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Modular Nanotransporters for Targeted Intracellular Delivery of Drugs: Folate Receptors as Potential Targets

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Abstract

The review is devoted to a subcellular drug delivery system, modular nanotransporters (MNT) that can penetrate into target cells and deliver a therapeutic into their subcellular compartments, particularly into the nucleus. The therapeutics which need such type of delivery belong to two groups: (i) those that exert their effect only when delivered into a certain cell compartment (like DNA delivered into the nucleus); and (ii) those drugs that are capable of exerting their effect in different parts of the cells, however there can be found a cell compartment that is the most sensitive to their effect. A particular interest attract such cytotoxic agents as Auger electron emitters which are known to be ineffective outside the cell nucleus, whereas they possess high cytotoxicity in the vicinity of nuclear DNA through the induction of non-reparable double-strand DNA breaks. The review discusses main approaches permitting to choose internalizable receptors permitting both recognition of target cells and penetration into them. Special interest attract folate receptors which become accessible to blood circulating therapeutics after malignant transformation or on activated macrophages which makes them an attractive target for both several oncological and inflammatory diseases, like atherosclerosis. *In vitro* and *in vivo* experiments demonstrated that MNT is a promising platform for targeted delivery of different therapeutics into the nuclei of target cells.

Keywords

Modular nanotransporters; subcellular drug delivery; cell nucleus; receptor overexpression; EGFR; folate receptors; melanocortin receptors; cancer treatment; activated macrophages; atherosclerosis; Auger electron emitters; photosensitizers

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

INTRODUCTION

Development of targeted drug delivery approaches is crucial for the treatment of various diseases where abnormal cells can be distinguished from healthy ones by differences in molecular composition presented on their cell surface and/or on a subcellular level. Over the last few years, great attention is being focused on drug delivery, aiming not only to accomplish delivery into the target cell, but also into the subcellular target compartment where maximal therapeutic efficacy can be achieved [1–13]. A drug that lacks specificity to the required compartment itself either should possess high stability and membrane penetration ability or requires imparting of specific properties ensuring targeting to the necessary site. The latter becomes even more significant if the drug possesses high affinity for any other cellular compartments [6]. Both ways are employed in attempts to solve these problems. For instance, use of the first approach resulted in the development of substances with specific physical-chemical properties providing their penetration into acidifying endosomes. However, the same result can be achieved by the second approach by exploiting receptor-mediated endocytosis [5, 9]. This field seems to be particularly significant for the attainment of cell-specific drug delivery, which is of great practical significance because it can reduce toxicity. One of the most promising ways to accomplish precise targeting to appropriate intracellular sites is the engagement of natural intracellular transport pathways. In this case, it is easier to solve the problem of combining such generally incompatible properties such as extremely specific molecular recognition of target cells based on cell surface peculiarities and the maximal drug efficacy that requires penetration into the cell or even into a specific cell compartment [14].

Creating a drug delivery system that can penetrate into target cells and deliver the therapeutic into their appropriate intracellular compartments represents a significant challenge for the treatment of numerous diseases. The practical consequences of the implementation of such an approach could include the ability to treat diseases caused by dysregulation of a specific cell type, including malignancy [1, 2, 4, 8, 12, 15, 16], neurodegenerative diseases [7, 17, 18], cardiomyopathy [19, 20] and many other pathological conditions including disorders caused by subcellular miss-localization of proteins [21]. A promising drug delivery strategy that possesses the required properties for specific intracellular delivery within target cells is the modular nanotransporter (MNT), which has demonstrated manifold increases in efficacy for a variety of cytotoxic drugs evaluated both *in vitro* and *in vivo* [8, 12, 14, 16, 22–30]. As described below, we have created MNT targeted to several types of cancer cells. Our current efforts are focused on folate receptors whose overexpression is characteristic of many types of cancer as well as of activated macrophages specific for atherosclerosis and several other inflammatory diseases. Folate receptors on the above-mentioned cells are accessible to blood circulating pharmaceuticals. In contrast, the folate receptors expressed at lower levels on certain normal cells of epithelial origin are generally not accessible for blood circulating agents, which makes folate receptors particularly attractive for creating new MNT and possibly other drug delivery agents.

DRUGS FOR INTRACELLULAR TARGETING

Compounds requiring targeted intracellular delivery for meaningful therapeutic impact represent a medley of diverse functional molecules: nucleic acids (DNA, siRNA, miRNA, shRNA, antisense oligonucleotides) used for gene therapy; various polypeptides and natural proteins that interact with intracellular targets; low molecular weight substances (photosensitizers, inhibitors of intracellular processes, radionuclides with a short range of emitted particles) and others. For most of them, passive transport into cells is either ineffective or associated with high toxicity to non-target cells. All these pharmaceutical compounds fall into two groups [29]. The first one comprises substances that exert their effect only when delivered into a certain cell compartment (for example, DNA delivered into the nucleus). The second group includes anticancer drugs that are capable of exerting a cytotoxic effect in different parts of the cell; however, there is a cell compartment that is the most sensitive to their effects. As an example of such substances, one can mention photosensitizers (PSs) [13], which are used for photodynamic therapy of a number of diseases, especially oncological, and radionuclides - α -particles [31, 32] or Auger electron [33] emitters utilized for endoradiotherapy of cancer. PSs produce reactive oxygen species (ROS) damaging DNA, cell membranes and macromolecules under irradiation with light of a suitable wavelength. Depending on their nature, these drugs penetrate into different subcellular structures where they exert their photodynamic effect [13]. To increase selectivity for target cells (usually cancerous) PSs can be attached to various vehicles that specifically interact with these cells. ROS generated after irradiation of PSs can cover distances limited to several tens of nanometers. Therefore, the problem of creating the optimal PS distribution in the cell becomes critical, especially taking into account that the most sensitive target for PSs is the cell nucleus [13, 22–24], where uptake of free PSs has not been detected [13, 25]. Thus, there exists a real possibility to significantly increase the effectiveness of PSs by achieving their delivery to the nucleus of target cells, reducing doses required to reach a therapeutic threshold, and thus diminishing side effects [8, 26–28].

Radionuclides that decay by emission of short range particles including α -particles and Auger electrons are also of great interest as therapeutic agents. It is well known that the nucleus is the most sensitive cellular compartment to ionizing radiation. Upon delivery of radionuclides inside the nucleus, a fortiori, the probability of interaction of the emitted particle track with DNA increases, leading to an enhanced likelihood of fatal damage to the cell. Furthermore, the α -decay recoil nucleus has linear energy transfer (LET) about 10 times higher than the α -particles themselves. These recoil nuclei travel short distances (less than 100 nm), thus requiring intranuclear or at least close to the cell nucleus localization of the α -emitting radionuclide to exploit their high LET [34]. Auger electron emitters are known to be ineffective outside the cell nucleus [35], however, they possess high cytotoxicity in the vicinity of nuclear DNA through the induction of double-stranded DNA breaks, which are almost non-repairable [36]. Because the mean range of the Auger electrons lies generally within nanometer dimensions, their cytotoxic effect is limited to direct site of their decay. These characteristics make them potentially highly appealing as anti-cancer agents provided that strategies can be devised for their delivery into the nuclei of target cells with high specificity.

The use of MNTs is designed to be beneficial in the cases when the internalizable receptor is overexpressed specifically on the target cell population. Therefore, MNT-mediated delivery could be used in cancer treatment, as well as potentially applied in cardiology, for example, for the ablation of atherosclerotic plaques; in the treatment of viral diseases, for example, for the elimination of host cells (inhibition of virion synthesis); gynecology, for example, for endometriosis treatment, to name but a few potential medical applications.

PENETRATION INTO THE TARGET CELLS VIA RECEPTOR MEDIATED ENDOCYTOSIS

When cell specificity is not required, the role of drug vehicles for active transport is limited to maintenance of therapeutic concentration or provision of drug penetration through lipid membranes. The situation changes dramatically when it is necessary to affect only a specific type of cells in order to avoid undesirable side effects to normal tissue. The most suitable substances for the recognition of a particular cell population are molecules that can interact with cell surface receptors or antigens with high affinity. The vast majority of such compounds are macromolecules that cannot penetrate plasma membrane and only a fraction of them are capable of entering the cells efficiently enough for effective drug delivery. As for any macromolecule, its subsequent transport follows natural intracellular pathways. Therefore, the use of active transport for drug delivery into the desired cell type via binding to surface receptors triggering receptor-mediated endocytosis and subsequent intracellular transport to target compartment looks attractive [32–34].

Currently, several kinds of MNTs [8, 16, 29], including the most extensively studied versions with ligands for melanocortin receptor type 1 (MC1R) [12, 24, 26, 28] and epidermal growth factor receptor (EGFR) [22, 23, 25–27, 37, 38] have been created. However, numerous growth factor receptors are known whose expression is highly increased during tumorigenesis. In addition, there are other potential therapeutic disease targets characterized by enhanced receptor expression, such as cardiovascular diseases, neurodegenerative diseases, inflammation, HIV, and others. A list of potential ligands for cell surface internalizable receptors with modified properties that enable distinguishing target cell from non-target cell populations, includes mutated oncogenes and overexpressed proteins on the surface of malignant cells, as well as receptors suitable for guiding delivery into specific cell types (Table 1).

An important subset of oncogenic genetic alterations is caused by mutations, deletions and translocations in genes encoding surface receptors for growth factors and hormones. These alterations can lead to the appearance of new unique amino acid sequences in the receptor [39–47], which are attractive targets for anticancer therapy as well as those resultant from receptor gene amplification and product overexpression. However, creation of specific approaches for many different cases of therapeutics is still a matter of the future; production of the most applicable drugs should be tackled first. A suitable top-priority target for the optimization of complex drug vehicles should be focused on a frequently occurring abnormality, for example, folate receptor overexpression, which is observed in many types of disease.

FOLATE RECEPTORS AND THEIR EXPRESSION IDIOSYNCRASIES IN DIFFERENT DISEASES

In their efforts to develop therapeutic and diagnostic systems with improved lesion selectivity properties, researchers are attempting to identify the ideal lesion-specific surface antigens for targeting and drug delivery. A perfect candidate should possess a set of necessary and desirable characteristics, adjusted to the lesion type as well as other factors. In all cases, this “ideal” surface molecule should be presented in a form accessible to the blood circulation only on abnormal cells, e.g. in case of tumor – only on cancer cells [103]. One of the surface molecules meeting this requirement is the folate receptor (FR), which is frequently overexpressed on many cancer cells. Although being expressed to some extent on certain normal cells of epithelial origin, FR generally becomes accessible to blood circulating agents after malignant transformation [67].

Folate Receptor Structure

Folate receptors are glycoproteins responsible for high affinity folate binding and subsequent transport into cells via endocytosis. The FR family currently includes three (hFR- α , hFR- β and hFR- γ/γ') well-characterized human receptor types [104] that share 70–80% homology in amino acid sequence. hFR- α and hFR- β are attached to the cell membrane via a glycosylphosphatidylinositol anchor, while hFR- γ and hFR- γ' (a truncated form of hFR- γ) both lack the specific sequence for the attachment of this anchor, and represent secreted isoforms of hFR [105, 106]. Recently, distinct crystallographic models of hFR- α and hFR- β corresponding to their different binding states, as well as the crystal structure of hFR- α in complex with folic acid, were published [107, 108]. These data shed more light on the mechanisms and peculiarities of folate binding and trafficking, suggesting models for the receptor's pH-dependent conformational changes [108].

The expression profile of each FR subtype depends on differentiation stage and tissue type [67]. FR- γ/γ' expression is limited to very low levels on lymphoid cells (bone marrow, spleen, thymus) [105]. FR- β is expressed on a number of hematopoietic precursor cells and differentiated cells of myelomonocytic lineage; however, it is present in a functionally inactive form, unable to bind folate or its derivatives [109]. Functional FR- β can be found on placenta [66], activated macrophages, and can be found at various lesion sites (psoriasis, rheumatoid arthritis, atherosclerosis, etc. – See Table 1) and some types of leukemia [110].

Although there are a number of normal tissues and cell types (proximal tubules in kidney, choroid plexus, uterus, fallopian tube, epididymis, the acinar cells of breast, submandibular salivary and bronchial glands, type I and II pneumocytes in the lung, trophoblasts in placenta) with significant levels of FR- α [111, 112], its expression is generally limited to the apical membrane of polarized normal cells of epithelial origin (excepting retinal pigment epithelial cells [113, 114]), thus making the receptor inaccessible for blood circulating agents. Consequently, following intravenous administration the accumulation of low molecular weight folate-radionuclide conjugates in normal tissues is limited to kidney (due to glomerular filtration) and peripheral blood macrophages (including activated Kupfer cells) [67]. FR-targeted macromolecules (antibodies, proteins of appropriate size,

nanoparticles, etc.) that are not subjected to glomerular filtration are believed to overcome the limitation of excessive renal uptake. Indeed, it was demonstrated that the modification of a folate radioconjugate with an albumin binding moiety reduced its accumulation in kidney from ~70% ID/g to less than 30% ID/g [115].

Folate Receptor Overexpression in Oncologic Diseases

Contrary to its negligible exposure to the blood pool in normal tissues, FR- α can be overexpressed and readily accessible to blood-borne agents after malignant transformation. Its expression level has been reported to be highly elevated in a great variety of different malignancies, including but not restricted to cancers of the: uterus, cervix and ovary [116, 117], breast [118], brain [119], lung [120], head and neck [121], mesothelium [122], colon [123], skin (melanoma) [124], pancreas, kidney [125]. Moreover, a positive correlation has been demonstrated between the FR- α expression level and the stage [121, 126], metastatic potential [127], histological grade [126], resistance to chemotherapy [124] and poor outcome [118] of the disease. In summary, high FR- α expression levels are associated with the most aggressive and difficult to cure cancers, making this receptor a potentially valuable target for molecularly directed therapeutic approaches.

FR- α expression up-regulation can be tuned by treatment with the glucocorticoid receptor agonist dexamethasone or the estrogen antagonist tamoxifen in malignancies positive for the widely expressed glucocorticoid receptor or estrogen receptor- α , respectively [128, 129]. Using a murine tumor xenograft model with HeLa cell line authors demonstrated an increase of tumor and serum FR- α by dexamethasone treatment along with the lack of *de novo* FR expression in different FR-negative murine tissues [128]. These findings provide a potential strategy for enhancing the efficacy of FR-targeted therapy of malignancies that possess low/moderate or nonuniform expression patterns of FR- α in tumors. Moreover, another FR isoform – FR- β – is up-regulated in several oncological conditions, including acute myelogenous leukemia and chronic myelogenous leukemia [130, 131] and sarcomas [67]. Taken together, these features make FR-targeting a highly attractive approach for cancer and anti-inflammation therapy, imaging or combining both applications with theranostics.

FR-targeting can be easily accomplished through its natural ligand – folic acid, which possesses a number of attractive characteristics including high affinity binding ($K_d \sim 0.01\text{--}3$ nM depending on FR subtype) [108], ease of chemical modification in different organic or aqueous solvents, long shelf-life, low toxicity and immunogenicity, as well as ready availability at reasonable cost [67]. Because of these positive qualities, a great variety of drug and imaging agent delivery approaches have been developed that utilize folate as the FR-targeting ligand. With most of them, significantly increased accumulation was demonstrated in target cells *in vitro* and some showed promising results *in vivo* [67, 115]. However, critically relevant to therapeutic application, the rate of internalization is slow (up to $1\text{--}3 \times 10^5$ molecules/cell/h) likely irrespective of FR number on cellular surface [132] and only 15 to 25% of receptor-bound conjugates are released inside the cell [67]. Unfortunately, these characteristics can narrow the range of feasible approaches for using either smart delivery of highly cytotoxic agents precisely into the most vulnerable cellular compartment or delivery systems with extremely high capacity.

Folate Receptor Over-Expression on Activated Macrophages Associated with Atherosclerosis and other Inflammatory Diseases - Use for Diagnostics and Therapy

FR- β has been detected on both CD34+ bone marrow cells and normal human neutrophils but it is functionally inactive there and unable to bind folate or its derivatives. In contrast, a functional FR- β with nanomolar affinity for folate is expressed on activated macrophages [105] that accumulate in diseases such as rheumatoid arthritis, atherosclerosis, systemic lupus erythematosus, Crohn's disease, and osteoarthritis [133]. For example, it has been shown that in contrast to resting macrophages, FR- β is expressed on activated rat and human synovial macrophages [134, 135] that are present in large numbers in arthritic joints, where they play an active role in rheumatoid arthritis. Macrophages present within atherosclerotic lesions of apolipoprotein E knockout (apoE2/2) mice demonstrated selective uptake of radioactive EC20 [136], a folate-containing peptide consisting of pteronic acid, D-Glu, β -L-diaminopropionic acid, Asp, and Cys [137]. Compared with control arterial samples, expression of FR- β and CD68 is significantly increased in atherosclerotic plaques from human carotid arteries; moreover, FR- β expression significantly correlated with expression of the activated macrophage marker CD68 [138]. It has also been reported that macrophages are perhaps differentially activated between stable and vulnerable atherosclerotic plaques [138, 139]. In addition, activated macrophages participate in the pathogenesis of allergic asthma [140, 141]. Finally, Low and colleagues have observed that alternatively activated macrophages from the lungs of asthmatic mice express FR- β and this receptor is functionally active on these macrophages [133].

These features allow not only diagnosis but also treatment of these diseases without compromising the essential housekeeping functions of tissue-resident macrophages [142–144]. FR-targeted imaging agents have been synthesized and used as diagnostics for determining the location and severity of inflammatory diseases that are accompanied by FR- β overexpression. These include folate conjugated radiopharmaceuticals, fluorescent dyes, MRI contrast agents, and PET imaging agents [133, 137–139, 142, 145–149]. In general, both the contrast and sensitivity obtainable with these agents were good [142].

Low and colleagues [150, 151] described a so-called antibody-mediated cellular cytotoxicity approach that promotes destruction of antibody-marked macrophages in order to treat experimental rheumatoid arthritis. Following immunization against a potent hapten, the arthritic rats were injected with a folate conjugated to the same hapten on days 1, 4, 7, 10, 13, 16 and 19 following arthritis induction. Folate-mediated decoration of the macrophage surface with the attached hapten then promoted recognition of the macrophage by anti-hapten antibodies. The marked macrophages were then eliminated by natural killer cells, B-cells and macrophages. The treatment attenuated systemic and peri-articular inflammation, bone and cartilage degradation, and arthritis-related body weight loss. In another approach, different folate-targeted nanoparticles carrying indomethacin or methotrexate have been used for the treatment of arthritis in experimental animals (reviewed in [152, 153]). Interestingly, targeted elimination of alternatively activated tumor-associated macrophages improved survival of tumor-bearing mice (subcutaneous CT-26 colon carcinoma) [154]. The authors used a fusion peptide consisting of a peptide, M2pep (YEQDPWGVKWWY), which preferentially binds to murine alternatively activated macrophages, and a pro-apoptotic

peptide KLAKLAKKLAKLAK. Intravenous injection of the fusion peptide increased life span of the tumor-bearing mice and selectively reduced the population of alternatively activated tumor-associated macrophages.

Based on the data that FR is a marker of macrophage activation and that folate accumulates in the atherosclerotic lesions of apolipoprotein E (apoE)-deficient mice [136], Furusho *et al.* [155] decided to deplete FR- β -expressing macrophages via a dsFv anti-FR- β -PE38 immunotoxin. In this construct, PE38 is a truncated form of the *Pseudomonas* exotoxin A and the other part of the chimeric immunotoxin is a disulfide-stabilized anti-FR- β antibody. Treatment with the immunotoxin resulted in 31% and 22% reduction in atherosclerotic lesions in 21- and 41-week-old mice, respectively. A similar approach has been used to treat a murine model of bleomycin-induced skin fibrosis [156]; anti-FR- β -PE38 treatment led to a dramatic reduction in the number of FR- β -expressing macrophages, skin thickness and hydroxyproline content, thus alleviating the bleomycin-induced fibrosis.

If FR endocytosis is being considered as a means for intracellular drug delivery, it should be mentioned that neither FR occupancy nor folate conjugate valence has a significant effect on FR levels at the cell surface [157]. Furthermore, these authors concluded that FR endocytosis occurs at a constitutive rate, regardless of FR occupancy or cross-linking due to multivalent ligand binding. Thus, selection of an optimal frequency for folate conjugate dosing need not involve consideration of folate conjugate concentration or valence.

One needs to keep in mind that only $1-3 \times 10^5$ FR molecules per hour are delivered inside most cell types via FR-mediated endocytosis, irrespective of number of receptors on the cell's surface [132]. Therefore, weakly active chemotherapeutic agents probably will not be effective if delivered via FR-mediated endocytosis, so one needs to select a highly potent chemotherapeutic drug to assure measurable therapeutic efficacy [158] or use special “nanocontainers” in order to deliver effective amounts of weakly active therapeutics. It is a more complicated approach compared with use of typical cytotoxic drugs to transport via FR-mediated endocytosis agents that either can produce many cytotoxic molecules, such as bacterial toxins with enzymatic properties (e.g. *Pseudomonas* or diphtheria toxins which possess enzymatic domains catalyzing the synthesis of toxic products) and photosensitizers (which produce many reactive oxygen species upon illumination), or can act on unique molecules at very low concentrations like alpha-particles or Auger electrons (which cause irreparable DNA damage).

THE DEVELOPMENT OF TARGETED DRUG DELIVERY SYSTEMS BASED ON THE MODULAR PRINCIPLE: MODULAR NANOTRANSPORTERS

To accomplish the goal of precise drug delivery into the intended cellular compartment of target cells (particularly the nucleus), a “smart” vehicle, called the modular nanotransporter (MNT) has been devised. MNT are recombinant molecules composed of multiple functional domains to achieve receptor binding and internalization, endosomal escape and, finally, nuclear translocation, thereby offering the prospect of facilitating the selective delivery of short range, highly cytotoxic drugs from the cell surface to the nucleus. As part of this process, the MNT utilizes the natural intracellular machinery of protein sorting and

transport. Initial experiments were carried out to demonstrate “proof of principle” for the proposed modular transporter concept. Crosslinking of four protein/peptide components with bifunctional reagents [159–161] resulted in modular polypeptides in which each module retained its biological function. These MNT were able to deliver PS into target cells specifically, and undergo internalization, release from intracellular vesicles, and, finally, intranuclear targeting, providing greatly enhanced cell specificity and cytotoxic efficiency for the PS.

After proof of principle had been confirmed, the expensive and time-consuming chemical ligation process used to construct the MNT was replaced by the use of a bacterially produced recombinant modular transporter strategy. This resulted in the design, production and finally, characterization of bacterially expressed modular nanotransporters (MNTs) comprising (Fig. 1) (1) as the internalizable ligand, either α -melanocyte-stimulating hormone (MSH) for MC1R overexpressed on human and murine melanoma cells, or epidermal growth factor (EGF) for EGFR overexpressed on a wide variety of cancer cells, including those from human head and neck, lung, brain, bladder and breast tumors, (2) the optimized nuclear localization sequence (NLS) from the SV40 large tumor antigen, (3) the *Escherichia coli* hemoglobin-like protein HMP as a carrier module, and (4) the translocation domain of diphtheria toxin as an endosomolytic amphipathic module (DTox) [22, 24, 162]. Subsequently, a few MNTs possessing other ligand modules (somatostatin, interleukin-3) targeted to somatostatin receptors that are overexpressed on neuroendocrine tumors or against interleukin receptors that are overexpressed on acute myeloid leukemia cells were produced [8]. Optimization of MNT expression and purification procedures has now led to high yield of the recombinant transporters with 90–99% purity.

Functional activity of the ligand module in EGFR-targeted MNT, assessed by evaluating its binding to EGFR on A431 human epidermoid carcinoma cells [22] was mainly preserved, as the dissociation constant for EGF-containing MNT was somewhat lower but generally close to that measured for native EGF. Ligand functionality of the MSH containing MNT was tested on mouse melanoma B16-F1 cells overexpressing MC1R using the melanogenesis assay [24]. The concentration producing a half-maximal receptor-mediated melanogenesis (EC_{50}) for MSH-containing MNT was higher than that for native MSH. However, similar recombinant peptides lacking the MSH module did not induce any melanogenesis in B16-F1 cells [24]. Thus, both ligands generally retained their function within their respective chimeric MNT molecule.

When bound to an internalizable receptor, MNT has been confirmed to follow its planned intracellular dynamics – first being internalized by receptor-mediated endocytosis and routed into the endosomes, gradually turning into lysosomes, where the proteins are susceptible to degradation. However, the importins that mediate the nuclear import of NLS-containing polypeptides are located in cytoplasm, making it necessary for the MNT to escape from the endosomes to deliver its cytotoxic cargo to its intended nuclear target. To accomplish this requirement, an additional module, DTox – a non-toxic part (translocation domain) of the Diphtheria toxin that is responsible for the endosomal escape of the toxin [163], was considered an essential component for MNT design.

The functionality of the DTox module within the MNT was examined by determining the ability of the transporters to make pores in membranes at lysosomal pH, using three independent approaches: 1) leakage of calcein loaded liposomes, 2) formation of ion channels in planar bilayer lipid membranes, and 3) atomic force microscopy AFM [29]. MNTs caused liposome leakage in the slightly acidic pH interval 4.5 to 6.5, close to endosomal pH [22, 24]. Moreover, HMP modulated the membranolytic action of the DTox module synergistically, leading to the formation of membrane defects at significantly lower MNT concentrations [22, 24]. An electrophysiological study of membrane defects revealed the formation of large conductance states in the range of 2–5 nS at pH 5.5, corresponding to pores of several nanometers diameter, which were large enough to enable permeation of calcein and larger molecules [37]. Truncated MNT lacking the DTox module had a significantly reduced ability to form ion channels at pH 5.5, and no channels were registered with either MNT at pH 7.5.

In addition, MNT-mediated membrane defects were characterized by AFM on supported egg lecithin bilayers [22, 37]. At pH 5.5, the addition of MNT yielded two types of defects in previously intact parts of the bilayer, consisting of big fluctuating holes with typical diameters ranging from 10 to 150 nm and structured small depressions or holes with a mean diameter of about 40 nm surrounded by circular ramparts. According to the data obtained by biospecific AFM examination, these “heights” and “ramparts” are formed by MNT molecules incorporated into or absorbed by the bilayer [37]. Thus, it was demonstrated that at pH 5.5, corresponding to endosomal pH, the addition of DTox module containing MNT to the bilayers led to formation of pores that were bordered with MNT molecules. Importantly, the dimensions of these pores are sufficiently large to enable translocation of an MNT molecule. Finally, using image ratio video microscopy, it was demonstrated that full-sized MNT localized in the neutral microenvironment in live cells, while truncated MNT lacking the DTox module was only detected in vesicles with weakly-acidic and acidic content [24].

Finally, the functionality of the NLS module within the recombinant transporter was characterized by assessing the interaction of the complete MNT construct with the importin dimer, which is responsible for mediating nuclear import of molecules [22]. The affinity constant, characterizing MNT binding to the importin dimer was very close to the constant for free NLS [164], suggesting that the NLS within the MNT fully retains its functionality. On the cellular level, confocal microscopy visualization of the intracellular distribution of the MNT in target cells confirmed its intranuclear localization [22]. Taken together, the results of the experiments described in this and the preceding paragraphs demonstrate that all modules within the MNT retain their anticipated functions, contributing to the provision of maximal efficiency of MNT-mediated drug delivery the into nuclei of target cells *in vitro*.

Attempting to achieve specific targeting *in vivo* of a drug delivery system into its targeted compartment is far more difficult than accomplishing this *in vitro* because of the many additional barriers on the way, including a number of blood and immune system traps, which must be overcome, before the MNT it reaches the intended cells [165]. With regard to the *in vivo* behavior of MNT, ¹²⁵I-labeled MSH-guided MNT accumulated in B16-F1 mouse melanoma tumors with optimal tumor: muscle and tumor: skin ratios of 8:1 and 9.8:1, respectively, reached 3 h after its intravenous (i.v.) injection. These results confirmed the

ability of MNT to be selectively delivered to tumors bearing the receptor of interest *in vivo* [26, 28]. Immunohistochemical analyses of tumor and neighboring normal tissues isolated from Cloudman S91 melanoma bearing mice injected i.v. with MC1R-targeted MNT established its selective accumulation in cancer cells, particularly in their cell nuclei [26, 28]. Similarly, EGFR-targeted MNT was demonstrated to localize within the nuclei of cancer cells after i.v. injection into Balb/c ByJco-nu/nu mice bearing EGFR-expressing A431 human epidermoid carcinoma xenografts [26]. Another significant problem for protein therapeutics potentially arising at the *in vivo* level is their toxicity and immunogenicity. Preliminary studies suggest that MNTs possess negligible toxicity and a low capability for evoking delayed type hypersensitivity on mice [26]. Taken together, these experiments confirm that MNT molecules are capable of being transported from the blood pool to their intended subcellular target in receptor expressing tumor cells *in vivo*.

MNT for Targeted Delivery of PSs

Once the capability of MNTs to reach their intended intracellular target in receptor-positive cancer cells *in vitro* and *in vivo* had been confirmed, the potential utility of MNT-drug conjugates for targeted cancer therapy was evaluated. When used for photodynamic therapy, PSs are a class of drugs that would derive significant benefit from being precisely delivered into the nuclei of target cells. The cytotoxicity of PSs is critically dependent on their delivery into the cell nucleus, because it is the most vulnerable intracellular compartment to ROS, which are the active agent for PS photodynamic action but have a tissue range of only 20–40 nm [166] (See: Drugs for intracellular targeting). Because of the potential for making major gains in therapeutic effectiveness of the PSs by enhancing its nuclear delivery, the efficacy of PS-MNT conjugates was studied *in vitro* and *in vivo* [29].

The PS bacteriochlorin *p* was covalently attached to MNT via a 1,5-diaminopentane spacer and completely retained its ability to produce active oxygen forms, as shown with spin traps for hydroxyl radicals and singlet oxygen [22]. Compared with free PS (either chlorin *e*₆ or bacteriochlorin *p*), PS conjugated to MNTs exhibited greatly enhanced photocytotoxicity (more than factors of 3000 and 200 for EGF-containing MNT and MSH-containing MNT, respectively) on target cancer cells overexpressing EGFR (A431 epidermoid carcinoma cells) or MC1R (B16-F1 melanoma cells). In contrast to free PS, which exhibited almost the same photocytotoxicity for cells overexpressing either EGFR or MC1R, expressing low levels of EGFR (NIH 3T3 cells), or no MC1R (NIH 3T3 and C3H/10T1/2 cells), MNT-PS mediated photocytotoxicity was cell specific, being toxic for the appropriate receptor-positive target cells at much lower concentrations than required for killing non-target (NIH 3T3 or C3H/10T1/2) cells [22, 24].

With regard to *in vivo* therapeutic potential, photodynamic treatment with bacteriochlorin *p* conjugated to MC1R-targeted MNT increased survival of mice with B16-F1 and Cloudman S91 melanomas by two fold and inhibited tumor growth by 89 to 98%. Similarly, therapy utilizing chlorin *e*₆ conjugated to EGFR-targeted MNT resulted in 94% tumor growth inhibition compared to free chlorin *e*₆ and 75% survival at 3 months compared to 0% and 20% survival for untreated and free chlorin *e*₆ treated groups, respectively [26]. In summary,

the efficacy of PS, drugs requiring nuclear localization for maximum effectiveness, can be appreciably improved via MNT mediated delivery *in vitro* and *in vivo*.

MNT for Targeted Delivery of Radionuclides Emitting Short Range Radiation, Particularly, Auger Electrons

Due to the very short range of Auger electrons (generally just a few nm or less), these radionuclides require precise delivery in close proximity to DNA within the nucleus to achieve efficient killing of cancer cells with the benefit of minimal non-specific damage to normal cells in which nuclear translocation does not occur. Likewise, a consequence of α -particle decay is the simultaneous emission of α -particle recoil nuclei, which also traverse short distances (less than 100 nm) [34] from their decay site, thus requiring nuclear localization for maximal efficacy so that the cytotoxic potential of both the α -particle recoil nuclei and the α -particle can be harnessed.

During the last few years, the potential utility of MNT for enhancing the nuclear delivery and cytotoxicity of the α -particle emitter ^{211}At , as well as two different Auger electron emitters – the prototypical emitter ^{125}I , and ^{67}Ga , possessing a nearly ideal half-life for ultimate clinical application for cancer therapy – have been studied. The EGFR-targeted MNT was labeled either via the residualizing agent, *N*-succinimidyl 4-guanidinomethyl-3- ^{125}I iodobenzoate (^{125}I SGMIB), or its ^{211}At analogue prosthetic group, SAGMB, yielding labeled proteins that do not undergo appreciable dehalogenation *in vivo* [167]. The MNT also was labeled with ^{67}Ga via 2,2',2''-(1,4,7-triazonane-1,4,7-triyl)triacetic acid (NOTA), a macrocycle known for its high stability constant for Ga(III) complex formation [168]. The ^{125}I SGMIB-MNT conjugate bound to EGFR-expressing A431 and D247 MG cells with an affinity comparable to that of native EGF [27]. Both the ^{125}I - and the ^{67}Ga -labeled MNT internalized effectively into EGFR-expressing human cancer cells, with more than half (55–60%) of the internalized radioactivity revealed within the cell nuclei after a 1 h incubation [23, 27]. Retention of radioactivity in the cell nuclei reached a maximum during the first few hours of incubation, and then decreased over time to 25–50% of peak accumulation values [23, 27]. These intracellular routing data indicate that for radionuclide applications, MNTs are likely better suited for the delivery of Auger electron emitters possessing short half-life, where a higher percentage of total decays would occur during the first few hours, resulting in more efficient cell kill. Moreover, the use of shorter half-life radionuclides (half life less than about 1 week) is more compatible with the constraints of ultimate application to patient treatment.

Consistent with their rapid intracellular and intranuclear accumulation, ^{125}I SGMIB-MNT [^{211}At]SAGMB-MNT, and ^{67}Ga -NOTA-MNT demonstrated significantly enhanced cytotoxicity on various EGFR-expressing human cancer cell lines, compared to either similarly radiolabeled EGF or as a radiolabeled control, bovine serum albumin (BSA). Specifically, [^{211}At]SAGMB-MNT resulted in 10–20 times higher cytotoxicity than [^{211}At]astatide control on A431 human epidermoid carcinoma cell and two human glioma cell lines, with A_{37} values (Activity concentration required to reduce survival to 37%, a standard radiobiological parameter) between 3.8–19.7 kBq/mL (0.1–0.5 $\mu\text{Ci/mL}$) depending on the cell line [25]. Consistent with the much shorter range of Auger electrons compared

with α -particles, the cytotoxicity of [^{125}I]SGMIB-MNT was demonstrated to be considerably more specific – [^{125}I]SGMIB-MNT killed human A431 epidermoid carcinoma cells with more than 3700-times higher efficiency than control ^{125}I -labeled BSA control and also 5–18 times higher efficiency than [^{125}I]SGMIB-EGF, which shares the EGF domain with the MNT but lacks its endosome escape and importin binding capabilities [19]. Likewise, ^{67}Ga -NOTA-MNT exhibited 17- and 385-fold higher cytotoxicity than non-specific ^{67}Ga -NOTA-BSA and ^{67}Ga -EDTA on A431 cells along with 13- and 72-fold enhanced cytotoxic potency in comparison to ^{67}Ga -NOTA-EGF on A431 cells and the EGFR-expressing human glioma U87MGWTT cell line, respectively [23]. Taken together, these results suggest that MNT warrant further evaluation, particularly *in vivo* models of human cancer, as a promising platform for the targeted delivery of Auger electron emitters into the nuclei of cancer cells.

CONCLUSION AND FUTURE DIRECTIONS

Summarizing the data obtained, we note that the MNT impart cellular specificity and high efficiency to several therapeutics owing to the fact that they are created of modules with tailor-made properties that ensure the “recognition” of the necessary target cell and the subsequent transport to the subcellular compartment of choice, particularly into the cell nucleus which is very sensitive to many anti-cancer drugs. The modular principle of the MNT structure enables the replacement of modules or a change in their position within the MNT as the task changes: changing a target cell type, a target intracellular compartment, etc., depending on the purposes of personalized therapy. In our opinion, the MNT may be considered as an artificial transporting platform for different bioactive agents and not only for cancer treatment. Of particular interest are folate receptors which are overexpressed and readily accessible to blood-borne agents after malignant transformation and in several pathologies like atherosclerosis in contrast to their negligible exposure to the blood pool in normal tissues. We consider a combination of MNT concept with folate receptor targeting as a new possible approach for treatment diseases characterized by folate receptor overexpression.

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LIST OF ABBREVIATIONS

MNT(s)	Modular nanotransporter(s)
AFM	Atomic force microscopy
apoE	Apolipoprotein E
BSA	Bovine serum albumin
DTox	The translocation domain of diphtheria toxin as an endosomolytic amphipathic module

EGFR	Epidermal growth factor receptor
FR	Folate receptor
hFR	Human folate receptor
HMP	<i>Escherichia coli</i> hemoglobin-like protein
[¹²⁵I]SGMB	<i>N</i> -succinimidyl 4-guanidinomethyl-3-[¹²⁵ I]iodobenzoate
LET	Linear energy transfer
MC1R	Melanocortin receptor type 1
MSH	α -melanocyte-stimulating hormone
NLS	Nuclear localization sequence
NOTA	2,2',2''-(1,4,7-triazonane-1,4,7-triyl)triacetic acid
PS(s)	Photosensitizer(s)
ROS	Reactive oxygen species
SAGMB	<i>N</i> -succinimidyl 3-[²¹¹ At]astato-5-guanidinomethylbenzoate

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Fig. 1.

A schematics for the estimated use of a modular nanotransporter (MNT) for the delivery of drugs to the nucleus of target cells. After administration, MNT consisting of four modules binds *via* its ligand module to receptors expressed on the surface of target cells (thus accumulating at the target site) following subsequent internalization *via* receptor-mediated endocytosis, undergoes endosome escape through its endosomolytic module, binds *via* its nuclear localization signal (NLS) module to importins in the cytoplasm to be transported through the nuclear pore into the target cell nucleus.

Table 1

Examples of internalizable surface receptors with altered structure and expression in various pathologies.

Name	Protein	Natural ligands	Damage	Diseases	References
Anaplastic lymphoma kinase (Ki-1, ALK tyrosine kinase receptor, CD246)	Q9UM73	Pleiotrophin, midkine	Overexpression, gene translocation, mutations	Neuroblastoma, anaplastic large-cell lymphoma, non small cell lung cancer, etc.	[48–50]
Cytokine receptor-like factor 2 (CRLF2; thymic stromal-derived lymphopoietin receptor, TSLPR)	Q9HC73	Thymic stromal lymphopoietin	Overexpression, rearrangement, mutations	Acute lymphoblastic leukemia, asthma, nasal polyps	[51–54]
Epidermal growth factor receptor (EGFR, proto-oncogene c-ErbB, HER1)	P00533	Epidermal growth factor, transforming growth factor α , etc.	Overexpression, mutations	Head and neck, breast, bladder, ovarian, renal, colon cancers, glioblastoma, etc.	[55–57]
Erythroblastic leukemia viral oncogene homolog 2 (erbB2, HER2)	P04626	none	Overexpression, mutations	Breast, ovarian, gastric, non small cell lung cancers, etc.	[57, 58]
Fibroblast growth factor receptor 1 (FGFR1, Basic fibroblast growth factor receptor 1, BFGFR, Fms-like tyrosine kinase 2, FLT-2, CD331)	P11362	Fibroblast growth factors	Amplification, mutations, overexpression	Myeloproliferative disorders, non-Hodgkin lymphoma, squamous cell lung cancers, craniosynostosis, Antley-Bixler & Kallmann syndromes, etc.	[59–61]
Fibroblast growth factor receptor 2 (FGFR2, keratinocyte growth factor receptor, KGFR, CD332)	P21802	Fibroblast growth factors	Amplification, mutations	Gastric, breast, endometrial cancers, etc.; acne and other skin diseases, craniosynostosis	[59, 62, 63]
Fibroblast growth factor receptor 3 (FGFR3, CD333)	P22607	Fibroblast growth factors	Mutations	Bladder cancer, cervical cancer, multiple myeloma, skeletal dysplasias	[59, 64]
Fibroblast growth factor receptor 4 (FGFR4, CD334)	P22455	Fibroblast growth factors	-	Breast cancer, rhabdomyosarcoma	[59]
Fms-like tyrosine kinase (fms-like tyrosine kinase, FLT3)	P36888	FLT3 ligand	Tandem duplication	Acute lymphocytic leukemia, acute myeloid leukemia	[65]
Folate receptor alpha (FR- α)	P15328	Folate	Overexpression	Ovary, brain, kidney, breast, lung cancers, etc.	[66, 67]
Folate receptor beta (FR- β)	P14207	Folate	Overexpression	Leukemias, sarcomas, activated monocytes/macrophages at inflammations, including rheumatoid arthritis, atherosclerosis and psoriasis	[66, 67]
Hepatocyte growth factor receptor (met proto-oncogene, MET, scatter factor receptor)	P08581	Hepatocyte growth factor	Mutations, gene amplification, transcriptional up-regulation	Papillary renal, head-neck squamous cell tumor, glioblastoma stem cells, etc.	[68–70]
Insulin-like growth factor-I receptor (IGFIR, CD221)	P08069	Insulin, IGF1, IGF2	Overexpression	Epithelial cancers, sarcomas, multiple myeloma, etc.	[71]
Insulin receptor (CD220)	P06213	Insulin, IGF1, IGF2	Overexpression	Breast, thyroid, colon, ovary, prostate cancers, sarcomas	[72]

Name	Protein	Natural ligands	Damage	Diseases	References
Interleukin 3 receptor alpha (IL-3Ralpha, CD123)	P26951	Interleukin 3	Overexpression	Acute myeloid leukemias	[73]
Interleukin 21 receptor (IL21R, CD360)	Q9HBE5	Interleukin 21	Translocation	Lymphomas	[74]
Immunoglobulin superfamily receptor translocation associated (IRTA1, Fc receptor-like protein 4, FCRL4, CD307d)	Q96PJ5		Translocation	Lymphomas	[75]
Mast/stem cell growth factor receptor Kit (SCFR, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, c-KIT, CD117)	P10721	Stem cell factor	Overexpression, mutations	Gastrointestinal stromal tumour, acute myeloid leukemia, melanoma, epitheliomas, mastocytosis	[76–79]
Melanocyte-stimulating hormone receptor (MSH-R, Melanocortin receptor 1, MC1-R)	Q01726	Melanocyte-stimulating hormones	Overexpression	Melanoma	[80, 81]
Nerve growth factor receptor, (Neurotrophic tyrosine kinase, receptor, type 1, NTRK1, tyrosine kinase receptor A, TRKA)	P04629	Nerve growth factors	Translocation	Thyroid, breast cancers, etc.	[82, 83]
NT-3 growth factor receptor (neurotrophic tyrosine kinase, receptor, type 3, NTRK3)	Q16288	Nerve growth factors	Translocation	Congenital fibrosarcoma, secretory breast cancer	[84, 85]
Platelet-derived growth factor, alpha receptor (PDGFR-alpha, Platelet-derived growth factor receptor 2, CD140a)	P16234	Platelet-derived growth factors	Translocation, mutations, amplification	Gastrointestinal stromal tumors, idiopathic hypereosinophilic syndrome, pediatric glioblastoma multiforme, fibrosis and atherosclerosis, etc.	[61, 79, 86–88]
Platelet-derived growth factor receptor beta (PDGFR-beta, platelet-derived growth factor receptor 1, CD140b)	P09619	Platelet-derived growth factors	Translocation, mutations, amplification	Myeloid leukemias, dermatofibrosarcoma protuberans, atherosclerosis, etc.	[61, 86, 87, 89]
Proto-oncogene tyrosine-protein kinase receptor Ret (proto-oncogene c-Ret, cadherin family member 12)	P07949	Glial cell derived neurotrophic factors	Mutations, translocation,	Thyroid cancers pheochromocytoma, non-small cell lung cancer, etc.	[90, 91]
Somatostatin receptor 2 (SSR2, SSSTR2)	P30874	Somatostatin	Overexpression	Neuroendocrine tumors	[92]
Thrombopoietin receptor (TPO-R, myeloproliferative leukemia protein, c-Mpl, CD110)	P40238	Thrombopoietin	Overexpression	Myeloproliferative neoplasms	[93]
Thyroid stimulating hormone receptor (TSHR)	P16473	Thyroid stimulating hormone	Mutations	Thyroid adenoma, Graves' disease, etc.	[94, 95]
Transferrin receptor (p90, CD71)	P02786	Transferrin	Overexpression, translocation	Esophageal squamous cell carcinoma, non-Hodkin lymphoma, etc.	[96–98]

Name	Protein	Natural ligands	Damage	Diseases	References
Tyrosine-protein kinase Tec (Angiopoietin-1 receptor; tunica interna endothelial cell kinase, TIE 2, CD202b)	Q02763	Angiopoietins	Overexpression	Myeloid leukemias, gastric cancer, glioma	[99, 100]
Vascular endothelial growth factor receptor 3 (Fms-like tyrosine kinase 4, vascular permeability factor receptor, FLT4, VEGFR3, VPF)	P35916	VEGF-C, VEGF-D	Mutations	Angiosarcoma, Milroy disease	[101, 102]