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Mini review

Recent advances in cell-penetrating, non-peptide molecular carriers

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Abstract

The intracellular delivery of proteins and other bioactive molecules by employing membrane-permeable carrier peptide vectors, e.g. HIV-1 Tat, Antp-HD, and related arginine-rich peptides are well known for a number of years. Because of some real and potential problems associated with these peptide carriers, such as instability due to various endogenous peptidases, uncertain *in vivo* delivery efficiency, potential neurotoxicity and immunogenicity, an urgent need exists for the development of efficient, non-peptide molecular carriers. This review briefly summarizes the structural characteristics and the delivery properties of the newly developed non-peptide carriers, in particular the ones developed in the author's laboratory, together with their potential as delivery vectors for poorly bioavailable drugs including small molecules, proteins, and nucleotides.

Keywords: Cell-penetration; Molecular carrier vectors; Organelle selectivity; Tissue selectivity; Blood–brain barrier

1. Introduction

Discovery of a new drug is only a part of the pharmaceutical R&D efforts. The drug, as an active ingredient of a medicine must somehow get to the right place at the right time at the right dosage level in order to have the desired therapeutic effects. Normally this issue is examined during the late developmental phase of a drug in connection with the pharmaceutics research determining dosage, formulation and administrative route. In reality, however, many drug candidates with promising *in vitro* activity fail to proceed to the developmental phase because of poor absorption, distribution, metabolism or excretion (ADME) profiles, and in addition, many drugs have limited administrative routes, e.g. injection, limited bioavailability, and limited efficacy, thus achieving only a limited commercial success. Available data suggests that about one out of 10 candidates entering clinical development survive, and more than 30% of the failures in early clinical phase are attributable to inadequate ADME properties (Kola and Landis, 2004). This is where the drug delivery technology can make major contributions. The traditional drug delivery technology, however, has paid much attention to developing controlled-release devices such as polymer-based caplets and mini-pumps, and developing alternative administrative routes such as pulmonary (inhalation), oral, and transdermal (patches) formulations to a substantial commercial success; annual sales of drug delivery systems in the U.S. was estimated to be more than $20 billion in 2000 (Langer, 2001). Thus, substantial scientific and technical challenges and tremendous commercial potentials remain in the new drug delivery technology. Even many of small molecule drugs are not utilized to their full therapeutic potential because of either the poor aqueous solubility and/or inadequate delivery properties. Anti-cancer agents such as vinblastine, doxorubucin, taxol, antisense drugs, antibiotics such as vancomycin, and anti-viral agents such as nucleoside phosphate derivatives may be cited as good examples of such cases. At present many potential protein/peptide drugs and DNA drugs or genes cannot be properly delivered to the desired sites because of poor uptake by the cells or nuclei.

Cellular and nuclear membranes do not allow crossing to a drug molecule searching for its therapeutic target, when a drug does not show an optimum balance of lipophilicity and hydrophilicity combined with an optimum molecular size. Although the cell membrane is constructed through an association of proteins and lipids, and the membrane lipid bilayer is only 7–10 nm thick, it tightly controls the cross-membrane traffic, entry as well as exit, of foreign molecules. Many of the membrane bound molecular transporters thus identified are unique to particular organs and selective to specific molecules to be ferried. The ideal solution to the membrane permeation

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problem might be based on harnessing cell's active transport processes of various kinds, but unfortunately our understanding of the cross-membrane transport machinery and mechanism is grossly incomplete (Alper, 2002).

Thus, for the foreseeable future the drug delivery technology development is expected to take advantage of various non-specific uptake mechanisms available in the cell or intracellular organelles such as nucleus and mitochondria. Typically, a drug molecule has to be water-soluble in order to travel through an aqueous environment to reach its target cells. However, crossing of the cell membrane by the drug molecule requires a certain degree of lipophilicty. Only a very small subset of molecules possesses the required solubility–lipophilicity profile. Thus, a large proportion of drug candidates fall victim to this hydrophilicity–lipophilicity paradox and fail to proceed to further developmental stages. A number of approaches have been examined to overcome this difficulty, and a typical solution known as "pro-drug technology" involves conjugation through an easily hydrolyzable linkage of the drug molecule to a carrier that can provide the needed hydrophilicity or lipophilicity. For example, a carbohydrate or a lipid can serve as the carrier molecules conferring the necessary hydrophilicity and lipophilicity to a drug candidate, respectively (Lambert, 2000; Allen and Cullis, 2004). In the cases of protein drug and DNA, polyethyleneimine, polylysine, polyamidoamine dendrimers and cationic liposomes, and β-cyclodextrin-based polymers, etc. have been extensively studied as molecular carriers, but toxicity liabilities remain as a persisting problem with these carrier materials (Goldberg and Gomez-Orellana, 2003).

2. Cell-penetrating peptides

More than a decade ago a seminal discovery was made that the HIV-1 Tat protein (86 amino acid residues), a viral transactivator, could cross cell membranes in a receptor independent manner, and the relatively short domain of the peptide sequence, the nine amino acid residue (Tat 49–57; RKKRRQRRR; Tat peptide) required for the transmembrane localization was determined (Vives and Lebleu, 2002). Similar discoveries have transpired with Antennapedia homeodomain protein, Antp (43–58; RQIKIWFQNRRMKWKK) (Dupont et al., 2002), viral protein VP22 (267–300; 34 AA residues) (Elliott and O'Hara, 1997), nuclear localization signal sequences (NLS) (Ragin et al., 2002) and others. These peptide sequences of various membrane translocating peptides are known as various names such as cell-penetrating peptide (CPP), protein transduction domain (PTD), and membrane translocating sequence (MTS), and may be classified into three groups: basic peptides such as Tat peptide, basic/amphiphilic peptides such as Antp, and hydrophobic peptides such as MTS (Futaki et al., 2003).

In the case of Tat and related peptides, the salient structural feature is that they are in general very rich in basic or cationic amino acids, in particular arginine and lysine. Futaki and his coworkers have studied various arginine-rich viral and synthetic peptides by means of fluorescence probe attached to the peptides and confocal microscopy with mouse macrophage RAW264.7 cells, and found that they all have translocating activity similar to the Tat peptide. These peptides have no specific primary or secondary structural motif in common, except that they all have several arginine residues in the sequence. Using arginine oligomers of various lengths, they have determined the optimum number of residues to be around eight or nine for efficient translocation (Futaki et al., 2001).

Wender, Rothbard et al. have studied cellular uptake into Jurkat cells of a series of synthetic analogues of the Tat peptide, and found that all truncated and alanine-substituted analogues (in which each amino acid was replaced by A) showed diminished cellular uptake, suggesting again the cationic residues of the peptides played a critical role in the uptake. However, charge alone apparently was insufficient for transport, as oligomers of several other cationic amino acids such as histidine, lysine, and ornithine were less effective than the Tat peptide. They observed that 9-mer of arginine was 20 times more efficient than Tat peptide itself, again obtaining evidence for a key role played by the guanidinium groups of the arginine residues. Based on these results they synthesized a series of polyguanidine peptoid derivatives, and found that a subset of peptoid analogues exhibited significantly enhanced cellular uptake compared to Tat (49–57) or arg_9 (Wender et al., 2000; Rothbard et al., 2002).

The uptake mechanisms of CPPs are still unsettled and controversial at best, since a range of mechanistic pathways such as conventional endocytosis (Vives, 2003; Brooks et al., 2005; Futaki, 2005), involvement of inverted micelles (Prochiantz, 2005), caveolae (Ferrari et al., 2003), macropinocytosis (Wadia et al., 2004), formation of ion pair complexes (Rothbard et al., 2005), and others including interactions with cell surface heparin sulfate proteoglycans (Tyagi et al., 2001) have been proposed. It now seems very likely that different CPPs utilize different uptake mechanisms and that there might be multiple mechanisms in operation depending not only on the nature of peptide carriers, but also the size and nature of cargoes to which the carriers are conjugated, membrane composition, and physiological state of the target cells (Joliot and Prochiantz, 2004; Tunnemann et al., 2006). For examples, Tat-mediated intracellular delivery of large molecules and nanoparticles proceed via the energy-dependent macropinocytosis with a subsequent enhanced escape from endosome into the cytoplasm, whereas individual CPP or CPP-conjugated small molecules tend to cross the plasma membrane via electrostatic interactions and hydrogen bonding in the energy-independent manner (Gupta et al., 2005). And liposomes modified with a low arg_8 density are taken up mainly through clathrin-mediated, conventional endocytosis, whereas those liposomes modified with a high density arg_8 are taken up by macropinocytosis (Prochiantz, 2005; Khalil et al., 2006).

Despite the lack of clear understanding of the uptake mechanisms, many research groups have tried to utilize arginine-based oligomers as drug delivery vectors. For example, CPPs have been used in order to improve the pharmacological properties of poorly bioavailable drugs and drug candidates, such as small molecules (Rothbard et al., 2000), proteins (Wadia and Dowdy, 2003; Wadia et al., 2004; Jo et al., 2005), nucleotides and genes (Torchilin et al., 2001; Kogure et al., 2004). However, this CPP-based drug delivery approach is potentially fraught with a number of scientific and technical problems which include insta-

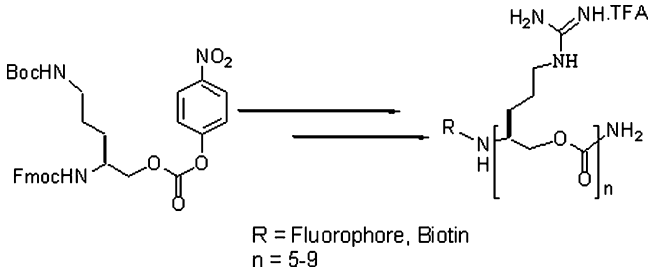
bility of the peptide carriers by endogenous peptidases, uncertain in vivo delivery efficiency (transduction between cells) as well as potential toxicity and immunogenicity liabilities.

3. Non-peptide carrier vectors

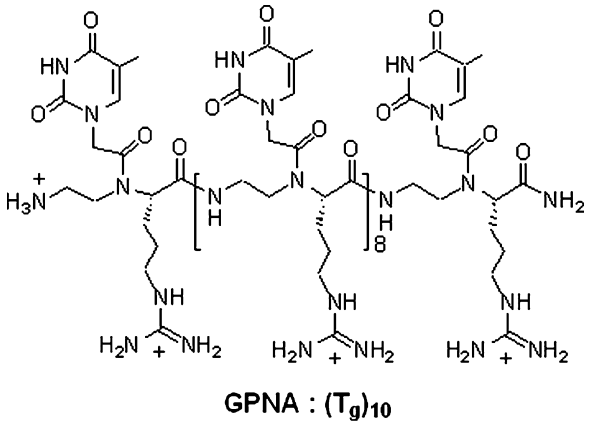
More recently there have been several reports on the development of synthetic molecular transporters mimicking natural and unnatural CPPs, especially in the area of polycationic arginine-rich peptides. In connection with the structure–activity relationships of the Tat peptide, Wender and Rothbard et al. have examined D-amino acid based peptides and found that these D-peptides translocate themselves as efficiently as or better than the L-counterparts (Wender et al., 2000; Wright et al., 2003). Gellman and Seebach groups have independently synthesized β-peptide analogues of the Tat peptide and oligoarginines, and found them to be capable of translocating across cell membranes in HeLa and 3T3 mouse fibroblast cells, respectively. In addition, it was observed that the uptake was not affected by temperature variations between 37 and 4 °C, and they were taken up by bacterial strains such as *E. coli* (Gram negative) and *B. megaterium* (Gram positive), which lack of endocytic apparatus, suggesting some uptake mechanisms other than the conventional endocytosis (Umezawa et al., 2002; Rueping et al., 2002; Geueke et al., 2005).

In 1998, the Chiron group investigated a combinatorial library of cationic peptoids (*N*-substituted glycine oligomers) in order to discover efficient delivery vector for genes and found that a 36-mer peptoid having 12 cationic aminoethyl side chains was most active, with efficiencies comparable to lipofectamine (Murphy et al., 1998). The Wender and Rothbard group prepared a peptoid library consisting of a varying number of guanidine groups as well as a varying chain length of the methylene linker (Wender et al., 2000). They synthesized a series of oligocarbamates ($n = 5$–9) on Rink resin by iterative coupling with monomer, which was prepared from an ornithine derivative (Scheme 1). The membrane permeability of these oligocarbamates was examined with Jurkat cells at 23 °C, and the nonamer was found to be most efficiently internalized, even better than Arg_8. They also demonstrated that the biotin-labeled octamer together with fluorescein-labeled streptavidin could penetrate across the cornified layers of the epidermis and into layers of the skin in 30 min (Wender et al., 2002).

Peptide nucleic acids (PNA) might be a useful tool in research as well as therapy, for they can sequence-selectively bind to the complementary DNA or RNA strand. However, they are not



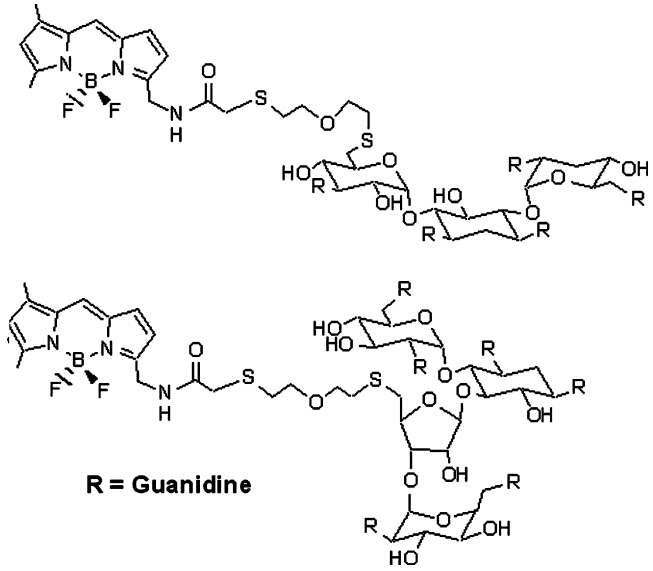
Scheme 1. Oligocarbamate carriers.



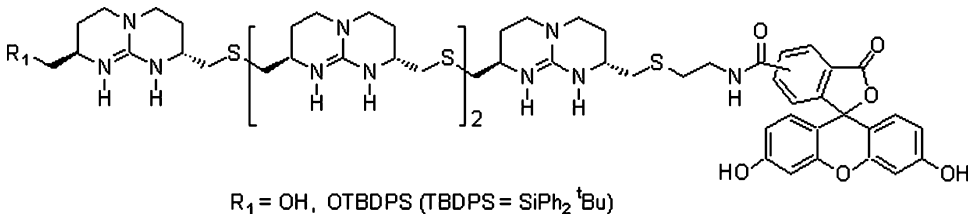
Scheme 2. Cell-penetrating peptide nucleic acid.

readily taken up by mammalian cells. Ly and his coworkers have prepared guanidine-based peptide nucleic acids by starting out with arginine in the synthesis of PNA and then introducing thymine via *N*-acylation (Scheme 2). The GPNA:$(T_g)_{10}$ was shown to bind to complementary A_{10} sequence with as high a sequence specificity as regular glycine-based PNA. Furthermore, the GPNA showed excellent uptake properties in human HCT116 (colon) and Sao-2 (osteosarcoma) cell lines. It was suggested that the uptake mechanism was neither endocytosis-driven nor receptor-mediated, since no difference in the uptake was observed between 37 and 4 °C (Zhou et al., 2003).

Aminoglycoside antibiotics function by inhibiting peptide synthesis through their interaction with rRNA, and their affinity for RNA is believed to be due to electrostatic interactions mediated by ammonium groups of the antibiotic. In an attempt to increase the RNA binding affinity and selectivity, Tor and his coworkers have replaced the ammonium groups of tobramycin (5 amino groups) and neomycin (6 amino groups) with guanidinium groups (Scheme 3). The guanidine-modified aminoglycosides were found to exhibit excellent



Scheme 3. Guanidinylated aminoglycosides.



Scheme 4. Heterocyclic guanidinium oligomers.

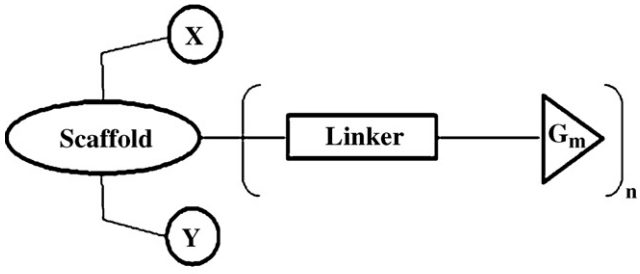
cellular membrane translocation abilities. Thus, BODIPY-tagged guanidino-tobramycin showed approximately the same transport efficiency as BODIPY-Cys(Arg)$_9$, and BODIPY-guanidino-neomycin better than BODIPY-Cys(Arg)$_9$. They suggested that the pre-organization of these guanidine groups on the semi-rigid aminoglycoside core may be responsible for the better efficiency of translocation across the cell membrane (Luedtke et al., 2003). This suggestion, however, is contrary to the uptake efficiency observed with poly-Arg peptoids with variable methylene chain lengths (Wender et al., 2000).

Recently, Giralt and his coworkers have synthesized heterocyclic guanidinium oligomers as potential delivery vectors (Scheme 4), and observed that they also internalized well into HeLa cells in 1 h. The internalization efficiency was found to be temperature dependent, and furthermore, a preferential localization was found within mitochondria. Although significant toxicity was associated with these compounds even at 10 μM concentration, these findings are very interesting. But no possible mechanistic explanation was offered (Fernandez-Carneado et al., 2005).

Various nonpeptidic carriers discussed above are technically interesting and informative. However, they do not provide all the answers necessary for them to be used as general delivery vectors in the sense that the ideal delivery vector themselves should be easily and economically prepared, applicable for a wide range of cargoes in terms of easy covalent conjugation, balanced hydrophilicity and lipophilicity, and adequate aqueous solubility, and be completely devoid of any discernible toxicity. Recently, our group has investigated possible development of such carrier vectors by using the structural design shown in Scheme 5. The novel, non-peptide molecular carriers consist of multiple (*m* times *n*) units of guanidine residues that are attached to the scaffold structure (monomer or dimer of carbohydrate or cyclitol) via a linker made up of a variable length of methylene chain or dendrimeric branch together with one or two functionality arms to which cargo and probe may be covalently conjugated. For the scaffold structure carbohydrate and cyclitol (inositol) have been chosen, since they have the highest density of functionality among organic compounds (thus, minimizing the molecular size and weight) with diverse stereochemical variations in the hydroxyl group, and they are cheap, naturally occurring, and devoid of any significant toxicity. In these regards, they represent an excellent platform to build a large molecular diversity by appending the requisite number of guanidine residues around the scaffold.

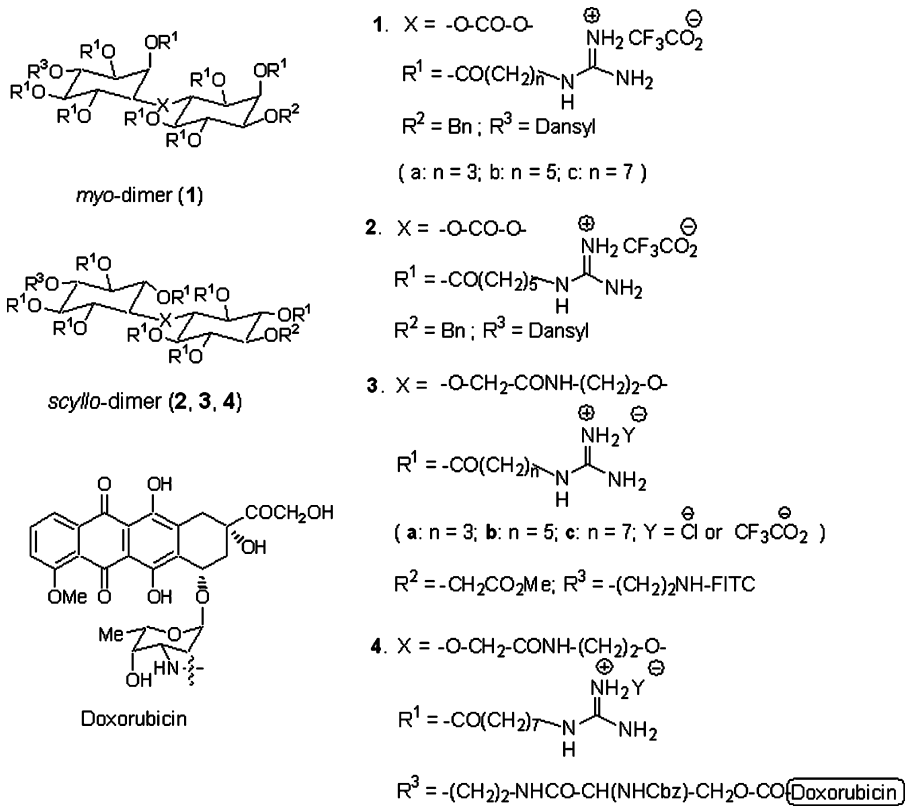
Initially, we synthesized molecular carriers based on dimeric inositol scaffolds, both *myo*- and *scyllo*-inositol (Scheme 6). Starting from readily available myo-inositol, a suitably protected dimer was formed via a carbonate linkage, and then guanidine-containing ω-amino acid was attached to give carrier **1**(**a**–**c**). The C2-OH configuration of *myo*-inositol was selectively inverted to provide *scyllo*-inositol, which was similarly transformed to carrier **2**. The structure of *scyllo*-inositol was further elaborated to have one amino and one carboxyl group on the same molecule, and then two units of the molecule were coupled to a *scyllo*-inositol dimer connected via an amide bond. This dimer was again acylated to give carrier **3**(**a**–**c**). The uptake properties of these carriers were examined in three cell lines: simian kidney COS-7, mouse macrophage RAW264.7 and HeLa cells. After 3–5 min exposure at 37 °C to the cultured cells, the cellular uptake was visually compared without fixing in comparison with Arg$_8$ or Arg$_9$. Carriers **1**–**3** all showed excellent translocation properties comparable to or better than Arg oligomers, and in general the carriers with a longer methylene chain displayed higher efficiencies than the carriers with shorter chains (Maiti et al., 2007a).

A more detailed studies carried out on **3**(**a**–**c**) with HeLa cells at 37 °C for 1 h followed by FACS analysis revealed that **3b** and **3c** were internalized 1.8 and 2.5 times as much as Arg$_8$, whereas the amount of **3a** internalized was about half that of Arg$_8$. A more precise kinetic study of internalization performed on **3c** in comparison with Arg$_8$, indicated that the amount of Arg$_8$ internalized reached saturation in 3 h, while the internalization of **3c** kept increasing even after 6 h, and the total uptake of **3c** was almost three times more than that of Arg$_8$. These results might suggest that carriers with this particular structural framework are helped by an increasing chain length either due to the increased



Scaffold: Carbohydrate, Cyclitol
Linker: linear or branched chain of variable lengths
G : guanidine (m = 1-3, n = 4-9)
X, Y : functionalities capable of conjugating with cargo (drug, Diagonostic device) and probe (fluorescent tag, targeting group, etc)

Scheme 5. Design features of novel molecular carriers.



Scheme 6. Dimeric-inositol-based carriers and conjugate.

hydrophobicity or higher flexibility for the interaction with cell membrane. The intracellular localization pattern studies on **3c** with HeLa cells in the presence of tetramethylrhodamine-labeled transferrin, tetramethylrhodamine-labeled Tat peptide, MitoTracker, and LysoTracker at 37 and 4 °C showed little similarities or co-localization, thus strongly suggesting that **3c** may use different mechanisms of internalization and cellular localization from the arginine-rich transporters reported to date. Compound **3c** also showed a rather unique *in vivo* tissue distribution pattern compared with the Tat-related peptides, which were previously reported to show a pretty much nonselective tissue distributions in liver, kidney, lung, heart muscle, and spleen (Schwarze et al., 1999). When injected intraperitoneally (*ip*) into 8-week-old mice, significantly lower extents of internalization of **3c** were observed into liver, kidney, and spleen, whereas heart, lung and brain tissues showed much higher distributions of **3c**. At present the reasons for the uneven or selective distribution are not defined; it is not clear whether it is due to kinetics or tissue themselves. In the repeated tissue distribution experiment of **3c** with mouse, the brain cortex region showed strong fluorescence in 20 min as well as 1 h after the *ip* injection, suggesting that the carrier clearly crossed the blood–brain barriers (BBB) rapidly and efficiently (Fig. 1).

Development of carrier vectors to help cross BBB is one of the major challenges in drug delivery, and these observations should be highly relevant to developing organ selective delivery technologies. Doxorubicin hydrochloride (Adriamycin) is extensively used in the clinic for the treatment of a variety of neoplastic diseases including leukemia, breast, ovarian, and solid cancers, but not brain cancer, since it does not overcome the

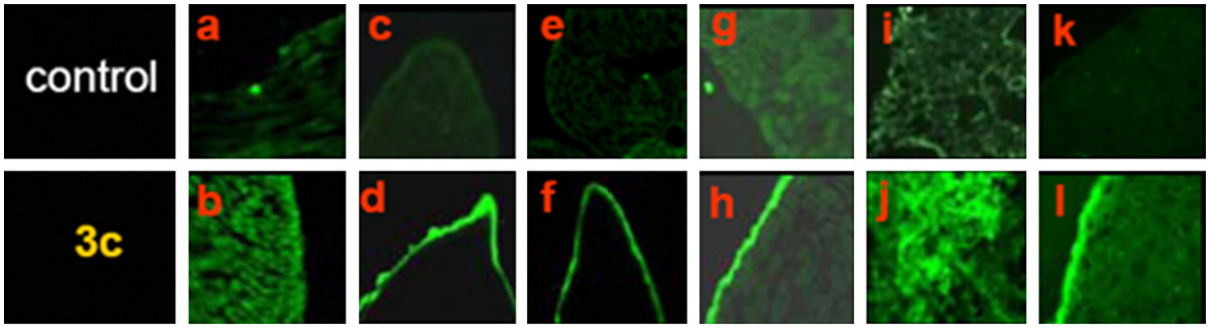
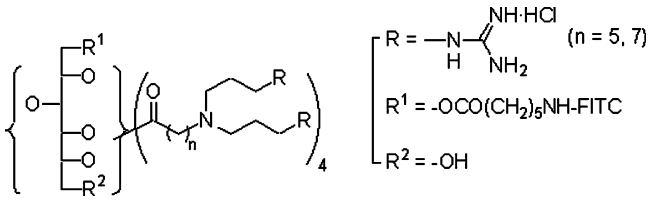


Fig. 1. Mouse tissue distribution pattern of carrier **3c** (Maiti et al., 2006). Fluorescence micrographs of heart muscle (a and b), spleen (c and d), liver (e and f), kidney (g and h), lung (i and j) tissue sections, and coronal brain section (k and l) isolated from 20 min after *ip* injection.



Scheme 7. Sorbitol-based carriers (Maiti et al., 2007b).

BBB (Rousselle et al., 2001). Doxorubicin has good UV–vis absorption bands and also highly fluorescent. The cellular uptake studies of the conjugate **4** with HeLa cells revealed that both at 10 and 30 μM concentrations it showed much-enhanced translocation into the cytoplasm, effectively killing cells at 10 μM, a concentration where neither doxorubicin nor carrier **4** caused significant cell damages. At present it is not clear whether the efficient cell killing is due to cleaved doxorubicin or the conjugate itself, nor whether due to more efficient delivery of doxorubicin or higher toxicity of the conjugate. The *in vivo* experiments with mice convincingly showed that the conjugate **4** was also extensively distributed in the cortex region of the mouse brain in 20 min after *ip* injection, whereas very little amount of doxorubicin translocated into the brain cortex in the same time frame. These *in vivo* results suggest that the brain uptake of doxorubicin through BBB is very inefficient, and that conjugation to the transporter significantly increases the brain uptake as well as the intercellular permeation of doxorubicin in the brain tissue (Maiti et al., 2006). The possibility that the carrier-doxorubicin conjugate might be clinically used in treating brain cancers is currently under investigation.

More recently we designed and synthesized a series of molecular carriers based on sorbitol scaffold and bifurcated linkers each of which having two residues of guanidine functionality (Scheme 7). Internalization characteristics of these carriers have been similarly studied and found to show comparable efficiency as Arg_8, and more significantly, highly selective co-localization with Mitotracker Red after 1 h incubation at 37 °C in HeLa and $CD34^+$ stem cell-like KG1a leukaemia cells (Maiti et al., 2007b). These observations are highly important since Tat and related peptides do not show discernible mitochondrial affinity, and molecular carriers capable of selectively delivering drug or diagnostic devices to mitochondria are in urgent need for the diagnosis and treatment of various mitochondrial diseases (Weissig et al., 2004; Dawson, 2004; Lane, 2006). Tissue distribution patterns of these sorbitol-based carriers in mice were also highly interesting in that better distribution was found in the heart muscle and brain sections than other tissues examined, a pattern substantially different from those observed for the inositol-based carriers. It might be somehow related to their organellar selectivity toward mitochondria, since heart and brain tissues are presumably very active in energy metabolism.

4. Prospect and conclusion

The novel molecular carriers developed by us as described above provided scientifically interesting and technically useful information for further development of viable carrier vectors. However, the inositol-based molecular carriers are synthetically arduous and uneconomical. Thus, we have synthesized a large number of additional novel molecular carriers by employing a variety of carbohydrate scaffolds such as sucrose, lactose, and alditols (e.g. sorbitol) as well as inositols, and syntheses of these carriers are much less demanding and proceed with much higher efficiencies. In the case of disaccharide scaffolds, synthetic protocols similar to those used for the dimeric inositol carriers have been employed, and for alditol and inositol scaffolds, branched forms of linkers were used in order to attach a suitable number of guanidine residues. Their internalization, cellular localization and tissue distribution studies are beginning to show some highly interesting and useful features. Unpublished results from the author's laboratory indicates that some of these carriers can be successfully employed for the delivery of small molecule drugs such as doxorubicin and taxol into mouse brain, and the lipid attached versions of the carriers for the delivery of large biomacromolecules (e.g. genes and *i*RNA) for transfection and expression inhibition, respectively, with high efficiencies. Notwithstanding the poor definition and complexity of the uptake mechanisms, it is now hoped that availability of these novel and versatile, non-peptide-based molecular carriers might possibly provide a solution to a variety of practically important delivery problems such as intracellular organelle selective and tissue selective deliveries in the foreseeable future.

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