ELSEVIER

Antiviral Research 28 (1995) 81–91

Antiviral Research

Inhibition of HIV-1 in vitro by C-5 propyne phosphorothioate antisense to *rev*

Ole Søgaard Lund, Jens Ole Nielsen, John-Erik Stig Hansen *

Laboratory for Infectious Diseases, Department 144, Hvidovre Hospital, 2650 Hvidovre, Denmark

Received 12 January 1995; accepted 3 April 1995

Abstract

A 15-mer C-5 propyne modified phosphorothioate oligodeoxynucleotide antisense to *rev* was approximately 5-fold more effective in providing viral inhibition compared to a 28-mer unmodified phosphorothioate oligodeoxynucleotide targeted to the same sequence and previously shown to inhibit HIV-1 in a sequence-dependent manner. The antiviral effect was obtained by lipofection or simple addition of 0.2–1 μM modified oligodeoxynucleotide to the culture medium of H9 cells chronically infected with the HIV-1_{LAI} isolate of human immunodeficiency virus type 1. We conclude that C-5 propyne oligodeoxynucleotides in accordance with previous findings by others are superior to unmodified phosphorothioates in providing inhibition of HIV-1 in a sequence-dependent manner and that this inhibition can be conferred by oligodeoxynucleotides in free solution.

Keywords: Human immunodeficiency virus (HIV); Phosphorothioate; C-5 propyne; Antisense; *rev*; Chronic infection

1. Introduction

Several DNA analogues have been developed in order to obtain gene-specific inhibition mediated by oligodeoxynucleotides (ODNs) (Milligan et al., 1993). Among these, phosphorothioate has so far gained the most widespread attention. Although phosphorothioate DNA is resistant to nuclease attack (Stein et al., 1988; Morvan et al., 1993) and to some extent crosses cellular membranes (Iversen et al., 1992), it appears as a serious drawback that the affinity between phosphorothioate and target RNA is rather low (Vickers et al., 1991; Morvan et al., 1993). In order to improve the antisense

* Corresponding author: Fax: +45 (31) 47 49 79. E-mail: infejesh@inet.uni-c.dk.

SSDI 0166-3542(95)00041-0

potential, a modified phosphorothioate, C-5 propyne, has been introduced (Froehler et al., 1992). In this modification of phosphorothioate, thymine groups are replaced by uracil, and propynyl groups have been added to pyrimidines at position C-5. These modifications strongly increase the affinity to RNA and C-5 propyne oligodeoxynucleotides elicit significant sequence-specific inhibition of reporter genes when introduced in cultured cells by microinjection or lipofection (Wagner et al., 1993). Wagner et al. reported that C-5 propyne ODNs injected into cells were several orders of magnitude more efficient in providing antisense inhibition of transfected reporter genes than unmodified phosphorothioate ODNs.

The aim of the present study was to test C-5 propyne ODNs in another biological system, which previously has been described as sensitive to sequence-dependent inhibition by phosphorothioate ODNs: the human H9 T-lymphocyte cell line chronically infected with HIV-1_{LAI} (Matsukura et al., 1989).

With respect to antisense inhibition of HIV by phosphorothioate ODNs, chronic infection is a more reliable system than acute infection, since phosphorothioate ODNs are multipotent inhibitors of the process of infection independent of sequence (CD4 interaction and reverse transcription) (Stein et al., 1991).

However, in chronically infected cells, several investigators have found sequence-dependent and -specific inhibition of HIV antigen production using a 27- to 28-mer phosphorothioate antisense oligo targeted to the 5′-terminal of the open reading frame of the *rev* gene. The term, '*sequence dependent*' means: dependent on the oligonucleotide sequence, without necessarily being due to an antisense mechanism. The term, '*specific*' means antiviral without being cytotoxic. Results ranging from 20 to 70% sequence-dependent and -specific viral inhibition have been reported targeting this particular sequence with 10 μM phosphorothioate in free solution (Matsukura et al., 1989; Vickers et al., 1991; Kinchington et al., 1992; Zelphati et al., 1994). A concentration of 10 μM phosphorothioate oligodeoxynucleotide exceeds by more than one order of magnitude the IC_{50} of HIV acute infection in vitro (0.2–0.8 μM) (Morvan et al., 1993; Zelphati et al., 1994). For this reason, an increase of potency with respect to gene-specific inhibition seems to be necessary if this mechanism should contribute significantly to the overall inhibition of acute viral replication. In addition, 10 μM phosphorothioate ODN corresponds to approximately 100 mg/kg and since infusion of 10 mg/kg can induce hemodynamic changes and complement activation in monkeys (Galbraith et al., 1994), an increment of the antisense potential of oligodeoxynucleotides seems also, for this reason, to be needed if a gene-specific mechanism should be relevant in vivo.

Our experimental approach in this study addressed two issues concerning the C-5 propyne analogue applied in cell culture: (1) the ability of 15-mer C-5 propyne to confer sequence-dependent and -specific inhibition of established HIV-1 infection either by simple addition to the culture medium or by transfection mediated by lipofectin (a membrane permeabilizing agent used by Wagner et al. (1993) and Fenster et al. (1994)); and (2) the efficiency of inhibition conferred by 15-mer C5-propyne compared to the 28-mer phosphorothioate anti-*rev* ODN previously shown to confer sequence-dependent and -specific inhibition of viral antigen production.

The reason for choosing a 28-mer phosphorothioate for comparison in this study is that only oligonucleotides of this length (27–28 nt) have been published to be inhibitory

in chronically infected cells. The paper presenting the C-5 propyne oligonucleotides as mediators of antisense inhibition (Wagner et al., 1993) states the increase in affinity to complementary RNA as the reason for improvement of antisense inhibition relative to unmodified phosphorothioates. The T_m of 28-mer anti-rev_{AUG} (unmodified phosphorothioate) against complementary RNA has been published to be 66.2°C (Zelphati et al., 1994). In comparison, T_m of 15-mer C-5 propyne oligodeoxynucleotides of similar composition as the one tested in this study has been published to be 70–72°C (Wagner et al., 1993). Thus, if the affinity to complementary RNA according to Wagner et al. (1993) is the explanation of C-5 propyne antisense improvement, the inhibition conferred by 15-mer C-5 anti-*rev* propyne could be expected to be comparable or even greater than inhibition conferred by the 28-mer unmodified phosphorothioate.

2. Materials and methods

2.1. Synthesis and preparation of oligodeoxynucleotides

ODNs were obtained from the Department of General and Organic Chemistry, University of Copenhagen, where they were synthesized by the standard phosphoramidite method at 1-μmol scale using a Biosearch 8750 DNA synthesizer. Monomers of C-5 propyne nucleotides were obtained from Glenn Research, USA. C-5 propyne ODNs contained modified pyrimidines (5-(1-propynyl)-dC and 5-(1-propynyl)-dU, respectively) at internal and 5'- positions. ODN stock solutions of 200 μM were prepared by ethanol precipitation, solution in PBS buffer and dialysis at 4°C for 2×24 h against > 1000 vols. of PBS buffer in a Spectra/POR dialysis membrane (molecular weight cut-off = 3500 Da) obtained from Spectrum, USA. Concentrations of ODNs were spectrophotometrically quantified at 260 nm using extinction coefficients for S-dT = 8.8; S-dC = 7.3; S-dG = 11.8; S-dA = 15.2; propyne S-dU = 3.2 and propyne S-dC = 5.0 (Brian C. Froehler, Gilead Sciences, USA, personal communication, 1994). Before dilution in RPMI 1640, stock solutions of ODNs were heated to 70°C for 10 min. Four different ODNs were tested: two 28-mer phosphorothioates and two 15-mer C-5 propyne ODNs. Antisense sequences were targeted to the *rev* gene (nt 5552–5579, HIVBRUCG, GenBank, Wain-hobson et al., 1985) as shown in Fig. 1. Control ODNs of C-5 propyne and unmodified phosphorothioate were additionally tested. The phosphorothioate control ODN was a 28-mer degenerate sequence synthesized by random incorporation of all 4 nucleotides simultaneously. The C-5 propyne control was a 15-mer ODN representing a non-homologous sequence containing the same number of pyrimidines and purines as the antisense sequence. The reason for not using a degenerate C-5 propyne control ODN was that no experience of C-5 propyne monomer coupling efficiency was available.

An additional 15-mer C-5 control oligonucleotide, anti-*gal*, was synthesized as described above. The sequence of anti-*gal*, 3'-CGCCCUUCUACUCCG, is complementary to the region at the AUG-codon of the human gene encoding β-1,4-galactosyltransferase (Masri et al., 1988).

2.2. Cell culture

Human T-lymphoid CD4-positive H9 cells infected with the HIV-1_{LAI} isolate (Popovic et al., 1984) were maintained in RPMI 1640 (GIBCO BRL, 72400) containing 2 mM L-alanyl-L-glutamine, 25 mM HEPES and supplemented with gentamicin 20 mg/l, penicillin 100 IU/ml and streptamycin 100 mg/l. Unless specifically stated, all culture media were supplemented with 10% v/v of fetal bovine serum. Cell cultures were kept in a humidified CO_2-atmosphere at 37°C.

2.3. Experimental regimen

2.3.1. Lipofection experiments

Cells were seeded out in a single culture flask at 0.15×10^6 cells/ml. Twenty-four hours postseeding, cells were resuspended in fresh medium and seeded out in triplicate aliquots of 180 μl on a 96-well microplate. Before addition to the cells, lipofectin (GIBCO BRL, Life Technologies) and ODNs were mixed in 10 times final concentration and allowed to complex for 15 min in RPMI 1640 (according to the manufacturer's manual). After addition of the lipofection mix, cells were incubated for 48 hours before analysis of HIV antigen.

2.3.2. Time-course experiments

Cells were seeded in duplicate at 0.1×10^6 cells/ml on a 96-well microplate containing 180 μl per well. A volume of 20 μl of culture medium containing ODN at 10 times final concentration (0, 0.04, 0.2, 1 and 5 μM) was added directly to the culture medium. Cells were harvested and analyzed after 48 and 96 h of incubation.

Phosphorothioate ODNs

rev mRNA A UG----------
Anti-Rev : 3′ ACCGTCCTTCTTCGCCTCTGTCGCTGCT 5′

Control : N_{28}

C-5 propyne ODNs

rev mRNA A UG----------
Anti-rev : 3′ ACCGUCCUUCUUCGC 5′

Control : 3′ TCUACCGUGCUUCUU 5′

Oligodeoxynucleotide sequences

Fig. 1. Nucleotides in the target sequence of *rev* mRNA downstream of the AUG-codon are indicated by a dashed line.

2.4. HIV antigen ELISA and cell viability assays (MTT)

The HIV antigen concentration in supernatants of cultured cells was quantitated by an in-house ELISA based on polyclonal antibodies obtained from HIV-1-infected patients (Hansen et al., 1990). Frozen stock dilutions of supernatants from H9 cells chronically infected with HIV-1_{LAI} were used as internal standards.

Viability of cells was quantified by the MTT method which measures cellular dehydrogenase activity (Hansen et al., 1989). Dehydrogenase activity is quantified by the addition of a diffusible tetrazolium salt to cells, which take up the salt and convert it into a formazan dye by the action of dehydrogenases.

MTT assays were performed on aliquots of 50 μl cell suspension in a total volume of 100-μl culture medium according to MB Hansen et al (1989). Cells were incubated for 3 h at 37°C before the addition of extraction buffer. Following 24 h of extraction, absorbance at 570 nm of 100 μl lysate was measured on an ImmunoReader NJ-2000 (InterMed).

3. Results

3.1. Lipofection experiments

Three experiments were performed initially in order to establish the experimental conditions of C-5 propyne sequence dependent inhibition of HIV-1 antigen production (antisense compared to control sequence). In one experiment (Table 1, Expt. A) a range of different concentrations of control and antisense C-5 propyne ODNs was tested by lipofection (lipofectin: 6 μg/ml) in RPMI containing 5% serum. After 1 day of culture, ODNs and lipofectin were added to the cells and 48 h later HIV antigen concentration was determined in supernatants.

From this experiment a clear dose–response of sequence dependent inhibition by the anti-*rev* C-5 ODN was evident. However, it also appeared that mock treatment with

Table 1
Lipofection experiments

(A) Variable C-5 propyne concentration (serum = 5% v / v; lipofectin = 6 μg / ml)				
C-5 propyne concentration (μM)	0.04	0.2	1	5
Specific inhibition (%)	9	31	43	61
(B) Variable lipofectin concentration (serum = 5% v / v; C-5 propyne = 1 μM)				
Lipofectin concentration (μg/ml)	0	2/3	2	6
Specific inhibition (%)	31	38	33	42
(C) Variable serum concentration (C-5 propyne = 1 μM; lipofectin = 6 μg / ml)				
Serum concentation (v/v%)	0	2	5	
Specific inhibtion (%)	53	55	44	

Lipofection experiments using different concentrations of C-5 propyne, lipofectin and serum. Values of specific inhibition are means of triplicate determinations (inhibition of HIV antigen production in cells treated with C-5 anti-*rev* ODN compared to cells treated with C-5 control ODN).

lipofectin alone significantly reduced the level of antigen production (results not shown) and in order to reduce non-specific toxicity due to lipofectin two additional experiments were performed. In these experiments, different concentrations of fetal bovine serum and lipofectin were tested (transfection of DNA by lipofectin is according to the manufacturer inhibited by the presence of serum). These experiments used a constant concentration of the C-5 propyne ODN of 1 μM. It turned out that the efficiency of viral inhibition was not significantly affected by application of lipofectin (Table 1, Expt. B). Reduction of serum (Expt. C) only slightly increased the observed inhibition (56% compared to 44%). We therefore decided not to use lipofectin and to use standard culture medium with 10% fetal bovine serum for further experiments.

3.2. Time-course experiments

It has previously been shown (Matsukura et al., 1989) that the inhibitory effect conferred by unmodified phosphorothioate antisense to *rev* in chronically infected cells increases when ODNs are present for more than 2 days. In order to test the C-5 propyne ODN in this respect we performed a time-course experiment in which HIV antigen in supernatants was analyzed at 48 and 96 h after seeding and addition of ODNs. Phosphorothioate and C-5 propyne ODNs were tested in the same experiment in order to compare their antiviral efficiency. The time-course experiment was carried out twice using duplicate cell cultures in each experiment.

In order to test whether the observed reduction in HIV antigen production was due to gene-specific inhibition or cytotoxic effects, a viability assay, MTT, was carried out in parallel with HIV antigen determination.

Mean viability (MTT colorimetric determination) is shown in Fig. 2 and it can be seen that a significant difference existed between the anti-*rev* C-5 propyne and the

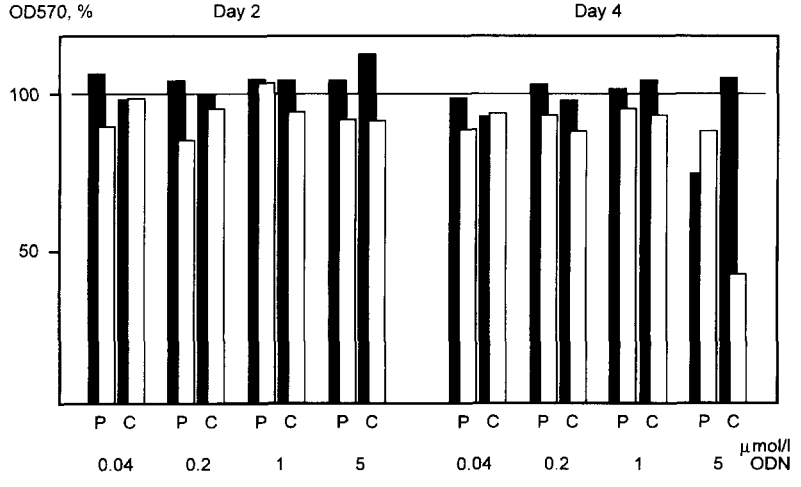

Fig. 2. MTT viability assay. Values are given as percentage relative to untreated control (mean values of two independent experiments each performed in duplicate). Dark columns, control ODN; light columns, anti-*rev* ODN; P, phosphorothioate; C, C-5 propyne.

control C-5 ODN at 5-μM concentration on day 4. In contrast, no significant difference in toxicity was observed between the phosphorothioate ODNs. Due to the observed toxicity of the C-5 propyne anti-*rev* ODN at 5 μM, we confined the following investigation of antiviral effects to concentrations which displayed either no detectable or equal toxicity between anti-*rev* and control ODN, i.e., C-5 $\leqslant 1$ μM and phospho-

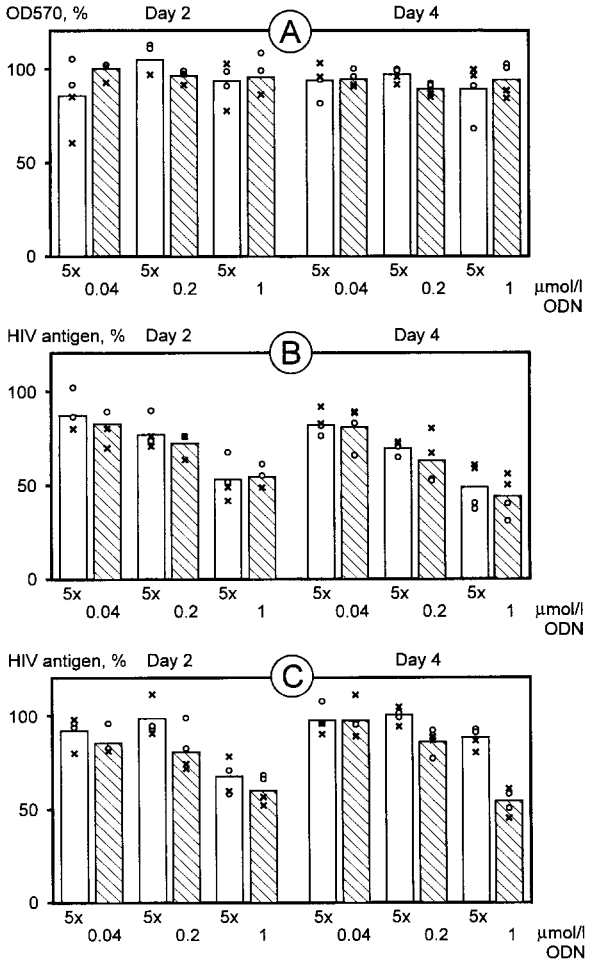

Fig. 3. Comparison of anti-*rev* ODNs. In these figures, unmodified phosphorothioate anti-*rev* is compared to C-5 propyne anti-*rev* at a concentration ratio of 5 : 1. Concentrations of C-5 propyne anti-*rev* are indicated. Dark columns, C-5 propyne anti-*rev*; light columns, unmodified phosphorothioate at 5 times the concentration indicated. All values are mean values of two independent experiments each performed in duplicate. Individual measurements of the two experiments are indicated by crosses and circles, respectively. A: absolute viability. MTT values are given as percentage relative to untreated control. B: absolute inhibition of HIV antigen. Values are given as percentage relative to untreated control. C: specific inhibition of HIV antigen. Values are given as percentage relative to cells treated with control ODNs.

rothioate $\leqslant 5$ μM. In an additional experiment, we tested the cytotoxicity of the C-5 propyne anti-*rev* ODN in uninfected cells to see if the toxic effect was dependent on the presence of HIV RNA. However, we found a similar toxicity in uninfected H9 cells, suggesting that cytotoxicity was independent of HIV infection. With respect to inhibition of HIV antigen production, our results show that a similar overall inhibition was obtained with one-fifth the concentration of C-5 propyne compared to unmodified phosphorothioate. In order to show this clearly, Fig. 3 A–C compare the data of $1 \times$ concentration of anti-*rev* C-5 propyne to $5 \times$ concentration of anti-*rev* unmodified phosphorothioate.

No difference in toxicity was detected between the anti-*rev* ODNs by this comparison (Fig. 3A). IC_{50} on both day 2 and 4 was approximately 1 μM for C-5 and 5 μM for phosphorothioate (Fig. 3B). However, from Fig. 3C it is seen that the sequence dependent inhibition, i.e., the inhibitory effect of anti-*rev* ODNs relative to the control ODNs, on both day 2 and 4 was more evident with the C-5 anti-*rev* than observed for unmodified phosphorothioate anti-*rev* at 5-fold higher concentrations. Sequence-dependent inhibition conferred by the C-5 anti-*rev* was maximal on day 4 at 1 μM reaching an approximate value of 45% inhibition (Fig. 3C). Regarding the unmodified phosphorothioate, sequence-dependent inhibition was only significant on day 2 at 5 μM reaching a sequence-dependent inhibition of 35%. This indicates that in chronically infected cells, a similar overall inhibition of HIV antigen production and an increased sequence-dependent inhibition can be obtained with one-fifth the concentration of a 15-mer C-5 propyne phosphorothioate compared to a 28-mer unmodified phosphorothioate.

Using the same data as presented in Fig. 3B, C, a comparison of C-5 propyne and unmodified phosphorothioate applied in the same concentrations (0.2 and 1 μM) can be summarized in the following way: at 0.2–1 μM the overall inhibitory effect of unmodified phosphorothioate anti-*rev* was weak (15–30%, Fig. 3B) and no sequence dependence of this inhibition was evident ($< 10\%$, Fig. 3C). In contrast, 0.2-1 μM C-5 propyne anti-*rev* gave rise to a more significant overall inhibition (25–50%, Fig. 3B) and the major part of this inhibition seemed to be sequence dependent (20–45%, Fig. 3C).

In order to further investigate our finding of sequence-dependent effects conferred by the C-5 propyne anti-*rev* ODN, we performed an additional experiment testing the C-5 propyne anti-*rev* ODN against another 15-mer C-5 propyne ODN: anti-*gal*. This ODN is targeted towards the gene encoding the Golgi enzyme: β-1,4-galactosyltransferase. The experiment was carried out according to the time-course experiment describe above except that only two concentrations (1 and 5 μM) were tested and only 96 h of incubation was used. Our results from this experiment showed that the anti-*gal* ODN was cytotoxic to at least the same extent as the anti-*rev* ODN but only at 5-μM concentration (anti-*gal* 88% reduction in cell viability; anti-*rev* 72% reduction of cell viability, MTT assay). Despite the cytotoxicity of both ODNs at 5 μM concentration, only the anti-*rev* ODN showed significant inhibition of HIV antigen production at 1 μM concentration (anti-*rev* 59% inhibition; anti-gal 5% inhibition). These results clearly indicate that the observed cytotoxicity is not restricted to the anti-*rev* ODN and is probably not dependent on sequence. On the other hand, the observed antiviral effect of the C-5 propyne anti-*rev* at low concentrations (0.2–1 μM) does not seem to be linked

to the observed cytotoxicity at 5-μM concentration, since the anti-*gal* ODN did not inhibit HIV antigen production at 1-μM concentration. The observed antiviral effect of C-5 anti-*rev* thus seem to be truly dependent on sequence and specific with respect to HIV.

4. Discussion

In this study, HIV-infected cells were exposed to a maximum of 5 μM ODN for 48 and 96 h. In accordance with previous studies, we found a sequence-dependent, specific but low-grade inhibition of HIV antigen production conferred by the phosphorothioate anti-*rev* ODN (range 10–35%). Sequence-dependent and -specific inhibition of HIV antigen in chronically infected cells by anti-rev_{AUG} phosphorothioate has been addressed by several studies. A 28-mer anti-rev_{AUG} phosphorothioate ODN identical to the one used in this study has been shown to confer approximately 70% sequence-dependent, specific inhibition of HIV antigen production after 5 days of incubation at 10 μM concentration (Matsukura et al., 1989). Another study, also in chronically infected H9 cells, reported 20% sequence-dependent and -specific inhibition of HIV antigen production at 10 μM ODN concentration (27 nt anti-rev_{AUG} was compared to poly dC_{28}; results concerning specificity were stated but not shown) (Kinchington et al., 1992). Two studies in chronically infected CEM cells reported 30–60% sequence-dependent inhibition by 27- and 28-mer anti-rev_{AUG} phosphorothioates respectively (Vickers et al., 1991; Zelphati et al., 1994). IC_{50} (antisense relative to untreated control) of the 28-mer anti-rev_{AUG} unmodified phosphorothioate used in our study has previously been determined in chronically infected cells to be 5–7.5 μM (Matsukura et al., 1989; Zelphati et al., 1994). Our findings are also consistent with this, but in contrast to Matsukura et al. using the same cell line, we found some of this inhibition to be independent of sequence. Wagner et al. (1993) and Fenster et al. (1994) did not see any significant inhibition by injection of unmodified phosphorothioate into cells even at 30-μM concentration. We suggest the reason for this to be their use of short ODNs (15–20 mer) in contrast to other researchers demonstrating sequence-dependent effects using longer phosphorothioate ODNs (27–28 mer). This is indicated by another study using an experimental system similar to the one used by Fenster et al. (1994) (*rev*-dependent reporter plasmids) (Li et al., 1993). In this study, 60% sequence-dependent inhibition using a 27-mer anti-rev_{AUG} unmodified phosphorothioate was reported.

Regarding C-5 propyne ODNs, the IC_{50} of the 15-mer C-5 anti-*rev* was obtained in our study at an approximately 5-fold lower concentration compared to 28-mer unmodified phosphorothioate. The antiviral effect obtained with C-5 propyne was clearly dose-dependent and in contrast to phosphorothioate, the inhibitory effect appeared both on day 2 and day 4 to be mainly dependent on sequence. Gene-specific inhibition of 20–45% observed at 0.2–1 μM concentration of 15-mer C-5 propyne in free solution approximates the non-sequence-dependent inhibition of phosphorothioate ODNs during acute HIV infection (IC_{50} 0.2–0.8 μM; Morvan et al., 1993, Zelphati et al., 1994). By this comparison, gene-specific inhibition by C-5 propynes could be therapeutically relevant compared to other antiviral effects conferred by phosphorothioates (Stein et al.,

1991). We suggest the reason for this apparent improvement of sequence-dependent inhibition to be due to the increase of target affinity as explained in the introduction.

At the highest concentration tested, 5 μM, two out of three C-5 propyne ODNs displayed significant cytotoxicity after 96 h of incubation. However, among these, only the anti-*rev* ODN conferred antiviral effect at 1 μM concentration, indicating that the observed cytotoxicity was not linked to the observed antiviral effect. The antiviral effect of the C-5 propyne anti-*rev* thus seems to be dependent on sequence. We have not been able to identify the cause of the cytotoxicity of C-5 propyne anti-*rev* and anti-*gal*, but since two out of three ODNs showed significant toxicity, we do not consider this phenomenon to be dependent either on sequence or the propyne modification per se.

Antisense effects targeting HIV-1 sequences on reporter plasmids (RRE/*env*) with C-5 oligodeoxyribonucleotides at the 0.1-μM scale have been demonstrated using microinjection and lipofection of ODNs (Fenster et al., 1994). In contrast to our findings, Fenster et al. did not see any inhibitory effect with simple addition of oligonucleotides to the culture medium. In the experimental system used in our study, specific inhibition was obtained by simple addition of ODNs to the culture medium, and the specific inhibitory effect observed by lipofection was only slightly higher in our hands. A recent study showing significant inhibition on Dengue virus replication by injection of 15-mer antisense C-5 propyne failed to inhibit viral replication by lipofection of C-5 propyne ODNs with lipofectin (Raviprakash et al., 1995). Thus, some disunity seems to exist concerning transfection of ODNs by lipofectin versus application in free solution. Together with our own findings, we suggest further work on this issue before drawing definitive conclusions.

In conclusion, the overall antiviral effect caused by a 28-mer phosphorothioate ODN antisense to *rev* applied in free solution as described by Matsukura et al. (1989) can be obtained by a 5-fold lower concentration of a 15-mer anti-*rev* C-5 propyne ODN. The inhibition appears to be specific and mainly dependent on sequence, but the involvement of an antisense mechanism cannot be inferred from this study.

Acknowledgements

The authors wish to thank Henriette Buch and Anders Sørensen for excellent technical assistance. This research was supported by the John and Birte Meyer Foundation, the Mrs. Lily Benthine Lund Foundation and the Danish Medical Research Council.

References

Agrawal, S., Ikeuchi, T., Sun, D., Sarin, P.S., Konopka, A., Maizel, J. and Zamecnik, P.C. (1989) Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues. Proc. Natl. Acad. Sci. USA 86, 7790–7794.

Fenster, S.D., Wagner, R.W., Froehler, B.C. and Chin, D.J. (1994) Inhibition of human immunodeficiency virus type-1 *env* expression by C-5 propyne oligonucleotides specific for *Rev*-Responsive Element stem-loop V. Biochemistry 33, 8391–8398.

Froehler, B.C., Wadwani, S., Terhorst, T.J. and Gerrad, S.R. (1992) Oligodeoxynucleotides containing C-5 propyne analogs of 2′-deoxyuridine and 2′-deoxycytidine. Tetrahedron Lett. 33, 5307–5310.

Galbraith, W.M., Hobson W.C., Giclas, P.C., Schechter, P.J. and Agrawal, S. (1994) Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. Antisense Res. Dev. 4, 201–206.

Hansen, J.-E.S., Clausen, H., Nielsen, C., Teglbjærg, L.S., Hansen, L.L., Dabelsteen, E., Nielsen, C.M., Mathiesen, L., Hakomori, S. and Nielsen, J.O. (1990) Inhibition of human immunodeficiency virus (HIV) infection in vitro by anticarbohydrate monoclonal antibodies: peripheral glycosylation of HIV envelope glycoprotein gp120 may be target for virus neutralization. J. Virol. 64, 2833–2840.

Hansen, M.B., Nielsen, S.E. and Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119, 203–210.

Iversen, P.L., Zhu, S., Meyer, A. and Zon, G. (1992) Cellular and subcellular distribution of phosphorothioate-oligonucleotides in cultured cells. Antisense Res. Dev. 2, 211–222.

Kinchington, D., Galphin, S., Jaroszewski, J.W., Ghosh, K., Subasinghe, C. and Cohen, J.S. (1992) A comparison of *gag*, *pol* and *rev* antisense oligodeoxynucleotides as inhibitors of HIV-1. Antiviral Res. 17, 53–62.

Li, G., Lisziewicz, J., Sun, D., Zon, G., Daefler, S., Wong-Staal, F., Gallo, R.C. and Klotman, M.E. (1993) Inhibition of *Rev* activity and human immunodeficiency virus type 1 replication by antisense oligonucleotide phosphorothioate analogs directed against the *Rev*-responsive element. J. Virol. 67, 6882–6888.

Masri, K.A., Appert, H.E. and Fukuda, M.N. (1988) Identification of the full-length coding sequence for human galactosyltransferase (β-*N*-acetylglucosaminide: β1,4-galactosyltransferase). Biochem. Biophys. Res. Commun. 157, 657–663.

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. (1989) Regulation of viral expression of human immunodeficiency virus in vitro by an antisense phosphorothioate oligodeoxynucleotide against *rev* (art/trs) in chronically infected cells. Proc. Natl. Acad. Sci. USA 86, 4244–4248.

Milligan, J.F., Matteucci, M.D. and Martin, J.C. (1993) Current concepts in antisense drug design. J. Med. Chem. 36, 1923–1937.

Morvan, F., Porumb, H., Degols, G., Lefebvre, I., Pompon, A., Sproat, B.S., Rayner, B., Malvy, C., Lebleu, B. and Imbach, J.-L. (1993) Comparative evaluation of seven oligonucleotide analogues as potential antisense agents. J. Med. Chem. 36, 280–287.

Popovic, M., Sarngadharan, M.G., Read, E. and Gallo, R.C. (1984) Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224, 497–500.

Raviprakash, K., Liu, K., Matteucci, M., Wagner, R., Riffenburgh, R. and Mitchell, C. (1995) Inhibition of dengue virus by novel, modified antisense oligonucleotides. J. Virol. 69, 69–74.

Stein, C.A., Subasinghe, C., Shinozuka, K. and Cohen, J.S. (1988) Physicochemical properties of phosphorothioateoligodeoxynucleotides. Nucleic Acids Res. 16, 3209–3221.

Stein, C.A., Tonkinson, J.L. and Yakubov, L. (1991) Phosphorothioate oligodeoxynucleotides – anti-sense inhibitors of gene expression? Pharmac. Ther. 52, 365–384.

Wagner, R.W. (1994) Gene inhibition using antisense oligodeoxynucleotides. Nature 372, 333–335.

Wagner, R.W., Matteucci, M.D., Lewis, J.G., Gutierrez, A.J., Moulds, C. and Froehler, B.C. (1993) Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. Science 260, 1510–1513.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. and Alizon, M. (1985) Nucleotide sequence of the AIDS virus, LAV. Cell 40, 9–17.

Vickers, T., Baker, B.F., Cook, P.D., Zounes, M. and Buckheit, R.W. Jr., Germany, J., Ecker, D.J. (1991) Inhibition of HIV-LTR gene expression by oligonucleotides targeted to the TAR element. Nucleic Acids Res. 19, 3359–3368.

Zelphati, O., Imbach, J.-L., Signoret, N., Zon, G., Rayner, B. and Leserman, L. (1994) Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms. Nucleic Acids Res. 22, 4307–4314.