of micrometres, it is clear that the intracellular action of PS is restricted to the site of their subcellular location and the surrounding radius of not more than 40 nm.3–5 That uneven intracellular distribution of PS can lead to differences in toxicity has been shown using laser microbeam irradiation.6 In contrast to cell membranes and other cytoplasmic organelles, the cell nucleus7–9 is known to be a very sensitive target for reactive oxygen species. In order to reduce the dose of PS administered to patients and hence minimize the harmful side-effects of PDT, new approaches have been devised to increase the effectiveness of tumour-cell killing through targeted delivery of PS to hypersensitive subcellular sites. These approaches are the focus of the present review.

Subcellular distribution of photosensitizers

Insomuch as most mammalian cells span tens of micrometres, it is clear that PS efficiency will depend not only on the relative distribution of PS between the tumour and surrounding tissues and between malignant and normal cells, but also on the intracellular distribution of the PS. Furthermore, membranes divide the interior of the eucaryotic cell into compartments that differ markedly in their sensitivity to reactions induced by PS-generated reactive oxygen species, in the ability of damaged molecules to be replaced/recycled and in the extent to which such damage affects cell viability and/or the capability of the cell to divide. It is noteworthy that preferential PS accumulation in tumours is itself not a guarantee of selective photoinduced tumour damage and successful PDT. In experiments on rats with gliosarcoma 9L,10 for example, it has been found that despite a 13-fold higher accumulation of the PS Photofrin in the tumour compared with...
the surrounding healthy tissue, the latter was found to be more sensitive to photodynamic injury. Thus, it is clear that not only the distribution of the PS in various types of tissue and its ‘affinity’ for certain cell types, but also its penetration into various subcellular compartments and accumulation therein are critical factors in the efficiency of PDT in situ in the whole organism.

The distribution of a PS within the cell depends on the route by which it enters, as well as on its physicochemical properties: that is, its hydrophobicity/hydrophilicity; the type, number and arrangement of its charged groups; the number of rings; the presence of a central atom in the tetrapyrrole structure; its aggregation state etc. In the in vivo situation where a PS may be introduced into the bloodstream, the picture is even more complicated. This is because many PSs interact strongly with blood proteins and lipoproteins, such that their penetration into particular cells may be mainly determined not so much by the properties of the PS itself, but rather by those of the blood component(s) carrying it. That PS redistribute readily from the liposomes, emulsions or complexes administered to the organism to the blood compartment is not at all surprising, especially in view of the highly localized mode of PS action, that the distribution within intracellular compartments is of great importance even for tetrasulfonated PS, which are known to preferentially localize at the plasma membrane.\textsuperscript{25,26}

The localization of PS in the cell is dependent on the mode of cellular uptake and subsequent redistribution. Aluminium phthalocyanine mono- and disulfonates, for example, have been found to be diffusely distributed over the cytoplasm of cultured LOX human melanoma cells, whereas the corresponding tri- and tetrasulfonates accumulate in granular lysosomal structures at the cell periphery.\textsuperscript{27} In cultured human meningioma cells, aluminium phthalocyanine di- and tetrasulfonates accumulate in the lysosomes, while aluminium phthalocyanine itself is diffusely distributed in the cytoplasm.\textsuperscript{28} Hydrophobic tetraoctylamine Zn(II) phthalocyanine\textsuperscript{29} and 5,10,15,20-tetra(m-hydroxyphenyl) chlorin\textsuperscript{30} show diffuse localization within the cytoplasm of cultured cells. Comparison of the distribution of meso-tetraphenylchlorin sulphonates (TPPSn) in NIH3T3 cervical carcinoma cells has shown TPPS2 (both isomers) and TPPS4 to be in the lysosomal/endosomal structures.\textsuperscript{31} A similar distribution has been found for TPPS4 in CT26 murine colon carcinoma cells, whereas TPPS1 is concentrated in small perinuclear vesicles, most probably the trans-Golgi.\textsuperscript{32} The PS lutetium texaphyrin also accumulates preferentially in lysosomes in EMT6 murine mammary sarcoma cells.\textsuperscript{33}

Intracellular tracking of Photofrin and N-monoaspartyl chlorin e6 has revealed that the former enters the cell by diffusion and localizes in mitochondria, while the latter is endocytosed and then localized in lysosomes.\textsuperscript{29} Incubation of bladder carcinoma cells with chlorin e6 leads to localization of the latter in the plasma membrane and other peripheral cell membranes,\textsuperscript{34} whereas lysyl chlorin p6 is largely found in endosomes.\textsuperscript{21} Lysyl chlorin p6 diester and its triester analogue also accumulate in the lysosomes of L1210 murine leukaemia cells, whereas lysyl chlorin e6 imide tends to localize in mitochondria and plasma membranes.\textsuperscript{35} As shown using several cell lines, lysosomes are also the destination of 9-acetoxy-2,7,12,17-tetrakis(beta-methoxyethyl)-porphycene.\textsuperscript{36} Benzoporphyrin derivative monoacid ring A is accumulated in trans-Golgi,\textsuperscript{37} and zwitterions, such as Nile Blue and some of its derivatives, tend to localize in lysosomes and trans-Golgi.\textsuperscript{38} Long chains and high concentrations appear to predispose PS to lysosomal localization, whereas PS with short chains accumulate predominantly in mitochondria.\textsuperscript{39}

Mitochondria quite specifically accumulate sulfonated aluminium chlorophthalocyanine,\textsuperscript{40} boronated porphyrin,\textsuperscript{41} the lipophilic cationic porphyrin derivative 5,10,15,20-tetrakis(1-decylpyridinium-4-yl)-21H,23H-porphine tetramethylammonio zinc(II) phthalocyanine tetramethylsulfate.\textsuperscript{42} Protoporphyrin IX, formed subsequent to the introduction of 5-aminolevulinic acid, localizes both in the endoplasmic reticulum (ER) and mitochondria of melanoma cells, whereas exogenously added protoporphyrin IX is found in lysosomes/endosomes (aggregated PS) or in the outer membrane (monomeric form).\textsuperscript{43} Protoporphyrin IX is synthesized in the cell interacts with the mitochondrial (peripheral) benzodiazepine receptor, which is a high-affinity binding site for dicarboxylic porphyrins and thereby an important determinant of photoactivation-dependent cytotoxicity.\textsuperscript{44}

**Subcellular distribution of photosensitzers in vitro**

Different PSs, depending on their properties, enter cultured cells through different mechanisms, thereby determining their subsequent subcellular localization and distribution and ultimately the efficacy of PDT in vitro. Photosensitizer molecules may permeate into the cell by diffusion both across and along the membranes, by non-specific endocytosis, or even by pinocytosis. Large aggregates or PS-carrying particles can also be internalized via phagocytosis.

Passive diffusion-controlled transfer into the cell has been shown for PS such as haematoporphyrin derivatives,\textsuperscript{14} hydroxyethyldeuteroporphyrin,\textsuperscript{15} benzoporphyrin derivative monoacids\textsuperscript{16} and zinc phthalocyanine.\textsuperscript{17} Passive diffusion also partly accounts for the entry into cultured cells of 5-aminolevulinic acid, localizes both in the endoplasmic reticulum (ER) and mitochondria of melanoma cells, whereas exogenously added protoporphyrin IX is found in lysosomes, endosomes (aggregated PS) or in the outer membrane (monomeric form).\textsuperscript{44} Protoporphyrin IX is synthesized in the cell interacts with the mitochondrial (peripheral) benzodiazepine receptor, which is a high-affinity binding site for dicarboxylic porphyrins and thereby an important determinant of photoactivation-dependent cytotoxicity.\textsuperscript{45}
Charged anionic and cationic PS are usually associated with membrane components, while neutral water-soluble PS are more diffusely distributed throughout the whole cell. Pyridinium Zn(II) phthalocyanine and tetratosulfonated Zn(II) phthalocyanine, carrying four positive and four negative charges, respectively, appear to localize in lysosomes, whereas the neutral tetraethanolamine Zn(II) phthalocyanine is diffusely localized through diverse intracellular membranes, with some preference for the Golgi complex.

Subcellular redistribution of photosensitizers after photoirradiation in vitro

As mentioned earlier, many PS translocate to membrane-delimited subcellular compartments subsequent to cell entry. The photochemical reactions induced by irradiation give rise to lipid peroxidation, which may damage membrane integrity and thereby cause the release of PS from their primary loci and subsequent redistribution. Both continuous and fractionated irradiation can thus result in the damage of secondary targets by PS in the cell.

Anionic meso-tetraphenylporphyrin di- and tetrarsulfonates (TPPS2o, 2a and 4, respectively), which initially accumulate in lysosomes, change their localization on cell irradiation; TPPS2o and 2a are spread diffusely throughout the cytoplasm, while TPPS4 concentrates in the nuclear region. Interestingly, the irradiation-induced redistribution of these PS appears to depend on the cell-cycle state of the cells; photoirradiation leads to the redistribution, for example, of TPPS4 from lysosomes to nuclei in growing but not in stationary cells. Cationic pyridinium zinc phthalocyanine initially localizes in the lysosomes of RIF-1 cells, but on photoirradiation diffuses into the cytoplasm as well as selectively staining the nucleoli. Anionic tetratosulfonated Zn(II) phthalocyanine and tetracycline Zn(II) phthalocyanine have been shown to migrate from lysosomes mainly to the region of the nucleus after low-level light irradiation, whereas hydrophobic tetradecylamine Zn(II) phthalocyanine, tetra(m-hydroxyphenyl) chlorin and amphiphilic polyhematoporphyrin do not change their location. The redistribution of PS from lysosomal compartments can be very rapid, taking minutes or even seconds, as found for polysubstituted Zn(II) phthalocyanines and sulfonated aluminium phthalocyanines.

Subcellular distribution of photosensitizers administered in vivo

On in vivo administration (e.g. by systemic injection), PS form complexes with the protein components of the blood and only a minor portion thereof may exist in a free state. Experiments where different PS were incubated with serum in vitro have demonstrated that practically all PS can bind to a range of blood proteins, albeit with differing affinities. This clearly implies that the intracellular localization of PS in vivo may be strongly influenced by their complexation with blood proteins, and redistribution between them and other factors. The data thus far available on the in vivo subcellular distribution of PS are much more fragmentary than those for cultured cells due to the understandable experimental difficulties in performing such studies. It should also be said that on account of the indirect action of PDT on the tumour vascular epithelium, the concept of a specific PDT target cell in vivo may be simplistic.

An important event in the systemic distribution of a PS to target tissues via the blood, and subsequent uptake by tumour and normal tissues, is its binding to serum components, including lipoproteins and albumin. Albumin-bound PS can enter epithelial cells by liquid-phase as well as receptor-mediated endocytosis and then either localize in lysosomes or, to a lesser extent, traverse the cell by transcytosis. Another type of albumin-accepting cell is macrophages, which trap radical-damaged albumin particularly rapidly. Albumin has also been shown to carry PS to the stromal elements of tumour tissue. Low-density lipoprotein (LDL)-bound PS can enter target cells via receptor-mediated endocytosis. In the case of chlorin e6 covalently bound to LDL, the PS has been detected in enzymatically active lysosomes. Subcellular fractionation of rat liver after intraperitoneal injection of haematoporphyrin has revealed the latter's presence in the mitochondrial and plasma membrane fractions.

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. Boronated porphyrin localizes in the mitochondria of glioma cells, for example, both in vivo and in vitro. However, in contrast to their pronounced subcellular accumulation in vitro, Photofrin II, meso-tetraphenylporphine disulfonate and Al(III) phthalocyanine trisulfonate mainly stain the plasma membranes of grafted LOX human melanoma cells in nude mice in vivo. Similarly, hydrophilic PS, such as meso-tetraphenylporphine tri- and tetrarsulfonates and Al(III) phthalocyanine tetrarsulfonate, do not permeate into the cells in vivo, but remain bound to the tumour stroma.

The redistribution on irradiation shown in vitro for many PS that initially localize in the lysosome has been confirmed in animal experiments in situ. In vivo redistribution of Al(III) phthalocyanine tetrarsulfonate, but not zinc phthalocyanine, has been observed in RR1022 rat bladder tumour cells, correlating with in vitro results. It should be pointed out, however, that for many PS there is no clear correlation between the ability to bind to particular serum proteins and in vivo PDT efficiency.

Systems for photosensitizer delivery into cells

Approaches to enhance PDT efficacy and selectivity have proceeded in several different directions. First, researchers have attempted to generate an array of new PS in the perhaps somewhat naïve hope that the new compounds would prove capable of accumulating in particular, desired subcellular compartments. A second approach has been to use various carriers, such as polymers, microspheres, liposomes and emulsions, to ‘target’ the PS both to, and more importantly into, the cell. Liposomes have been widely used as a means of drug delivery, and in most cases (see, however, Reddi), the incorporation of PS into liposomes has been shown to enhance their photocytotoxicity appreciably. However, PS injected into the bloodstream in liposomes ultimately reach cells in complexes with blood lipoproteins, to which they redistribute on contact of the liposomes with serum proteins.
Intracellular delivery of photosensitizers

proteins.\textsuperscript{12,13,48} Analogous events occur with PS administered as emulsions.\textsuperscript{64}

The use of other vehicles, such as microspheres\textsuperscript{14} or nanoparticles,\textsuperscript{65} enables the intracellular delivery of PS to phagolysosomes. The covalent attachment of the PS chlorin e\textsubscript{6} to 1 µm polystyrene microspheres results in its entry into MGH-U1 human transitional cell bladder carcinoma cells via phagocytosis. The fluorescence due to microspheres and/or free chlorin e\textsubscript{6} has been detected, respectively, in phagolysosomes and on cell membranes, but no free chlorin e\textsubscript{6} has been found in the cytoplasm or within the nucleus or lysosomes. The photodynamic activity of chlorin e\textsubscript{6}–microsphere conjugates in MGH-U1 cells has proved to be higher than that of free PS, although the singlet oxygen-generating capacity of chlorin e\textsubscript{6} in the free state is ninefold greater than in microspheres.

The specificity of photodynamic action can be improved by conjugating PS to monoclonal antibodies (mAb) or ligands specific for receptors or antigens on the tumour cell surface. Chlorin e\textsubscript{6} conjugates with the mAb OC125 accumulate to up to threefold higher levels than free chlorin e\textsubscript{6} in murine ovarian tumour.\textsuperscript{66} Immunoconjugates of chlorin e\textsubscript{6} monoethylene diamine monoamide with the mAb IG12 specific for uveal melanoma cells produce fourfold greater damage in the latter than the free PS at the same concentration.\textsuperscript{67} 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, on incubation of melanoma cells with mAb conjugates of Sn-chlorin e\textsubscript{6}, photoinduced cell lysis is caused by point lesions of the plasma membrane.\textsuperscript{68} However, it is known that, in contrast to the plasma membrane, lysosomes and especially nuclei are extremely susceptible targets for reactive oxygen species.\textsuperscript{7,69} It thus seems desirable to create a selective form of PS that can penetrate into the cell interior where it may exert its photodynamic action.

One means to attain this goal may be to modify mAb–PS conjugates to include additional components that confer on them an ability to be endocytosed by the cell. The attachment of polyclone to a conjugate containing mAb OC125 directed against ovarian cancer cells enhances PS uptake up to 17-fold,\textsuperscript{70} and similarly, internalization of meso-chlorin e\textsubscript{6} mono(N-2-aminoethylamide) by OVCAR-3 ovarian carcinoma cells could be achieved by coupling it to MAb OV-TL16 using N-(2-hydroxypropyl)methacrylamide.\textsuperscript{71} Furthermore, it has recently been shown that m-tetrahydroxyphenylchlorin attached to internalizable mAb 425 specific for head and neck squamous cell carcinoma is more effective than a conjugate with a non-internalizable antibody (U36).\textsuperscript{72} There certainly appears to be much promise for enhancing PDT in using tumour cell-specific internalizable antibodies.

The use of immunoliposomes has also been shown to improve the efficacy of antibody conjugated PS by facilitating penetration into the target cells. Comparison of conjugates of the PS sulfonated aluminum phthalocyanine with antibodies or in combination with monolayer immunoliposomes has revealed that the photodynamic activity of immunoliposomes on bladder carcinoma cells \textit{in vitro} is 13-fold higher than that of the PS–antibody conjugates alone.\textsuperscript{73} Pheophorbide \textit{a} in immunoliposomes with antibodies to T-24 tumour bladder cells has proved to be more efficient \textit{in vitro} on these cells than on those of the MGH-U1 human bladder line; in this case, the PS was found to accumulate selectively in the lysosomes.\textsuperscript{74} It is not clear how practicable the use of immunoliposomes may be, however, in view of the observed \textit{in vivo} redistribution of PS from liposomes to blood components such as lipoproteins (see earlier).

Targeted intracellular delivery of photosensitizers

The fact that a PS introduced into the body 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 PS that bind with high affinity to particular blood proteins that possess the desired cell-specific ‘addressing’ capabilities; or (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, because in this case the researcher is not constrained by the existing set of serum proteins or the particular features of their interaction or lack thereof with tissues/particular cell types. It is also facilitated by the extensive knowledge gained over the last decade and a half with respect to the signals determining subcellular localization and in particular the cellular machinery responsible for recognizing such signals and delivering the corresponding molecules to their destinations.

\textbf{Internalizable ligand–photosensitizer complexes and their subcellular redistribution}

As already mentioned, sufficiently hydrophobic PS can be readily incorporated into lipoproteins such as LDL and high-density lipoproteins (HDL),\textsuperscript{63,75} with the receptors for the former being more abundant in cancerous cells than in surrounding normal tissues.\textsuperscript{54,76–78} Experiments with PS pre-incorporated into lipoproteins have shown that simple incorporation into LDL provides for effective photodynamic action.\textsuperscript{79} \(\alpha\)2-Macroglobulin/LDL receptors are widely found in cells of various types, with LDL uptake most pronounced in adrenals, liver and spleen.\textsuperscript{54,78} Proliferating vascular epithelial cells are also rich in \(\alpha\)2-macroglobulin receptors, which may be the basis of preferential PS accumulation observed in neovascularized tissues.\textsuperscript{56}

The use of lipoproteins as ‘intermediary’ transporters for PS delivery to target cells imposes certain limitations connected with redistribution in the blood, depending on the properties of the PS and blood components, the dynamics of the interactions of which are not understood.\textsuperscript{80} Furthermore, such a mode of delivery predetermines to a large degree the subsequent subcellular distribution of the PS and thereby its sites of action. An increasingly popular alternative approach has made use of conjugates comprising a PS and an addressed internalizable ligand.

By attaching a PS to a macromolecular internalizable ligand, one can explicitly predetermine not only the specificity of its uptake only by certain cells, but also to some extent its subsequent intracellular location. Experiments with cultured cells have shown that such manipulation can greatly potentiate the photodynamic effect.\textsuperscript{5,69,81–83} Not all internalizable ligands can be simply conjugated with PS without the
loss of receptor-binding ability. To overcome this, both the ligand and the PS can be attached to an additional carrier. The intracellular fate of the ligand after internalization is also an important factor in the photodynamic effect achieved; ligands entering the cell by receptor-mediated endocytosis may be dispatched either back to the cell surface (recycling) or to other compartments of the cell through specific sorting systems.

We have shown that a PS delivery system comprising insulin as an internalizable ligand, a proportion of which has been shown to reach the nucleus, and chlorin e6 covalently linked to bovine serum albumin (BSA) is highly efficient in killing cultured PLC/PRF/5 human hepatoma cells or C6 rat glioma cells. The conjugate has been shown to have high affinity for the insulin receptor and to be internalized efficiently by PLC/PRF/5 cells. Fluorescence video-intensified microscopy (VIM) has been used to visualize the fluorescein-labelled conjugate mainly in the perinuclear space and to a small extent within the nucleus. Similar localization has also been observed using 2',7'-dichlorofluorescin diacetate to assay for the reactive oxygen species generated on cell irradiation. 2',7'-Dichlorofluorescin diacetate penetrates into living cells and is deacetylated by intracellular esterases; because reactive oxygen species react with 2',7'-dichlorofluorescin to yield fluorescent 2',7'-dichlorofluorescin, this can be used to visualize the sites of intracellular production of reactive oxygen species and sites of PS subcellular localization, as well as being an indicator of intracellular photodynamic activity in situ. The insulin–BSA–chlorin e6 conjugate at low concentrations suppresses the propagation of hepatoma cells (half-maximal effect at about 1 μmol/L). Other ligands that have been used in internalizable PS conjugates include transferrin, epidermal growth factor and maleylated BSA, a ligand for ‘scavenger’ receptors.

Approaches to deliver photosensitizers to the nucleus

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 what is known about the mechanisms of directed intracellular transport that ensure the delivery of biomolecules to certain intracellular compartments. The following section concentrates largely 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 and because it is the compartment to which PS have been delivered most successfully.

Targeting proteins to the nucleus

All passive and active transport into and out of the nucleus occurs through the nuclear envelope-localized nuclear pore complex (NPC), a multisubunit, complex structure. As its name implies, the NPC has a pore/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 a specific targeting signal in order to pass through the NPC; in the case of nuclear import, a nuclear localization sequence (NLS) 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 of a particular protein may be required to function several times and subsequent to several cell divisions. Proteins are also able to be translocated to the nucleus in their native conformation, again in contrast to targeting to mitochondria/chloroplasts etc.

Nuclear localization sequence-dependent nuclear protein import can be inhibited by antibodies specific to the soluble NPC components, the nucleoporins (nups). The nups are believed to represent docking sites for components of the nuclear import machinery during transport through the NPC. Certain nups are able to bind the cytosolic factors mediating association with and transport through the NPC; RanBP2 (nup358), for example, is able to interact with importin β (see later), which mediates the docking of signal-containing transport substrates at the NPC, as well as the monomeric guanine nucleotide-binding protein Ran (in its GTP-bound form), which plays a key role in translocation through the NPC, in part through interaction with importin β.

Nuclear targeting signals are defined as the short modular peptide sequences that are necessary and sufficient for nuclear localization of their respective proteins. The process of signal-mediated transport into the nucleus involves two basic steps. The first step involves energy-independent recognition of the targeting signal of the transport substrate and docking at the NPC, while the second is energy-dependent and involves translocation through the pore and into the nucleus. Types of nuclear targeting signals conferring transport between the cytoplasm and nucleus include conventional or ‘directional’ NLS that mediate nuclear protein import exclusively; nuclear export sequences (NES) that mediate protein transport in the reverse direction, and what are best termed ‘shuttle signals’, which can mediate both nuclear import and export of the proteins carrying them.

Conventional NLS fall into several classes, two of which are predominantly basic in nature. They are those resembling the NLS of the simian virus SV40 large tumor antigen (T-ag, PKKRRKVLV\textsuperscript{132}, comprising a short stretch of basic amino acids, and bipartite NLS, which consist of two stretches of basic amino acids separated by a spacer of 10–12 amino acids. Other types include those resembling the NLS of the yeast homeodomain containing protein Mat2, where charged/polar residues are interspersed with non-polar residues, or that of the protooncogene c-myc (PAAKRVKLD\textsuperscript{528}) where proline and aspartic acid residues either side of the central basic cluster are important for nuclear targeting. All classes of NLS are held to be recognized specifically by the ‘NLS-receptor’, the α/β-importin heterodimer, during the first step of nuclear transport, as has been shown directly for the importins from several species.

Certain apparently conventional NLS have been demonstrated to be recognized specifically not by the α/β-importin heterodimer, but by importin β (importin β1) alone, such as the T cell protein tyrosine phosphatase, the HIV-1 Rev protein, the yeast transcriptional activator GAL4 and parathyroid hormone related protein (PTHrP). All of these proteins appear to be able to be...
transported to the nucleus in the absence of importin α, which has been shown directly for PTHrP. The nuclear targeting signals recognized by importin β, as opposed to importin α, have been purported to be Arg-rich (rather than Lys-rich), but because the minimal PTHrP sequence recognised by importin β (YLTQETNKEVYKEQPLKTGKKKKGKP) is clearly T-ag-like and completely lacking in Arg residues, it seems clear that the situation is rather more complicated, with at least two distinct types of importin β-recognized nuclear targeting signal.

It has recently become clear that there are a large number of different importin β homologues in eucaryotic cells with apparently specific transport roles for particular classes of proteins. Importin β4 (Kap123p/Yrb4p) mediates the import of ribosomal proteins into the nucleus, as does β3 (Kap121p/Pse1p/Imp5-RanBP5), whereas importin β2 (Kap104p/transportin) mediates the nuclear import of mRNA-binding proteins. Interestingly, a region from ribosomal protein rpL23a (the BIB domain, VHSHIKKKKIRT-SPTFTTPKTLRLRRQPKYPKRSPRNRPNDY) has been shown to be able to be recognized specifically by any of four distinct importin β homologues, including β1–3, each of which can mediate nuclear import. Analogously to the BIB domain, amino acids 1–41 of rpL25 (MAP-SAKATAAKAVVKTGTNKAKLKRATSATFRPLKTLK-LAR) can be recognized by either importin β3 or β4. The best defined ‘shuttle sequence’, sufficient and necessary to mediate transport in both directions through the NPC, is the largely hydrophobic M9 sequence (NQSSNFGPMKGNFGGRSSGPYGGQYGAFKPRNQGGY) from the human mRNA-binding protein hnRNP A1. The target sequence requirements of other importin β homologues (of which there are at least 13 in yeast) largely remain to be determined (see Jans et al.).

**Figure 1** Visualization (a) and quantification (b,c) of photoactivation in situ in living PLC/PRF/5 human hepatoma cells incubated with insulin and nuclear localization signal (NLS)-containing photosensitizing conjugates. Cells were incubated with 50 nmol/L BSA-P101 Lys-chlorin e6-insulin construct (BSA-P101Lys-Ins-chl e6, see Table 1), BSA-P101Thr-(chlorin e6)-insulin construct (BSA-P101Thr-Ins-chl e6, see Table 1) or chlorin e6 alone for 18 h at 37°C, washed, incubated with 5 µmol/L 2',7'-dichlorofluorescin diacetate for 5 min at 37°C and then washed and irradiated (96 kJ/m²). Fluorescence due to the production of 2',7'-dichlorofluorescein from 2',7'-dichlorofluorescin diacetate resulting from photodynamic action was visualized after 5 min using confocal laser scanning microscopy (a), and then fluorescence was quantified from the digital images using image analysis (b,c; NIH Image 1.60 public domain software) to give an estimate of nuclear (Fn, ■) and cytoplasmic (Fc, □) fluorescence (b) or the nuclear to cytoplasmic ratio (Fn/c) (c). Measurements represent the average from at least five separate measurements for each of Fn and Fc, with background fluorescence subtracts. Arrows indicate the cell nuclei.
Table 1  Photodynamic activity of internalizable, NLS-containing chlorin e6 constructs.8,9

<table>
<thead>
<tr>
<th>Construct†</th>
<th>Signal sequence‡</th>
<th>EC50 ± SD (nmol/L)‡</th>
<th>PLC/PRF/5 cells</th>
<th>C6 glioma cells</th>
</tr>
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<tbody>
<tr>
<td>Chlorin e6</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-Galactosidase-chlorin e6-insulin (1:5:7)</td>
<td>None</td>
<td>2.0 ± 0.4</td>
<td>PLC/PRF/5 cells</td>
<td>&gt; 1000</td>
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</table>

| Chlorin e6 | None | 320 ± 2 | PLC/PRF/5 cells | > 1000 |
| P10-chlorin e6-insulin (1:3:8) | Includes T-ag amino acids 111–135: Ser112-Asp-Asp-Glu-Ala-Thr-Ala-Asp-Ala-Gln-His-Ala124-Pro-Pro-Lys-Lys125-Lys-Arg-Lys-Val-Glu-Asp-Pro135 | 0.13 ± 0.06 | PLC/PRF/5 cells | ND |
| β-galactosidase cholin e6-insulin (1:2:8) | None | 2.0 ± 0.4 | PLC/PRF/5 cells | ND† |

†The ratio of components in the constructs8 is indicated in parentheses. †Numbers refer to the T antigen (T-ag) amino acid sequence, with the nuclear localization sequence (NLS; amino acids 111–125, see Table 1), which has been shown to enhance T-ag nuclear transport (see Jans and Huebner,† Jans et al.114,118). ‡Determined from colony formation test. EC50, effective concentration for half maximal effect; ND, not determined.

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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.9,118 The modular sequences conferring phosphorylation-regulated nuclear protein import, phosphorylation-regulated NLS (pNLS),9,118 which are able to confer phosphorylation-regulated nuclear import on heterologous proteins, have been identified for a number of transcription factors and other proteins (see Jans et al.114,118) and have been used to target other molecules of interest to the nucleus (see Jans et al.118 and below).

Modular vehicles for targeted photosensitizer delivery

In order to carry out cell-specific targeted nuclear delivery of chlorin e6, we have prepared conjugates containing insulin as a model internalizable ligand and modified versions of the T-ag NLS.8,9 Conjugates were constructed using either BSA, β-galactosidase or the 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 are listed schematically, together with the sequences of the derivatives of T-ag NLS used, in Table 1.

We first compared the photodynamic activity of constructs carrying or lacking the minimal T-ag NLS (amino acids 126–132),8,9 with or without the amino-terminal flanking sequence of T-ag (amino acids 111–125, see Table 1), which has been shown to enhance T-ag nuclear transport (see Jans and Huebner,11 Jans et al.114,118). The CK2 phosphorylation site (Ser112) within this flanking region increases the rate of T-ag nuclear import by approximately 50-fold, whereas phosphorylation by the cyclin-dependent kinase cdc2 at another site (Thr128) reduces the maximal level of transport. An optimized NLS for efficient nuclear targeting (P101Lys), spanning T-ag amino acids 111–132, including the enhancing CK2 site, but lacking the inhibitory cdk site (see Table 1), was thus designed.8

Photosensitizing activity was measured using the insulin receptor-bearing PLC/PRF/5 hepatoma and C6 glioma lines (Table 1; Fig. 1). 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 effective concentration for half maximal effect (EC50) was 0.13 nmol/L on PLC/PRF/5 cells, while the β-galactosidase construct, completely lacking an NLS, exhibited an EC50 value of 2.0 nmol/L; free chlorin e6 had an EC50 value 2500-fold higher (Table 1).
In experiments using confocal laser scanning microscopy (CLSM) and VIM in conjunction with 2',7'-dichlorofluorescin diacetate (see earlier), in situ photoactivation due to BSA-P101Lys-(chlorin e6)-insulin was found both within the nucleus and the cytoplasm (Fig. 1a, right panel), while that due to BSA-P101Thr-(chlorin e6)-insulin and chlorin e6 alone (Fig. 1a, left panel) was mainly within the cytoplasm. Quantification of fluorescence from the digitized CLSM images using image analysis (Fig. 1b) indicated that the nuclear fluorescence (Fn) in cells treated with the NLS-containing conjugate were 15-fold higher than those treated with PS alone, or the non-functional NLS-containing conjugate (Fig. 1b, left panel). The nuclear cytoplasmic ratio (Fn/c, Fig. 1b, right panel) for BSA-P101Lys-chlorin e6-insulin was almost twice as high as that for BSA-P101Thr-chlorin e6-insulin, clearly indicating a higher level of nuclear accumulation due to the functional NLS. This differential localization is presumably the basis of the difference between the EC50 of the two constructs (see Table 1). P10-chlorin e6-insulin and β-galactosidase-chlorin e6-insulin exhibited subcellular localization in PLC/PRF/5 cells very similar to that of BSA-P101Lys-chlorin e6-insulin and BSA-P101Thr-chlorin e6-insulin, respectively. That the results with respect to intracellular localization of NLS-containing constructs were not attributable to photodynamic action after irradiation damaging the nuclear envelope and leading to enhanced nuclear transport entry seems unlikely, based on kinetic considerations (see Akhlynina et al.), as well as the fact that the T-ag NLS is exclusively a nuclear entry signal like other NLS and is unable to mediate nuclear accumulation in the absence of an intact nuclear envelope. The results (see Table 1) show that nuclear targeting of photosensitizing constructs through the inclusion of NLS can enhance photodynamic activity maximally approximately 2500-fold that of free chlorin e6. This has been confirmed by our more recent work and that of Bisland et al.

It is known that only a small proportion of internalized insulin is transported to the nucleus under normal conditions. 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 is able to disrupt the membrane of endocytotic vesicles during receptor-mediated internalization, enabling access to the cytosol and delivery of its DNA into the nucleus. Adenoviruses have been used to increase the delivery of a variety of proteins into cells, 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. We have used attenuated Ad5 adenovirus strain dL312 (deleted for the tumourigenic E1A region) in combination with the most potent photosensitizing NLS-containing construct, P10-chlorin e6-insulin (see Fig. 2a), to demonstrate that the nuclear accumulation of the construct and its photodynamic efficiency in situ can be significantly increased (see Fig. 2b). Hence, even though the constructs and adenovirus are internalized through distinct cellular receptors, their occurrence within the same endocytotic vesicles results in escape of the PS-containing conjugates into the cytosol through adenovirus-mediated disruption of the endosomal membrane. The NLS of the conjugates in the cytoplasm are subsequently recognized by the cellular nuclear import machinery to effect the nuclear delivery. Enhanced endosomal exit thus leads ultimately to increased photodynamic activity in the nucleus. The data clearly indicate that NLS-mediated nuclear localization of PS enhance their activity, confirming the nucleus to be a hypersensitive site for photodynamic damage. The precise mechanism of nuclear photodamage-induced cell death, and in particular how this may relate to processes such as the destruction of tumour vasculature, is unclear at this stage.

**Figure 2** A schematic of a modular photosensitizer (PS)-transporting construct including adenovirus as an endosomolytic component (a) and visualization of construct-induced photoactivation in situ in living PLC/PRF/5 human hepatoma cells (b). (a) The basic components of the modular construct are shown: carrier, internalizable ligand (insulin), nuclear localization signal (NLS) and photosensitizer, with the additional endosomolytic component (adenovirus). (b) Cells were incubated with 20 nmol/L P10-chlorin e6-insulin construct (see Table 1) in the presence of human adenovirus serotype 5, strain dL312 (3.7 × 10¹⁰ virions/mL) for 18 h at 37°C, washed, incubated with 5 µmol/L 2',7'-dichlorofluorescin diacetate for 5 min at 37°C and then washed and irradiated (96 kJ/m²). Fluorescence due to photodynamic action was visualized as described in the legend to Fig. 1a using video-intensified microscopy. Predominantly nuclear fluorescence is evident (compare with Fig. 1a).
Future prospects

The data summarized here indicate that it is possible to design highly efficient molecular constructs that possess specific and distinct sequence modules conferring cell-specific targeting, internalization, targeted delivery to the nucleus and even intracellular vesicle escape (see Fig. 2). Individual sequence components/modules can retain their activities and contribute to the overall goal of achieving cell-specific, efficient PDT of tumour or other conditions. Although addressed intracellular delivery systems have thus far proved to be highly efficient in vitro, as indicated earlier (see Table 1), it must be admitted that they represent first steps toward successful therapy founded on this principle. Based on the advances detailed earlier an idealized scheme for a modular delivery system would incorporate three basic components: (i) an internalizable ligand conferring cell-specific delivery; (ii) a sequence with endosomolytic activity; and (iii) a nuclear targeting signal, in addition to the photosensitizer. Future prospects with respect to each of these modular components are discussed later, as well as the nature of the conjugate itself.

Although insulin has been used as a model ligand able to confer both cell-type specificity and receptor-mediated endocytosis on a photosensitizing construct, the approaches above are clearly applicable to a wide variety of ligands and cancer cell types. To date, internalizable peptide ligands for which receptors on tumour cells are more abundant than on the surrounding normal cells include α-melanocyte-stimulating hormone (MSH), specific for a number of melanomas, or insulin-related (neuroblastomas and osteosarcomas) or nerve (neuroblastomas or gliomas) growth factors, can be placed in the same category, while tumour cell-specific internalizable antibodies may be another alternative. An additional prospect in this context is polypeptide ligands, such as acidic and basic fibroblast and platelet-derived growth factors and interleukin-1, -2, and -5, which are themselves nuclear localizing through the possession of functional NLS. Depending on the tissue (e.g. brain), growth factor receptors, such as those for insulin or insulin-related (neuroblastomas and osteosarcomas) or nerve (neuroblastomas or gliomas) growth factors, can be placed in the same category, while tumour cell-specific internalizable antibodies may be another alternative. An additional prospect in this context is polypeptide ligands, such as acidic and basic fibroblast and platelet-derived growth factors and interleukin-1, -2, and -5, which are themselves nuclear localizing through the possession of functional NLS. These ligands thus may confer not only cell-specific delivery, but also efficient subsequent nuclear translocation of PS.

In view of the earlier results indicating that endosomal exit subsequent to receptor-mediated endocytosis is a limiting step in the nuclear delivery of PS, possibilities to ‘improve’ intracellular transport include the introduction of a component effecting the release from endosomes, such as amphipathic oligopeptides that have already been shown to promote the liberation of macromolecules from the endocytotic pathway compartments appreciably. Specific examples include fusogenic peptides from influenza virus haemagglutinin and synthetic acidic derivatives, such as the peptide GALA. In terms of nuclear delivery itself, despite the success in using modifications of the T-ag prNLS, alternative nuclear targeting signals may be used, including modular sequences such as M9 or the BIB domain (see earlier). 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 optimized T-ag NLS in targeting PS 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 and perhaps 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 chimaeric 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 recently succeeded in producing bacterially expressed, modular recombinant polypeptide vehicles for PS, comprising: (i) MSH as the internalizable ligand; (ii) the optimized prNLS of T-ag; (iii) the \textit{Escherichia coli} haemoglobin-like protein HMP as a PS carrier; and (iv) an endosomolytic amphipathic peptide. These vehicles delivered the PS into the nuclei and peri-nuclear space of M3 murine melanoma cells and provided for a much greater photodynamic effect than free PS. Analysis of the M3 cell survival versus the irradiation dosage (at equimolar PS concentrations) demonstrated that the D0 for the PS-vehicle complex (67 kJ/m²) is 10-fold less than that for free PS (620 kJ/m²), that is, the melanoma cell nuclei are 10 times more sensitive to photodynamic injury than the sites affected by free PS. These results are also indicative of the prospects of using recombinant chimaeric multicomponent vehicles for PS (Smirnova et al. unpubl. data, 2000).

Problems may reside 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, although ways and means to attenuate the immunogenicity of macromolecules have been described. The incorporation of human or ‘humanized’ proteins into the types of constructs mentioned above to reduce immunogenicity is a focus of future work in our respective laboratories. This should enable us to advance towards our long-term goal of killing tumour cells rapidly and efficiently, with a minimum of normal cell and tissue damage.

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