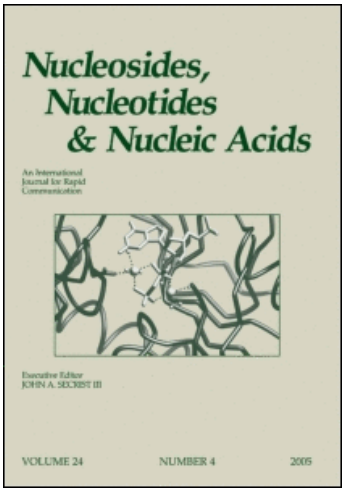

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Muthiah Manoharan[a]; Kathleen L. Tivel[a]; Laura K. Andrade[a]; V. Mohan[a]; Thomas P. Condon[a]; C. Frank Bennett[a]; P. Dan Cook[a]
[a] Isis Pharmaceuticals, Carlsbad, CA, USA

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OLIGONUCLEOTIDE CONJUGATES: ALTERATION OF THE PHARMACOKINETIC PROPERTIES OF ANTISENSE AGENTS

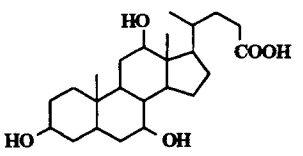
Muthiah Manoharan*, Kathleen L. Tivel, Laura K. Andrade, V. Mohan, Thomas P. Condon, C. Frank Bennett and P. Dan Cook

Isis Pharmaceuticals, Carlsbad CA 92008 USA

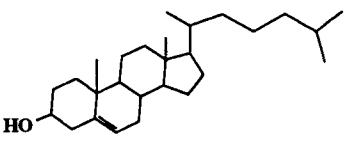
Abstract: Cholic acid, cholesterol, several polyamines and polyethylene glycols were conjugated to antisense oligonucleotides targeted to human or murine intercellular adhesion molecule-1 (ICAM-1) mRNA to study their effects on cellular absorption.

A major aspect of drug discovery research directed at the conversion of oligonucleotides into useful antisense agents might involve modifications to enhance their pharmacokinetic properties.[1] While oligonucleotides are inherently highly negatively charged and hydrophilic, the cell membranes through which they must pass are lipophilic and polyanionic in nature. For these reasons, oligonucleotides may not be sufficiently absorbed and distributed for maximum *in vivo* activity. Unfortunately, the appropriate physicochemical parameters of oligonucleotides that would relate to enhanced absorption and distribution are not well understood.

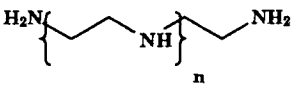
We have employed oligonucleotide modification strategies[2] to improve the uptake of oligonucleotides. These include the conjugation of various pendant moieties to the oligonucleotide to affect its overall physical properties such as hydrophobicity, charge, and amphipathicity. Certain pendants may also mediate absorption by binding to certain cellular receptors which internalize specific ligands. Our laboratory has prepared oligonucleotides conjugated to cholic acid, cholesterol, polyamines, and polyethylene glycols (**FIGURE 1**) to study their effects on enhancing absorption of antisense agents. These conjugates were targeted against human or murine intercellular adhesion molecule-1 (ICAM-1) mRNA.[3] Bioavailability of the conjugates was determined by measuring the inhibition of gene expression in target cells. In some cases, a fluorescent molecule was



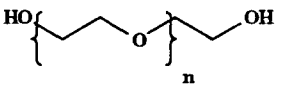
Cholic acid



Cholesterol



Polyamines



Polyethylene glycols

FIGURE 1. Ligands conjugated to oligonucleotides

attached to the conjugate to assess its subcellular distribution by fluorescence microscopy.

ICAM-1 assays -- The ICAM-1 antisense oligonucleotide 1570 has been shown[3] to inhibit cytokine-induced ICAM-1 expression in a sequence-specific and concentration-dependent manner in the presence of cationic liposomes.[4] In the ICAM-1 antisense assay, human lung carcinoma (A549) cells, human umbilical vein endothelial cells (HUVEC), or murine endothelioma cells (BEND.3), grown in 96 well plates, were washed in serum-free medium and then incubated with different concentrations of oligonucleotides in serum-free medium in the presence or absence of cationic liposomes. ICAM-1 expression was induced by adding tumor necrosis factor (5 ng/ml) and ICAM-1 expression was determined by ELISA or flow cytometry. For fluorescence microscopy, cells were washed with serum-free or serum-containing medium and incubated with fluorescein-labeled oligonucleotide (1 μM) for 4 h at 37°C in the presence or absence of cationic liposomes (10 mg/ml).

Cholic Acid Conjugates -- Cholic acid, a lipophilic steroid-like molecule which is internalized by a bile acid receptor system,[5] was conjugated to ICAM oligonucleotides at either the 5'- or 3'-terminus using an aminolinker.[6] This was achieved by first converting cholic acid into its N-hydroxysuccinimide ester or pentafluorophenyl ester, which, in excess, was allowed to react in DMF at pH 8.0 with ICAM oligonucleotides containing an aminolinker.

In the absence of cationic liposomes, neither 1570 nor its 3'-cholic acid conjugate (1570C) significantly inhibited ICAM-1 expression at concentrations up to 30

μM in HUVEC cells. Similar results were obtained with A549 cells. In the absence of cationic lipids, both fluorescein-1570 and fluorescein-1570C accumulated in vesicular structures in the cytoplasm of cells. In the presence of cationic liposomes, both 1570 and 1570C had similar activity; fluoresceinated-1570 and fluoresceinated-1570C readily accumulated in the nucleus of cells; this is consistent with previously published data.[3] These data suggest that conjugation of cholic acid to ICAM oligonucleotides does not significantly change their pharmacological activity, nor does it markedly affect their cellular pharmacokinetics.

Polyamine Conjugates -- Polyamines were conjugated to oligonucleotides to form amphipathic molecules and to potentially reduce the net negative charge on oligonucleotides.[7] These modified oligonucleotides might also be ligands for polyamine receptors present in certain cells.[8]

Polyamines were conjugated to oligonucleotides via aminolinkers at the 5'- or 3'-end of oligonucleotides using the homobifunctional linker disuccinimidyl suberate (DSS). In this two-step reaction, DSS converts the aminolinked oligonucleotide into an electrophilic intermediate which subsequently condenses with the polyamine.

Oligonucleotides conjugated to polyamines at the 3'-end exhibited increased nuclease resistance compared to the parent oligonucleotide. NMR and molecular modeling studies showed that the polyamine portion of the conjugate is a free floating chain that does not interact with the anionic backbone.

Incubation of A549 cells with free polyamine [pentaethylene hexamine (PEHA) and spermine, 0 to 4 μM], polyamine-conjugated oligonucleotides, or the unmodified parent compound (1570) in the absence of cationic lipid failed to inhibit ICAM-1 expression. Conjugation of PEHA to the oligonucleotide also did not change its subcellular distribution compared to 1570, consistent with the observed lack of activity. However, incubation of A549 cells with these same oligonucleotides in the presence of cationic liposome resulted in a concentration-dependent inhibition of ICAM-1 expression, with the PEHA conjugate exhibiting the greatest activity.

PEG Conjugates -- Polyethylene glycols (PEGs) are known to play an important role in the pharmacokinetic behavior of therapeutic proteins.[9] Multistep chemical methods have been reported to attach oligonucleotides to PEGs.[10] We employed a simpler method: aminolinked 1570 was conjugated to a series of PEG active esters of average molecular weight 550, 2000, and 5000, corresponding to 12, 44 and 110 ethylene glycol residues. T_m analyses of these conjugates indicated that the PEG group does not interfere with the hybridization of the oligonucleotide to its DNA or RNA complements. The energy minimized molecular modeling structure was in accordance with this observation.

Biological activity of these conjugates observed in this study was unexpected and, in certain respects, disappointing: in the presence and absence of cationic lipids the conjugates exhibited less activity than the parent compound. Very little cellular localization of 5'-fluoresceinated PEG-oligonucleotide conjugates was observed compared to the parent compound. This implies that the PEG moieties interfered with uptake of the oligonucleotides. In view of the higher hydrophobicity of the conjugates compared to the parent compound and the fact that hydrophobicity increases with the number of PEG groups, it is rather surprising that PEG conjugation caused reduced cell association even for larger PEG conjugates. PEG groups may divert oligonucleotides into non cellular compartments or bind proteins present in the biological milieu.

Cholesterol Conjugates[11] -- While the aforementioned ligands were attached after oligonucleotide synthesis, cholesterol attachment was accomplished at the nucleoside stage. 5'-*O*-Dimethoxytrityl-2'-*O*-hexylaminouridine was condensed with cholesteryl chloroformate. The resultant 2'-cholesterol nucleoside was then converted to the corresponding phosphoramidite. The amidite was used to incorporate cholesterol at the 5'-end of the bioactive oligonucleotide 3082. This oligonucleotide is targeted to murine ICAM-1 mRNA. Cholesterol greatly increased the lipophilicity of the oligonucleotide and, at 1-10 μM, was shown to be a potent inhibitor of ICAM expression in the absence of cationic lipids. Fluorescence microscopy of the 3'-fluoroscein analog revealed increased cellular concentration of oligonucleotides compared to unconjugated parent oligonucleotide, presumably due to the interaction of cholesterol with its receptor system. Cellular association of the oligonucleotide was more pronounced in the presence of 10% fetal bovine serum.

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