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Oligodeoxynucleotide analogs with 5′-linked anthraquinone

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We report here the synthesis of novel 5′-linked oligodeoxynucleotides, both normal phosphodiester and phosphorothioate analogs, in which a covalently attached group at the 5′-terminus is an anthraquinone. These compounds represent a new class of antisense compounds in which the base sequence of the oligodeoxynucleotide serves to deliver a nuclease-resistant reactive drug-like molecule to a cellular target nucleic acid (mRNA or DNA).

Oligodeoxynucleotide, antisense; Nuclease resistance; Anthraquinone, 5′-linked

1. INTRODUCTION

The prospects of antisense oligodeoxynucleotides (ODNs) has attracted a great deal of attention in view of the possibility of selective gene regulation, and the consequent therapeutic implications [1,2]. The selection of methylphosphonate (P-CH_3) [3,4] or phosphorothioate (P-S) [5,6] modified ODNs to provide nuclease resistance has greatly enhanced the possibility that such antisense ODNs could be used in vivo. Indeed, an antisense effect has been shown to occur in vitro for HIV infection in chronically infected T cells [7]. Similar antisense effects have been reported with normal and S-ODNs in cancer cells in which oncogenes are overexpressed [8,9], although complete inhibition of expression was not achieved. In these cases, the S-ODNs were not found to be toxic to the cells, although some of the normal ODNs were somewhat toxic, presumably due to nuclease digestion to mononucleotides [7,10].

These results, while encouraging for the general strategy of using antisense ODNs as drugs, ignore two important factors, one of which is the high cost of producing such drugs, making the approach interesting, but possibly impractical. The other concerns the fact that although a cell surface receptor protein that may be involved in ODN transport has recently been identified [11,12], the S-ODNs are very slowly transported by this mechanism [11,13]. Thus, for both these reasons, cost and cellular uptake, there is a need to increase the potency of the antisense compounds.

Since ODNs appear to produce translation arrest by an RNase H mechanism, it may be argued that the destruction of the mRNA [14,15], the main target in the antisense oligo approach, occurs without the need for an attached chemical moiety. Nevertheless, several types of covalently attached compounds have been described. Helene and coworkers [16,17] have used 3′-linked intercalative groups (mainly acridines) in order to increase the binding capability of a short ODN in binding to its complementary target sequence. They have also linked proflavin to a terminal thiophosphate group at either the 3′- or 5′-end of an octadeoxy-

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Abbreviations: ODN, oligodeoxynucleotide; S-ODN, phosphorothioate oligodeoxynucleotide; AQ, anthraquinone; p-, piperazinyl linker; m-, methylene linker; α-rev, antisense rev sequence of HIV; HIV, human immunodeficiency virus; T_m, melting temperature

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thymidylate [18]. The proflavin was then activated by light irradiation. Knorre and co-workers [19,20] have produced a series of covalently attached alkylating groups that are designed to modify the complementary strand. These workers have also attached cholesteryl groups to oligos in an attempt to increase the rate of cellular uptake [21]. EDTA or other chelating agents have also been attached to the oligo in order to bind a metal ion and bring about destruction of DNA [22–25].

Our strategy is to attach groups onto oligos at the 5′-terminus using the intermediate linked phosphoramidite synthons in the automatic synthesizer [13,26]. This is the most efficient approach since the 3′-terminus is attached to the solid support, and the entire synthesis occurs in the synthesizer. In some cases the linkage to the group is not stable under the conditions of acid and base used in the automated synthesis, and therefore a linker attached to a phosphoramidite was used, followed by subsequent attachment of the group [26]. In the case of the 9-aminoacridine bond, which is susceptible to cleavage by NH_4OH, substitution of NaOH prevents cleavage of the linked group [27,28].

We have previously attached acridines using these methods [13,26], and describe here the products containing anthraquinones (fig.1). This group was chosen because it is a potential radical-producing moiety, that does not necessarily require the presence of a metal ion or optical activation, and can act very much like a series of well-known radical-producing anti-cancer drugs [29], such as adriamycin. Given the possibilities of chemical variation, including the length of the linker arm, as well as the ODN itself, and the attached group, this compound represents a novel approach to the increase in potency of the antisense method.

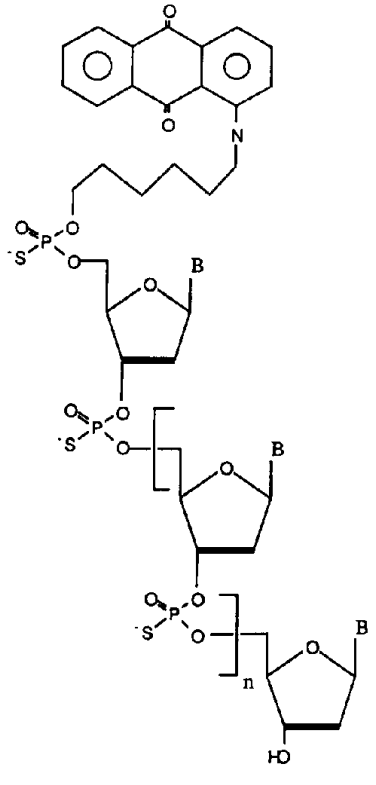

Fig.1. Structure of an anthraquinone 5′-linked to a phosphorothioate oligodeoxynucleotide analog via a methylene linker (AQ-m-S-ODN).

2. MATERIALS AND METHODS

2.1. *Preparation of anthraquinone-piperazinyl derivative*

A mixture of 1-chloroanthraquinone (1 g) and 1-(2-hydroxyethyl)piperazine (5 g) was heated at 150°C for 30 min. After cooling to room temperature, water was added, and the material filtered. Recrystallization from chloroform gave 1-[1-(2-hydroxyethyl)piperazinyl]anthraquinone (fig.2) in a yield of 79% (1.1 g); m.p. 168°C; ^{1}H NMR spectrum ($CDCl_3$): 8.28 (1H, doublet, C8), 8.24 (1H, doublet, C5), 7.97 (1H, doublet, C4), 7.75 (2H, multiplet, C6,7), 7.66 (1H, triplet, C3), 7.41 (1H, doublet, C2), 3.71 (2H, triplet, CH2), 3.35 (4H, triplet, 2 × CH2), 2.87 (4H, triplet, 2 × CH2), 2.71 (2H, triplet, CH2), 1.61 (1H, singlet, OH) ppm; MS: 337 (M + 1, 100%), 338 (M + 2, 21%), 339 (M + 3, 7%).

2.2. *Synthesis of anthraquinone-piperazinyl phosphoramidite*

1-[1-(2-Hydroxyethyl)piperazinyl]anthraquinone (336 mg, 1 mmol) was dissolved in CH_2Cl_2 (2 ml), and *N*-ethyldiisopropylamine (760 μl, 4 mmol) and *N,N*-diisopropylmethylphosphonamidic chloride (194 μl, 1 mmol) were added. After 30 min the solution was poured into ethyl acetate (5 ml, previously washed with 2 × 5 ml of 5% $NaHCO_3$, and 2 × 5 ml saturated NaCl). The ethyl acetate phase was dried over Na_2SO_4 and evaporated to an oil. TLC on silica gel (ethyl acetate/triethylamine, 9:1) showed complete reaction (R_f of starting material 0.3, R_f of product 0.7). ^{31}P NMR spectroscopy of the product at 162 MHz gave a single peak at 148.048 ppm of the product *N*-anthraquinonyl-*O*-methoxydiisopropylamino phosphite 1-(2-hydroxyethyl)piperazine (fig.2).

2.3. *Preparation of anthraquinone methylene-linked phosphoramidite*

Preparation of the methylene-linked anthraquinone phosphoramidite was accomplished as described above, but

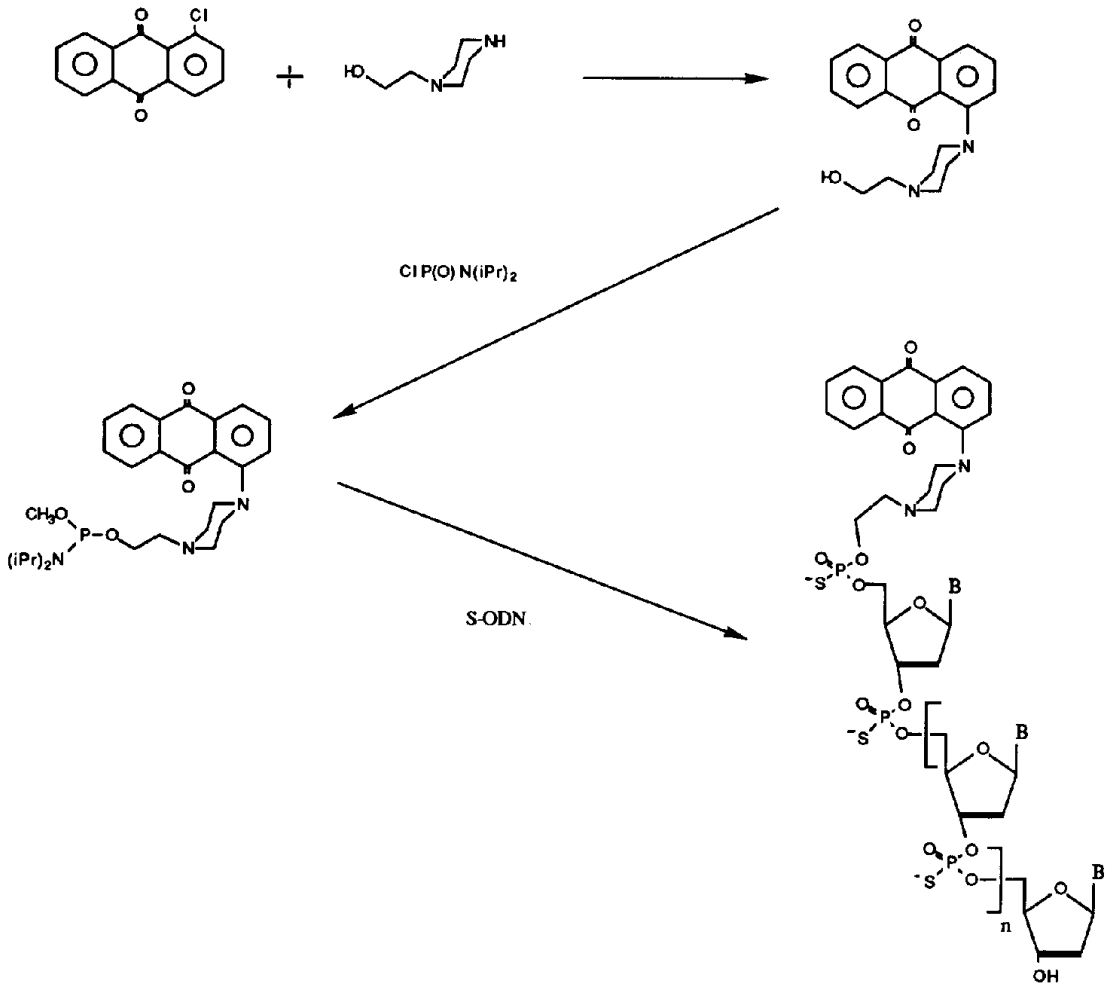

Fig.2. Synthesis of an anthraquinone 5′-piperazinyl-linked oligodeoxynucleotide via an intermediate linked phosphoramidite.

with substitution of the hexamethylene linker, AQ-$(CH_2)_6$-OH. ^1H and ^{31}P NMR spectra and MS were consistent with the purified product (fig.1).

2.4. *Synthesis of anthraquinone-linked ODNs*

Without further purification the anthraquinone-linked phosphoramidites were dissolved in the appropriate amount of acetonitrile and used directly in oligonucleotide preparation. Oligonucleotides, including phosphorothioate analogs, were synthesized as in [30] on an Applied Biosystems 380B synthesizer with the anthraquinone group attached at the 5′-end; a 15-mer all-PO (60% yield), the same all P-S (40% yield), a 28-mer all-PO (40% yield), the same all-PS (30% yield). The anthraquinone-containing oligomers were characterized by UV, and ^1H and ^{31}P NMR. A ^1H NMR spectrum of the aromatic region of anthraquinonylpiperazinyl-5′-dT_4 is shown in fig.3, in which the protons of both the thymine and anthraquinone moieties are assigned by comparison with precursors.

2.5. *Melting temperatures*

Melting curves were determined as described [30], using a Gilford Response II automatic temperature-adjusting spectrophotometer. In fig.4 we present the melting curves of normal and phosphorothioate piperazinyl-linked anthraquinone oligothymidines. The reduction in T_m due to sulfur substitution is similar to the reported value for these compounds [30]. The T_m values of both piperazinyl- and methylene-linked anthraquinones, compared to the appropriate oligo controls, are increased by approx. 6–12°C (table 1). These results indicate that the anthraquinone moiety is intercalated in each case, and that the difference between heterocyclic and methylene linkers is minimal. We also report the T_m of the α-rev HIV sequence, which shows that, as expected for a 28-mer, the increase in T_m due to intercalation is small, but not negligible in the case of the persulfurized oligo. It is noteworthy that the ΔT_m is approx. 2-fold greater for the phosphorothioated anthraquinone compounds vs the normal linked oligodeoxynucleotides.

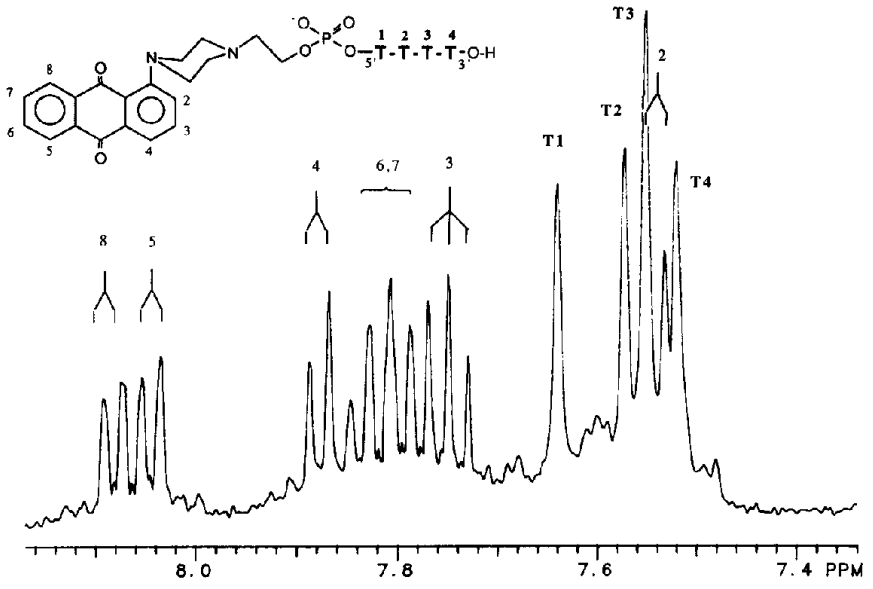

Fig.3. ^{1}H NMR spectrum at 400 MHz of the aromatic region of the anthraquinone 5′-piperazinyl phosphorothioate tetrathymidylate (AQ-p-S-dT$_4$). The individual anthraquinone and thymidine resonances are of equal area.

3. RESULTS AND DISCUSSION

Our main objective is to obtain oligodeoxynucleotides and analogs with covalently attached groups that confer biological activity upon the product. To accomplish this we synthesized oligodeoxynucleotides in the automatic synthesizer with 5′ covalently attached chemical groups via intermediate linked phosphoramidites.

This approach was chosen as an efficient method of obtaining reasonable yields of the desired linked product. Other more standard procedures, including synthesis of the oligo followed by covalent attachment of the linker and/or the desired chemical group outside the automatic synthesizer, or linkage to positions other than the 5′-terminus (such as 3′- and/or at intermediate

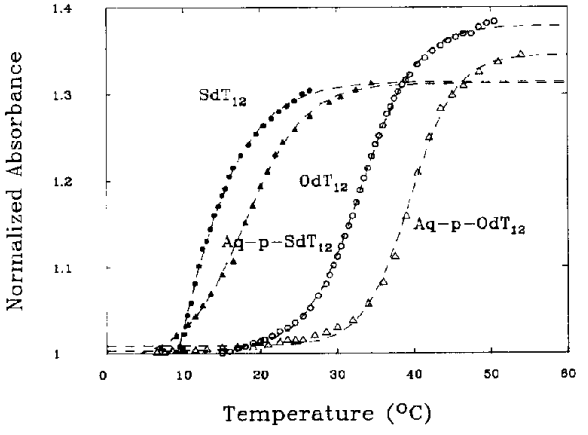

Fig.4. Melting curves of normal and phosphorothioate oligodeoxynucleotides with and without attached 5′-piperazinylanthraquinones (AQ-p-dT$_{12}$) with poly(rA). The T_m values are listed in table 1. Conditions and curve fitting were as described in [30].

Table 1

Melting temperatures of oligodeoxynucleotides with poly(rA)

Oligo	T_m	ΔT_m	ΔH
O-dT$_{12}$	33.1		33.6
S-dT$_{12}$	7.5	−25.6	32.5
AQ-m-O-dT$_{12}$	41.3	8.2	60.6
AQ-m-S-dT$_{12}$	19.2	11.7	31.9
AQ-p-O-dT$_{12}$	39.5	6.4	65.0
AQ-p-S-dT$_{12}$	18.0	10.5	43.0
AQ-p-O-dT$_8$	27.5		50.8
AQ-p-S-dT$_8$	–		–
O-α-rev	78.6		
S-α-rev	66.8	−11.8	
AQ-O-α-rev	81.4	2.8	
AQ-S-α-rev	71.5	4.7	

T_m and ΔT_m are expressed in °C, ΔH in kcal/mol

points within the oligo) could also be used to attach such groups.

A novel example described here is the synthesis of anthroquinone 5′-linked to a series of oligodeoxynucleotides of different base sequences by two kinds of linkers, methylene and piperazinyl. This is an example of a wide range of feasible compounds of general form R-L-ODN, where R represents the attached group, L is a linker arm, and ODN is the oligodeoxynucleotide. The ODN in this case can be either a normal oligo or a chemically modified analog, such as a phosphorothioate (containing PS), or a copolymer combination of both. An important and novel aspect of our work is that such covalently linked groups have not previously been attached to phosphate-modified (such as PS-containing oligos), but only to normal phosphate oligos.

In the present work, an anthroquinone derivative was linked via either a hydroxyethylpiperazinyl (fig.2) or a methylene bridge (fig.1) to an ODN. Piperazinyl was chosen as a tertiary amine that was expected to stabilize the link. A diisopropyl phosphoramidite (diisopropylamino phosphite) *O*-methyl ester was prepared from these compounds. This was then attached via the standard nucleotide cycle to the 5′-end of an oligonucleotide chain in the automatic synthesizer. The products were characterized by the usual methods (^{1}H NMR, ^{31}P NMR, UV, MS), and purified by HPLC. Measurements of T_m values indicate that intercalation of the anthraquinone occurs with both linkers (table 1). Specific base sequences that were synthesized with 5′-linked anthroquinone are: dT_1, dT_4, dT_8, dT_{12}, dT_{15}, dC_{15}, and anti-sense phosphorothioate oligos: c-*myc* 15-mer [8,9] and the anti-rev (S-α-rev) HIV sequence: d(5′-AQ-TCG TCG CTG TCT CCG CTT CTT CCT GCC A) [7].

The anthraquinone-ODNs described here exhibited anti-HIV activity in an in vitro assay system [31], and can be expected to show other antiviral properties. The anthroquinone group is an oxidizing agent and radical-producing moiety, and the oligonucleotide is regarded as a means of delivering the group (R) to a biological site (mRNA, for example) where it can cause destruction and/or bind (intercalate) to prevent normal biological functioning. Other groups that could be attached in a similar manner to that described here include other anthraquinones [32], and known drugs, such as adriamycin and bleomycin.

These compounds would then be used as drugs to inhibit or to attenuate viral activity or mammalian gene (oncogene) expression. The choice of base sequence of the ODN and/or the type of ODN analog would depend on the target sequence in the target gene chosen. The results of inhibition by the S-α-*rev* sequence in an in vitro T-cell viral *gag* protein assay have been compared with the same sequence 5′-linked to acridine [13] and anthraquinone. The latter was the most effective (10-fold vs unlinked sequence) at inhibiting HIV expression [31]. While the anthraquinone increases the stability of the S-α-rev hybridization (table 1), the difference is not thought to be sufficient to give rise to such a biological effect. Further studies regarding the mechanism of this effect and the synthesis and testing of these and other linked compounds are in progress.

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