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Targeted Intracellular Delivery of Antisense Oligonucleotides via Conjugation with Small-Molecule Ligands

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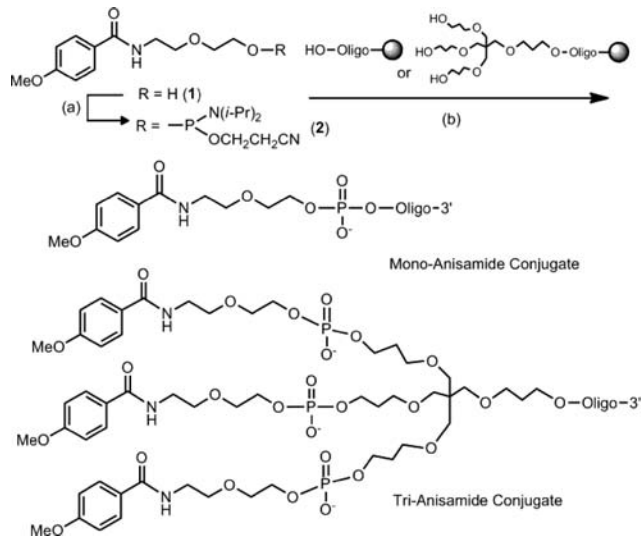
Targeted delivery is a key issue for the pharmacology of antisense and siRNA oligonucleotides.[1] Receptor-specific ligands can be linked to various nanocarriers that contain oligonucleotides, or they can be directly conjugated to the oligonucleotide itself. For example, we recently showed that conjugation of a dimeric RGD (arginine-glycine-aspartic acid) peptide, a high-affinity ligand for the integrin $\alpha v\beta 3$, to an oligonucleotide (ON) increased cellular uptake via receptor-mediated endocytosis and enhanced the biological effect of the ON.[2] Thus, direct mono- or multivalent ligand conjugation to ONs seems an attractive strategy for enhancing receptor-specific delivery of ONs to cells and tissues while using well-defined chemical moieties. In this report, we describe the synthesis of mono- and multivalent oligonucleotide conjugates using anisamide, a high-affinity ligand for sigma receptors, and evaluate the function of these conjugates in tumor cells in culture.

Sigma receptors ($\sigma 1$, $\sigma 2$) are transmembrane proteins, found on the endoplasmic reticulum and on plasma membranes, that seem to play a role in regulating ion channels.[3] High-level expression of sigma receptors has been observed for a diverse set of human and rodent tumor cell lines.[4] Small molecules such as haloperidol, SA4503, and opipramol have been reported as sigma-receptor ligands.[3] Several $\sigma 1$ ligands have been developed as radioimaging agents for tumors and successfully tested *in vivo*.[5] These observations suggested that sigma-receptor ligands could also be used for targeted drug delivery. Huang and colleagues have reported that the high-affinity sigma-receptor ligand anisamide, when conjugated to lipid nanocarriers, could be used to deliver doxorubicin[6] or siRNA[7] to tumors in animals. Mukherjee et al. reported that haloperidol conjugated lipoplexes showed 10-fold greater delivery of DNA to breast carcinoma cells than did control lipoplexes.[8]

For liposome-conjugated anisamide [*N*-(2-aminoethyl)-4-methoxybenzamide], the 4-methoxybenzamide unit itself is very important for binding activity to sigma receptor. The aminoethyl moiety, especially the terminal nitrogen, is also important.[6,9] Interestingly, DeSimone *et al.* developed a new anisamide ligand that used a modification with an oxygen atom, instead of a nitrogen atom, in the aminoethyl moiety.[10]

With the intent of establishing an efficient strategy for ligand−oligonucleotide conjugation, we designed the direct incorporation of anisamide ligand into antisense oligonucleotides using a DNA synthesizer. Thus, we used *N*-[2-(2-hydroxyethoxy)ethyl]-4-methoxybenzamide (**1**) for direct conjugation to ONs. This allows solid-phase synthesis of anisamide−ON conjugates, since a protective group is not needed during DNA synthesis (Scheme 1 and Supporting Information). The phosphitylation of the anisamide **1** successfully afforded the phosphoramidite **2**. The anisamide phosphoramidite **2** was incorporated into oligonucleotides using conventional phosphoramidite chemistry on an automated DNA synthesizer. For the monoanisamide conjugate, **2** was directly incorporated at the 5′-terminal of the ON. The trivalent conjugate was synthesized using a three-branched linker, readily introduced into the 5′-terminal of the ON. The facile synthesis described here should be readily applicable to various other types of oligonucleotides such as siRNA, miRNA, triplex-forming oligonucleotides, and molecular decoys.

Scheme 1. Synthesis of 5′ Mono- and Trivalent Anisamide-Conjugated Oligonucleotides[a]



[a] ON (623): 5′-GTT ATT CTT TAG AAT GGT GC-TAMRA-3′ (2′-*O*-Me-RNA with phosphorothioate backbone). Reagents and conditions: (a) iPr$_2$NP(Cl)OCH$_2$CH$_2$CN, iPr$_2$NEt, CH$_2$Cl$_2$; (b) DNA synthesizer, HPLC purification.

To evaluate the biological activity of the conjugates, we utilized sigma-receptor-expressing cells (PC3, human prostate carcinoma cells) that contain a luciferase reporter gene interrupted by an abnormal intron that prevents expression of functional luciferase protein.[11] Upon adequate delivery of an appropriate splice-switching antisense oligonucleotide (an SSO, here designated ON 623) to the nucleus, the intron is spliced out and luciferase is expressed.[11] The 623 ONs were 2′-*O*-methyl-RNA with a phosphorothioate backbone and a 3′-TAMRA fluorophore. Uptake of the fluorescent ONs was monitored in cells by flow cytometry.[2] As shown in Figure 1A, there was slightly higher uptake of the monoanisamide conjugate as compared to the unconjugated ON control, while the trivalent anisamide conjugate showed significantly greater uptake than control, possibly reflecting greater avidity of the multivalent version for the sigma receptor. The increased uptake could be partially blocked by co-incubation with excess free haloperidol (Figure 1B), a strong sigma-receptor antagonist. Since the overall uptake process likely involves both receptor-mediated endocytosis and nonspecific fluid-phase pinocytosis, it is expected that the blocking effect of an antagonist would be partial.

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The biological effect (luciferase induction) paralleled the uptake data but was more pronounced. The trivalent conjugate displayed a significantly greater effect than the monovalent compound or the unconjugated control (Figure 1C). Consistent with our previous observations,[2] the biological effects of oligonucleotides entering cells by a receptor-mediated process seem to be greater than those of oligonucleotides entering by pinocytosis. Thus, as compared to the unmodified oligonucleotide, the trivalent conjugate displayed an approximately 2-fold increase in uptake but a 4-fold increase in luciferase induction. No toxicities were apparent with use of these conjugates, as judged by the retention of normal cell morphology and lack of effect on total cell protein recovered.

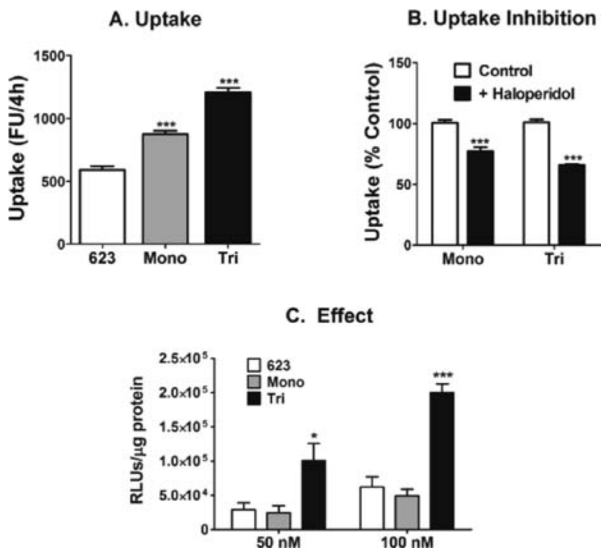

Figure 1. (A) Initial cellular uptake. Cells were treated with 50 nM monoanisamide-623-TAMRA, trianisamide-623-TAMRA, or 623-TAMRA for 4 h in OptiMEM at 37 °C. The cells were rinsed in buffered saline solution and then trypsinized. Total cellular uptake of the TAMRA-labeled conjugate was measured by flow cytometry. (B) Effect of sigma-receptor inhibitor on initial uptake. Cells were treated with 50 nM mono- or trianisamide-623-TAMRA in the absence or in the presence of 50 μM haloperidol. After 4 h, the cellular uptake was measured by flow cytometry. Results are normalized on the basis of cells receiving no inhibitor as 100%. (C) Luciferase induction. Cells were treated with monoanisamide-623-TAMRA, trianisamide-623-TAMRA, or 623-TAMRA for 24 h, and luciferase activity was determined 48 h after treatment. Results are the means and standard deviations of triplicate determinations.

Thus, we have demonstrated the facile production of mono- and trivalent anisamide-conjugated oligonucleotides via conversion of the ligand to a phosphoramidite followed by conventional solid-phase ON synthesis. The multivalent anisamide conjugate significantly enhanced receptor-specific cell uptake and biological effect. While there has been considerable work on peptide−oligonucleotide conjugates,[12] relatively little has been done with oligonucleotides linked to small organic molecules. The novel conjugation approach described here should create opportunities to utilize a variety of highly selective small-molecule ligands to target various receptor types, including members of the vast G-protein-coupled receptor family,[13] thus enhancing possibilities for receptor-selective ON delivery to a wide variety of cells and tissues.

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Supporting Information Available: Synthesis of mono- and trivalent conjugated oligonucleotides and cell culture experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Whitehead, K. A.; Langer, R.; Anderson, D. G. *Nat. Rev. Drug Discovery* **2009**, *8*, 129–138. (b) Juliano, R.; Bauman, J.; Kang, H.; Ming, X. *Mol. Pharm.* **2009**, *6*, 686–695. (c) Bennett, C. F.; Swayze, E. E. *Annu. Rev. Pharmacol. Toxicol.* **2010**, *50*, 259–293. (d) Corey, D. R. *J. Clin. Invest.* **2007**, *117*, 3615–3622. (e) Tiemann, K.; Rossi, J. J. *EMBO Mol. Med.* **2009**, *1*, 142–151.
(2) (a) Alam, M. R.; Dixit, V.; Kang, H.; Li, Z. B.; Chen, X.; Trejo, J.; Fisher, M.; Juliano, R. L. *Nucleic Acids Res.* **2008**, *36*, 2764–2776. (b) Alam, M. R.; Ming, X.; Dixit, V.; Fisher, M.; Chen, X.; Juliano, R. L. *Oligonucleotides* **2010**, *20*, 103–109.
(3) (a) Maurice, T.; Su, T. P. *Pharmacol. Ther.* **2009**, *124*, 195–206. (b) Cobos, E. J.; Entrena, J. M.; Nieto, F. R.; Cendan, C. M.; Del Pozo, E. *Curr. Neuropharmacol.* **2008**, *6*, 344–366.
(4) Vilner, B. J.; John, C. S.; Bowen, W. D. *Cancer Res.* **1995**, *55*, 408–413.
(5) John, C. S.; Vilner, B. J.; Geyer, B. C.; Moody, T.; Bowen, W. D. *Cancer Res.* **1999**, *59*, 4578–4583.
(6) Banerjee, R.; Tyagi, P.; Li, S.; Huang, L. *Int. J. Cancer* **2004**, *112*, 693–700.
(7) (a) Li, S. D.; Chono, S.; Huang, L. *Mol. Ther.* **2008**, *16*, 942–946. (b) Chono, S.; Li, S. D.; Conwell, C. C.; Huang, L. *J. Controlled Release* **2008**, *131*, 64–69.
(8) Mukherjee, A.; Prasad, T. K.; Rao, N. M.; Banerjee, R. *J. Biol. Chem.* **2005**, *280*, 15619–15627.
(9) Ablordeppey, S. Y.; Fischer, J. B.; Glennon, R. A. *Bioorg. Med. Chem.* **2000**, *8*, 2105–2111.
(10) DeSimone, J. M.; Murphy, A. J.; Galloway, A.; Petros, R. A. Patent WO/2008/045486, 2008.
(11) Sazani, P.; Kole, R. *J. Clin. Invest.* **2003**, *112*, 481–486.
(12) (a) Juliano, R. L. *Curr. Opin. Mol. Ther.* **2005**, *7*, 132–136. (b) Abes, R.; Arzumanov, A. A.; Moulton, H. M.; Abes, S.; Ivanova, G. D.; Iversen, P. L.; Gait, M. J.; Lebleu, B. *Biochem. Soc. Trans.* **2007**, *35*, 775–779. (c) Juliano, R.; Alam, M. R.; Dixit, V.; Kang, H. *Nucleic Acids Res.* **2008**, *36*, 4158–4171. (d) Cesarone, G.; Edupuganti, O. P.; Chen, C. P.; Wickstrom, E. *Bioconjugate Chem.* **2007**, *18*, 1831–1840.
(13) Armbruster, B. N.; Roth, B. L. *J. Biol. Chem.* **2005**, *280*, 5129–5132.

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