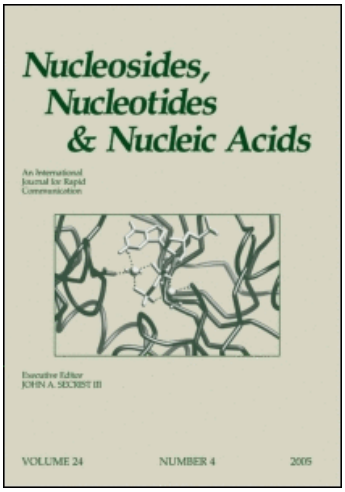

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SYNTHESIS OF BASE MODIFIED PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES AS INHIBITORS OF HIV-1

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Summary. By the H-phosphonate approach on a solid support we have synthesised a 28-mer homopolymer of 2'-deoxycytidine (SdC_{28}), a 28-mer phosphorothioate oligodeoxynucleotide (*Srev*) complementary to the HIV-1 regulatory gene and a number of its base modified derivatives. Anti HIV-1 activity of $S\text{-}dC_{28}$ and Srev in chronically infected T-cells was studied. Melting temperatures of *rev*-comp *rev* and *Srev*-comp *rev* were also measured.

Introduction

Synthetic oligodeoxynucleotides with base sequences complementary (or antisense) to various regions of the viral mRNA, have been found to inhibit viral expression[1-4]. Furthermore, it has been found that phosphorothioate oligodeoxynucleotides (S-ODNs) which have a modified backbone, in which a single sulphur atom is replaced for an oxygen atom on each phosphate, are more effective anti-HIV agents[5-7]. It is thought that this substitution confers resistance to nucleases in the host cell without significantly impairing hybridization of the S-ODN with mRNA[8]. In addition, the hybrids between S-ODNs and mRNA are effective substrates for RNase H in which the mRNA strand is digested.

Since the mode of action of antisense S-ODNs has been shown to involve complementary base pairing, chemical modifications to S-ODNs which increase the S-ODN/mRNA duplex thermal stability, would be expected to increase their anti-viral activity. A number of alterations to nucleic acid bases are known to increase duplex stability[9], notably: 5-bromo or 5-iodo cytosine in place of cytosine and 5-bromo uracil in place of thymine. The reasons for increased duplex stability are not fully understood and the observed stabilisation varies with the immediate base- stacking environment. However, halogenation of pyrimidines increases acidity of the base, which is likely to increase the strength of inter-base hydrogen bonds and dipole-dipole base stacking interactions. Bulky halogen atoms also change the pattern of major grove hydration. Halogenated bases would be more hydrophobic and therefore might also have increased cellular permeability.

In an attempt to develop S-ODNs with increased anti-HIV activity, synthesis of a number of these base modified S-ODN was undertaken. *Srev* (S-ODN complementary to the 5′ coding region of the HIV-1-IIIB*rev* gene[7]) was chosen because this sequence has the highest reported anti-HIV activity. Also, with 22 pyrimidines in its structure, *Srev* would be ideal for substituting halogenated bases. Furthermore, halogenated base modified *Srev* would be more hydrophobic and therefore might also have increased cellular permeability.

Synthesis

Synthesis of three H-phosphonate monomers for incorporation into base modified S-ODNs was achieved by benzoylating the exocyclic amines, tritylating the 5′-hydroxyls and phosphonylating the 3′-hydroxyls of 5-bromo 2′-deoxycytidine, 5-methyl 2′-deoxycytidine and 5-bromo 2′-deoxyuridine. Benzoylation was achieved by

reacting the nucleosides with 2,3,4,5,6-pentafluorophenyl benzoate in pyridine[10] or with benzoyl chloride in pyridine followed by alkaline hydrolysis of the product[11]. Tritylation was carried out with dimethoxytrityl chloride in pyridine in the presence of DMAP[11]. For the phosphonylation of 3′-hydroxyls, *tris* (1,1,1,3,3,3-hexafluoro-2-propyl)phosphite[12] in pyridine was used as the phosphitylating reagent.

All S-ODNs were synthesised and deprotected on an Applied Biosystems Model 380B DNA Synthesiser that employed manufacturer supplied reagents and solvents. The H-phosphonates were used with modification to manufacturer supplied cycles for 'trityl-on' synthesis on 1 μM scale CPG column supports. After the chain elongation cycles, the oxidation of the support-bound oligodeoxynucleotide H-phosphonate to the phosphorothioate internucleotide linkage was carried out by manual sulphurization using a solution of sulphur in a mixture of CS_2/pyridine/Et_3N. Base protecting groups were removed by heating the solution of the S-ODNs in conc. ammonium hydroxide at 55°C for 12 hours. For the synthesis of S-ODNs yields per coupling step, as determined by trityl cation released at each detritylation step, were ⩾97%. The ammonia from the deprotection process was removed in the presence of Et_3N (to preserve the trityl group) in the usual manner and the resultant crude product was purified by HPLC using a reversed-phase (C8) column and a linear gradient of increasing acetonitrile concentration (20-50%) over 30 min., in 0.1 M-triethylammonium acetate buffer at pH 7. Detritylation of S-ODNs was accomplished in 3% aq. acetic acid (15 min.). The product was purified twice over Sephadex (NAP) column, equilibrated and eluted with Milli-Q water. The S-ODNs were then deionized using Dowex-Na^+ form and Milli-Q water as eluent. Finally, the products were frozen in dry ice and lyophilized. This procedure was used to produce non-cytotoxic oligodeoxynucleotides in several milligram quantities.

Table 1. Sequences of Synthesised S-ODNs

Sequence No.	Sequence (Abbreviation)
1	28-mer homopolymer of 2'-deoxycytidine (SdC28)
2	28-mer complementary to the 5'coding region of the HIV-1 IIIB *rev* gene (S*rev*) 5'-TCG.TCG.CTG.TCT.CCG.CTT.CTT.CCT.GCC.A-3'
3	S*rev* in which C = 5-Bromo 2'-deoxycytidine (S*rev* 5-BrC)
4	S*rev* in which C = 5-Methyl 2'deoxycytidine (S*rev* 5-MeC)
5	S*rev* in which T = 5-Bromo 2'-deoxyuridine (S*rev* 5-BrU)
6	S*rev* in which C = 5-Bromo 2'deoxycytidine and T = 5-Bromo 2'deoxyuridine (S*rev* 5-BrC 5-BrU)
7	S*rev* in which C = 5-Methyl 2'-deoxycytidine and T = 5-Bromo 2'deoxyuridine (S*rev* 5-MeC 5-BrU).

For biological experiments phosphorothioates were used as mixtures of diastereomers.

Biological Evaluation

In agreement with the results of Matsukura et al[3], we have found that S*rev* inhibits the accumulation of HIV-1 p24 gag protein in culture supernatants of chronically infected H9 cells, without cytotoxic effects. In addition, the homopolymer SdC_{28}, which we have previously found to inhibit *de novo* infection of H9 cells, has no inhibitory effect in chronically infected cells. Figures 1 and 2 show the time course of inhibitory effects and results of cytotoxicity studies. The different effects of S*rev* and SdC_{28} suggests that virus expression in chronically infected cells is inhibited through an antisense effect. The anti-viral activity of base modified S-ODNs, designed to increase duplex thermal

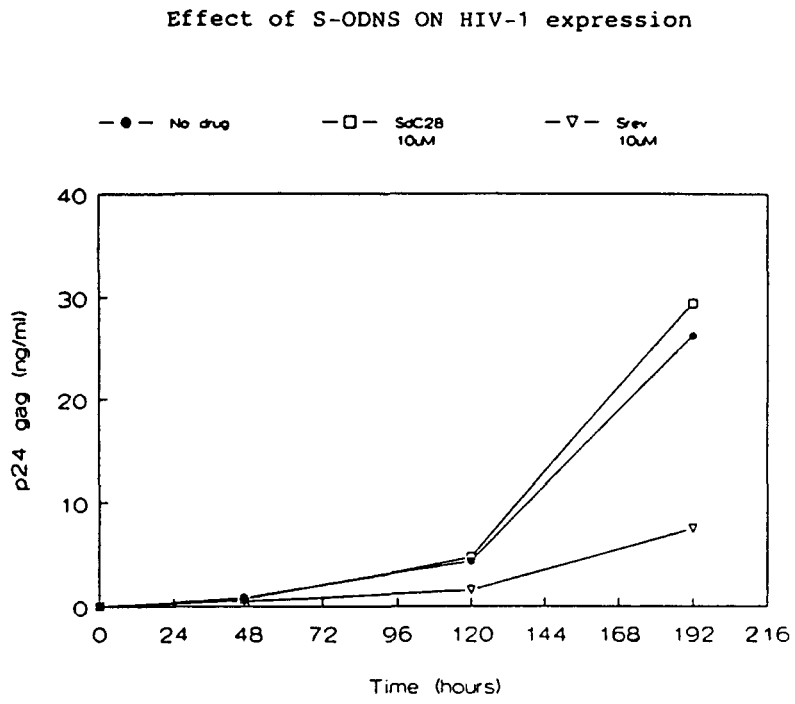

Figure 1. After 120 hours growth of H9/IIIB in the presence of SdC_{28} or Srev (see Figure 1), paraformaldehyde was added to a final concentration of 0.5%. After thorough mixing, cells were counted by haemocytometry. The cell densities of three separate cultures were determined for each addition.

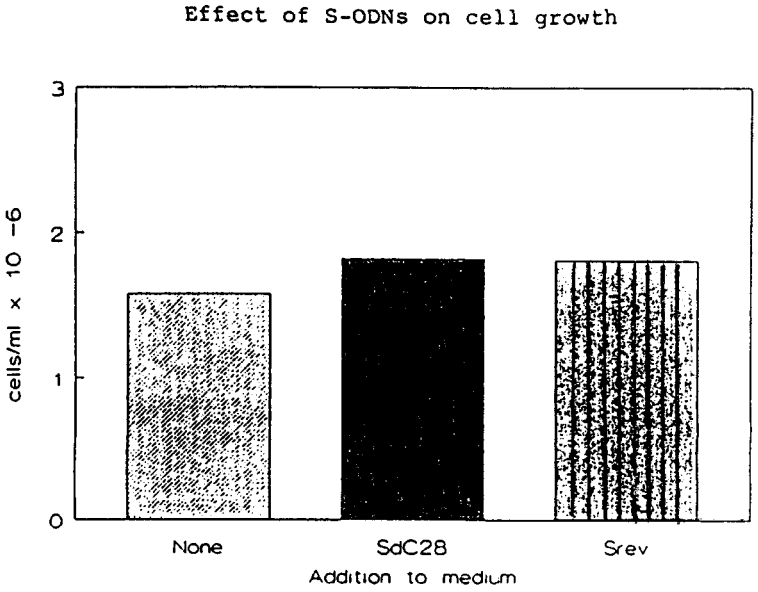

Figure 2. H9 cells, chronically infected with HIV-1 IIIB were washed to remove extracellular virus and cultured (1,250 cells/well in a 96-well culture plate) in the presence of 10 uM, SdC_{28} and Srev in 250 ul of medium (RPMI 1640 with 15% foetal calf serum, 4 mM L-Glutamine, 50 mM 2-mercaptoethanol and 50 u/ml of both penicillin and streptomycin). At intervals 150 ul of culture supernatant were taken for p24 gag ELISA (Coulter).

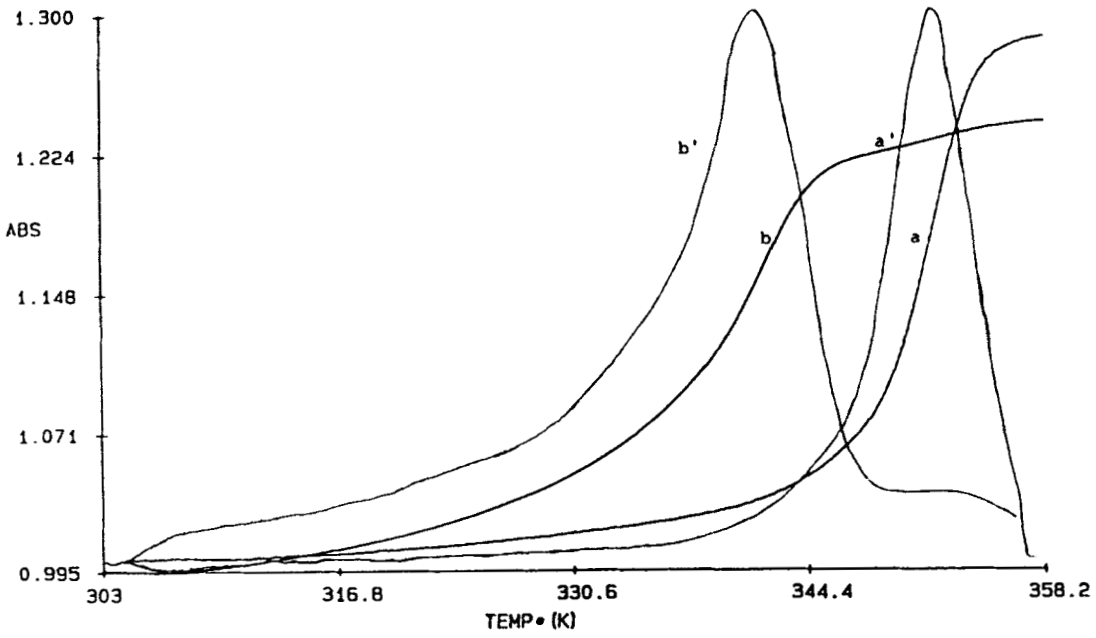

Figure 3. Melting curves of (a) rev-comp rev duplex (T_m= 350°K); a' = first derivative of a. (b) Srev-comp rev duplex (T_m = 340.8°K); b' = first derivative of b. These were measured in triplicate at 264 nm in 0.1M NaCl buffer containing 10 mM NaH_2PO_4 and 1 mM EDTA at pH 7.3. All data was processed using the Pecss system of Perkin Elmer.

stability, is in progress using the activity of *Srev* as a baseline.

Melting Temperatures

Preliminary u.v. melting studies carried out on *rev*-comp-*rev* and *Srev*-comp *rev* duplexes revealed that the respective melting temperatures (T_m) were 340.8°K and 350°K at 15 μM concentration as shown in Figures 3a and 3b. Detailed studies on thermodynamic properties of these duplexes as well as those of base modified *Srev* with comp *rev* are in progress.

Acknowledgements

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References

1. Goodchild, J.; Agrawal, S.; Civiera, M.P.; Sarin, P.; Sun, D. and Zamecnik, C., *Proc.Natl.Acad.Sci. USA*, 1988, 85, 5507.
2. Zamecnik, P.C. and Stephenson, M.L., *Proc.Natl.Acad. Sci. USA*, 1978, 75, 280.
3. Zamecnik, P.C.; Goodchild, J.; Taguchi, Y. and Sarin, P.S., *Proc.Natl.Acad.Sci. USA*, 1986, 83, 4143.
4. Cohen, J.S. in "Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression", Macmillan Press, 1989.
5. Matsukura, M.; Shinozuka, K.; Zon, G.; Mitsuya, H.; Reitz, M; Cohen, J.S. and Broder, S., *Proc.Natl.Acad. Sci. USA*, 1987, 84, 7706.
6. Matsukura, M.; Zon, G.; Shinozuka, K.; Stein, C.A.; Mitsuya, H.; Cohen, J.S. and Broder, S., *Gene*, 1988, 72, 343.
7. Matsukura, M.; Zon, G.; Shinozuka, K.; Robert-Guroff, M.; Shimada, T.; Stein, C.A.; Mitsuya, H.; Wong-Staal, F.; Cohen, J.S. and Broder, S., *Proc.Natl.Acad.Sci. USA*, 1989, 86, 4244.
8. Stein, C.A.; Subasinghe, C.; Shinozuka, K. and Cohen, J.S., *Nucleic Acids Res.*, 1988, 16, 3209.
9. Hayakawa, T.; Ono, A. and Ueda, T., *Nucleic Acids Res.*, 1987, 1, 219.
   Razin, A. and Szyf, M., *Biochim.Biophys.Acta*, 1984, 782, 331.
   Doerfler, W., *Angew.Chem.Int.Ed.Engl.*, 1984, 23, 919.
   Kemp, J.D. and Sutton, D.W., *Biochem.Biophys.Acta*, 1976, 425, 148.
10. Finlay, M.; Debiard, J.P.; Guy, A.; Molko, D. and Teoule, R., *Synthesis*, 1983, 303.
11. Gait, M.J. in "Oligonucleotide Synthesis: A Practical Approach", pp. 24-27, IRL Press, 1984.
12. Sakatsume, O.; Ohtsuki, M.; Takaku, H. and Reese, C.B.; *Nucleic Acids Res.*, 1989, 17, 3689.