Bioorganic & Medicinal Chemistry Letters, Vol.3, No.6, pp. 1223-1228, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

ANTI-HIV ACTIVITIES AND PHYSICOCHEMICAL PROPERTIES OF PHOSPHOROTHIOATE ANALOGUES COMPLEMENTARY TO HIV SEQUENCES

Sang-Gug Kim, Kenji Kanbara, Hideki Nakashima[1], Naoki Yamamoto[1], Kunichika Murakami[2], and Hiroshi Takaku*

(*Received in USA* 6 *July* 1992; *accepted* 5 *January* 1993)

Department of Industrial Chemistry, Chiba Institute of Technology, Narashino, Chiba 275, Japan; [1]Department of Microbiology, Tokyo Medical and Dental University School of Medicine, Yushima, Bunkyo-ku, Tokyo 113, Japan; [2] Research Laboratory of Bioresources, Sanyo-Kokusaku Pulp Co., LTD., Iida, Iwakuni, Yamaguchi 740, Japan.

ABSTRACT: The modifications of oligodeoxyribonucleotides include replacement of the other chain either the whole-PS (S-ODNs) group, or group end-capped with PS (SO-ODNs) at both 3'- and 5'-ends. The melting temperature of the duplexes for one normal chain with modified phosphorothioate oligomers indicates diminished hybridization capability of the whole phosphorothioate oligomers relative to both the unmodified phosphodiester oligomers and the partially phosphorothioate-diester oligomers. In the assays of HIV, oligomers (S-ODNs) with complete replacement of the phosphodiesters with phosphorothioate groups were found to be very active. Finally of particular interest are the oligomers complementary to the HIV sequences (S-ODNs-rev or tat, 15 and 20mers) which possessed higher anti-HIV activity than the homooligomers (S-dC_{28} or S-dC_{20}) itself.

Recently, oligonucleotides complementary to viral RNA have been shown to inhibit viral replication in cell cultures with rous sarcoma virus[1], human immunodeficiency virus (HIV)[2,3], vesicular stomatitis virus[4,5], herpes simplex virus[6,7], and influenza virus[8,9]. Experiments with normal (phosphodiester-bonds) antisense oligodeoxyribo-nucleotides as inhibitors have indicated that these relatively short oligonucleotides can be taken up by cells in culture and may also be effective in producing modulating of gene expression. The relatively short half-lives of normal oligonucleotides in serum and in cells due to the presence of nucleases, and low permeability of these charged molecules into normal cells, limit their potential usefulness *in vivo*. To overcome these problems, some of antisense oligonucleotides have been modified on the backbone as methylphosphonates[4] or phosphorothioates (S-ODNs)[3], S-ODNs have been shown to inhibit the *de novo* infection of susceptible cells by the HIV and to inhibit viral expression and proliferation in already infected cells[2,3,10-13]. Matsukura et al. have reported that the inhibition of *de novo* infection by S-ODNs is both composition-dependent and length-dependent: as examples, S-dC_{28} is a better inhibitor than S-dA_{28} or S-ODNs (20mer, coding exon I of art/trs gene in HIV). To study the dependence of both composition and length of the phosphorothioates for anti-HIV activity, we have synthesized the phosphorothioate oligodeoxyribonucleotide analogues. We herein report the synthesis of one normal chain and the other chain either of whole-PS

group, or group end-capped with PS at both 3'- and 5'-ends in the sequences of anti-rev[14-16] and tat 20mers[17-19] (See Fig. 1), as well as their physicochemical properties (melting temperature and CD spectrum) and anti-HIV assay.[20]

The ability of oligonucleotides to hybridize to a complementary DNA or RNA strand is important for their use as antisense oligomers. We have measured the melting temperature

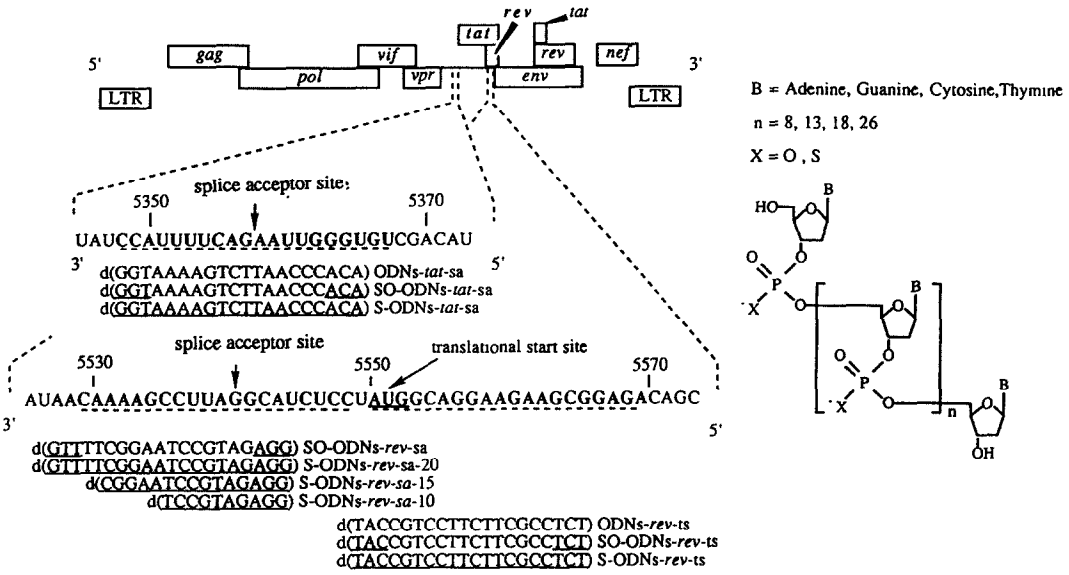

Fig. 1. General molecular structure of normal (X=O; ODNs), 3',5'-phosphorothioate end capped oligomer (X=S,O; SO-ODNs) and phosphorothioate (X=S; S-ODNs). The chemical structures of oligomers are shown at the top and their sequences corresponding to complementary regions of the HIV genome are shown. The phosphorothioate regions are underlined.

Table 1. Melting temperature and enthalpies duplexes of RNA with modified and unmodified DNA.

Duplexes	Tm(°C)	$-\Delta H$(Kcal mol^{-1})
ORNs/ODNs	48.1	67.7
ORNs/SO-ODNs	45.5	44.4
ORNs/S-ODNs	35.5	33.1

The duplexes are 5'AUGGCAGGAAGAAGCGGAGA3'(ORNs)/rev-ts-5'TCTCCGCTTCTTCCTGCCAT3'(ODNs); 5'AUGGCAGGAAGAAGCGGAGA3'(ORNs)/rev-ts-5'TsCsTCGCTTCTTCCTGCCsAsT3'(SO-ODNs), and 5'AUGGCAGGAAGAAGCGGAGA3'(ORNs)/rev-ts-5'TsCsTsCsCsGsCsTsTCsTsTsCsCsTsGsCsCsTs(S-ODNs).

of the duplexes of the analogues phosphorothioates and unmodified oligonucleotide, hybridized to their complementary RNA strand (Table 1).[21] Tm is depressed from unmodified phosphodiester oligomer to partially phosphorothioate-diester oligomer to whole phosphorothioate oligomer. The depression Tm per modified bond, ΔTm, for a rev-ts-20mer is ca. 0.5

°C for the whole phosphorothioate oligomer (S-ODNs) and ca. 1.6 °C for the partially phosphorothioate-diester oligomer (SO-ODNs). The whole phosphorothioste oligomer leads to markedly decreased values of ΔH as compared to the unmodified oligomer. The reason for the

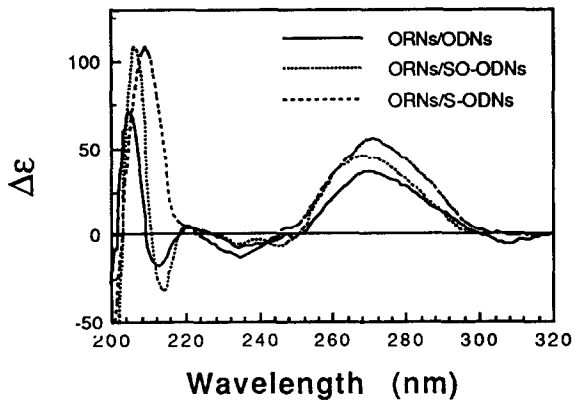

Fig. 2. Circular dichroism spectra for 5'AUGGCAGGAAGAAGCGGAGA3'(ORNs)/rev-ts-5'TCTCCGCTTC-TTCCTGCCAT3'(ODNs); 5'AUGGCAGGAAGAAGCGGAGA3'(ORNs)/rev-ts-5'TsCsTCGCTTCTTCCTGCCsAsT3'(SO-ODNs), and 5'AUGGCAGGAAGAAGCGGAGA3'(ORNs)/rev-ts-5'TsCsTsCsCsGsCsTsTCsTsTsCsCsTsGsCsCsTs (S-ODNs) performed in 10 mM sodium cacodylate (pH 7.0) containing 140 mM NaCl.

depression of the Tm of phosphorothioates analogues may be an altered conformation of the backbone due to the sulphur atoms and their stereoisomeric species.

The CD spectra of oligonucleotides are presented in Fig. 2.[22] We note a few interesting features in these CD spectra. The CD spectrum of the unmodified phosphodiester oligomer (ODNs), the partially phosphorothioate-diester oligomer (SO-ODNs) and the whole phosphorothioate oligomer (S-ODNs) showed the regular A type CD spectrum, almost no difference is found in the major absorption band. However, in S-ODNs, the sing of Cotton effect at the 255 nm changed to the positive band from the negative in ODNs. This can be explained by assumption that S-ODNs has stereoisomeric species. On the other hand, SO-ODNs and ODNs gave Cotton curves of the same profile. The result, including Tm's is apparent that all oligomers having the phosphorothioate bond showed a significant decrease in duplexes stability.

Oligonucleotides with chain lengths of 20 bases, complementary to two sites in HIV RNA were shown to be targets of inhibition, and were tested for inhibition of HIV replication. These sequences were a region at the start of translation (AUG) of rev-RNA and a splice acceptor site used to generate rev and tat RNAs (Fig. 1). Fig. 3 showns the results of the antiviral effect and cytotoxicity of oligomers of different phosphate backbones.[23] We could not detect any inhibitory effects of either the unmodified oligomers (ODNs-rev-ts) or the 3',5'-capped phosphorothioate substituted-oligomers (SO-ODNs-rev-ts) at a concentration of 0.5 μM. The ODNs or SO-ODNs 20mer complementary to a sequence of the

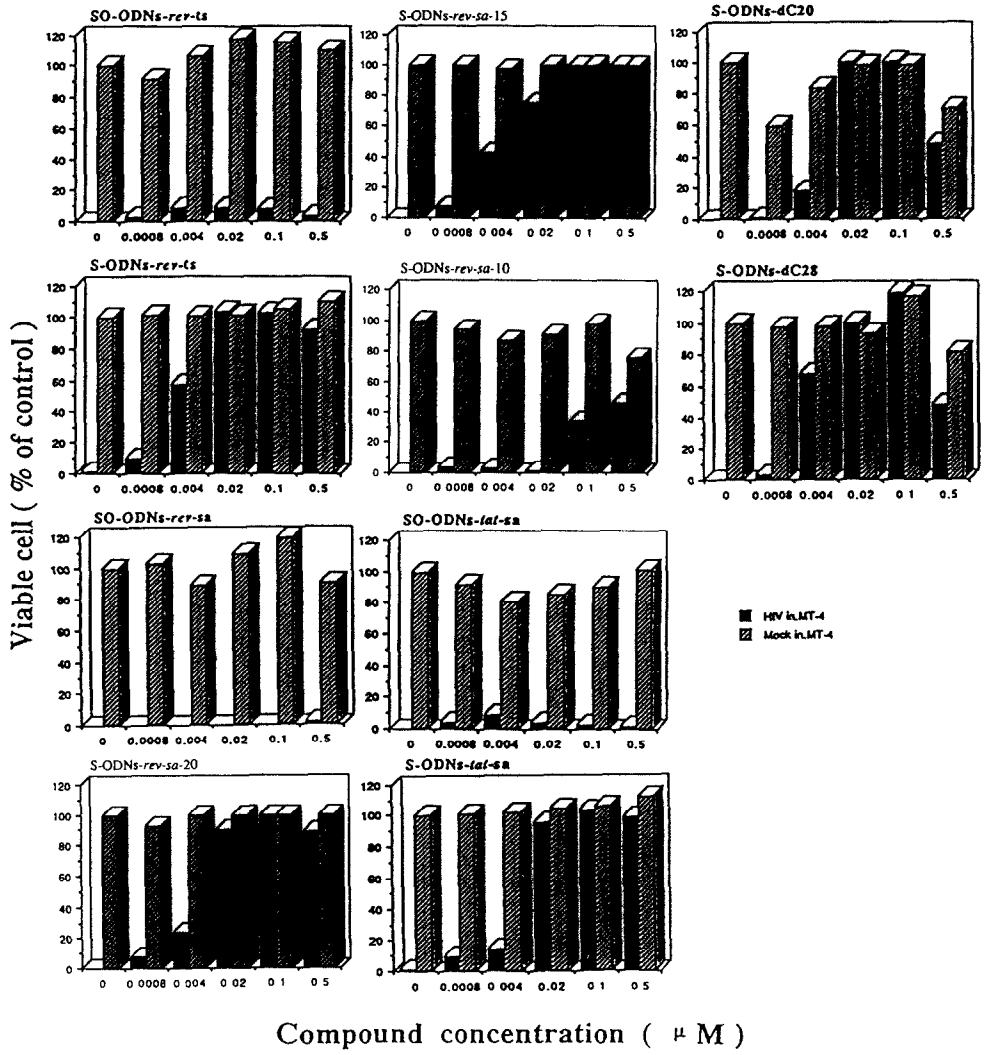

Fig. 3. Inhibitory effects of various oligomers. Filled columns represent virus-exposed cells and hatched columns represent non-virus exposed cells.

HIV tat gene were also used in the anti-HIV assay and gave similar results.

On the other hand, the whole phosphorothioate oligomers (S-ODNs-rev-ts or sa and tat-sa) tested showed very potent anti-HIV effects and exhibited significant inhibition of the cytopathic effect of HIV. The oligomers at a concentration as low as 0.02-0.5 μM gave complete protection against the virus induced CPE. The S-ODNs-tat-ts and rev-ts or sa were found to be more effective than the S-dC_{28} (28mer) and S-dC_{20} (20mer) at the same molar concentration of nucleotide unit. Furthermore, even at a concentration of 5 μM, no

cellular cytotoxicity was observed (data not shown). However, the S-dC_{28} (28mer) and S-dC_{20} (20mer) which were active, and activity was of the same order as for the antisense phosphorothioate oligomers with chain length of 20 (S-ODNs-rev or tat) at 0.02 μM but at 0.5 μM showed low activity and some toxicity. In the above assay, only whole phosphorothioate oligomers (S-ODNs-rev or tat) showed anti-HIV activity, and which is probably due mainly to the relative resistance of S-ODNs to nucleases that keeps them intact relative to S-ODNs and allows them to reach and remain at the target site. Surprisingly, the 3',5'-capped P-S oligomers did not show any anti-HIV activity in our assay. This finding suggests that they are digested by the enzyme in an endonucleolytic manner rather than exonucleolytic cleavage. In addition, the anti-HIV activity of antisense oligomers are influenced by the resistance of oligonucleotides to nucleases than the stability of RNA-DNA duplexes (Tm and CD data).

Finally, the different chain length of oligonucleoside phosphorothioates were studied and the target site on HIV RNA was used as with a splice acceptor site of HIV rev gene. The oligonucleoside phosphorothioate with chain length of 15 (S-ODNs-rev-sa-15) was equally active as the phosphorothioate oligomer with chain length of 20 (S-ODNs-rev-sa-20) but the oligonucleoside phosphorothioate with chain length of 10 (S-ODNs-rev-sa-10) showed no activity. Of particular interest is S-ODNs-rev-sa-15 which was active, and activity was of the same order as for the phosphorothioste oligomer with chain length of 20 (S-ODNs-rev-sa-20). However, the oligomer chain length should had greater effect on the antiviral activity, with longer compounds being more potent.

A mechanism of action other than antisense competitive hybridization might be operational. In addition, these findings open interesting questions concerning the mechanism by which the anitisense oligomers (20mer) inhibit HIV in this cellar system. The fact that antisense oligomers (20mer) should be considerably useful than the homooligomers.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas No.04269108 from the Ministry of Education, Science and Culture, Japan, and the Research Grant from the Human Life-Science Foundation and the Daiwa Health Care Foundation.

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21. Melting temperature was measured with a Shimadzu UV-160 spectrophotometer equipped with a temperature controller (TCC-240A, Shimadzu) in 10 mM sodium cacodylate buffer (pH 7.0) containing 140 mM NaCl. The temperature of the solution was increased linearly with time at a rate 15°C/h.
22. The CD spectra of oligonucleotides were measured on a JASCO J-6 spectropolarimeter in the same buffer melting temperature experiment described.
23. Anti-HIV activity of test compounds in fresh cell-free HIV infection were determined by the protection for HIV-induced cytopathic effects (CPE). Briefly, MT-4 cells was infected with HTLV-IIIB at the multiplicity of infection (MOI) of 0.01. HIV-infected or mock-infected MT-4 cells (1.5×10^5/mL, 200μL) were placed into 96 well microtiter plates and incubated in the presence of various concentrations of the test compounds (The dilution factor from one concentration to 2 or 5 times and examined with 9 different concentrations of each compound. All experiments were carried out in triplicate.). After 5 days-culture at 37°C in a CO_2 incubator, cell viability was quantified by a colorimtric assay by monitoring the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to blue formazan product. The absorbances were read by a microcomputer controlled photometer (Titertek Multiskan^R; Labsystem Oy, Helsinki, Filnland) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically substracted from the absorbance at 540 nm, so as to eliminate the effects of non-specific absorption. All data represent the mean values of triplicate well. These values were then expressed as percentage cytotoxicity and percent antiviral protection, from which the 50% cytotoxic concentration (CC_{50}), 50% effective concentration (EC_{50}) and selectivity indexes (SI) were calculated[24,25].
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