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Cholesteryl-conjugated oligonucleotides: Synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture

(antiviral agents/antisense polymers/amphiphiles)

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ABSTRACT **A family of oligonucleotides and phosphorothioate oligonucleotide analogues was synthesized with a cholesteryl group tethered at the 3′-terminal internucleoside link. This modification, introduced to enhance interaction of the polyanions with cell membranes, significantly increases the antiviral activity of the oligomers, as judged by inhibition of syncytia formation and expression of viral proteins p17, p24, and reverse transcriptase for human immunodeficiency virus 1 in Molt-3 cells. In the most favorable case, with a 20-mer cholesteryl-phosphorothioate derivative, complete inhibition by all assays was obtained with an oligomer concentration of 0.2 μM. Even decamers were active, and some antiviral activity was observed for a heptanucleotide cholesteryl-phosphorothioate derivative, which binds very poorly to complementary oligonucleotides. These facts, and the finding that the activity of the phosphorothioate decamers does not correlate with a specific sequence, suggests that a mechanism other than "antisense inhibition" may be operative in these systems.**

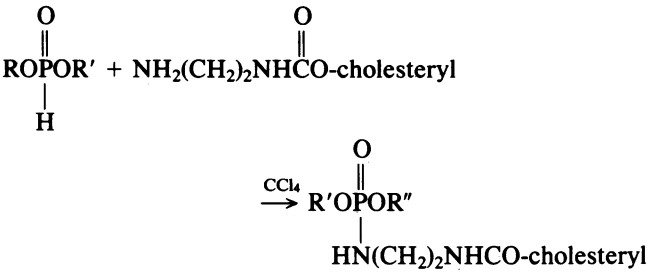
The pioneering work of Zamecnik and Stephenson on antiviral activity of oligonucleotides (1) and Miller and Ts'o on the chemistry and biochemistry of nonionic analogues (2, 3) has stimulated extensive research directed toward the therapeutic potential of nucleotide polymers (4). Oligonucleotide analogues with methylphosphonate (5–8), phosphorothioate (9, 10), and phosphoramidate (10) backbones, as well as unmodified oligonucleotides (11, 12) and a polylysine conjugate (13), have been reported to inhibit viral replication in cell culture. The viruses studied in this context include Rous sarcoma virus (1), simian virus (5), vesicular stomatitis virus (6, 13), human immunodeficiency virus (HIV) (8–12), herpes simplex virus (7), and influenza virus (14).

The concept underlying this work is that an oligonucleotide complementary to a unique segment of a viral genome, or an RNA derived from it, may selectively disrupt processes dependent on that segment by hybridization. This rationale is supported by a variety of experiments with cell-free systems and with cells to which "antisense" polynucleotides have been inserted by microinjection or transfection (15, 16). However, the actual mechanisms by which oligonucleotides and their analogues function as inhibitors in cell cultures are still far from clear. Even homooligonucleotides (9, 10) have been found to exhibit activity in some antiviral assays. Little is known about the interaction of the oligomers with cell membranes or the locus of their reactions within cells. It seems likely that nonionic oligomers, such as the methylphosphonate analogues, diffuse passively through cell membranes. Some evidence suggests an active transport mechanism for unmodified oligonucleotides (17).

We describe here the synthesis, physical properties, and activity as inhibitors of HIV replication in cell culture of a family of oligonucleotides with a modification designed to anchor the oligomer, at least transiently, at the cell membrane. Cholesterol was selected as the anchor since it is highly hydrophobic and membranes of HIV and HIV-infected cells are rich in this steroid (18).

MATERIALS AND METHODS

Synthesis of Oligomers. Oligonucleotides with cholesteryl groups covalently joined at the 3′-terminal internucleoside phosphorus were synthesized on commercial controlled pore glass (CPG) supports. A support-bound dinucleoside hydrogen phosphonate derivative (19) was first prepared manually; then a cholesteryl group was tethered to the internucleoside phosphorus by oxidative phosphoramidation (20) with carbon tetrachloride and cholesteryloxycarbonylaminoethylamine (Scheme I; R is a nucleoside linked at the 3′ O; R′ is a support-bound nucleoside linked at the 5′ O).



Scheme I

For extension of phosphodiester chains, nucleoside units were added subsequently by standard phosphoramidite chemistry (21) using a Biosearch 8600 Synthesizer. A second tethered cholesteryl group could be added at any desired position by repeating the cycle for coupling and oxidative amidation. For extension of phosphorothioate oligonucleotide chains, hydrogen phosphonate chemistry (19) was used, followed by a terminal oxidation with sulfur (10). At the end of a synthetic sequence, the derivatized CPG was removed and treated with ammonium hydroxide at 55°C for 5 hr. Concentration of the aqueous portion, chromatographic separation of the reaction products on a C-18 column, and lyophilization then afforded the cholesteryl-modified oligonucleotide. In each case, rechromatography confirmed the presence of a single band corresponding to the target compound. Greater than 50% of the nucleotidic product was

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Abbreviations: HIV, human immunodeficiency virus; CPG, controlled pore glass; t_m, melting temperature.

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labeled with cholesteryl, as indicated by the chromatographic profiles of the reaction mixtures obtained directly from the ammoniacal deprotection step.

Synthesis of 2-(Cholesteryloxycarbonylamino)ethylamine. Cholesteryl chloroformate (2 g) in dichloromethane (6 ml) was added dropwise to a solution of ethylenediamine (2.5 ml) in dichloromethane (6 ml) and pyridine (6 ml). The mixture was stirred for 2 hr; then the solvent was removed under vacuum and the residue was partitioned between water (150 ml) and dichloromethane (150 ml). The organic layer was washed with water, dried, and concentrated to give the title compound; 1.6 g (76%), mp 149°C–155°C. Recrystallization from cyclohexane afforded crystals melting at 152°C–155°C; R_f on silica (chloroform/methanol, 1:1; vol/vol) 0.15; positive ninhydrin test. Analysis calculated for $C_{30}H_{52}N_2O_2$: C, 76.22; H, 11.09; N, 5.93. Found: C, 75.96; H, 11.10; N, 5.86.

Preparation of Cholesteryl-Modified Dinucleoside Monophosphate on CPG Support. A sample of 5′-*O*-dimethoxytrityl-*N*-isobutyryldeoxyguanosine (DMT-ibG) linked through the 3′ O to a CPG support (Biosearch) (250 mg; 8 μmol of DMT-ibG) was placed in a Glenco gas-tight syringe (10 ml) equipped with a plug of glass wool at the inlet. Reactions and washings were carried out by drawing in and ejecting the desired solutions. Thus, the dimethoxytrityl group was removed with dichloroacetic acid (2.5% in dichloromethane), the support was washed repeatedly with acetonitrile/pyridine (4:1), coupling was effected by drawing in together DMT-ibG-hydrogen phosphonate (80 mg; 0.1 mmol; in 1.2 ml of acetonitrile/pyridine) and trimethylacetyl chloride (65 μl; 0.5 mmol; in 1.2 ml of acetonitrile/pyridine) (2 min), and the support was washed well with acetonitrile/pyridine. A solution of cholesteryloxycarbonylaminoethylamine (250 mg; 0.5 mmol) in carbon tetrachloride (5 ml) and pyridine (2 ml) was then drawn into the syringe and after 0.5 hr the solution was ejected and the solid was washed well with acetonitrile. Appropriate portions were then transferred to a cartridge for extension by machine synthesis or to a syringe for manual synthesis.

Characterization Procedures. High-performance liquid chromatography (HPLC) was carried out with a C-18 reversed-phase column (5-μm Hypersil ODS; 100 × 2.1 mm) on a Hewlett–Packard 1090 chromatograph using aqueous 0.1 M triethylammonium acetate (pH 7.3) and an acetonitrile gradient (1%/min; flow rate, 0.5 ml/min). Brinkmann silica gel plates (0.25 mm) with fluorescent indicator were used for TLC; they were developed with *n*-propyl alcohol/ammonium hydroxide/water (55:10:35; vol/vol). PAGE was carried out with denaturing cross-linked 20% gels [bisacrylamide/acrylamide, 1:28.4 (wt/wt); 1 × 140 × 170 mm] at constant voltage (400 V; 2 hr). Nucleotidic bands were visualized by UV shadowing and by staining with methylene blue (0.02%). Migration distances are reported relative to movement of bromophenol blue. Dissociation curves were obtained by following changes in A_{260} as aqueous solutions (0.1 M NaCl/0.01 M Tris buffer, pH 7.0) containing equimolar amounts of an oligomer and unmodified complement (total nucleotide concentration, ≈0.1 mM in base units) were warmed from 0°C to 80°C in 5°C increments with 5 min for equilibration at each step.

Assays for Inhibition of HIV by Oligonucleotides. HIV-1 inhibition assays (8) and HIV-1 p24 antigen expression and reverse transcriptase assays (22, 23) were carried out as described.

RESULTS

The modified oligomers and related parent oligonucleotides synthesized for this study are listed in Table 1. These compounds were characterized by HPLC, TLC, PAGE, thermal dissociation curves for hybrids formed with complementary strands (Table 1), and UV and NMR spectroscopy. No separation of stereoisomeric phosphoramidates could be achieved; consequently, the cholesteryl derivatives represent in each case a mixture of *R* and *S* stereoisomers at phosphorus. The ^{31}P NMR spectra exhibited the expected peaks near −0.8, +10.5, and +56 for the phosphodiester, phosphoramidate, and phosphorothioate functional groups, respectively (Table 1). A proton NMR spectrum of compound **2** showed the presence of the cholesteryl fragment. In addition, the hydrophobic nature of the cholesteryl-modified oligonucleotides was clearly revealed by the HPLC data (Table 1). For example, the elution times for samples analyzed by a reversed-phase C-18 column increased from 12 to 46 to 61 min for compounds **1** (parent), **2** (one cholesteryl), and **4** (two cholesteryls), respectively. Susceptibility to nuclease degradation was examined with compound **2**. In the presence of spleen phosphodiesterase and alkaline phosphatase, compound **2** was completely hydrolyzed to the expected

Table 1. Properties of oligonucleotides

Compound	Formula	HPLC, min†	TLC R_f	PAGE, Rm‡	t_m§
1	d(ACACCCAATTCTGAAAATGG)	12.2	0.26	0.64	60
2	d(ACACCCAATTCTGAAAATG*G)	46.0	0.41	0.55	60
3	d(ACTCCGAAATCAGATAAAG*G)	46.6	0.43	0.54	—
4	d(A*CACCCAATTCTGAAAATG*G)	61.0	0.58	¶	52
5	d(CAATTCTGAAAATG*G)	46.5	0.54	0.64	46.5
6	d(AsCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsAsTsGsG)	16.5	0.59	0.67	44
7	d(AsCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsAsTsG*G)	40.2	0.61	0.64	47.5
8	d(CsAsAsTsTsCsTsGsAsAsAsAsTsGsG)	17.3	0.47	0.67	29
9	d(CsAsAsTsTsCsTsGsAsAsAsAsTsG*G)	47.4	0.59	0.63	25
10	d(CsTsGsAsAsAsAsTsGsG)	15.6	0.52	0.76	29.5
11	d(CsTsGsAsAsAsAsTsG*G)	49.6	0.56	0.62	26
12	d(GsAsCsTsTsTsTsAsG*G)	45	0.60	0.79	7.5
13	d(CsTsGsAsTsTsTsTsG*G)	45.2	0.59	0.76	13
14	d(AsAsAsAsTsG*G)	47.5	0.66	0.62	‖
15	d(TsTsTsTsTsTsTsTsTsTsTsT*T)	50.0	0.60	0.65	16††

*, O=P—NH$(CH_2)_2$NHCO$_2$ cholesteryl; s, O=P—S$^-$. Altered nucleotides in compound **3** are underlined. Conditions for HPLC, TLC, PAGE, and t_m are given in *Materials and Methods*.

†Elution time.

‡Rm is distance of migration relative to bromophenol blue.

§t_m is the temperature (°C) at the midpoint of the maximum slope in a plot of A_{260} vs. *t*.

¶The sample appeared as a broad streak starting at Rm 0.2.

‖No break in the A_{260} vs. *t* curve was observed.

††The complement for compound **15** was poly(dA).

nucleosides (dI in place of dA) and a fragment corresponding to the terminal d(G*G) [*, the lipophilic phosphoramidate link O=P—$NH(CH_2)_2NHCO_2$ cholesteryl].

To assess the effect of the lipophilic side chain on hybridization, the dissociation of complexes formed from equimolar quantities of modified and unmodified complementary oligodeoxyribonucleotides was followed by changes in absorbance at 260 nm as a function of temperature. Melting temperature (t_m) curves typical for oligonucleotide duplexes were obtained. The data in Table 1 show that introduction of a single cholesteryl fragment at a terminal internucleoside position has only a minor effect on the stability of the hybrid duplex as measured by t_m values (compare compound **1** with **2**, **6** with **7**, and **8** with **9**), but that cholesteryl substituents at both termini appreciably destabilize hybridization (compare 20-mer compound **4**, t_m 52°C, with compounds **2** and **1**, t_m 60°C).

Inhibition of HIV-1 Replication. The parent oligonucleotide, compound **1**, is complementary to the splice acceptor site at nucleotides 5349–5368 in HIV (12) and has been reported to inhibit replication of this virus in Molt-3 cells (11, 12). The target compounds specified in Table 1 are structural variations of this basic sequence. They were designed to provide information on the anti-HIV properties of cholesteryl-conjugated oligonucleotides—specifically, with respect to changes in the number of cholesteryl substituents, the nature of the backbone, the length of the oligomers, and the sequence.

Data on inhibition of formation of syncytia and expression of HIV proteins p17, p24, and reverse transcriptase are given in Tables 2 and 3. There is some scatter in the data obtained from the different assay procedures; however, the patterns from the different tests are the same and the overall agreement is good. For convenience, the results are discussed here in terms of ID_{50} values for inhibition of syncytia [concentration of an oligomer (μg/ml) that reduces syncytia formation by 50%]. The LD_{50} value [concentration of oligomer (μg/ml) that kills 50% of the cell population] was >100 for each of the compounds listed in Tables 2 and 3.

Several conclusions may be drawn from these data. (*i*) The activity of the parent oligonucleotide, compound **1**, is low (ID_{50} > 100), in agreement with the previous report (12). (*ii*) Anchoring a cholesteryl fragment to the oligonucleotide significantly enhances the antiviral activity (to ID_{50} = 10 for compound **2**). (*iii*) Anchoring a second cholesteryl fragment near the other terminus does not help; indeed, the second cholesteryl fragment leads to a reduction in activity (to ID_{50} = 32 for compound **4**) relative to the monocholesteryl. (*iv*) The antiviral activity of the cholesteryl-modified 15-mer in the phosphodiester series is comparable to that of the related 20-mer (compare compounds **2** and **5**), as in the case of the

Table 2. Inhibition of HIV-1 by cholesteryl-oligonucleotide derivatives

Compound	Concentration, μg/ml	% inhibition			ID_{50}, μg/ml (syncytia)
		Syncytia	p24	RT	
1	0.16	0			>100
	0.8	3			
	4	20			
	20	34			
	100	45			
2	2	0	0		10
	5	4	0	0	
	10	51	63	48	
	20	95	88	90	
	50	100	100	92	
3	2	0	0	0	16
	5	2	13	0	
	10	22	70	0	
	20	77	69	0	
	50	100	100	84	
	100	100	100	100	
4	2	0	0	0	32
	5	3	0	0	
	10	7	0	0	
	20	28	32	26	
	50	85	88	75	
	100	100	100	100	
5	2	0	0	0	11
	5	5	0	0	
	20	92	100	82	
	50	100	100	100	
	100	100	100	100	

For HIV-1 inhibition assays, virus and oligonucleotide were added to Molt-3 cells once at time 0 and the assays were performed at 96 hr. For infection of Molt-3 cells, 500–1000 virus particles per cell were used. Compound **1** is the 20-mer (complementary to the HIV-1 tat splice acceptor site) with no cholesteryl, whereas compounds **2** and **4** have one and two cholesteryl residues, respectively. Compound **5** is a 10-mer with one cholesteryl group. RT, reverse transcriptase.

Table 3. Inhibition of HIV-1 by cholesteryl-phosphorothioate derivatives

Compound	Concentration, μg/ml	% inhibition				ID_{50}, μg/ml (syncytia)
		Syncytia	p17	p24	RT	
6	2.5	15	18	22	33	6.0
	6.25	56	67	81	70	
	10	90	89	89	85	
	25	100	100	100	100	
7	0.25	0	12	19	23	0.8
	1.0	74	69	70	68	
	1.5	100	100	100	100	
	6.0	100	100	100	100	
8	1.6	0	0	0	0	14.5
	6.25	15	16	26	26	
	25	95	84	82	67	
	100	97	96	96	78	
9	1.6	28	39	43	47	3.2
	6.25	98	92	96	73	
	25	98	96	96	76	
	100	98	96	96	88	
10	1.6	0	0	0	0	>100
	6.25	0	4	4	0	
	25	0	20	22	25	
	100	0	24	33	28	
11	1.6	30	42	47	45	3.5
	6.25	97	86	88	61	
	25	97	92	92	70	
	100	98	92	96	89	
12	1.6	28	33	40		3.4
	6.25	93	60	67		
	25	100	100	100		
	100	100	100	100		
13	1.6	20	23	31		3.6
	6.25	89	56	67		
	25	100	100	100		
	100	100	100	100		
14	1.6	18	21	25		13
	6.25	35	30	32		
	25	90	70	66		
	100	100	100	100		
15	25	80	20	25		
	50	99	99	90		
	100	100	100	95		

HIV-1 inhibition assays were carried out as described in *Materials and Methods* and in Table 2 legend. Compounds **6–15** are phosphorothioate derivatives with or without cholesteryl residues as described in Table 1. RT, reverse transcriptase.

unmodified oligonucleotides. (*v*) Anchoring a cholesteryl fragment to a phosphorothioate oligonucleotide analogue enhances the antiviral property of the phosphorothioate derivative (compare compounds **6** and **7**, **8** and **9**, and **10** and **11**). This result is significant since work from several laboratories has shown that phosphorothioate oligonucleotides are more effective inhibitors of protein synthesis than natural type phosphodiester oligomers with the same sequence (9, 10, 24). It is noteworthy that the cholesteryl modification further enhances the relatively high activity of the phosphorothioate derivatives. (*vi*) The activity of the cholesteryl-modified phosphorothioate derivatives shows a downward trend as the length of the oligomer is decreased. Thus, the ID_{50} values increase from 0.8 for the 20-mer (compound **7**) to $\approx$3.5 for the 10- to 15-mers (compounds **11** and **9**) to 13 for the 7-mer (compound **14**). However, the decrease in activity with decreasing size for unmodified phosphorothioate derivatives, also noted by others, is greater [ID_{50} increases from 6 to 14.5 to >100 with progression from the 20-mer (compound **6**), to the 15-mer (compound **8**), to the 10-mer (compound **10**)]. As a consequence, the influence of the cholesteryl modification on activity of the phosphorothioate derivatives is especially striking with the shorter oligomers. For example, attachment of the cholesteryl fragment to the 10-mer (compound **10**) drops the ID_{50} from >100 to $\approx$3.5. (*vii*) Appreciable antiviral activity is found for the 7-mer, d(AsAsAsAsTsG*G) (s, phosphorothioate link O=P—S^-) (ID_{50} = 13), even though binding to the complementary sequence must be very weak (no interaction with the complement, d(CCATTTT), could be detected down to 0°C under our conditions for the binding assays). (*viii*) Inhibition of HIV by the cholesteryl-modified oligonucleotides is not strongly dependent on the nucleotide sequence. This conclusion applies to both the phosphodiester and the phosphorothioate cholesteryl derivatives (compare the data for compound **2** with that for compound **3**, which has six mismatched base sites; and the data for compound **11** with that for **12** and **13**, which have 8 and 3 mismatches). Indeed, for the phosphorothioate derivatives, the activity of all three 10-mers is similar ($ID_{50} \approx 3.5$), although the sequences differ markedly.

DISCUSSION

A convenient procedure is described for preparing oligonucleotides and phosphorothioate oligonucleotide derivatives with a cholesteryl group linked to an internucleoside phosphorus. These cholesteryl-conjugated oligonucleotides are readily soluble in water and form complexes with complementary oligonucleotides that exhibit normal thermal dissociation curves. A striking feature of these compounds is the high activity they exhibit as inhibitors of replication of HIV in tissue culture. The tethered cholesteryl fragment enhances the antiviral activity of both phosphodiester and phosphorothioate oligonucleotides. Of the oligomers examined, ranging from 7-mers to 20-mers, the highest activity was found with a phosphorothioate 20-mer bearing one cholesteryl group (compound **7**). Expression of viral proteins p17, p24, and reverse transcriptase and formation of syncytia were all completely inhibited by this compound at a concentration of $\approx$0.2 μM (1.5 μg/ml). Although the shorter oligomers in this family were less active, the relative impact of the cholesteryl modification was greater, since the unmodified parent phosphorothioates in the decamer range and below exhibit little antiviral activity.

The unusual activity of the cholesteryl-conjugated oligonucleotides makes such amphiphilic oligomers promising targets for further study as potential therapeutic agents in the treatment of AIDS and related viral diseases. In addition, these findings open interesting questions concerning the locus of reaction and the mechanism by which the cholesteryl derivatives inhibit HIV in this cellular system. More data are needed to answer these questions. However, the fact that very short cholesteryl-phosphorothioate derivatives (e.g., heptamer **14**), which bind poorly to complementary strands, exhibit appreciable activity and the observation that the activity of the decamers is not dependent on sequence point toward some mechanism other than "antisense inhibition," at least in the case of the shorter oligomers.

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