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A comparison of gag, pol and rev antisense oligodeoxynucleotides as inhibitors of HIV-1

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Summary

Sequences from the gag, pol and rev regions of the RF strain of HIV-1 (HIV-1_{RF}) were chosen as targets for antisense phosphorothioate oligodeoxynucleotides (S-oligos). These sequences were the p18/p24 junction in gag, the active site of HIV protease in pol; a sequence from the first exon of the rev gene and *S*-oligodeoxycytidylic acid controls. Compounds were tested against HIV-1 in both acutely and chronically infected cells. The results show that these phosphorothioate analogues tested in acutely infected cells were active in the 0.1–2 μM range, were dependent on chain length but had no sequence specificity. To study the mechanism of action, the time of addition of *S*-oligos to acutely infected cells was delayed for up to 48 h post-infection. It was found that antiviral activity was lost when compounds were added to the cultures later than 10 h post-infection. With chronically infected cells only the antisense rev sequence showed activity at 30 μM and neither of the gag or pol antisense sequences has a significant effect on HIV replication at 50 μM. These results are consistent with previous in vitro studies which demonstrate that antisense *S*-oligodeoxynucleotides have several modes of action.

Antisense; Gag; HIV; Oligodeoxynucleotide; Phosphorothioate; Pol; Rev

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Introduction

Antisense oligodeoxynucleotides have been shown, in a number of studies, to be effective inhibitors of virus replication in tissue culture. Activity has been achieved against Rous sarcoma virus (Zamecnik and Stephenson, 1978), vesicular stomatitis virus (Agris et al., 1986; Lemaitre et al., 1987), herpes simplex virus type 1 (Smith et al., 1986), influenza virus (Zerial et al., 1987) and the human immunodeficiency virus (Zamecnik et al., 1986; Matsukura et al., 1987; Agrawal et al., 1988; Goodchild et al., 1988a; Sarin et al., 1988; Zaia et al., 1988; Matsukura et al., 1988; Matsukura et al., 1989). The various studies carried out to date in the use of antisense oligodeoxynucleotides as inhibitors of HIV show significant differences in their activities. Some unmodified 20-mers complementary to HIV RNA were active at 10 μM in CEM cells infected with a high multiplicity of infection (MOI) while the non-complementary controls showed no activity against HIV (Goodchild et al., 1988a). However, two other studies reported no antiviral effect with unmodified oligodeoxynucleotides complementary to HIV RNA (Matsukura et al., 1987; Shibahara et al., 1989); loss of activity of these unmodified compounds was attributed to nuclease activity (Matsukura et al., 1987). Some of these differences are addressed in this study.

A number of different types of oligodeoxynucleotides with modified backbones have been synthesized. Methylphosphonate and phosphorothioate modifications of the phosphodiester linkage of *O*-oligos are two such types of stabilized structures (Miller et al., 1985; Cohen, 1989). These modified compounds are stable to degradative nucleases present in culture medium and host cells. The physical and chemical properties of phosphorothioate structures, in particular, have major advantages when compared with other analogues: they are readily soluble in water, have a relatively high duplex melting temperature (T_m) which aids hybridisation (Miller et al., 1987) and synthesis is readily adapted from the standard automated synthesis of *O*-oligos (Stein et al., 1988).

Recent work has shown that an antisense *S*-oligo targeted to the initiation sequence of the rev gene of the IIIB strain of HIV-1, a gene essential for the replication of HIV, had two modes of action: the compound was active in HIV-1 acutely infected T-lymphoblastoid cells but activity was not sequence-dependent (Matsukura et al., 1987, 1988) In contrast, this analogue, which was complementary to the 5′–3′ initiation sequence of the rev gene, had a potent selective inhibitory effect on HIV transcription in chronically infected cells (Matsukura, 1989).

To extend these observations, the antiviral activity of *S*-oligos complementary to a gag sequence (680-701) and a pol sequence (1833-1854) of the Haitian strain (RF) of HIV-1 (Starcich et al., 1986) (Fig. 1) were compared with the activity of an analogue complementary to the rev initiation site (5488-5514) of the same strain. The three sequences are located respectively at the p18/p24 junction which is a substrate for HIV protease (Meek et al., 1990; Roberts et

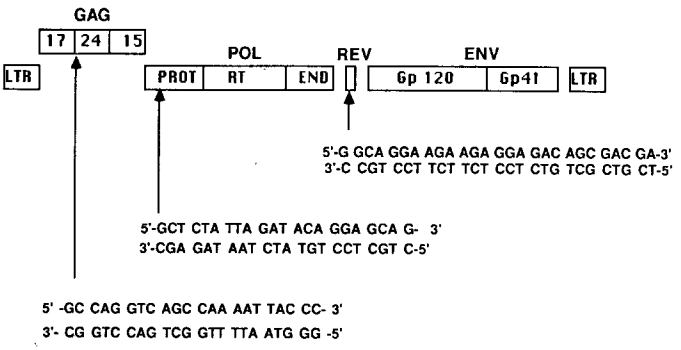

Fig. 1. The genomic structure of the RF strain of HIV-1. For simplification only the first exon of the rev regulatory gene is shown.

al., 1990); the active site of HIV protease (Pearl and Taylor, 1987) and the initiation sequence in the first exon of the rev gene (Sodroski et al., 1986). Even though HIV_{RF} is highly divergent from the IIIB strain of HIV with an overall sequence variation of approx. 20% (Hahn et al., 1985), the rev gene is highly conserved, although there is a single base change in the initiation sequence between the two strains.

To assess at what stage in the replication cycle the antisense compounds were active, delayed addition experiments were carried out in which these *S*-oligos were added to acutely infected cells up to 48 h post-infection. This report compares the specificity and chain length of *S*-oligos in inhibiting HIV-1 replication in acutely infected C8166 lymphoblastoid cells and H9 cells chronically infected with the same virus.

Materials and Methods

Synthesis and purification of O-oligos and S-oligos

Gag and pol phosphorothioate oligodeoxynucleotides were synthesized according to Stein et al. (1988). All sequences were made on an Applied Biosystems 380-B DNA synthesizer. The standard oxidation cycle using iodine was substituted by a sulphurization step. After cleavage from the column and deblocking in aqueous ammonia, phosphorothioate oligomer and blocked co-polymers were purified via reverse phase HPLC. The yield determined from absorbance at 260_{max} was approx. 40%.

Antigen reduction assay in acutely infected cells

Virus stocks ($HIV\text{-}I_{RF}$) were prepared from chronically infected $H9_{RF}$ cells grown in RPMI 1640 (Flow Laboratories) supplemented with 10% fetal calf serum. Cell debris was removed by low speed centrifugation and the supernatant stored in liquid nitrogen until required. The cells used in these

assays were the C8166 T-lymphoblastoid line. Cells were infected with 10 $TCID_{50}$ HIV-1_{RF} at 37°C for 90 min and washed three times with PBSA. Aliquots of 10^4 cells were resuspended in 200 μl growth medium in 96-well plates with each compound at log dilutions from 0.1 to 10 or 100 μM and incubated at 37°C for 72 h in a 95% air/5% CO_2 atmosphere. The HIV antigen was measured in the supernatants with a commercial ELISA using the steps described by the manufacturers (Coulter Electronics Ltd., Luton, U.K.) (Kinchington et al., 1989). AZT and ddCyd (Roche Products U.K., Ltd.) were the drug controls together with untreated cells. The appearance of syncytia was also noted. Assays were carried out in duplicate.

To test for compound toxicity, aliquots of 10^4 uninfected cells were cultured with the compounds at the same log dilutions for 72 h. The cells were then washed with PBSA and resuspended in 200 μl of growth medium containing ^{14}C protein hydrolysate. The cells were harvested after 16 h and the ^{14}C incorporation measured. Untreated cells were used as controls.

Delayed addition experiments

C8166 cells were acutely infected with HIV-1, as previously explained, and *S*-oligos were added at various times up to 48 h post-infection. *S*-oligos were added at a concentration of 10 μM to ensure that the EC_{50} value for each *S*-oligo was exceeded. Each sample supernatant was tested for HIV antigen after 72 h incubation.

Antigen reduction assay in chronically infected H9 cells

Aliquots of 5×10^3 cells chronically infected with HIV-1_{RF} were thoroughly washed in PBSA to remove extracellular virus and resuspended in 200 μl of

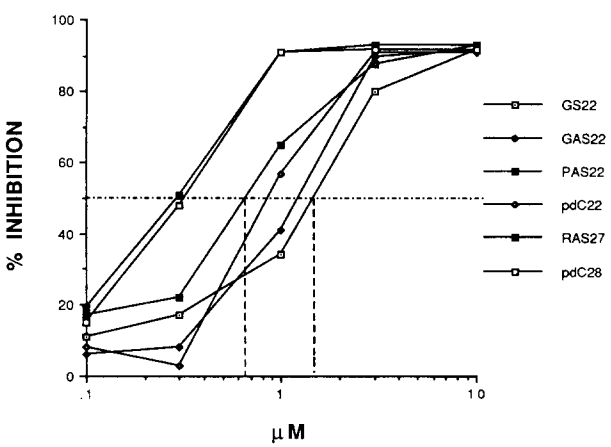

Fig. 2. Dose responses of six *S*-oligos tested in C8166 cells acutely infected with HIV-1 (RF). The two larger molecules are approximately ten times more active than the 22-mers.

growth medium in 96-well plates. Compounds were added in log dilutions from 0.1–100 μM. The supernatants were sampled at 96 h and tested for antigen reduction in the ELISA assay.

Results

Fig. 2 shows the dose response of six phosphorothioate oligodeoxynucleotides in acutely infected C8166 cells. There is no significant difference between the activities of the antisense, sense and poly-dC analogues which have chain lengths of 22 bases. These compounds have EC_{50} values which range from 0.8–1.5 μM. However, the 27-mer antisense rev sequence (RAS27) and the poly-dC28-mer show enhanced activities with EC_{50} values of approximately 0.3 μM. Syncytia were scored in this assay and the endpoints are similar (Table 1). None of these compounds showed a reduction in ^{14}C protein hydrolysate uptake at 10 μM when compared to the untreated controls in the toxicity assay.

A number of further assays were carried out to establish that the original end-points obtained were representative of this virus-cell system. Fig. 3 shows the dose response of four separate experiments for three of the 22-mer compounds: the EC_{50} values are in the 0.8–2 M range. Table 2 shows the average data obtained for the seven *S*-oligos investigated in this study.

Fig. 4 shows the dose response for the six *S*-oligos in a delayed addition experiment. At 10 h post-infection there was no significant difference in the activity of these compounds, but activity was lost between 10–15 h and all compounds were inactive at 20 h post-infection.

Six *S*-oligodeoxynucleotides were tested in the $H9_{RF}$ chronically infected cells and Fig. 5 shows the dose response. The only compound which showed

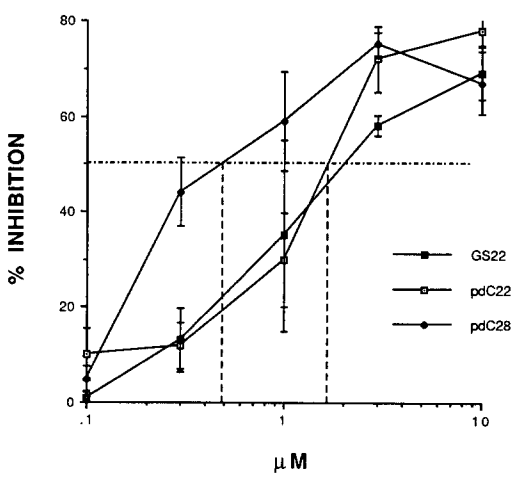

Fig. 3. Average values obtained for three *S*-oligos which were evaluated on four separate occasions. The bars show the standard error of the mean.

TABLE 1

Syncytia formation in the presence of six *S*-oligos

S-Oligo	μM				
	10	3	1	0.3	0.1
GS22	–	–	–	+	+
GAS22	–	–	–	+	+
PS22	–	–	–	+	+
PAS22	–	–	–	+	+
pdC22	–	–	–	–	+
RAS27	–	–	–	–	+
pdC28	–	–	–	–	+

Inhibition of syncytium formation coincided with p24 antigen reduction. GS: gag-sense; GAS: gag-antisense; PS: pol-sense; PAS: pol-antisense; pdC22: poly deoxycytidine (22-mer); RAS27: rev-

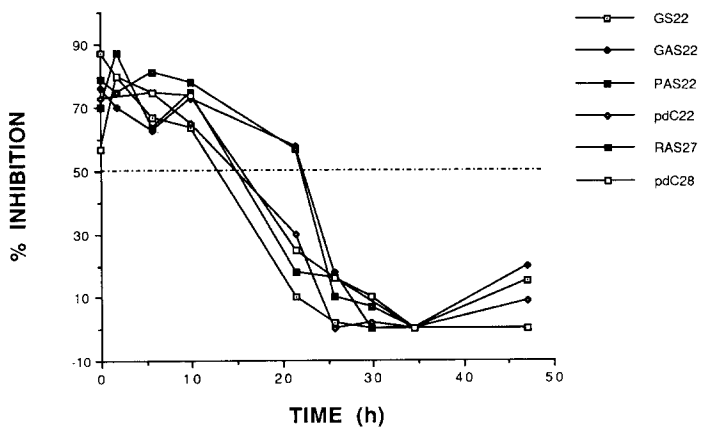

Fig. 4. Antiviral activity of six *S*-oligos in a delayed addition experiment. The concentration of each compound in the culture media was 10 μM; this concentration was approximately ten times above the EC_{50} values.

antisense; pdC28: poly deoxycytidine (28-mer).

TABLE 2

Total data obtained for all the analogues evaluated in acutely infected lymphoblastoid cells

S-Oligo	Average EC_{50} values						
	GS22	GAS22	PS22	PAS22	pdC22	RAS27	pdC28
n	5	2	2	2	4	2	4
EC_{50}	1.2	1	0.8	0.7	1.4	0.2	0.35

The five 22-mers consistently show lower activity than the 27-mer and 28-mer. Each assay is run in duplicate and the values given are the average of at least two experiments.

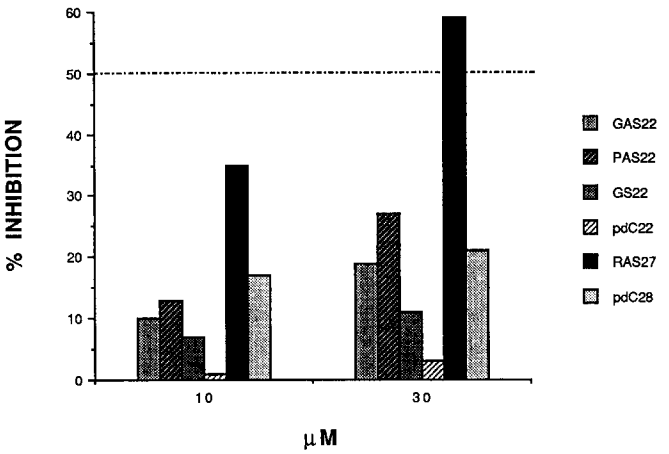

Fig. 5. Dose response of six oligos evaluated in the H9 cells chronically infected with HIV-1 (RF). Only the rev antisense analogue (RAS27) showed significant antiviral activity. Compounds were tested in duplicate at 10, 30 and 100 μM. Some toxicity was observed at 100 μM.

significant activity in this system was compound RAS27, antisense to the rev initiation site, which had an EC_{50} value of 30 μM. None of the other sense or non-specific controls showed significant anti-HIV activity, and none of the compounds were toxic at 30 μM with this cell line in culture over a four-day period. At 100 μM these compounds have some toxic effect on the H9 cells.

Discussion

The sequences used in this investigation are key sites involved in HIV protease activity and HIV gene regulation. The EC_{50} values of all the *S*-oligos evaluated in the C8166/HIV_{RF} system are of the same order as those values previously reported for acutely infected cells with TT12/HIV_{IIIB} and ATH/HIV_{IIIB} cell-virus systems (Matsukura et al., 1987; Matsukura et al., 1988), which are in the 0.5–1.5 μM range. In our study, a reduction in chain length from 28 to 22 bases caused a ten-fold drop in antiviral activity. This agrees also with previous work which investigated differences in chain length of *S*-oligos on HIV replication with both antisense and non-specific analogues, whereby efficacy was lost as chain length was reduced (Matsukura, 1987; Stein et al., 1989). This may be expected on the basis of lower duplex hybridisation. In comparative studies between *S*-oligos and *O*-oligos carried out in this laboratory, no antiviral activity was observed with the latter (data not shown), although *O*-oligos have not been tested as rigorously as the phosphorothioate analogues.

In order to investigate the mode of action of these compounds, six *S*-oligos were added to acutely infected C8166 cells at increasing times post-infection.

All the compounds essentially have the same response in that they lose activity between 10 and 20 h (Fig. 4). This type of response is similar to that observed with both TIBO and ddC in this cell-virus system (data not shown). Addition of compounds, which act late in the replication cycle, may be delayed for 18–20 h in this system before activity is lost. These data indicate that *S*-oligos act on intermediate stages of HIV replication, which is consistent with activity against reverse transcriptase. This is supported by other data (Majumdar et al., 1989) which showed that *S*-oligos act on the HIV reverse transcriptase. However, it has to be established if these compounds have other roles in inhibiting HIV during acute infections of cells.

A different mode of action of these analogues is highlighted in experiments with H9 cells chronically infected with the RF strain of HIV 1 (Fig. 5), in which only the antisense compound targeted against the initiation sequence of rev (RAS27) shows significant activity at 30 μM. This activity is similar to that previously published for HIV_{IIIB}, in which a 50% reduction in HIV replication was achieved at 25 μM. (Matsukura, 1989). The two 22-mer antisense sequences targeted against the gag and pol are essentially inactive in these chronically infected cells. This data is in accord with previous comparative studies in which only those analogues targeted against the rev gene had significant activity (Matsukura, 1989). Thus it may be that translation arrest is efficient only when directed against early regulatory genes which are transcribed in low copy numbers, or it may be that signal sequences such as primer binding sites or splice sites have a higher affinity for antisense molecules. The mode of action of these compounds may be entirely attributable to translation arrest and not to other activities such as RNase H activity: studies with å-form of these *S*-oligodeoxynucleotides show them to have anti-HIV activity even though these configurations are not recognized by RNase H (Rayner et al., 1989).

In conclusion, the results presented here show that these phosphorothioate analogues have a marked effect on the replication of HIV_{RF} in acutely infected C8166 cells. The activity of these molecules is independent of sequence specificity but is dependent upon chain length. With the chronically infected $H9_{RF}$ cells, the activity of the *S*-oligos was approximately twenty times lower than in acutely infected C8166 cells. It should be emphasized that the sequence-non-specific effect of phosphorothioate oligomers in which de novo infection of HIV is blocked would appear to be fundamentally different from the sequence-specific antiviral effect in chronically infected cells.

Acknowledgements

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