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SEMINAR ARTICLE

Novel modular transporters delivering anticancer drugs and foreign DNA to the nuclei of target cancer cells

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Summary

A major challenge in the development of specific and effective cancer treatments is the paradoxical situation that exploiting a molecular target that is accessible (surface membrane or extracellular matrix) is critical for achieving tumor selectivity whereas delivery of the therapeutic inside the cell, to the cell nucleus is generally required for maximizing the therapeutic effect. Photosensitizers, alpha-particle emitting radionuclides and foreign genetic material could be considered as such therapeutics if they possessed cellular and subcellular specificity. The author describes a novel approach of using modular recombinant transporters to target the therapeutics to the nucleus of cancer cells, where their action is most pronounced or can only be expressed. Photosensitizer-transporter conjugates displayed up to 3000 times greater efficacy than free photosensitizers and acquired cell specificity in contrast to free photosensitizers. Alpha-emitting radionuclides, conjugated with the modular transporters, acquired similar properties. DNA complexed with analogous transporters efficiently transfected target cells. The different modules of the transporters are interchangeable, meaning that they can be tailored for particular applications.

Key words: alpha-particles, drug delivery, modular transporters, photosensitizers, radionuclides, transfection

Introduction

Definitions

Modular transporters can be defined [1] as engineered polypeptides consisting of several interchangeable parts, or modules designed for delivery of anticancer drugs to the target cancer cell and its specific subcellular compartment. Modular transporters can also be considered as nanomedical drug vehicles which recognize the cancer cells of choice, and once in those cells, they are transported to the most sensitive compartment of the cell (e.g. nucleus). In order to reach the desired compartment of the cancer cell, the modular transporters are first passively delivered to the surface of the cell in the blood stream. Once within the cell, depending upon the nature of the polypeptide modules, they are transported to a particular subcellular compartment utilizing the cell's intrinsic transport machinery. To minimize side effects, many anti-tumor agents need to be delivered not only to the target cancer cell but also into a specific subcellular compartment, usually into the most sensitive/vulnerable site of the cancer cell. Examples of such antitumor agents are: a) foreign DNA for cancer gene therapy; b) photosensitizers for photodynamic therapy; or c) radionuclides emitting alpha-particles for endoradiotherapy. All of the above should be delivered into the nuclei where they can perform their specific function, and all these 3 groups will be considered in this paper. On the other hand, d) toxins, most of which are active in the cytosol, require a different modular transport strategy to retain in the cytoplasm. This goal 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.

Necessity of different modules

It is determined by the following considerations. First, cell type specificity together with internalization

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into the target cell can be achieved if the engineered transporter possesses a ligand module, which has high binding affinity to the receptor over-expressed on the target cell but not on non-target 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 (NLS) to target the cell nucleus. Finally, the modules as well as the antitumor agent should be integrated into one moiety; this goal can be achieved by inclusion of the fourth module, a carrier module. Therefore, modular transporters for nuclear drug delivery should include the following parts: (i) an internalizable ligand module providing for target cell recognition and subsequent receptor-mediated endocytosis; (ii) an endosomolytic module ensuring escape of the transporter from endosomes; (iii) 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 (iv) a carrier module for attachment of an antitumor agent (Figure 1). In the case of nucleic acids, the carrier module should also function as a condensation component.

Anticancer agents (1): photosensitizers

Photodynamic therapy 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 non-optimal 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 is 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 [2-4]. Uneven intracellular distribution of PSs determines the difference in subcellular toxicity as it was shown by laser microbeam irradiation [5]. In contrast to cell membranes and other cytoplasmic organelles, the cell nucleus [6-8] is known to be a very sensitive

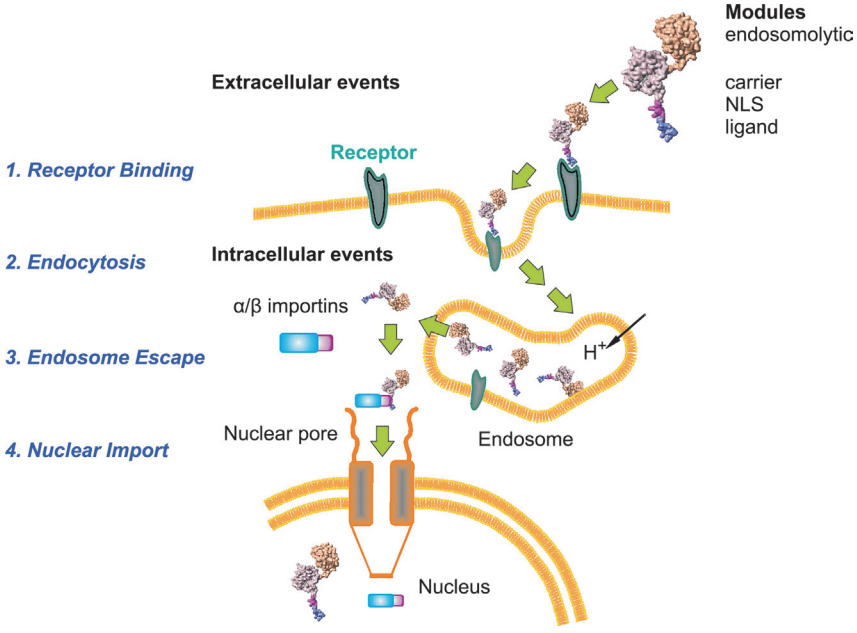

Figure 1. Steps in targeting of modular recombinant transporter (MRT) from surface to nucleus of cancer cell (after [29] with kind permission of Elsevier).

target for reactive oxygen species. Investigations carried out in different laboratories clearly demonstrate that (*i*) PSs localize *in vitro* and *in vivo* in different cellular compartments excluding the cell nucleus; (*ii*) illumination can cause redistribution of PSs within the cells; (*iii*) *in vivo* subcellular localization of PSs does not often correlate with that revealed *in vitro*; (*iv*) 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; (*v*) photophysical properties of PSs bound to serum proteins may differ from those of the free PSs [9]. These data lead to an obvious conclusion that PSs need to be transported to the most sensitive compartment of target cancer cell, presumably the nucleus, in such a way that would permit to avoid interaction with undesirable biomolecules and retain PS properties necessary for photodynamic therapy.

Anticancer agents (2): radionuclides emitting particles with low range

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. Targeting alpha-emitting radionuclides (AERs) such as ^{211}At to cancer cells has emerged as a particularly promising approach to cancer radiotherapy [10,11]. 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 intra-nuclear 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.

Anticancer agents (3): DNA

Though viruses as foreign gene vehicles have entered several clinical trials, with a significant degree of success, their therapeutic use for delivery is problematic because of the dissemination of modified but potentially replicable genomes that could integrate or recombine with cell DNA. Upon virus administration, serious side effects from inflammation, immune reactions, induced by preceding doses of viruses or previous infections, up to death have been observed [12]. In an attempt to meet biosafety requirements and to retain the viral properties that are important for gene therapy (Table 1), many different artificial constructs have been generated, among them a number of modular polypeptides have been produced to deliver foreign DNA into the nuclei of target cells. Thus, again, the case in point is a multifunctional vehicle possessing the modules listed above which permit to fulfill virion functions by mimicking its properties.

Analyzing these three examples of anticancer approaches, one can conclude that, despite significant differences among their chemical nature, molecular mechanisms of actions etc., they have a significant common feature: they need to be transported only to a specific or most vulnerable subcellular compartment of target cancer cells, the cell nucleus, where the effect of these agents can be revealed. So, their vehicles should have common/similar properties in order to achieve the same goal, the nuclei of specific cancer cells.

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

Our earlier investigations in 1989-1999 analyzed and summarized elsewhere [13], with polypeptide transporters produced by cross-linking of the modules mentioned above (ligand, endosomolytic, carrier, and NLS - containing modules) gave an experimental evidence that the cell nucleus is a hypersensitive site for photodynamic action of PSs and verified feasibility of the modular principle of PS transporters. PSs transported to the cell nucleus by the modular conjugates proved to be several orders of magnitude more efficient than non-modified, free PSs. 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. Notably, internalized PSs are more efficient in cell killing than those localized at the cell surface, whereas PSs transported to

Table 1. A list of viral properties that are relevant to gene therapy [12]

1. Nucleic acid condensation and stabilization
2. Receptor recognition, attachment and cell internalization
3. Endosomal escape
4. Nuclear transport
5. Uncoating
6. Integration (tumor viruses)
7. Replication

the nuclei are more efficient than those internalized and, as just mentioned, substantially more efficient than free, non-modified PSs. The nucleus is thus a hypersensitive site for photodynamic damage.

An important aspect is a technological feasibility of producing the transporting constructs. Recombinant technology is usually more flexible, reproducible and cost-effective in comparison to many alternative methodologies. It is thus expedient to develop recombinant vehicles that would include modules for addressed delivery both to specific target cells and into the nuclei thereof.

We designed, produced, and characterized bacterially expressed modular recombinant transporters (MRTs) comprising (Figure 2) 1) α-melanocyte-stimulating hormone (MSH) or epidermal growth factor (EGF) as the internalizable ligand modules to either melanocortin-1 over-expressed receptors on human and murine melanoma cells, or ErbB1 over-expressed receptors on human head & 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) [14-16]. Recently, other MRTs possessing either somatostatin (against somatostatin receptor over-expressing neuroendocrine tumors etc.) or interleukin-3 (against interleukin receptor over-expressing acute myeloid leukemia) as ligand modules have been produced (Figure 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 [16] using A431 human epidermoid carcinoma cells over-expressing ErbB1 receptors [17], and ligand-receptor interaction of MSH-containing MRTs [14] 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 [14].

MRTs delivered to cells by receptor-mediated endocytosis are internalized into endosomes (enclosed membranous structures with weakly acidic internal pH),

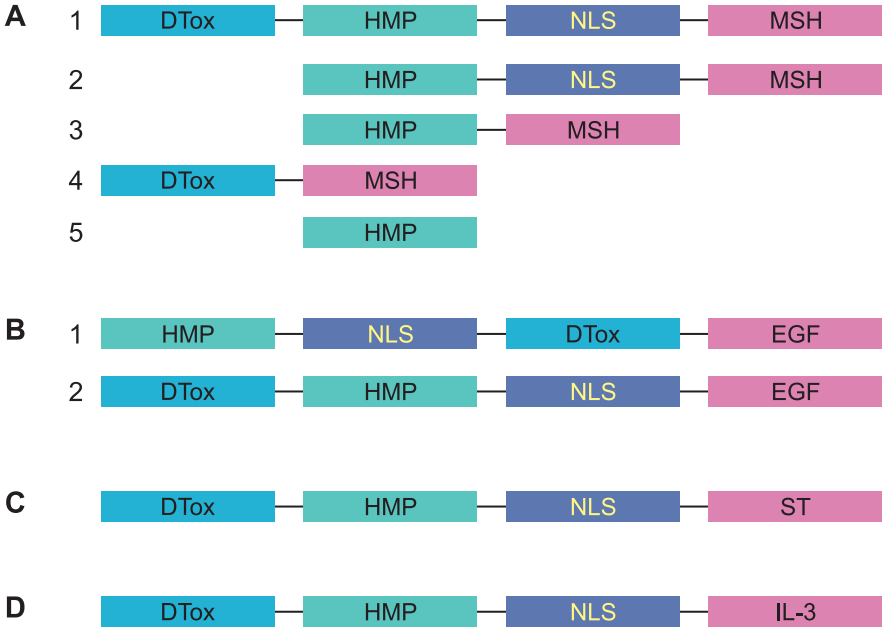

Figure 2. Schemes of modular recombinant transporters (MRTs). **A:** MRTs with α-melanocyte stimulating hormone (MSH) as a ligand module [14]: 1: a complete MRT; 2-5: different truncated MRTs served as controls. **B:** MRTs with EGF as a ligand module [16] possessing identical modules but placed in different positions (1 and 2). **C:** MRT with somatostatin (ST) as a ligand module (Lunin VG, Sergienko OV, Sobolev AS, unpublished). **D:** MRT with interleukin-3 (IL-3) as a ligand module (Lunin VG, Soboleva TA, Sergienko OV, Rosenkranz AA, Young IG, Sobolev AS, unpublished). HMP: E-coli hemoglobin-like protein, DTox: diphteria toxin.

which they must exit to be targeted subsequently to their final intracellular 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. 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 [14], and 5.5 to 6.5, which is close to the endosomal pH, and was attributable to the activity of the DTox moiety [14]. EGF-containing MRTs showed similar properties [16].

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 [16,18]. Interestingly, DTox included in different parts of the MRTs caused similar defects in lipid membranes [16], which suggests a possibility to use the DTox as an endosomolytic module in different polypeptide contexts, which agrees with the findings made by Nizard et al. [19].

Results for probing the pH of the intracellular microenvironments of the MRTs in living mouse melanoma cells by image-ratio video-intensified microscopy [14] were consistent with the above results. A truncated MRT, HMP-NLS-MSH lacking an endosomolytic module was found in acidic vesicles in the cells; no such acidic regions were revealed in the vicinity of a full-size MRT, DTox-HMP-NLS-MSH, meaning that the MRT escaped from endocytotic vesicles.

Assessment [16] of the recognition of the MRTs by the nuclear transport-mediating α/β-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 [20], and can be attributed to proteins with functional NLSs [21].

As a result, full-size MRTs were detected 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 [14,16].

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 [16].

Evaluation of the photocytotoxic effect on human A431 epidermoid carcinoma cells, which over-express 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 (Figure 3A, C, and D) and bacteriochlorin *p* (Figure 3B). 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 over-expressing ErbB1 receptors (A431) and expressing a few [22] ErbB1 receptors (NIH 3T3 cells; Figure 3D), 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 (Figure 3C) [16].

Qualitatively similar results [14] were obtained during evaluation of the photocytotoxic effect of PSs carrying by MSH-containing MRTs on mouse B16-F1 melanoma cells, which over-express MSH receptors, a property of many melanomas [23-26]. 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*. (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. The difference in efficacy of MSH- and EGF-containing MRTs may result from different number of corresponding over-expressed receptors in each study (ca. 10^4 and $>10^6$ receptors per B16-F1 melanoma and A431 carcinoma cell, respectively). (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. A comparison of efficacies of the full-size MRT and its truncated variants clearly shows that availability of all modules is a necessary condition for MRT to reveal its full potential [14].

It is well known that melanoma is considered as an inappropriate tumor for photodynamic therapy [27], 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 [14] 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

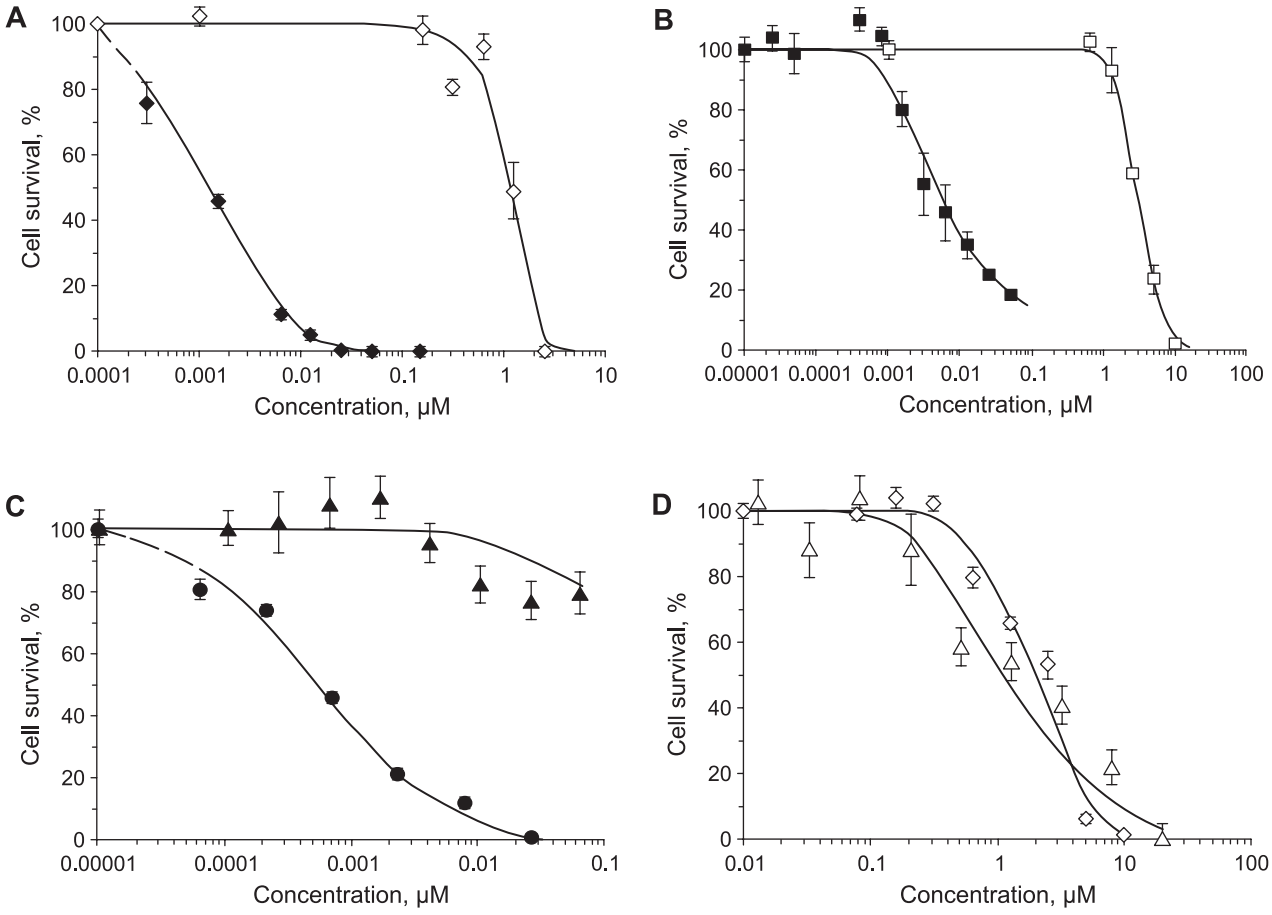

Figure 3. Photocytotoxicity of PS-MRT conjugates compared with free photosensitizers [16]. **A:** (chlorin e_6)-HMP-NLS-DTox-EGF conjugate (◆) and free chlorin e_6 (◇). **B:** (bacteriochlorin *p*)-HMP-NLS-DTox-EGF conjugate (■) and free bacteriochlorin *p* (□). **C:** photocytotoxicity of (chlorin e_6)-DTox-HMP-NLS-EGF conjugate estimated on target A431 cells (●) and non-target NIH 3T3 cells (▲). **D:** photocytotoxicity of free chlorin e_6 estimated on target A431 cells (◇) and non-target NIH 3T3 cells (△).

B16-F1 s.c. melanoma tumors selectively accumulated within the tumor cells and their nuclei even 3 h after i.v. injection as was revealed with immunofluorescence microscopy. Bacteriochlorin *p* did not influence tumor growth and mean lifespan of the mice even after 3 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 the mean lifespan of the mice (by 68 ± 4%) and inhibited tumor growth (9-day delay) [16].

Modular transporters for targeted subcellular delivery of alpha-particle emitting radionuclides

These results are indicative of the prospects of using recombinant chimeric multicomponent vehicles for these and, possibly, for other locally acting antitumor agents 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 $^{211}At^-$ [28].

Recently [29], the DTox-HMP-NLS-EGF MRT described above was labeled [30] with AER ^{211}At. Binding, internalization and clonogenic assays were performed with A431, D247 MG and U87 MG human cancer cell lines over-expressing ErbB1 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 3 cell lines (Figure 4). With D247 MG glioma cells and a 4 h exposure, the D_0 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 [31], demonstrating that intranuclear

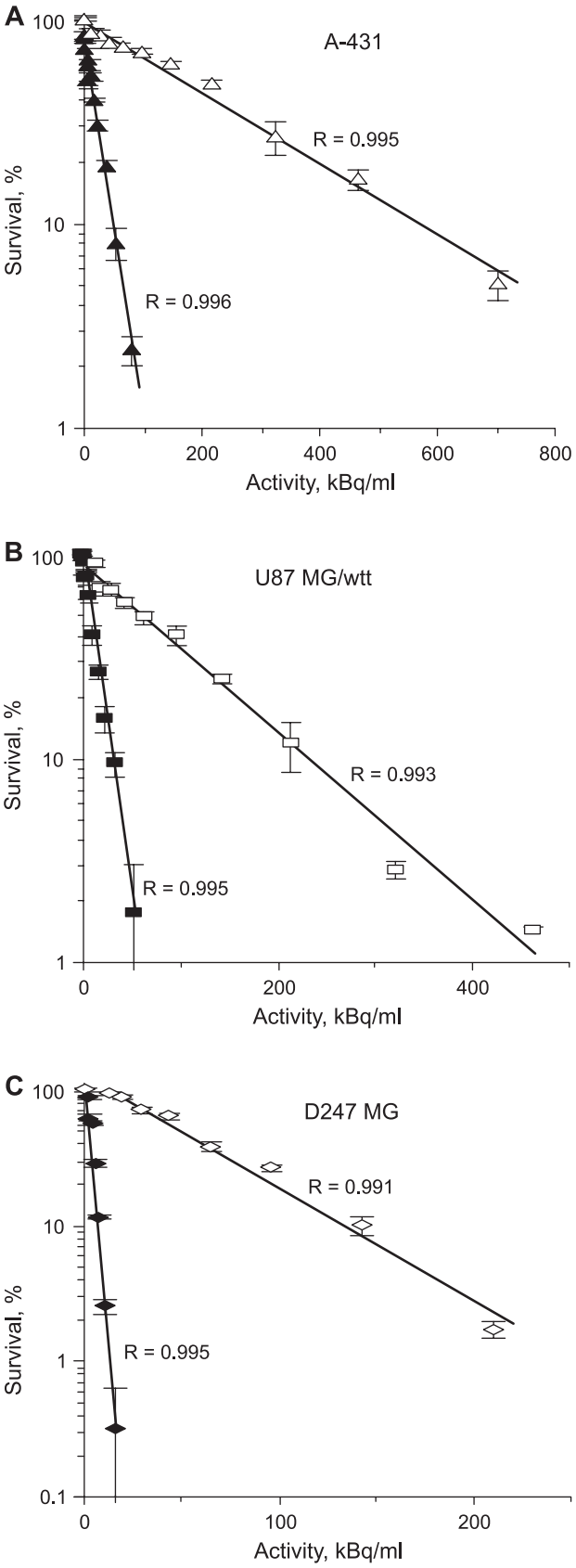

Figure 4. Clonogenic survival of **(A)** A431 human epidermoid carcinoma cells and **(B)** U87MG.wtEGFR and **(C)** D247 MG human glioma cells after exposure for 4 h to varying activity concentrations of [211At]SAGMB-MRT (closed symbols) and [211At]astatide (open symbols). From [29] with kind permission of Elsevier.

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 over-expressed ErbB1 receptors like anaplastic astrocytomas (up to 94% of cases) and glioblastoma multiforme (~90% of cases) [32,33].

Modular transporters for targeted subcellular delivery of foreign DNA

Concerning modules of DNA transporters (Table 2), special requirements are imposed on the carrier module: it should reversibly condensate the DNA to be transported in such a way as to permit internalization of the whole DNA-transporter complex and subsequent sufficient gene expression. Two types of carrier modules

Table 2. Peptides used for gene delivery [12,42,43]

Function	*Peptide*
DNA condensation	Polylysine
	Polylysine-containing peptides
	Protamine
	Histones
	Basic domain of HIV Tat protein
	Segment of the Antennapedia homeodomain
	Tetracycline repressor protein TetR
Endosomolytic/Fusogenic	Histidine-rich peptides
	Influenza HA-2
	Melittin
	TAT (48-60)
	Penetratin
	Transportan
	GALA
	KALA
Nuclear localization sequences	
Monopartite	SV40 T antigen
	SV40Vp3
	Adenovirus E1a
	Human c-*myc*
Bipartite	Nucleoplasmin
	Mouse FGF3
	PARP
Nonclassical	M9
Cellular targeting	RGD
	Integrin binding
	Secretin
	GE7 (from EGF)
	Neurotensin
	LOX-1binding

FGF3: fibroblast growth factor 3, PARP: poly (ADP-ribose) polymerase, RGD: Arg-Gly-Asp, EGF: epidermal growth factor, LOX-1: lectin-like oxidized LDL receptor

were used for this purpose: cationic peptides/proteins, and sequence-specific DNA-binding proteins (Table 2). DNA can also be linked to non-cationic, streptavidin-conjugated protein vehicles through biotinylated polylysine molecules [34,35] or similarly. Oligolysines of a defined length are preferable to polylysines because of precise control of synthesis and homogeneity of peptide length and lower toxicity. Thus, an optimal oligopeptide containing 18 lysine residues, tryptophan and an alkylated cysteine (AlkCys-Trp-Lys_{18}) gave small complexes with DNA with higher transfection efficiency than polylysine [36]. Other parts of Table 2 show qualitative similarity to the modules used for MRT transporting other types of drugs (see above).

An interesting peculiarity of usage of modular transporters for DNA delivery by several research groups is an attempt to use NLS-containing modules not only for their direct purpose but also as DNA carrier modules and *vice versa*, oligo- or polylysines as NLS-mimicking modules, thus reducing the number of modules within MRT (examples are in [12]). Nevertheless, it should be kept in mind that transporters containing polylysine and functional NLS demonstrated significantly higher transfection efficacy than similar transporters with mutated and, thus, non-functional NLS [37,38]. Similar results were shown with NLS and another polycation, polyethyleneimine [39]. One can set off another group of attempts to reduce the number of MRT modules from known publications devoted to MRT. These attempts tried to exploit an ability of several natural hormones (e.g., insulin, EGF etc.) not only to be internalized by cells but also to be transported, at least partially, into their nuclei, thus trying to obviate the need for NLS. Our experiments, as an example, showed that insulin, as a ligand module, can be effectively used in a modular transporter lacking special NLS-containing module [40] but inclusion of NLS into insulin-containing transporters significantly enhanced its intranuclear accumulation [7]. An interesting example of MRT was designed recently by Xavier et al. [41]. They constructed a polypeptide MRT without cell specificity, thus not requiring ligand modules. It contained synthetic NLS resembling SV40 large T antigen NLS, DNA binding/condensing module (MRRAHHRRRRASHRRMRGG) and penetrating polypeptide TAT (YGRKKRRQRRR) which accomplished membrane penetration function. DNA-MRT complexes transfected MCF-7, COS, CHO and HepG2 cells with high efficiency. Addition of lysosomotropic chloroquine significantly enhanced transfection efficiency: it became higher than obtained with lipofectins. These data indicate also that lysosomal degradation of DNA-MRT complexes is significant and needs prevention of this degradation by including endosomolytic modules in MRT. These data together with those presented under the heading of "Modular recombinant transporters for cell-specific targeted subcellular delivery of PSs" demonstrate the necessity of at least 4 modules (ligand, endosomolytic, NLS-containing, and carrier) for full MRT efficiency. Some other types of truncated MRT are analyzed elsewhere [42,43].

Following Aris and Villaverde [12], we differentiate two alternative strategies for the construction of full-size modular transporters for gene therapy. First, when modules are combined by linear fusion in single-chain modular vehicles. Second, when modules, as short functional peptides, are inserted into a carrier protein. Both approaches can be exemplified by constructs GD5 [44] and NLSCt [45], respectively. In GD5, the DNA is bound to the transporter by a segment of the yeast transcription factor GAL4 and is condensed by non-recombinant polylysine peptides, added separately (so, two carrier modules). An anti-Erb2 single-chain antibody fragment (scFv) functions as a ligand module for cell-specific binding and receptor-mediated internalization of the complexes, and the translocation domain of the diphtheria toxin plays a role of the endosomolytic module. In NLSCt, a N-terminal lysine tail supports DNA binding and condensation (one of two carrier modules), a foot-and-mouth disease virus RGD motif (the ligand module inserted between residues 249 and 250 of *E. coli* β-galactosidase, the second carrier module) supports integrin-targeted cell binding and internalization, and a carboxy terminal NLS from SV40 large T-antigen (NLS-containing module) supports nuclear targeting of the whole DNA–protein complexes. The second carrier, *E. coli* β-galactosidase, a tetrameric enzyme, provides technical functions that allow the single-step purification of the whole construct by affinity chromatography, as well as its detection and quantification by a simple enzymatic assay.

Conclusions

Cell specificity and high efficacy of many antitumor agents 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. 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 local-

izing 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. polycations, in order to carry nucleic acids or micelles in order to enlarge its loading capacity. Keeping in mind the tumor cell heterogeneity, one may assume that using different MRTs with different ligand modules could enhance the efficacy of drugs with short ranges of action. 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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