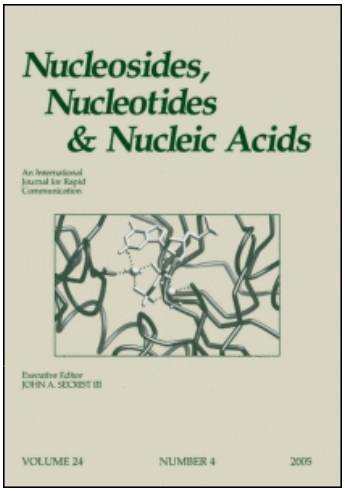

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Conjugated Antisense Oligonucleotides

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To cite this Article Manoharan, Muthiah , Tivel, Kathleen L. , Condon, Thomas P. , Andrade, Laura K. , Barber-Peoch, Isabelle , Inamati, Gopal , Shah, Shefali , Mohan, Venkatraman , Graham, Mark J. , Bennett, C. Frank , Crooke, Stanley T. and Cook, P. Dan(1997) 'Conjugated Antisense Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 1129 — 1138

To link to this Article: DOI: 10.1080/07328319708006147

URL: http://dx.doi.org/10.1080/07328319708006147

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1129-1138 (1997)

CONJUGATED ANTISENSE OLIGONUCLEOTIDES

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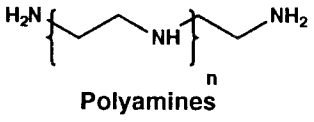
We have employed chemical modification strategies to improve cellular absorption of oligonucleotides. These include the conjugation of various pendant moieties to the oligonucleotide to affect its overall physical properties such as hydrophobicity, charge, and amphipathicity as well as pendants that may mediate absorption by binding to certain cellular receptors which internalize specific ligands. Our laboratory has prepared polyamines, polyethylene glycols and lipidic constituents conjugated to oligonucleotides in order to study their effects in enhancing absorption of antisense agents. These conjugates were targeted against human or murine Intercellular Adhesion Molecule-1 (ICAM-1) mRNA.

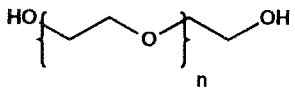
Polyamine Conjugates: Polyamines were conjugated to oligonucleotides to form amphipathic molecules and to potentially reduce the net negative charge on oligonucleotides. These modified oligonucleotides might also be ligands for polyamine

receptors present in certain cells. Polyamine conjugation improved the antisense activity of oligonucleotides.

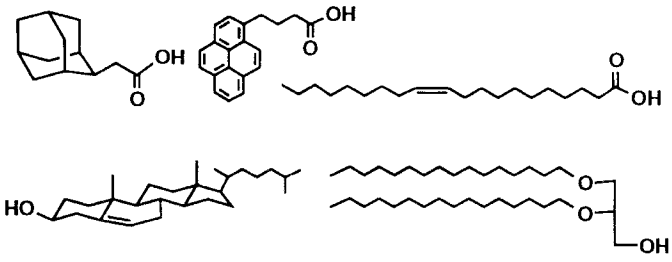
PEG Conjugates: PEGs (polyethylene glycols) are known to play an important role in the pharmacokinetic behavior of therapeutic proteins. Human ICAM-1 oligonucleotide was conjugated to a series of PEG active esters of average molecular weight 550, 2000, and 5000, corresponding to 11, 44 and 110 ethylene glycol residues. Our results indicate that PEGs seem to interfere with the cellular permeation of oligonucleotides *in vitro*.



Polyamines



Polyethylene glycols



Lipidic Nucleic Acid Building Blocks

Lipidic Oligonucleotides : Nucleoside synthons containing cholesterol at the 2' or 3' position were synthesized and incorporated into oligonucleotides targeted against the 3'-untranslated region of the mouse intercellular adhesion molecule-1 (ICAM-1) gene. In cell culture experiments, the ICAM-1 phosphorothioate cholesterol conjugated oligonucleotide (at the 5'- end via a 2'-*O*-hexylamino linker of the nucleoside) reduced the levels of ICAM-1 gene expression (both protein and mRNA) without cationic lipid formulation. This inhibition was specific to ICAM-1; in the same experiments, VCAM-1 protein expression was not affected. Other lipophilic molecular conjugates (adamantane, eicosenoic acid, pyrene, and a glyceride lipid) did not improve *in vitro* efficacy. Replacement of cholesterol with cholic acid did not improve pharmacological effects. Cholesterol conjugation also modified the pharmacokinetics and biodistribution of oligonucleotides. Concurrent *in vivo* experiments demonstrated that cholesterol conjugates can significantly reduce the ICAM-1 mRNA levels in mouse liver. The site of cholesterol conjugation plays an important role in the biostability of the resulting conjugate.

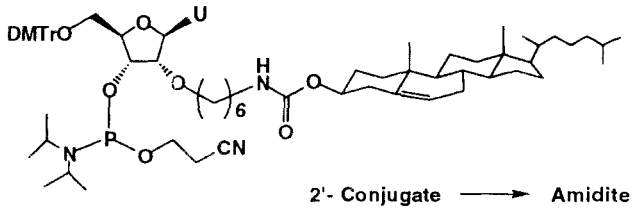
Conjugation of cholesterol via the terminal 3'-OH group of the oligonucleotide significantly improves the nuclease stability of the oligonucleotide by blocking exonucleases. This is correlated with greater efficacy of the 3'-conjugate compared to the 5'-regioisomer. Finally, we demonstrate that the observed antisense effects are sequence-specific. The observed data suggest that cholesterol conjugation may be enhancing the efficacy by improving the transport and bioavailability of oligonucleotides via a specific receptor-mediated process. All the observed biological effects were rationalized by a molecular modeling study employing energy minimization methods.

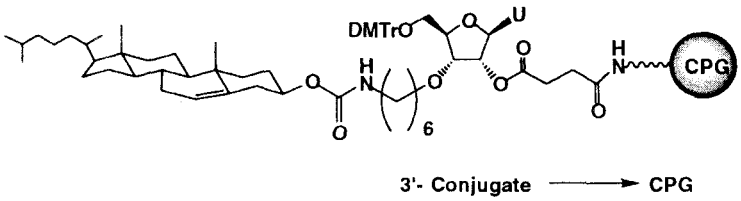
We have synthesized several lipidic oligonucleotide conjugates to modify the physicochemical properties of the ICAM-1 antisense oligonucleotide and carried out a systematic structure-activity relationship study correlating the observed biological activity as shown by mRNA and with the protein levels of ICAM-1. Our results illustrate the unique role of cholesterol among hydrophobic ligands and also indicate that the site of attachment of cholesterol conjugation determines the relative order of efficacy. These observations are consistent with known principles of mechanistic enzymology and explained on the basis of molecular modeling computational experiments.

Effect of cholesterol conjugated oligonucleotide on ICAM-1 expression

The nucleoside-cholesterol conjugate was incorporated into the 5'-end of the ICAM-1 antisense oligonucleotide developed for mouse model studies (ISIS-3082) (Stepkowski 1994). The resultant conjugate (ISIS-8005) was tested for inhibiting ICAM-1 expression. ISIS-3082 shows antisense inhibition in cell culture with an IC_{50} of 100nM when formulated with a cationic lipid for delivery. Lipofectin, a cationic liposome, is a mixture of a cationic lipid, N-[1-(2,3-dioleoyl)propyl-N,N,N-trimethylammonium choloride (DOTMA), and dioleoylphosphatidylethanol amine (DOPE). The function of this cationic lipid is believed to involve three steps: 1) condensation of oligonucleotide by cationic groups of lipids; 2) increased cellular uptake due to interaction of positively charged complexes with negatively charged cell surfaces; and 3) a fusion process in the

endosome followed by cytoplasmic delivery avoiding degradation in the lysosomal compartment. The cationic lipids, which are added as adjuvants in large molar excess to gain overall cationic charge, can have toxic properties at these high concentrations. It is also known that they are ineffective in the presence of serum.





An alternative to this approach is to conjugate oligonucleotides to small molecules and ligands, like cholesterol, fusogenic peptides or alkyl groups. The ultimate aim is to eliminate the need for cationic lipids for delivery in cell culture and improve the efficacy *in vivo* of the antisense compounds.

We observed that in cell culture comparison experiments evaluating the effect of ISIS-3082 and 8005 on controlling ICAM-1 expression without any cationic lipid adjuvant ISIS-8005 inhibited ICAM-1 in a dose dependent manner. ISIS-3082 did not show any activity at all, even when high concentrations were used. Furthermore, the inhibition of protein expression appears to be target specific. When analyzed for controlling the isotype protein VCAM-1, we observed that neither molecule showed significant inhibition of VCAM-1 expression. Since no sequence similarity exists between the mouse ICAM-1 sequence and the mouse VCAM-1 sequence, ISIS-3082 or its conjugate would not be

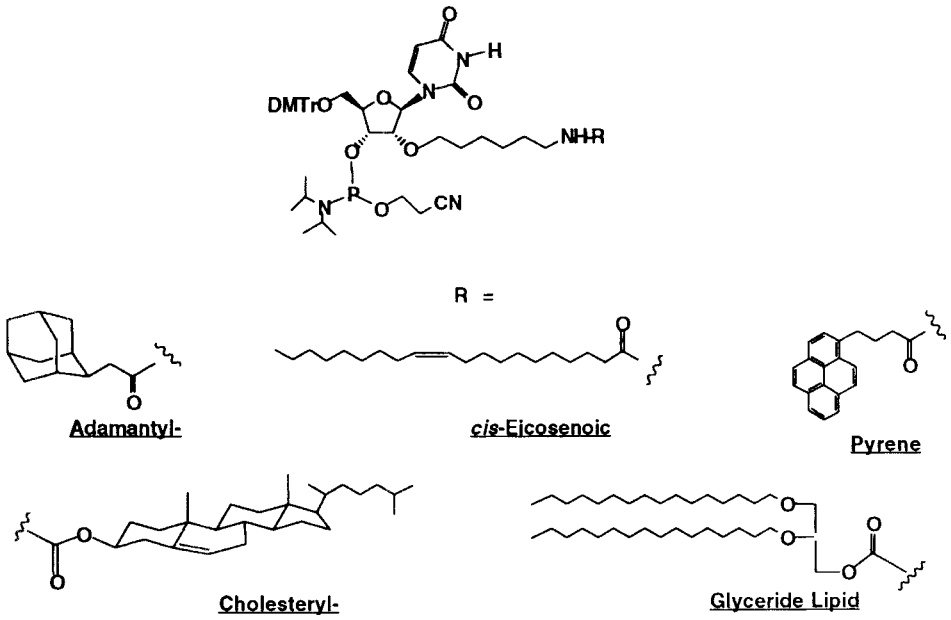
expected to influence the expression of VCAM-1 if they were working through an antisense mechanism.

We observed that treatment of murine endothelioma cells with a cholesterol conjugated ICAM-1 antisense oligonucleotide, ISIS 8005, resulted in a concentration dependent reduction of ICAM-1 expression in the absence of cationic lipids The unmodified oligonucleotide ISIS 3082 failed to inhibit ICAM-1 expression in the absence of cationic lipids. To demonstrate that the cholesterol conjugated oligonucleotide inhibited ICAM-1 expression by an antisense mechanism, the effect of the oligonucleotides on VCAM expression, also an inducible member of Ig superfamily, was determined.

Does cholesterol work by its lipophilicity alone?

To understand the role of cholesterol in improving the function of ISIS-3082, we asked if this molecule works merely by being a hydrophobic molecule or by potential specific protein-mediated (e.g., apo–ε) binding to LDL particles as cholesterol conjugated oligonucleotides can bind to lipoproteins. The answer can be obtained by synthesizing other lipophilic conjugates of ISIS-3082 and repeating the ICAM-1 inhibition experiments. Adamantane, pyrene, eicosenoic acid and a C_{16}-glyceride lipid nucleoside conjugates were synthesized and incorporated into ISIS-3082 in the same fashion as ISIS-8005.

A reverse-phase HPLC assay was used to measure the relative lipophilicities as a model for the cell membrane-antisense oligonucleotide interaction. The relative lipophilicity of the oligonucleotides was presumed to be reflected by the order in which they eluted from a C-18 reverse phase HPLC column. The retention time of the oligonucleotide (and presumably the lipophilicity) increases with the number of carbon atoms in the pendant group. There is a linear correlation between the percentage of acetonitrile needed for elution and the total number of carbons. The two compounds having the same number of carbons (pyrene and eicosenoic acid), the group having a greater number of hydrogens elutes later (eicosenoic acid). Thus a wide spectrum of lipophilicites was observed on going from ISIS 3082 to ISIS-3082-glyceride lipid conjugate, ISIS 11826.



In the antisense efficacy assays, again without any added cationic lipid formulation, relative order of lipophilicity was not reflected in efficacy. While the cholesterol conjugate can inhibit ICAM-1 expression, other conjugates failed to inhibit ICAM-1 expression at the concentration range of 1μM to 10μM of oligonucleotides. Cholesterol oligonucleotide shows a linear dose dependent response in controlling the ICAM-1 expression. This experiment illustrates that the lipophilicity is not the only factor affecting the observed activity of the cellular delivery of oligonucleotides, which is presumably determined by the cellular delivery of the oligonucleotides. The results suggest that a receptor-mediated process may be operating in the case of cholesterol-conjugated oligonucleotides. A similar observation was noted with cholic acid conjugated oligonucleotides. Conjugation of cholesterol, and not cholic acid, was able to improve the activity of oligonucleotides.

Cholesterol conjugation affects the biodistribution of the oligonucleotide

The effect of cholesterol conjugation on the pharmacokinetics properties of the oligonucleotide was determined in mice using ^{3}H radiolabeled ISIS 8005. This

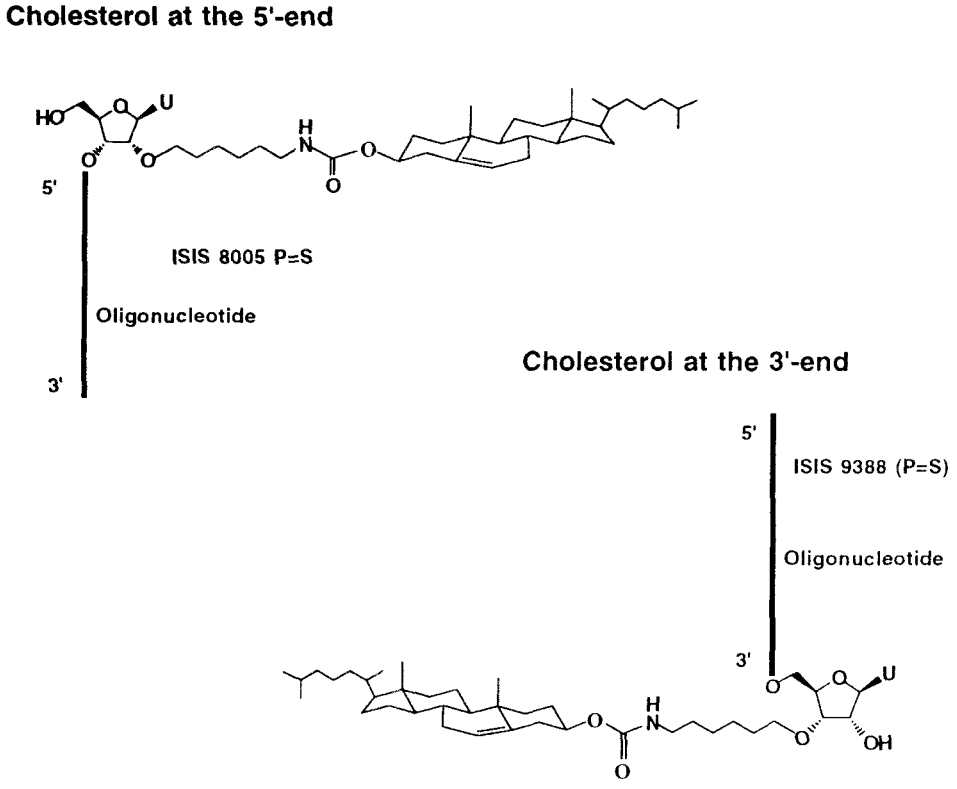
modification has a marked influence in the biodistribution of the oligonucleotide. ISIS-3082 was mainly distributed in liver, kidney, skeletal muscle and skin. In the case of ISIS-8005, more oligonucleotide was found in the liver. The amount was reduced in kidney, skeletal muscle and skin. It is not possible to determine if there was active transport of ISIS-8005 into the liver or whether the effects observed were simply due to changes in lipophilicity. ISIS-8005 was also retained in the plasma for longer periods of time than was ISIS-3082, which is consistent with the improved efficacy of ISIS-8005.

In vivo therapeutic efficacy of cholesterol conjugated oligonucleotides

The greater concentration in liver was correlated with the therapeutic effect of the drug as measured by ICAM-1 mRNA levels in mouse liver *in vivo* . In lipopoly saccharide induced expression of ICAM-1 mRNA by intravenous treatment of the mouse with ISIS 8005 at a dose of 10mg/kg 24 hours and 2 hours prior to polysaccharide treatment, we observed improved efficacy presumably due to cholesterol conjugation, as indicated by mouse ICAM-1 RNA levels in the liver. At this concentration, the unmodified oligonucleotide ISIS 3082 does not have any effect and we had to change the concentration range to 30 -100 mg/kg.

Improved nuclease resistance due to 3'-conjugation of cholesterol

Oligonucleotides ISIS 8005 (the 5'-cholesterol conjugate) and ISIS 9388 (the 3'-cholesterol conjugate) are regioisomers. We anticipated that the terminal 3'-modification as in 9388 would provide resistance to exonucleases in addition to facilitated transport due to cholesterol. The 3'-hydroxyl group, which is involved in the nucleophilic attack of the adjacent phosphate bond when the exonuclease enzyme makes a complex with the nucleic acid, is unavailable in ISIS 9388. As expected, we observed increased biostability of 9388 over 8005 and 3082.



Conclusions

The goal of this study was to find a method to deliver antisense oligonucleotides without the use of cationic lipid formulations. We have achieved this goal by conjugating oligonucleotides with cholesterol.

Oligonucleotide conjugation chemistry—attaching proper ligands to modulate the activity of oligonucleotides—has proven to be a valuable methodology. In this paper, we have shown that conjugating lipophilic and amphipathic components at the nucleoside level and incorporating them into synthetic oligonucleotides can influence their pharmacological properties. Among the several lipophilic oligonucleotides studied, cholesterol is a unique ligand in improving the efficacy of ICAM-1 antisense oligonucleotides.

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