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Oligocarbamate Molecular Transporters: Design, Synthesis, and Biological Evaluation of a New Class of Transporters for Drug Delivery

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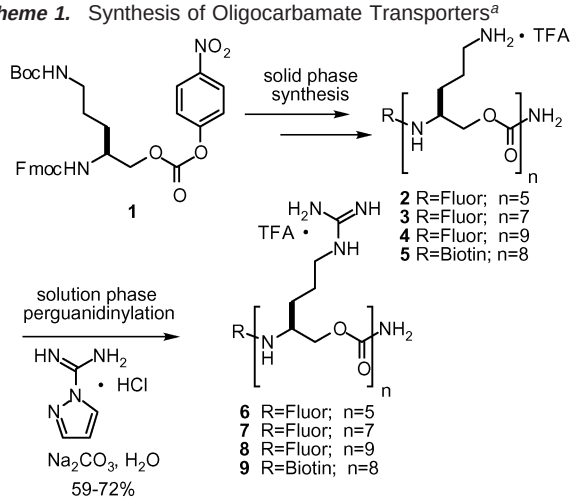
The effectiveness of drugs and probes aimed at intracellular targets is significantly determined by their ability to breach biological barriers. Polar molecules generally diffuse poorly through the nonpolar bilayer of a cell, while nonpolar compounds exhibit limited solubility in aqueous formulations and extracellular fluids. As a consequence, most drugs must be both water and lipid soluble, that is, conform to a narrow log *P* range. In contrast, certain naturally occurring molecules, irrespective of their log *P*, enter cells through a facilitated transport pathway mediated by a receptor or by a change in physical properties arising from cell surface interactions.[1−3] An exceptional example of such a molecule in this rapidly emerging field of molecular transporters is the nuclear transcription activator protein (Tat) encoded by HIV type 1 (HIV-1).[4,5] The sequence responsible for the cellular uptake of HIV-1 Tat is a highly basic region of nine amino acids (Tat_{49-57}: RKKRRQRRR).[6] Structure−function studies have shown that either truncation of the Tat 9-mer or replacement of any of its cationic residues with an alanine leads to significantly decreased cellular uptake. In contrast, replacement of the lysine and glutamine residues with arginine affords increased rates of uptake, leading to the identification of 9-mers of D- and L-arginine and related guanidine rich peptoids as superior transporters.[7−9] Conjugates of these transporters and various probes (e.g., fluorescein) or drugs (e.g., cyclosporin A) are highly water soluble and rapidly enter cells and tissues,[8,10−12] enabling their advancement into human trials.

Our previous work has shown that guanidine-rich peptides and peptoids with 1,4-spaced side chains are superior to Tat_{49-57} in cellular uptake.[8] The current study sought to explore whether the amide backbone common to peptide and peptoid transporters could be replaced with a carbamate and whether the resultant increased spacing (1,4 to 1,6) would enhance uptake into cells and tissues.

A series of novel guanidine-rich oligocarbamates was targeted for preparation. Our synthesis (Scheme 1) was designed to produce an oligoamine which upon perguanidinylation would be converted to the desired oligoguanidine. The required monomer **1** (Scheme 1) was efficiently synthesized by the reduction of Fmoc-Orn(Boc)-OH (1,1′-carbonyldiimidazole in THF with $NaBH_4$, H_2O, 92% yield),[13] followed by activation of the resulting amino alcohol as the *p*-nitrophenyl carbonate (96%).[14]

The oligocarbamate 5-, 7-, and 9-mers were synthesized on Rink amide resin by iterative coupling with monomer **1** on a PE Biosystems 433A automated peptide synthesizer using modified FastMoc chemistry. Incorporation of an aminohexanoic acid spacer and a fluorescein thiourea or biotin label followed by cleavage from the resin and purification by RP-HPLC afforded oligocarbamates **2**−**5** in 34−53% yield. Perguanidinylation[8,15,16] with 1*H*-pyrazole-1-carboxamidine hydrochloride[17] and Na_2CO_3 converted oligoamines **2**−**5** to the corresponding guanidine-rich oligomers **6**−**9** in 59−72% yield after purification by RP-HPLC (perguanidinylation was complete (MS), >95% pure by analytical HPLC).

Scheme 1. Synthesis of Oligocarbamate Transporters[a]



[a] Fluor = Fluorescein-NHCS-aminohexanoic acid; Biotin = D-biotin-aminohexanoic acid.

The ability of the oligocarbamates to enable cellular uptake was determined by FACS analysis of Jurkat cells that had been incubated with fluoresceinated oligomers for 3 min at 23 °C. In this assay, fluorescein itself does not enter cells (control not shown), while the pentacarbamate and pentaarginine conjugates of fluorescein (Figure 1: **6** and D-arg5, respectively) display very limited cellular uptake. In striking contrast, the hepta- and nonacarbamate conjugates of fluorescein (**7** and **8**) rapidly entered virtually all cells at each of the concentrations analyzed. The rate of uptake increased with guanidine content (**8** > **7**) and concentration as observed with other Tat-related transporters.[8,9,18,19] Representing one of the best guanidine-based transporters studied to date, carbamate 9-mer **8** translocated into cells 2.3 times faster than D-arg9, which is taken up into cells 100 times faster than Tat_{49-57}.[8]

The cellular uptake of the carbamate transporters and other guanidine-rich transporter molecules with nonnatural backbones[8,18,19] (guanidine-rich peptoids, β-Tat, β-Arg_9, D-Tat_{49-57}) is a further indication that the backbone of the transporter molecule is simply a scaffold from which the transport enabling guanidinium functionalities are displayed. Previous studies showed that homo-oligomers of arginine efficiently cross the cell membrane, whereas lysine, ornithine, and histidine oligomers do not.[7] In agreement with

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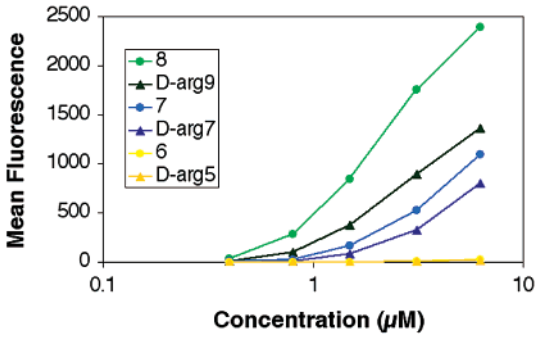

Figure 1. FACS determined cellular uptake of oligocarbamate transporters **6**−**8** and the corresponding fluoresceinated 5-, 7-, and 9-mers of D-arginine (D-arg5, D-arg7, D-arg9). Jurkat cells were incubated with transporter for 3 min at 23 °C.

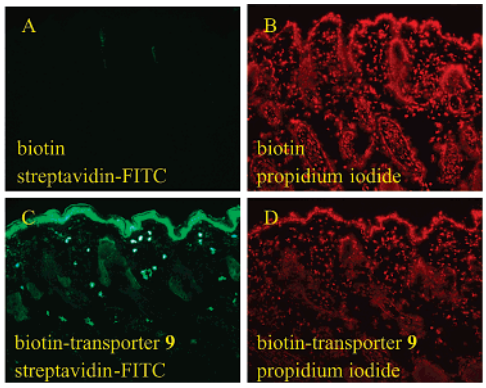

Figure 2. Uptake into mouse skin. Biotin and biotin-transporter conjugate **9** visualized with streptavidin-FITC (panels A and C). Skin architecture visualized with propidium iodide counter stain (panels B and D).

this earlier result and further underscoring the importance of the guanidinium group, nonacationic amine **4** exhibits poor uptake into cells (Supporting Information), supporting the generality of our hypothesis that multiple guanidinium groups and not just charge are required for uptake.

While the mechanism by which arginine-rich transporters cross the cellular membrane remains unknown, short arginine-rich peptides from the basic domain of Tat enter cells at 4 °C (conditions which typically inhibit endocytosis).[20] Similar to the Tat peptide, nonacarbamate **8** entered cells effectively at 4 °C but was significantly inhibited by preincubating cells with sodium azide.[7,21] These results are consistent with an energy dependent but nonendocytotic mechanism. While further investigation of this pathway is in progress, it is clear that the oligocarbamates exhibit exceptional cellular penetration.

To further evaluate oligocarbamate transporters as potential clinical agents for drug delivery, we sought to determine whether these agents could transport a drug or probe molecule into the formidable barrier represented by the skin. While most drug classes cannot penetrate the skin, intradermal drug delivery of therapeutic agents for the skin is especially attractive as it minimizes or eliminates systemic side effects and reduces the amount of drug needed.

Biotin was chosen as a model drug cargo, as it is unable to cross the cornified layer of the skin and penetrate the epidermis and its location can be readily visualized by complexation with fluorescein-labeled streptavidin. Biotin-labeled transporter **9** and biotin alone (control) were separately applied (0.5 mM) to the back of a nude mouse, and the residual solution was removed after 30 min. After a 2 h interval, dissected sections of the treated areas of skin were exposed to fluorescein-labeled streptavidin. While biotin alone did not penetrate the skin (Figure 2, panel A), visualization of skin treated with biotin-transporter **9** (panel C) revealed highly efficient penetration across the cornified layer of the epidermis and into all layers of the skin. Nuclear localization (consistent with the known polycationic nuclear localization signal) was dramatic in the intensely stained epidermis, and the various cells of the dermis were also highly stained (detail in Supporting Information).

In conclusion, the oligocarbamate transporters are found to be among the most efficient molecular transporters studied to date. They enable exceptionally efficient uptake into cells of a cargo that by itself does not enter cells. Significantly, they also enable uptake into skin of a probe molecule that by itself does not penetrate skin. Structurally these are the first nonamide linked oligoguanidine transporters and the first with a 1,6-spacing of side chains. The relationship of these structural features to transport efficiency and mechanism and the utility of these agents in drug delivery are subjects of ongoing studies.

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Supporting Information Available: Experimental data for the synthesis and biological evaluation of oligocarbamate transporters (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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