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CHOLIC ACID-OLIGONUCLEOTIDE CONJUGATES FOR ANTISENSE APPLICATIONS

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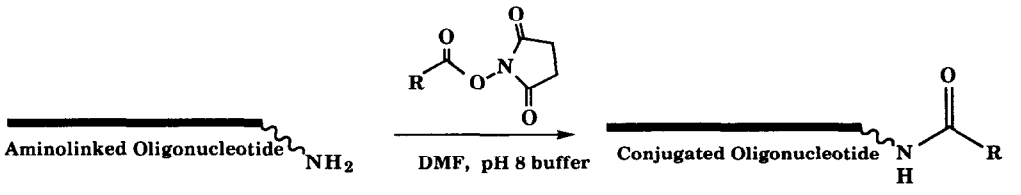
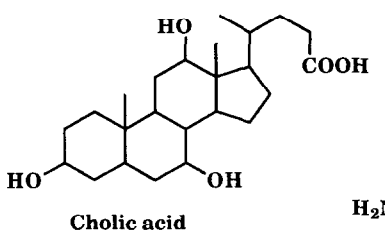
Abstract: To improve the pharmacokinetics of antisense oligonucleotides, cholic acid was conjugated to several biologically active sequences. Physical and biological properties of the conjugates were examined. While the cholic acid conjugation changes the lipophilicity of the oligonucleotides, the *in vitro* biological activity observed was dependent on the disease target, cell lines and the method of oligonucleotide treatment.

Pharmacokinetic properties of oligonucleotides will have great bearing on the efficacy of antisense agents[1]. Oligonucleotides are inherently highly negatively charged, hydrophilic molecules of approximately 20 nucleotides in length. Due to these properties the physical nature of oligonucleotides may not be conducive to sufficient absorption and distribution. Unfortunately the appropriate physical character of oligonucleotides that would relate to enhanced absorption and distribution are not well understood. Modification strategies to enhance absorption and distribution properties of oligonucleotides would include the conjugation of various pendant moieties to the oligonucleotide to affect the overall physical properties[2]. Various pendant groups could provide hydrophobicity, cations to alter charge effects, cell receptor binding and internalizing properties, and altered amphipathicity resulting from combining one or more of the above characteristics. Following this approach, a variety of molecules[3] have been attached to oligonucleotides to enhance absorption and distribution; these include phospholipids, cholesterol, alkyl groups, vitamin E, adamantane, targeting peptides, polyamides and poly(*L*-lysine). Among these ligands, cholesterol has been conjugated by

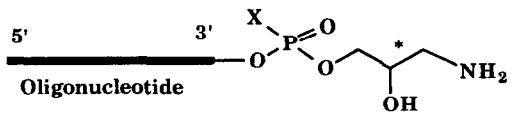


R-COOH

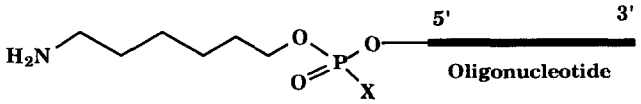


Cholic acid

3', 5' -AMINOLINKER CONTAINING OLIGONUCLEOTIDES



X = O or S; * = Chiral center



Scheme I

several methods and efficacy changes of different levels have been claimed in the literature[4]. Cholic acid, which has a similar steroidal architecture to offer lipophilicy but a different (bile acid) receptor system[5], has not been evaluated so far. For these reasons, we conjugated cholic acid to oligonucleotides at either the 5'- or 3'- terminus using an appropriate aminolinker. These conjugates were targeted against ICAM-1 (human intercellular adhesion molecule-1), HIV-1 (human immunodeficiency virus-1) and BPV-1 (bovine papillomavirus-1) messages. The ICAM-1 and BPV-1 oligonucleotides were 2'-deoxyphosphorothioates while the antisense HIV oligomers were 2'-*O*-methyl phosphodiesters. The absorption of the conjugates was measured by the inhibition of gene expression in target cells. In some cases, a fluorescent molecule was added to the conjugate to assess subcellular distribution of the oligonucleotides by fluorescence microscopy.

Synthesis, Analysis and Purification: Cholic acid was converted into its N-hydroxysuccinimide ester or pentafluorophenyl ester. Oligonucleotides containing an aminolinker in borate buffer (pH 8.0) were treated with an excess of the active ester dissolved in DMF (**Scheme I**). HPLC analyses of the conjugates on a reverse phase C-18 column[6] confirmed that the cholic acid considerably increases the lipophilicity of the oligonucleotide. The increased lipophilicity facilitates the analysis of the conjugation reaction and purification of the conjugate. An HPLC analysis of BPV oligonucleotides is shown in **Figure 1.** Peak A (33 min) is the parent ISIS-2324 oligonucleotide. Peak B (43 min) is the 3'-conjugate (2955C) which has a shorter tether than the 5'-conjugate (peak C; 2971C; 46 min). The *bis*-conjugate, derived from the oligonucleotide having aminolinked cholic acid at both ends, has the longest retention time (peak D; 2976C; 51 min). For conjugates having fluorescein and cholic acid, fluorescein was attached to the 5'-end of the oligonucleotide using a fluorescein phosphoramidite and cholic acid was attached to the 3'-end by conjugation via the linker.

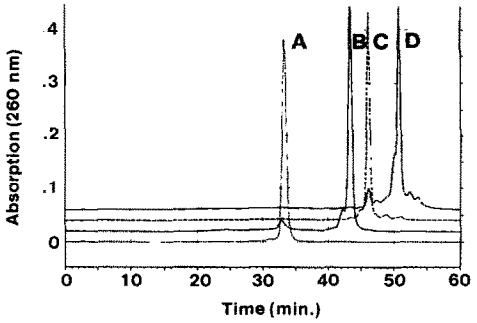


Figure 1. Reverse Phase (C-18 column) HPLC Analysis of Cholic Acid Conjugates.

NMR Characterization: NMR analyses of short oligonucleotide-conjugates confirmed the structure of 5'- and 3'-conjugates. ^{1}H and ^{31}P NMR of d(5'-GAG-3'-cholic acid), respectively in panels A and C, and d(5'-cholic acid-TCAG-3'), respectively in panels B and D, were recorded at 37°C (**Figure 2**). In the low field region of the proton spectra, the expected number of nucleobase protons and anomeric protons were observed; in the high field region, cholate methyl signals between 0.2 and 0.6 ppm were observed. For the 5'-conjugate, all four ^{31}P signals were clearly observed and the phosphate between the cholate-bearing linker (the most downfield signal) and the DNA is separated by > 1.3 ppm. For the 3'-conjugate the separation was only 0.7 ppm. This variation may be due to the difference in the structure and length of the tethers involved. Interestingly, in both ^{1}H and ^{31}P NMR of the 3'-conjugate, the diastereomeric nature of the conjugate, which arises due to the racemic linker (**Scheme I**), is borne out by the apparent doubling of peaks.

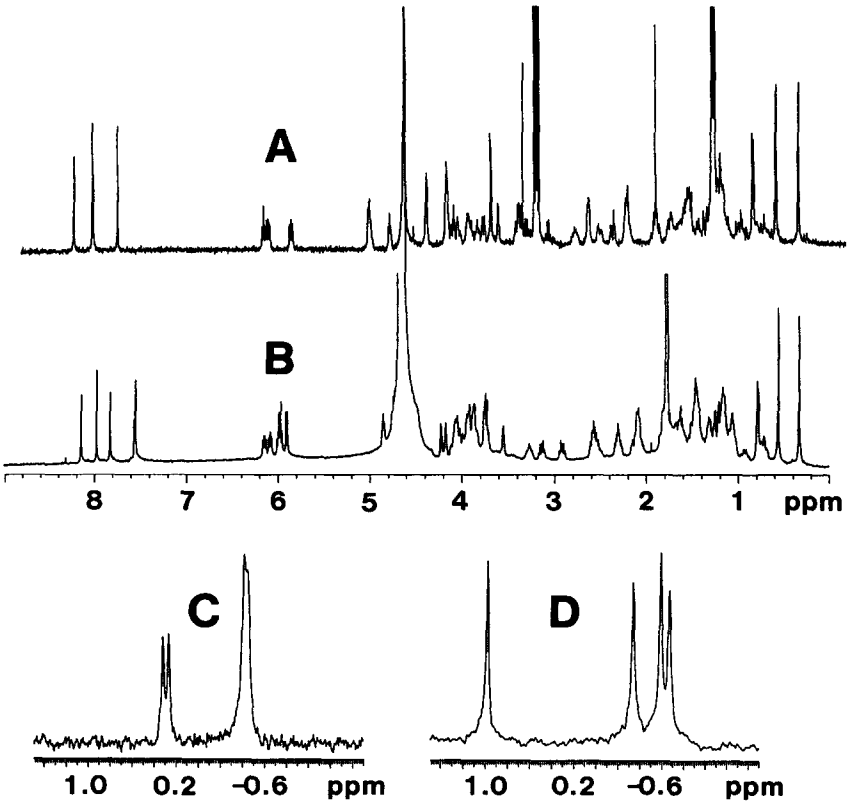


Figure 2. 400 MHz ^{1}H (A and B) 162 MHz ^{31}P (C and D) NMR Spectra of Cholic Acid Conjugates.

Nuclease stability: Nuclease stability of cholic acid conjugates in 10% fetal calf serum was assessed using gel analysis followed by laser densitometry. While the phosphodiester oligonucleotide 3133 had a half-life of about 0.5 hr., the corresponding conjugate, 5'-CTGTCTCCATCCTCTTCACT-cholic acid-3' (3141C) had a half-life of >24 hr. (**Figure 3**). Thus the 3'-conjugation offered significant nuclease stability against 3'-exonucleases and the lifetime of conjugated phosphodiester approaches that of unmodified thioates. Similar observations have been made by other laboratories[7] for other 3'-conjugates.

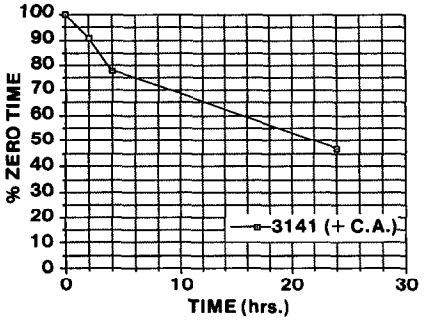
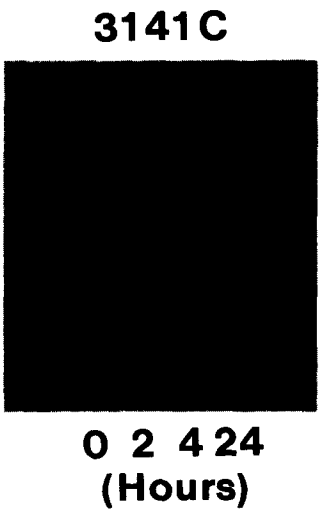


Figure 3



T_m Analyses: In evaluating hybridization properties of cholic acid conjugated oligonucleotides to DNA and RNA complements (**Table I**), we observed no unfavorable effects compared to the parent oligomers. In fact, we noted a small increase in T_m (averaging about 1° C). Such stabilizing interactions of lipophilic tails and tethers towards target strands have been reported[8].

Table I. T_m Values of Cholate Conjugates and Their Precursors[a]

Oligo	Sequence/Description	Backbone	T_m vs. RNA	T_m vs. DNA	ΔT_m[b] vs. RNA	ΔT_m[b] vs. DNA
1570	5'-TGG GAG CCA TAG CGA GGC-3'	P=S	54.6	59.8		
2875	1570+3'-linker	P=S	54.4	60.3		
1570C	1570+3'-cholate	P=S	56.1	60.7	+1.5	+0.9
3133	5'-CTG TCT CCA TCC TCT TCA CT-3'	P=O	64.9	60.8		
3141	3133+3'-linker	P=O	64.6	60.3		
3141C	3133+3'-cholate	P=O	66.6	61.6	+1.7	+0.8
3157	3133+5'-linker	P=O	65.1	60.8		
3157C	3133+5'-cholate	P=O	65.5	61.7	+0.6	+0.9
2221	5'-TCC AGG TGT CCG CAT C-3'	P=O	62.3	62.6		
2221C	2221+5'-cholate	P=O	62.4	63.4	+0.1	+0.8
1283	5'-TCC AGG TGT CCG CAT C-3'	P=S	54.9	55.4		
1283C	1283+5'-cholate	P=S	55.9	56.4	+1.0	+1.0
			Average ΔT_m		**+1.0**	**+0.9**

[a] In 0.1M Na^+, 10mM phosphate and 0.1 mM EDTA, pH 7.0 . [b](Cholate conjugate-Wild type).

Table II. Antisense Cholic Acid-Oligonucleotides Used for Biological Assays

ANTISENSE ICAM-1 OLIGONUCLEOTIDES (2'-H; Phosphorothioates, P=S)

1570: 5'-TGG GAG CCA TAG CGA GGC-3' (parent oligo)

1570C: 5'-TGG GAG CCA TAG CGA GGC-cholic acid-3'

4299C: 5'-Fluorescein-TGG GAG CCA TAG CGA GGC-cholic acid-3'

2971C: 5'-cholic acid-CT GTC TCC ATC CTC TTC ACT-3' (negative control)

ANTISENSE HIV-TAR OLIGONUCLEOTIDES, (2'-OMe, Phosphodiesters, P=O)

3281: 5'-CUC CCA GGC UCA GAU CU-3' (parent oligo)

3848C: 5-CUC CCA GGC UCA GAU CU-cholic acid-3'

3869C: 5'-cholic acid-CUC CCA GGC UCA GAU CU-3'

3864C: 5'-UGU GGC CCU CCA CUC AA-cholic acid-3' (negative control)

3874C: 5'-cholic acid-UGU GGC CCU CCA CUC AA-3' (negative control)

ANTISENSE BPV OLIGONUCLEOTIDES (2'-H; Phosphorothioates, P=S)

2324: 5'-CTG TCT CCA TCC TCT TCA CT-3' (parent oligo)

2955C: 5'-CTG TCT CCA TCC TCT TCA CT-cholic acid-3'

2971C: 5'-cholic acid-CT GTC TCC ATC CTC TTC ACT-3'

2976C: 5'-cholic acid-CT GTC TCC ATC CTC TTC ACT-cholic acid-3'

1570C: 5'-TGG GAG CCA TAG CGA GGC-cholic acid-3' (negative control)

BIOLOGICAL ASSAYS:

ICAM-1 assays: The ICAM-1 antisense oligonucleotide 1570 has been shown to inhibit cytokine-induced ICAM-1 expression in a sequence specific manner[9]. Human lung carcinoma (A549) cells, grown in 96 well plates, were washed in serum free medium. Cells were incubated with increasing concentrations of oligonucleotides 1570, 1570 conjugated to cholic acid (1570C), and a papillomavirus oligonucleotide conjugated to cholic acid (2971C) as a control in serum free medium in the presence or absence of cationic liposomes[10]. ICAM-1 expression was induced by adding interleukin 1ß (5 units/ml) and ICAM-1 expression was determined as previously described. In the presence of cationic liposomes, 1570 inhibited ICAM-1 expression in a concentration dependent manner. Conjugation of cholic acid did not significantly affect the activity of 1570 in the presence of cationic lipids. The control cholic acid oligonucleotide, 2971C, failed to inhibit ICAM-1 expression in either the presence or absence of cationic liposomes. In the absence of cationic liposomes, neither 1570 nor 1570C significantly inhibited ICAM-1 expression at concentrations up to 30 μM (**Figure 4**). Similar results were obtained with human umbilical vein endothelial cells (data not shown).

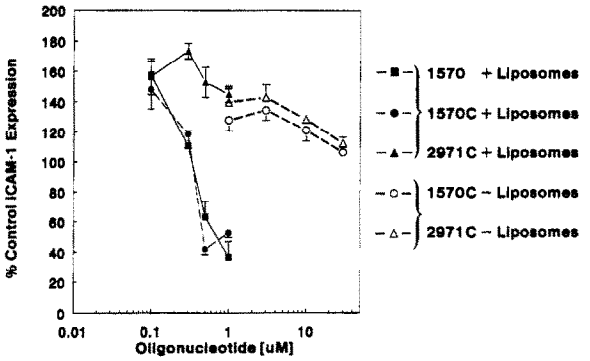


Figure 4: Effect of Antisense Oligonucleotides on ICAM-1 Expression.

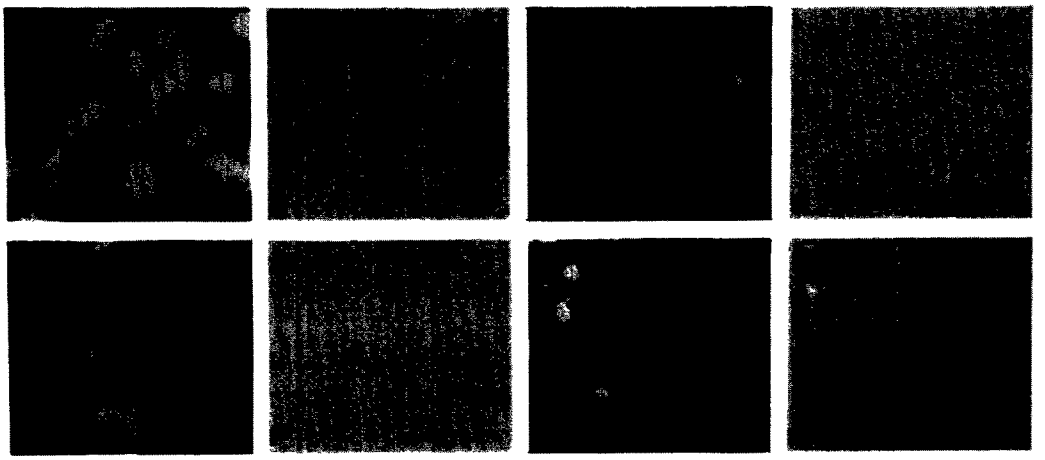


Figure 5: Subcellular distribution of fluorescein labeled oligonucleotides. Phase contrast (B, D, F, H) and fluorescence (A, C, E, G) images of human umbilical vein endothelial cells incubated with fluorescein labeled oligonucleotides 1570 (A, B) and 1570C (C, D) in the presence of cationic liposomes. Cells were also incubated in the presence of fluorescein labeled 1570 (E, F) and fluorescein labeled 1570C (G, H) in the absence of cationic liposomes.

Fluorescence microscopy: A549 cells were grown on glass microscope slides. Cells were washed with serum free medium and incubated with the indicated fluorescein labeled oligonucleotide (1 μM) for 4 h in the presence or absence of cationic liposomes (10 ug/ml) at 37°C. Localization of fluorescein labeled oligonucleotide in the cells was determined by fluorescence microscopy[9] . In the presence of cationic liposomes, fluorescein-1570 and fluorescein attached to 1570C (4299C) readily accumulated in the nucleus of cells (**Figure 5**) consistent with previously published data[11]. In the absence of cationic lipids, both fluorescein-1570 and fluorescein-1570C accumulated in the cytoplasm of cells within vesicular structures. These data suggest that conjugation of cholic acid to ICAM oligonucleotides did not significantly change the pharmacological activity of the antisense oligonucleotide nor did it markedly affect the cellular pharmacokinetics of the oligonucleotide.

HIV assays: HeLa cells were pre-incubated with oligonucleotides at 2μM and 7μM concentrations (**Figure 6**) for 20 hours. Oligonucleotides were removed and cells washed prior to transfection with pHIVluc (an HIV-luciferase reporter plasmid) and a plasmid expressing tat protein[12]. Cells were harvested 24 hours later and luciferase activity quantitated as described previously[11] . The 2'-*O*-methyl oligonucleotide 3281 forms a pseudo-half-knot structure when bound to the HIV tar element that is capable of disrupting tat binding and HIV mediated transcription[13]. The oligonucleotides 3848C and 3869C are derivatives of 3281, having cholic acid at the 3' and 5' ends respectively. Both oligonucleotides show enhanced inhibition of luciferase activity compared to the parent compound 3281. 3869C is approximately twice as active as 3281. Randomized control oligonucleotides (3864C and 3874C) show less activity compared to their non random counterparts (3848C and 3869C).

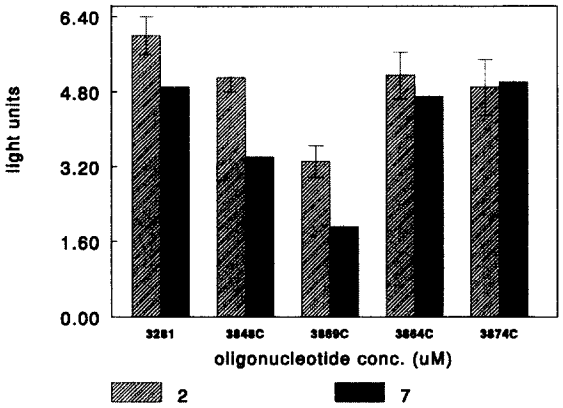


Figure 6: HIV Assays with Cholic Acid Conjugates.

BPV transactivation assays: The BPV-1 E2 antisense oligonucleotide 2324 has been shown[14] to inhibit E2 dependent transactivation and BPV-1 transformation. I-38 cells (BPV-1 transformed C127 cells) were plated 24 hours prior to transfection. After attachment, cells were treated overnight with oligonucleotide by direct addition to the medium. The media was aspirated and the monolayer washed once with fresh media. Growth media, without oligonucleotide, was added and the cells cotransfected with IPV110, the E2 dependent chloramphenicol acetyl transferase (CAT) expression vector and PCH110, a constitutive expressor of β-

glactosidase. Cells were glycerol shocked four hours after transfection. Growth media containing oligonucleotide was added. Cells were incubated for 48 hours, lysed and assayed for CAT and β-galatosidase activity. To control for transfection efficiency, CAT data were normalized to β-galactosidase activity. Data are reported as percent acetylation. In previous studies[13], 2324 has been shown to have a 50 percent inhibitory concentration (IC_{50}) of approximately 100 nM in the E2 transactivation assay. In this series of experiments, cells treated with 2324 (100nM) showed approximately 50 percent reduction in E2-dependent CAT activity. In this same assay conjugation of cholic acid to either the 3' end (2955C), the 5' end (2971C) or both the 5' and 3' ends (2976C) did not result in a significant increase in activity. (**Figure 7**).

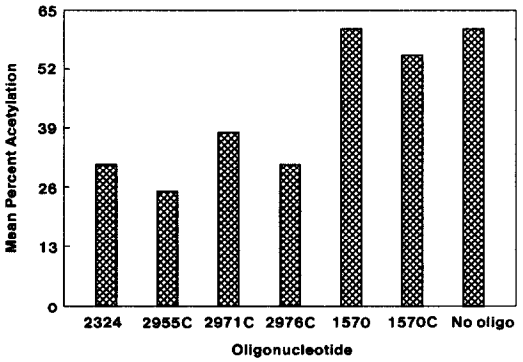


Figure 7: Inhibition of BPV by E2 Specific Antisense Oligonucleotides and their Cholic Acid Conjugates.

In the above preliminary experiments, the contrast between the apparently enhanced activity in the HIV assay and the lack of activity in the ICAM-1 assays in the absence of cationic lipids, may be explained by differences in the amount of oligonucleotide associated with cells during the pre-incubation period. When the reporter plasmids are transfected into cells, perhaps more oligonucleotide enters, producing an increase in activity. The apparent lack of activity in the BPV-1 system may have been the result of fewer cholic acid receptors on C127 cells. In the case of the ICAM-1 assay, there was no transfection and no increase in activity. When oligonucleotide entry into the cell was facilitated by the use of cationic liposomes, the cholic acid conjugate was as active as the non-conjugated oligonucleotide, thus demonstrating that it was capable of inhibiting ICAM-1 expression. These results are supported by the subcellular localization studies. In the absence of cationic liposomes, the cholic acid conjugate localized to vesicular structures in the cytoplasm while in the presence of cationic lipids the cholic acid conjugated oligonucleotide localized in the nucleus. In the HIV system, the activity of the conjugate may also come from the increased nuclease resistance. The different cells used may also have different numbers of cholic acid receptors which might also play an important role. As far as a specific utility of the cholate receptor system is concerned, cholic acid conjugates may be particularly useful in liver related disorders such as hepatitis. Other sites of attachment in cholic acid to maximize the recognition needs to be addressed as well. More experiments are necessary to corroborate these hypotheses.

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