Modular transporters for subcellular cell-specific targeting of anti-tumor drugs

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Summary

A major problem in the treatment of cancer is the specific targeting of anti-tumor drugs to these abnormal cells. Ideally, such a drug should act over short distances to minimize damage to healthy cells, and target subcellular compartments that have the highest sensitivity to the drug. Photosensitizers, alpha-emitting radionuclides and many other medicines could be considered as such drugs if they possessed cellular and subcellular specificity. The author describes a novel approach of using modular recombinant transporters to target photosensitizers and alpha-emitting radionuclides to the nucleus, where their action is most pronounced, of cancer cells. Photosensitizer-transporter conjugates have up to 3000 times greater efficacy than free photosensitizers and display cell specificity in contrast to free photosensitizers. Alpha-emitting radionuclides, conjugated with the modular transporters, acquired similar properties. The different modules of the transporters are interchangeable, meaning that they can be tailored for particular applications. *BioEssays* 30:278–287, 2008. © 2008 Wiley Periodicals, Inc.

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Abbreviations: AER, alpha-emitting radionuclide; D_0, a radiation dose which on the average yields a hit in a target and corresponds to the dose which reduces surviving fraction of cells by a factor of $1/e = 0.37$; DTox, translocation domain of diphtheria toxin; EC_{50}, concentration of an agent, which produces 50% of the maximum possible response; EGF, epidermal growth factor; ErbB1 receptor, the same as EGF receptor; HMP, E. coli hemoglobin-like protein; MRT, modular recombinant transporter; MSH, α-melanocyte-stimulating hormone; NLS, nuclear localization sequence; PDT, photodynamic therapy; PS, photosensitizer.

Introduction

Photodynamic therapy (PDT) is based on a predominant accumulation of photosensitizers (PSs) in a tumor and subsequent irradiation of the tumor with light of appropriate wavelength. Upon photoactivation, PSs generate reactive oxygen species (singlet oxygen and free radicals, such as $^{\cdot}OH$ and $^{\cdot}HO_2$), which are active principles of the PSs and able to damage proteins, nucleic acids, lipids, and other cellular components. However, photodynamic therapy has several considerable limitations. First, PSs are not cell-specific agents; that is, normal cells are also able to accumulate PSs, which results in a number of negative side effects (e.g. prolonged skin and retina photosensitization). Second, large doses of PSs are normally required for efficient tumor cell killing owing to their nonoptimal subcellular distribution.

PSs cause 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.[1–3] Uneven intracellular distribution of PSs determines the difference in subcellular toxicity as it was shown by laser microbeam irradiation.[4] In contrast to cell membranes and other cytoplasmic organelles, the cell nucleus[5–7] 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 paper.

Targeted radionuclide therapy is a promising strategy for cancer treatment that involves the use of a radiolabeled molecule to selectively deliver a cytotoxic level of radiation to a tumor. During the past few years, targeted radiotherapy has

made the transition to practical treatment, which is evidenced by the FDA approval and clinical use of two targeted radiotherapeutics for the treatment of lymphoma—Zevalin® and Bexxar®.[8] Targeting alpha-emitting radionuclides (AERs) such as ^{211}At to cancer cells has emerged as a particularly promising approach to cancer radiotherapy.[9,10] Mean range of alpha-particles in tissues is only a few cell diameters (about 50–70 μm for ^{211}At α-particles). The most-vulnerable site to radiation damage is the cell nucleus. Thus, AERs are the most-potent form of targeted radiation for cancer therapy, particularly when localized in close proximity to the highly radiosensitive cell nucleus. Moreover, when intranuclear delivery of AERs is achieved, it should be possible to also exploit the cytotoxic action of alpha-particle recoil nuclei, created during alpha decay, which possess a mean range in tissue considerably shorter than that of α-particles (less than 100 nm); furthermore, the linear energy transfer of the recoil nuclei is significantly higher.

Subcellular distribution of PSs

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. It is worth mentioning that preferential PS accumulation in tumors is not itself a guarantee of selective photoinduced tumor damage and successful PDT. In experiments with rat gliosarcoma 9L, it was found[11] 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. It is therefore clear that not only the distribution of PS in various types of tissue, and 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. Some PSs distribute very broadly in various intracellular membranes. An example is pyropheophorbide-*a* methyl ester that was reported to be localized in endoplasmic reticulum, Golgi apparatus, lysosomes and mitochondria, in NCI-h446 cells.[12]

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 tetrapyrrole structure, aggregation state, etc. In an in vivo situation where a PS is introduced into the bloodstream, the picture is significantly more complicated. Many PSs interact with blood proteins and lipoproteins, whereupon their penetration into particular cells is determined not so much by the PS proper as by the PS-carrying blood component(s). Thus, PSs may be redistributed between the liposomes, emulsions or complexes administered to the organism and the blood components.[13–15] For this reason, the subcellular distribution of PS observed in vitro may not always reflect the in vivo pattern. Thus, whilst PS hydrophobicity correlated with in vitro cytotoxicity and penetration into L1210 murine leukemia cells in serum-free medium, it did not correlate with photodynamic efficiency in vivo.[16] 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.

Subcellular distribution of PSs in vitro

Extended surveys of subcellular distribution of PSs in vitro have been published recently[17,18] and one can draw the following main conclusions from these surveys: (1) the localization of a PS in the cell will be altered according to its mode of cellular uptake and subsequent redistribution, (2) PSs localize to different cytoplasmic compartments excluding the cell nucleus, and (3) irradiation of the cells incubated with PSs usually causes redistribution of the PSs.

In vivo subcellular distribution of PSs

PSs administered in vivo form complexes with the protein components of the blood, and only a minor portion thereof may exist in free state.[19,20] Binding (affinity) constants, K_a's, of different PSs to some blood proteins have been estimated, they vary between 10^6 M^{-1} and 10^8 M^{-1}.[21–24] Serum proteins can inhibit in vitro cellular uptake of PSs[20] which is possibly the result of very slow kinetics of PS release from protein complexes to membranes.[25] Photophysical properties (e.g. quantum yield of singlet oxygen or triplet state) of a PS non-covalently bound to proteins may differ from those of the free PS.[26] Experiments on incubation of various PSs with serum in vitro demonstrated that practically all PS is bound to blood proteins.[19,27–31]

In vivo data about binding of PSs to different blood plasma proteins are summarized in Table 1. 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.

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,[34] then localize in lysosomes or, to a lesser extent, traverse the cell by transcytosis.[35] Macrophages are another type of albumin-accepting cells that trap radical-damaged albumin particularly rapidly.[36] Albumin has also been shown to carry PSs to the stromal elements of tumor tissue.[30] It should be mentioned that PSs are not stably bound to the serum components and dynamic equilibrium between different PS complexes can probably exist.

Table 1. In vivo distribution of administered PSs among human serum proteins

PS	Percentage of injected PS bound to: Lipoproteins	Heavy proteins[a]	Free[b]	References
Hematoporphyrin	21	75	4	19
Hematoporphyrin derivative	23	60	17	19
Tetraphenylporphine tetrasulfonate	5	94	1	19
N-Aspartyl chlorin e_6	35	65	—	32
2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-*a*	68	27.5	4.5	33

[a]Nonlipoprotein proteins including serum albumin.
[b]Presumably aggregated forms.

Low-density lipoprotein (LDL)-bound PSs can enter the target cells via receptor-mediated endocytosis.[37] In the case of chlorin e_6 covalently bound to LDL, the PS turns up in enzymatically active lysosomes.[38] Subcellular fractionation of rat liver after intraperitoneal injection of hematoporphyrin revealed the latter's presence in the mitochondrial and the plasma membrane fractions.[39,40]

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.[40] 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 not permeate into the cells in vivo, remaining bound to the tumor stroma.[41,42] For this reason, in vitro data about subcellular PS distribution can rarely be used for prediction of its in vivo subcellular distribution, possibly because of different and indefinite vehicles involved in PS binding and transportation in vitro and in vivo.

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.[43]

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.

Targeted cell-specific subcellular delivery of PSs

The data summarized above clearly demonstrate that (1) PSs localize in vitro and in vivo in different cellular compartments excluding the cell nucleus, (2) illumination can cause redistribution of PSs within the cells, (3) in vivo subcellular localization of PSs does not often correlate with that revealed in vitro, (4) after systemic administration, PSs bind to blood serum proteins which, presumably, determine PS cellular uptake to a greater extent than physico-chemical properties of the PS itself, and (5) photophysical properties of PSs bound to serum proteins may differ from those of the free PSs.

These data indicate a necessity of creating carriers with preset properties which, among other things, would ensure recognition of the desired target cell and subsequent directed transport to the necessary subcellular compartment. One way to accomplish this task is to employ modular polypeptide transporters possessing (1) an internalizable ligand module providing for target cell recognition and subsequent receptor-mediated endocytosis of the transporter by the cell, (2) an endosomolytic module ensuring escape of the transporter from endosomes, (3) a module containing a nuclear localization sequence (NLS) and thus enabling interaction of the transporter with importins, the intracellular proteins ensuring active translocation into the nucleus, and (4) a carrier module for attachment of the PSs (Fig. 1). The necessity of the modules is dictated by the following reasons. First, a cell specificity together with internalization into the target cell could be concomitantly achieved if the transporter uses highly specific ligand–receptor binding with a subsequent receptor-mediated endocytosis. In this case, the internalized transporter will turn out within endocytotic vesicles, endosomes, into the cytoplasm. Second, a specific intranuclear delivery can be achieved if the transporter possesses an NLS. Third, because the above-mentioned importins localize to the cytosol, whereas the internalized transporter moves along its endocytotic pathway being kept within endosomes etc. and, thus, being separated from the importins, it is necessary to provide the transporter with an endosomolytic module enabling tranporter's escape from the endosomes. Finally, the modules as well as PSs should be integrated into one moiety; this goal is achieved by inclusion of the fourth module, a carrier module (Fig. 1).

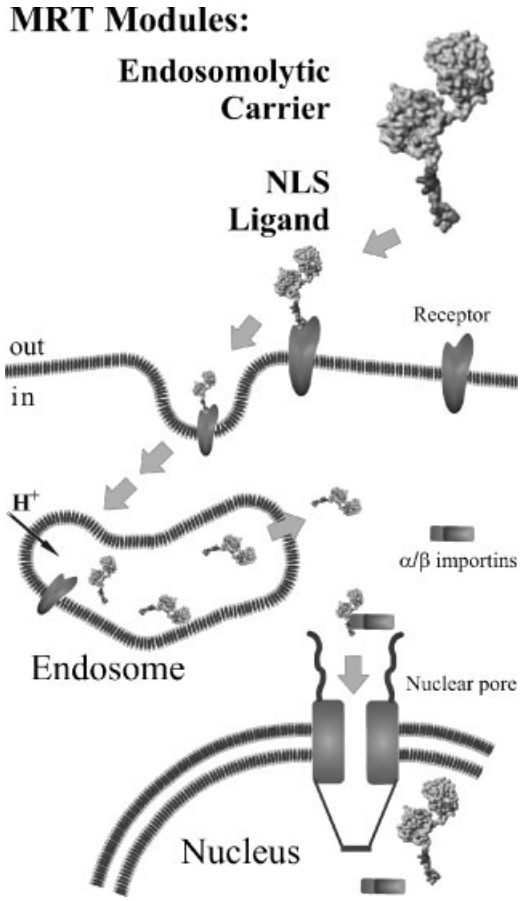

Figure 1. A schematic diagram depicting the MRT modules and stages of the MRT transport to the cell nucleus of the target cell. (Reproduced from Rosenkranz AA, Lunin VG, Gulak PV, Sergienko OV, Shumiantseva MA et al. 2003 FASEB J 17: 1121–1123, with kind permission of FASEB J.)

Our earlier experiments, with polypeptide conjugates consisting of the above-mentioned components/modules and produced by cross-linking of the modules, demonstrated feasibility of this approach. The PSs transported to the cell nucleus by the modular conjugates proved to be several orders of magnitude more efficient than non-modified, free PSs.[6,7,11,44,45] These data indicated that it is possible to design highly efficient molecular constructs that possess specific and distinct sequence modules conferring cell-specific targeting, internalization, intracellular vesicle escape 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. Notably, internalized PSs are more efficient in cell killing than those localized at the cell surface,[46–48] whereas PSs transported to the nuclei are more efficient than those internalized[6,7] and, as just mentioned, substantially more efficient than free, non-modified PSs. The nucleus is thus a hypersensitive site for photodynamic damage.

Modular recombinant transporters for cell-specific targeted subcellular delivery of PSs.

An important aspect is a technological feasibility of producing the transporting constructs since multicomponent transporters described above and produced in a laborious and expensive fashion through covalent linkage of different peptide modules via bifunctional cross-linking reagents are unlikely to find broad clinical application. It is thus expedient to develop recombinant vehicles that would include modules for addressed delivery both to specific target cells and into the most-vulnerable compartments thereof.

We designed, produced and characterized bacterially expressed modular recombinant transporters (MRTs) comprising (Fig. 2) (1) α-melanocyte-stimulating hormone (MSH) or epidermal growth factor (EGF) as the internalizable ligand modules to either melanocortin-1 overexpressed receptors on human and murine melanoma cells, or ErbB1 overexpressed receptors on human head and neck, bladder or breast cancer cells, respectively, (2) the optimized NLS from SV40 large tumor antigen, (3) the *Escherichia coli* hemoglobin-like protein HMP as a carrier module, and (4) a translocation domain of diphtheria toxin as an endosomolytic amphipathic module (DTox).[49–51] Recently, other MRTs possessing either somatostatin (against somatostatin receptor overexpressing neuroendocrine tumors etc.) or interleukin-3 (against interleukin receptor overexpressing acute myeloid leukemia) as ligand modules have been produced (Fig. 2).

The MRTs were obtained with 90–98% purities. The purified chimeric MRTs were tested to assess whether their individual modules retained their functional activities and were able to contribute to the overall goal of cell-specific nuclear PS delivery.

Binding of EGF-containing MRTs by ErbB1 receptors was assessed[51] using A431 human epidermoid carcinoma cells overexpressing ErbB1 receptors,[52,53] ligand–receptor interaction of MSH-containing MRTs[49] was evaluated using B16-F1 murine melanoma cells overexpressing receptors to MSH. Dissociation constants for HMP–NLS–DTox–EGF and DTox–HMP–NLS–EGF, obtained from displacement curves were close to that for free EGF. The concentrations producing a half-maximal receptor-mediated melanogenesis (EC_{50}) were similar for the two MSH-containing MRTs, HMP–NLS–MSH and DTox–HMP–NLS–MSH, but higher than for native MSH. Recombinant peptides designed similarly but not containing the MSH module did not induce melanogenesis in B16-F1 cells.[49]

MRTs delivered to cells by receptor-mediated endocytosis are internalized into endosomes (enclosed membranous structures with weakly acidic internal pH), which they must exit to be targeted subsequently to their final intracellular

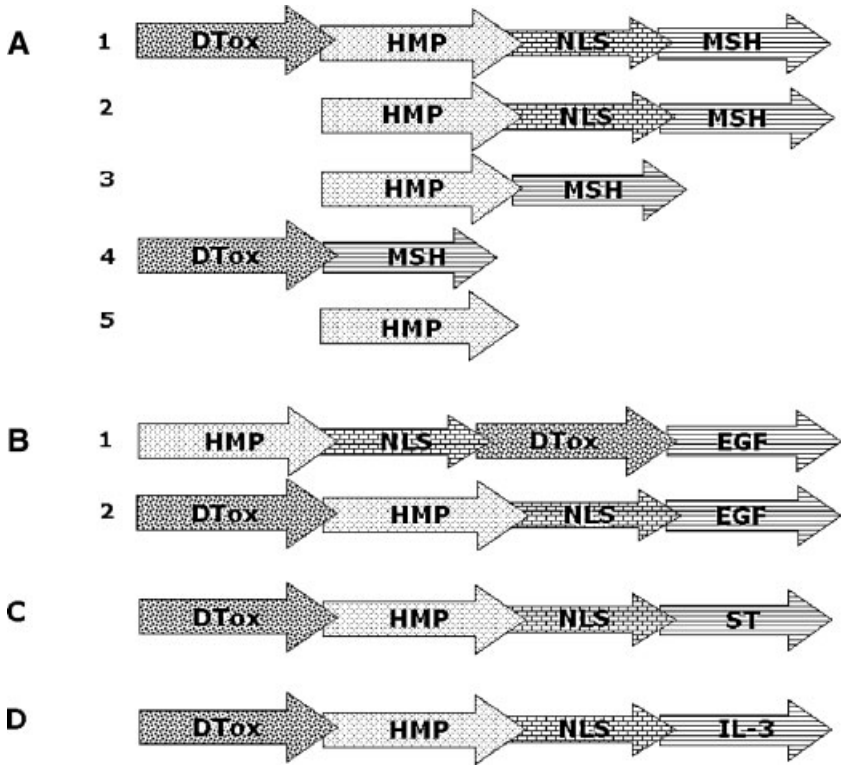

Figure 2. Schemes of MRTs. **A:** MRTs with MSH as a ligand module[49] 1, a complete MRT, 2–5, different truncated MRTs served as controls. **B:** MRTs with EGF as a ligand module[51] possessing identical modules but placed in different positions (1 and 2). **C:** MRT with somatostatin (ST) as a ligand module (Lunin V.G., Sergienko O.V., Sobolev A.S., unpublished); **D:** MRT with interleukin-3 (IL-3) as a ligand module (Lunin V.G., Soboleva T.A., Sergienko O.V., Rosenkranz A.A., Young I.G., Sobolev A.S., unpublished).

destination, in this case, the nucleus through the action of importins in the cytosol.

The propensity of a polypeptide to make pores in membranes in an acidic medium can be assessed from its ability to effect leakage of dye-loaded liposomes at different pHs.[54] Liposome leakage under the action of the MRTs was observed in two pH intervals: 3 to 4, which was attributable to the HMP because it alone showed a maximal activity at pH 3.5 to 4.5,[49] and 5.5 to 6.5, which is close to the endosomal pH,[55] and was attributable to activity of the DTox moiety.[49] EGF-containing MRTs showed similar properties.[51]

Membrane defects produced by DTox–HMP–NLS–EGF were assessed with the use of atomic force microscopy on supported egg lecithin bilayers. At pH 5.5, the MRT caused formation of two types of defects in previously intact parts of the bilayer: (a) fluctuating holes with typical diameters ranging from 10 to 150 nm and (b) structured small depressions or holes with mean diameter of ca. 40 nm surrounded by circular ramparts. The MRT did not cause the above-described defects at pH 7.5.[51]

Results for probing the pH of the intracellular environments of the MRTs in living cells by image-ratio video-intensified microscopy were consistent with the above results. Fig. 3 C,D, displays the pH-microenvironment of the PS-containing MRTs probed by the pH-sensitive dye 2′,7′-dichlorofluorescein produced intracellularily from 2′,7′-dichlorodihydrofluorescein by reactive oxygen species generated by the PS-MRTs upon illumination. Thus, a pH-specific fluorescence (shown in conventional colors in Fig. 3) develops at the subcellular sites where PS-MRT and the dye colocalize. The HMP-NLS-MSH conjugate lacking an endosomolytic module was found in acidic regions of the cells (reddish spots, presumably late endosomes, in Fig. 3C; see also Fig. 3E for match of conventional colors and pH values). No such acidic regions were revealed in the vicinity of DTox-HMP-NLS-MSH conjugate localization (Fig. 3D), meaning that the MRT and the dye do not colocalize within the acidic ($<$5.4) sites in the optical section.

Assessment[51] of the recognition of the MRTs by the nuclear transport-mediating $\alpha\beta$-importin heterodimer using a surface-plasmon resonance assay indicated that the NLS in the context of the MRTs is able to interact with the importins: their affinity constants turned out to be very close to that for the same NLS as a free oligopeptide,[56] and can be attributed to proteins with functional NLSs.[57]

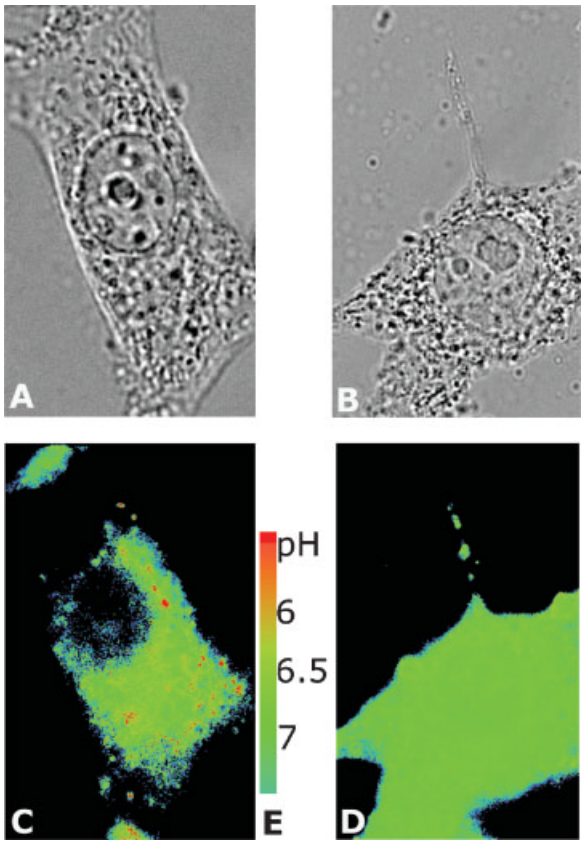

Figure 3. The pH of MRT intracellular microenvironment as an indicator of subcellular localization of the MRT. B16 melanoma cells were incubated either with **A,C:** a truncated MRT lacking the DTox module, (chlorin e_6)–HMP–NLS–MSH or **B,D:** a complete MRT, (chlorin e_6)–DTox–HMP–NLS–MSH (*B,D*). **A,B:** Brightfield images of the cells. The pH of the MRT microenvironment within the cells (**C,D,** respectively) is shown in conventional colors (refer to insert **E**) and was determined by image-ratio video-intensified microscopy. (After Rosenkranz AA, Lunin VG, Gulak PV, Sergienko OV, Shumiantseva MA et al. 2003 FASEB J 17:1121–1123, with kind permission of FASEB J.)

As a result, full-size MRTs were detected[49,51] in either A431 human epidermoid carcinoma cells (EGF-containing MRTs) or in murine melanoma B16-F1 cells (MSH-containing MRTs) and demonstrated a predominant nuclear localization.

Spin trapping either of singlet oxygen or hydroxyl radicals could not reveal any significant variations in spin adduct production kinetics between a PS covalently attached to MRT and free PS.[51]

Evaluation of the photocytotoxic effect on human A431 epidermoid carcinoma cells, which overexpress ErbB1 receptors, showed that the efficacy of PSs is greatly enhanced by their covalent attachment to MRTs in the case of both used PSs chlorin e_6 (Fig. 4A) and bacteriochlorin *p* (Fig. 4B). The most-efficient (chlorin e_6)–DTox–HMP–NLS–EGF conjugate ($EC_{50} = 0.53$ nM) displayed 3,360 times higher photocytotoxicity than free chlorin e_6 ($EC_{50} = 1{,}780$ nM). Moreover, the MRTs impart cell specificity to PSs: free chlorin e_6 is almost equally photocytotoxic for the cells overexpressing ErbB1 receptors (A431) and expressing a few[58] ErbB1 receptors (NIH 3T3 cells; Fig. 4D), whereas the same PS attached to the MRT was not photocytotoxic for non-target NIH 3T3 cells at the concentrations that were photocytotoxic for target A431 cells (Fig. 4C).[51]

Qualitatively similar results[49] were obtained during evaluation of the photocytotoxic effect of PSs carrying by MSH-containing MRTs on mouse B16-F1 melanoma cells, which overexpress MSH receptors, a property of many melanomas.[59–62] A half-maximal effect of (bacteriochlorin *p*)–DTox–HMP–NLS–MSH was attained at a concentration ($EC_{50} = 22$ nM), which is 230 times lower than that required for free bacteriochlorin *p* ($EC_{50} = 4{,}990$ nM). (Bacteriochlorin *p*)–DTox–HMP–NLS–MSH conjugate was not photocytotoxic to normal C3H/10T1/2 or NIH/3T3 mouse fibroblast lines which do not express melanocortin-1 receptors, demonstrating cell-specific activity of the MRT through the MSH module. (Bacteriochlorin *p*)– HMP–NLS–MSH conjugate, lacking the endosomolytic module, was 5.3 times less active than (bacteriochlorin *p*)–DTox–HMP–NLS–MSH, possessing this module; PS-MRT conjugates lacking NLS module showed less photocytotoxic activity than the above two conjugates. Free MRTs, not carrying PSs, did not affect viability of B16-F1 melanoma cells.[49]

These experiments showed that the modules of the chimeric MRTs retain their functional activities. Interestingly, DTox included in different parts of the MRTs caused similar defects in lipid membranes,[51] which suggests a possibility to use the DTox as an endosomolytic module in different polypeptide contexts, which agrees with findings made by Nizard et al.[63]

The difference in efficacy of MSH- and EGF-containing MRTs may result from different number of corresponding overexpressed receptors in each study (ca. 10^4 and $>10^6$ receptors per B16-F1 melanoma and A431 carcinoma cell, respectively).

These results are indicative of the prospects of using recombinant chimeric multicomponent vehicles for these and, possibly, for other locally acting anti-tumor drugs such as AERs where the dose of radioactivity necessary to kill 63% of cells (D_0), of 211-astatine, delivered to human hepatoma cell nuclei by our modular transporters, is one order of magnitude less than that of free $^{211}At^-$.[64]

Recently,[65] the DTox–HMP–NLS–EGF MRT described above was labeled[66] with AER ^{211}At. Binding, internalization and clonogenic assays were performed with A431, D247 MG and U87 MG human cancer cell lines overexpressing ErbB1

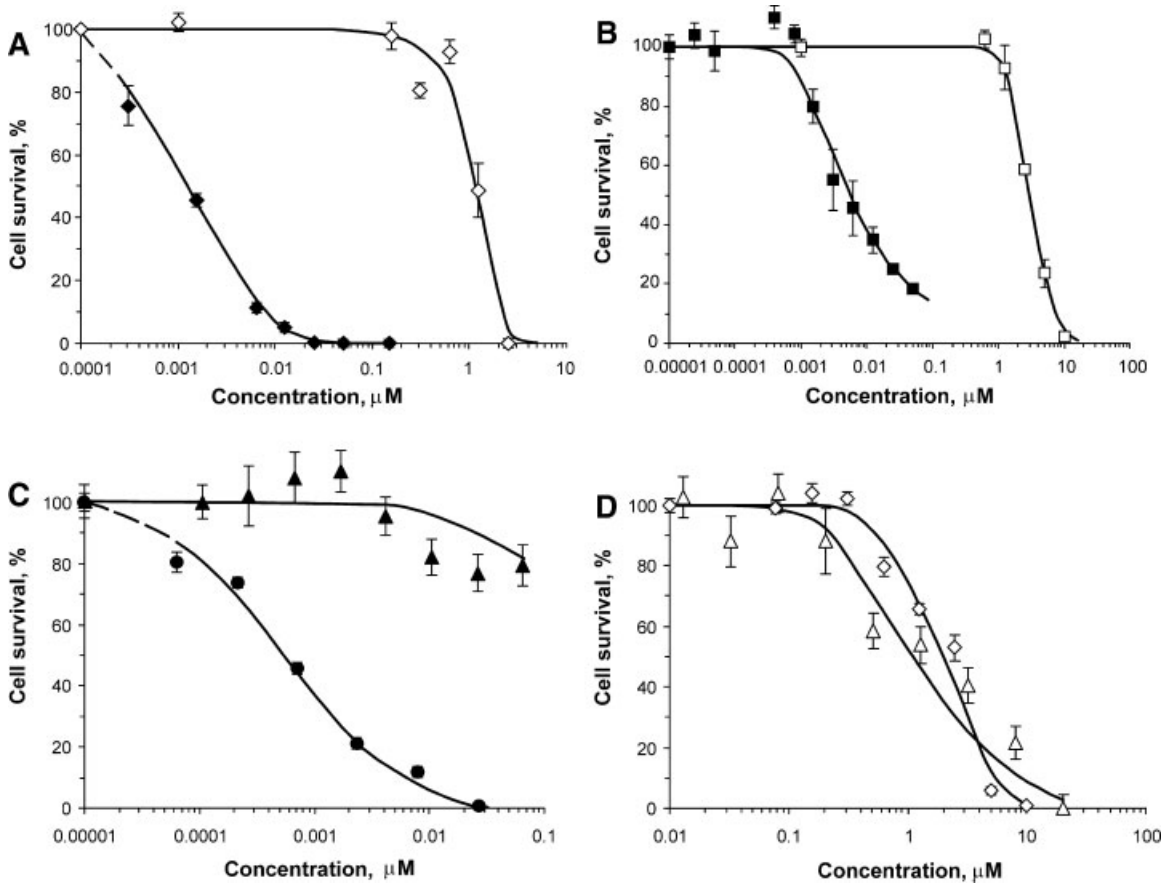

Figure 4. Photocytotoxicity of PS-MRT conjugates compared with free PSs.[51] **A:** (chlorin e_6)–HMP–NLS–DTox–EGF conjugate (◆) and free chlorin e_6 (◇). **B:** (bacteriochlorin *p*) –HMP–NLS–DTox–EGF conjugate (■) and free bacteriochlorin *p* (□). **C:** Photocytotoxicity of (chlorin e_6)–DTox–HMP–NLS–EGF conjugate estimated on target A431 cells (●) and non-target NIH 3T3 cells (π). **D:** photocytotoxicity of free chlorin e_6 estimated on target A431 cells (◇) and non-target NIH 3T3 cells (△).

receptors. The affinity of MRT to A431 cells did not change after radiolabeling. [^{211}At]astato–MRT was significantly more cytotoxic than [^{211}At]astatide control for all three cell lines. With D247 MG glioma cells and a 4 hour exposure, the D_0 (reduction in survival to 37% of untreated controls) for SGMAB–MRT and astatide were 0.07 and 1.3 μCi/ml, respectively, i.e. ^{211}At became 18.6 times more effective when transported into the nuclei of target cells. The number of decays required to achieve a D_0 level of cell killing for [^{211}At]astato–MRT with this glioma cell line was more than 13 times lower than that needed with DNA incorporated 5-[^{211}At]astato–2′-deoxyuridine,[67] demonstrating that intranuclear targeting further enhances cytotoxicity. The high cytotoxicity of [^{211}At]astato–MRT for human glioma cells is encouraging and provides motivation for developing ^{211}At-labeled MRT as a targeted radiotherapeutic for the treatment of brain cancers with overexpressed ErbB1 receptors like anaplastic astrocytomas (up to 94% of cases) and glioblastoma multiforme (~90% of cases).[68,69]

It is well known that melanoma is considered as an inappropriate tumor for photodynamic therapy treatment,[70] owing to almost complete light absorption by melanin. Keeping in mind that our MSH-containing MRT gave ca. 230-fold enhancement of bacteriochlorin *p* efficacy[49] together with the fact that this photosensitizer possesses absorption peak at the wavelength (761 nm), where light penetration is better, we carried out in vivo experiments with this type of the MRTs. The MSH-containing MRT given to C57/black mice bearing B16-F1 s.c. melanoma tumors selectively accumulated within the tumor cells and their nuclei even 3 hours after i.v. injection as was revealed with immunofluorescence microscopy. Bacteriochlorin *p* did not influence tumor growth and mean life span of the mice even after three administration/illumination cycles, whereas this PS, used according to the same scheme and at the same doses but conjugated with the MRT, significantly ($P < 0.001$) increased mean life span of the mice (by 68 ± 4%) and inhibited tumor growth (9-day delay).[51]

Conclusions

Cell specificity and high efficacy of many anti-tumor drugs can be achieved with the use of modular transporters with preset properties, which would ensure recognition of the desired target cell and subsequent directed transport to the subcellular compartment of choice. The necessity of different modules is determined by the following considerations (Fig. 1).

First, cell-type specificity together with internalization into the target cell can be achieved if the engineered transporter possesses a ligand module, which has high-binding affinity to the receptor overexpressed on the target cancer cell but not on non-cancer cells. This highly specific ligand–receptor binding will ensure recognition of the target cell as well as a subsequent receptor-mediated endocytosis. The internalized transporter will then be delivered to endocytotic vesicles, or endosomes, localized in the cytoplasm. Second, because the internalized transporter moves along the endocytotic pathway, it is necessary to provide the transporter with an endosomolytic module enabling the transporter's escape from the endosome. Third, a specific subcellular delivery can be achieved if the transporter has a specific localization amino-acid sequence, e.g. a nuclear localization sequence to target the cell nucleus. Finally, the modules as well as the anti-tumor agent should be integrated into one moiety; this goal can be achieved by inclusion of the fourth module, a carrier module. For these reasons, modular transporters for nuclear drug delivery should include the following parts: (1) an internalizable ligand module providing for target cell recognition and subsequent receptor-mediated endocytosis, (2) an endosomolytic module ensuring escape of the transporter from endosomes, (3) a module containing a nuclear localization sequence (a sequence of amino acids that is recognized by importins needed for the active translocation into the nucleus), and (4) a carrier module for attachment of an anti-tumor agent.

Fundamental to the success of this strategy is insuring that the modules are functional within the transporter, i.e. they retain their activities within the chimeric molecule. Depending on the type of target cancer cells, the ligand module can be replaced; the module with subcellular localization signal can be replaced or omitted (e.g. omission of the nuclear localizing signal will leave the transporter in the cytoplasm of the target cell). The carrier module can be replaced by inclusion other carrier systems, e.g. micelles, in order to enlarge its loading capacity. Keeping in mind tumor cell heterogeneity, one may assume that using different MRTs with different ligand modules could enhance efficacy of drugs with short ranges of action.

Thus, the MRTs can also be considered as nanomedical drug vehicles, which recognize the cancer cells of choice and, once in those cells, are transported to the most sensitive compartment of the cell (e.g. nucleus). MRTs of the type described here, capable of cell-specific targeting to particular subcellular compartments to increase drug efficacy, represent new pharmaceuticals with general application.

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