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Antiviral Research 38 (1998) 63–73

Antiviral Research

Synergistic inhibition of HIV-1 by an antisense oligonucleotide and nucleoside analog reverse transcriptase inhibitors

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Received 12 July 1997; received in revised form 23 December 1997; accepted 23 December 1997

Abstract

We have studied the effects of the *gag* antisense phosphorothioate oligonucleotide GEM 91® and mismatch antisense controls on the antiviral activities of ddC and other nucleoside analogs in HIV-infected MT-4 cells using a cytoprotection based assay. Under standard assay conditions, i.e. simultaneous incubation of drugs, HIV-1 IIIB and MT-4 cells, both GEM 91® and mismatch controls interacted synergistically with ddC resulting in an approximate 40-fold decrease in the IC_{50} value of ddC; this suggests a potent but sequence non-specific effect of GEM 91®. Under post-adsorption assay conditions, i.e. pre-incubation of virus and cells and removal of excess HIV before drug addition, GEM 91® exhibited synergism with ddC, with an approximate 5-fold decrease in ddC IC_{50} value. This favorable interaction was not seen with any of the mismatch oligonucleotides, suggesting the involvement of a sequence-specific mechanism of action. Similar results were seen with the thymidine analogs AZT and d4T in combination with GEM 91®. These data suggest a potential role for GEM 91® and future sequence-specific antisense drugs in combination with nucleoside analogs for the treatment of HIV infection. It is essential that potential interactions between new and existing classes of anti-HIV drugs are studied extensively as antiretroviral drug combinations become increasingly more complex.

Keywords: HIV-1; Combination therapy; Antisense; Oligonucleotide; GEM 91®; Nucleoside analog

1. Introduction

Over the course of the past few years, the use of combination therapy in the long-running battle against the human immunodeficiency virus (HIV),

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0166-3542/98/$19.00
PII S0166-3542(98)00004-7

has been shown to be significantly more effective than monotherapy in preventing disease progression and reducing mortality in patients with acquired immunodeficiency syndrome (AIDS) (Delta Co-ordinating Committee, 1996; Hammer et al., 1996). However, although impressive synergy has been seen both within and between the two major classes of anti-HIV drugs, i.e. the reverse transcriptase and protease inhibitors (St. Clair et al., 1995; Collier et al., 1996; Deminie et al., 1996), many other agents have exhibited less favorable interactions with those drugs currently licensed for use in HIV-infected individuals (Vogt et al., 1987; Perno et al., 1992; Clayette et al., 1997).

Antisense phosphorothioate oligonucleotides have been shown in numerous in vitro studies to be effective inhibitors of HIV replication (Matsukura et al., 1987; Agrawal et al., 1988; Agrawal and Tang, 1992; Lisziewicz et al., 1994). Recent studies carried out by our group with GEM 91®, a 25-mer phosphorothioate oligodeoxynucleotide complementary to the *gag* initiation site of HIV-1, suggested several mechanisms of action in vitro. We observed sequence-specific inhibition of virus entry/reverse transcription as well as sequence-independent inhibition of virus binding to the cell surface. In addition, attempts to generate drug resistance to GEM 91® in vitro were unsuccessful (Yamaguchi et al., 1997).

In the current study, we have carried out in vitro cell culture experiments to study the effects of GEM 91® and mismatch antisense controls on the activity of the nucleoside analog drug zalcitabine (2′,3′-dideoxycytidine; ddC), using a method recently shown to be appropriate for the evaluation of multidrug combinations against HIV-1 (St. Clair et al., 1995). We also present data from comparative studies carried out with GEM 91® in combination with the thymidine analogs zidovudine (3′-azido-2′,3′-dideoxythymidine; AZT) and stavudine (2′,3′-dideoxy-2′,3′-didehydrothymidine; d4T). Although previous studies suggested no interaction between AZT and a non-complementary homopolymer oligonucleotide (Chou et al., 1991), it is important to understand that the nucleoside analog drugs have different metabolic profiles leading to their activation and that their activities can be influenced markedly by changes in levels of different endogenous nucleotides (Gao et al., 1993; Sommadossi, 1993; Shirasaka et al., 1995). Also, individual antisense oligonucleotides may have different properties which may influence their efficacy in combination with other agents.

2. Materials and methods

2.1. Cells and virus

MT-4 cells were obtained from the AIDS Research and Reference Reagent Bank, Division of AIDS, NIAID, NIH contributed by Dr Richman (Pauwels et al., 1988). Cell cultures were propagated in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 250 U/ml penicillin, 250 μg/ml streptomycin and 2 mM L-glutamine at 37°C in a 5% CO_2-gassed incubator.

HIV-1 IIIB was originally obtained from Dr Robert Gallo, National Cancer Institute (Popovic et al., 1984). Virus stocks of HIV-1 were prepared from cell-free supernatant of chronically infected H9 cultures by the shaking method as previously described (Vujcic et al., 1988). Supernatant stocks were aliquoted into microtubes and stored at −80°C. Virus titers were determined by dilution assays for virus cytopathic effects (CPE) in MT-4 cells in 96-well microtiter plates under the same conditions as used for the standard assay experiments carried out in these studies, i.e. with the simultaneous incubation of cells and virus for a period of 6 days. The amount of HIV-1 IIIB that resulted in a 90% decrease in MTT absorbance under standard assay conditions was defined as the $TCID_{CPE\text{-}90\%}$ concentration, this was the optimal amount of virus for maximum sensitivity with this type of cytoprotection assay. In experiments carried out under post-adsorption conditions, i.e. after pre-incubation of cells and virus for 4 h followed by removal of excess virus by washing, a $10 \times TCID_{CPE\text{-}90\%}$ concentration was required to give the same level of inhibition.

2.2. Compounds

GEM 91®, a gag translation initiation site 25-mer oligodeoxynucleotide phosphorothioate (5′-d(CTC TCG CAC CCA TCT CTC TCC TTC T)-3′; 777–801), GEM 91® mismatch controls (GEM 91®-1 mis, 5′-d(CTC TCG CAC CCA TAT CTC TCC TTC T)-3′; GEM 91®-3 mis, 5′-d(CTC TCG CTC CCA TAT CTC ACC TTC T)- 3′; GEM 91®-5 mis, 5′-d(CTA TCG CTC CCA TAT CTC ACC TGC T)- 3′; mismatch positions represented by underline) and end-modified MBO (mixed backbone oligonucleotide control with no reverse transcriptase priming activity, 5′-d(**CGC** ACC CAT CTC TCT CC **UUCU**)-3′; bold text represents 2′-*O*-methyl modified nucleotides) were prepared by standard methods (Lisziewicz et al., 1994). The purity of the oligonucleotides was confirmed by polyacrylamide gel electrophoresis, hybridization melting temperature and ^{31}P-NMR analysis. Stock solutions (400 μM) were prepared in double distilled sterilized water and stored at −20°C until use. ddC, AZT and d4T were obtained from Sigma, St. Louis, MO (catalogue numbers D5782, A2169, D1413, respectively), dissolved in RPMI 1640 to prepare 400 μM stock solutions and stored at 4°C.

2.3. Inhibition of HIV-1 induced CPE in MT-4 cells

CPE based infection experiments using MT-4 cells were performed either under standard conditions, i.e. with the simultaneous incubation of HIV-1 IIIB and MT-4 cells or following pre-incubation of virus and cells, i.e. post-adsorption conditions (Pauwels et al., 1988; St. Clair et al., 1995). Serial dilutions (10-fold) of ddC (from 0.01 to 100 μM) were prepared in 96-well plates and infections were performed in the presence or absence of a constant concentration of GEM 91® or control oligonucleotides by adding MT-4 cells (2×10^4 cells/well) and either a $1 \times \mathrm{TCID}_{\mathrm{CPE\text{-}90\%}}$ (low) or a $125 \times \mathrm{TCID}_{\mathrm{CPE\text{-}90\%}}$ (high) concentration of HIV-1 IIIB directly to the wells. For post-adsorption assays, MT-4 cells (5×10^6 cells/ml) were pre-incubated with a $10 \times \mathrm{TCID}_{\mathrm{CPE\text{-}90\%}}$ (low) or $125 \times \mathrm{TCID}_{\mathrm{CPE\text{-}90\%}}$ (high) concentration of HIV-1 for 4 h at 37°C, 5% CO_2 in the absence of inhibitors, washed to remove non-adsorbed virus, then added to wells (2×10^4 cells/well) containing inhibitors. Tests using 10-fold dilutions of ddC or GEM 91® in the absence of other drugs were also performed to determine the IC_{50} and IC_{90} values for each drug alone in the presence of high and low concentrations of HIV-1 IIIB.

All cultures were incubated for 6 days at 37°C, 5% CO_2 and the CPE measured using the MTT dye method (Mosmann, 1983; Pauwels et al., 1988). Briefly, cells were incubated with 25 μl of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 7.5 mg/ml) for a period of 4 h, 100 μl of supernatant was then removed from each well and 130 μl of acidified isopropanol was added. When complete solubilization of the formazan crystals had taken place, the absorbance measured at a wavelength of 690 nm was subtracted from that measured at 540 nm for each well using a computer controlled photometer.

2.4. Cytotoxicity

MT-4 cells were incubated at a density of 2×10^4 cells/well with ddC or GEM 91® (from 0.1 μM to 1 mM) in 96-well plates in the absence of virus. Plates were incubated for 6 days at 37°C, 5% CO_2 and cell viability determined using the MTT dye method.

2.5. Experimental data analysis

Control wells containing MT-4 cells alone (Negative control: no virus, no inhibitors), MT-4 cells + virus (Positive infection control: no inhibitors) and MT-4 cells + virus + oligonucleotide (Second agent control) were included in the assays. The % inhibition was calculated using the formula $[1\text{-}((\mathrm{OD}_{\mathrm{negative\ control}}\text{-}\mathrm{OD}_{\mathrm{experimental}})/(\mathrm{OD}_{\mathrm{negative\ control}} - \mathrm{OD}_{\mathrm{positive\ control}}))] \times 100\%$. The % inhibition value of each experimental sample was calculated then the mean and standard deviation was calculated for each treatment group. Values greater than 100% were indicative of an increased growth of cells over experimental con-

trols, i.e. MT-4 cells + culture medium only, due to intra-assay variations. All assays were performed in sextuplicate and all experiments were repeated at least twice with comparable results being obtained.

The IC_{50} and IC_{90} concentrations for ddC alone were compared to the values obtained in the presence of GEM 91® or control oligonucleotides, the ratio of these values giving an indication of the strength of any interaction with ddC. The two sample Student's *t*-test was used to compare differences between the combined drug effect (% inhibition of drug 1 + drug 2) and the additive effect of the individual agents (% inhibition of drug 1 + % inhibition of drug 2). In vitro synergy is defined as a significantly greater effect of two drugs in combination than the sum of the two single drug effects. At drug concentrations where significant synergy was seen with two drugs in combination, the *t*-test was used to compare the GEM 91® plus ddC combination versus GEM 91® derivative plus ddC combinations.

3. Results

3.1. Anti-HIV effects of ddC and GEM 91® tested alone

The anti-HIV effects of ddC and GEM 91® when incubated in the presence of high ($125 \times TCID_{90\%\text{-CPE}}$) and low ($TCID_{90\%\text{-CPE}}$) concentrations of HIV-1 IIIB were determined using the MT-4 cell standard assay, i.e. simultaneous incubation of drugs, cells and virus. Anti-HIV activities were determined by measuring inhibition of HIV-induced CPE following a 6-day incubation with virus and drugs using the MTT dye assay. At low virus concentration, ddC exhibited an IC_{50} value of 0.30 μM and an IC_{90} value of 0.79 μM, these values shifted approximately 10-fold to 2.4 and 7.5 μM, respectively when incubated at the higher concentration of HIV-1 IIIB. For GEM 91®, IC_{50} values were 0.02 and 0.08 μM at low and high concentrations of HIV-1 IIIB, respectively; similarly the IC_{90} value for GEM 91® increased from 0.09 to 0.15 μM (Fig. 1).

3.2. Combined anti-HIV effects of ddC and oligonucleotides: standard assay

Fig. 2 shows the effect of GEM 91® and control oligonucleotides on the anti-HIV activity of ddC following a 6 day incubation with HIV-1 IIIB ($125 \times TCID_{90\%\text{-CPE}}$) under standard conditions, i.e. simultaneous incubation of drugs, cells and virus. The IC_{50} and IC_{90} values of ddC were significantly decreased in the presence of all oligonucleotides tested. For example, the HIV-induced CPE seen in MT-4 cells was not significantly inhibited by ddC at a concentration of 0.1 μM nor by GEM 91® at a concentration of 0.05 μM. When combined however, the same concentrations of drugs resulted in an approximate 60% protective effect as measured by an increase in cell viability.

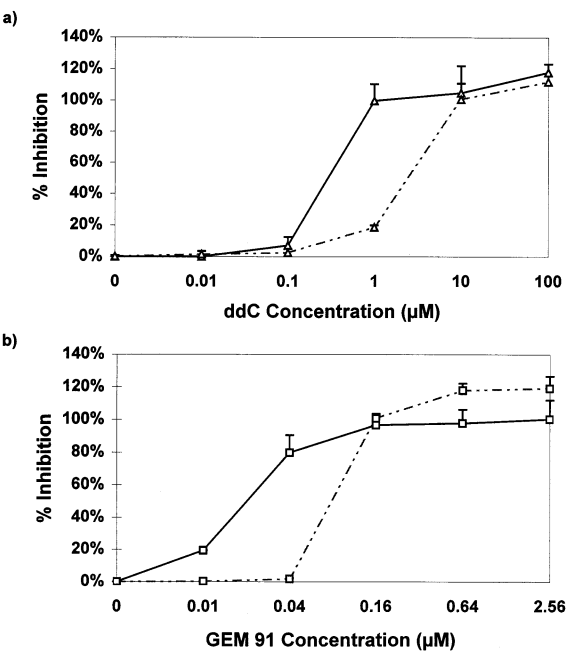

Fig. 1. Inhibition of HIV-1 IIIB induced CPE in MT-4 cells by (a) ddC and (b) GEM 91®. MT-4 cells were incubated with either a low concentration of HIV-1, i.e. 90% tissue culture cytopathic dose ($TCID_{CPE\text{-}90\%}$; shown by continuous line) or a high concentration of HIV-1, i.e. $125 \times TCID_{CPE\text{-}90\%}$ (broken line) under standard assay conditions. The percentage inhibition of CPE was determined by measuring cell viability after a 6-day incubation period using the MTT dye assay.

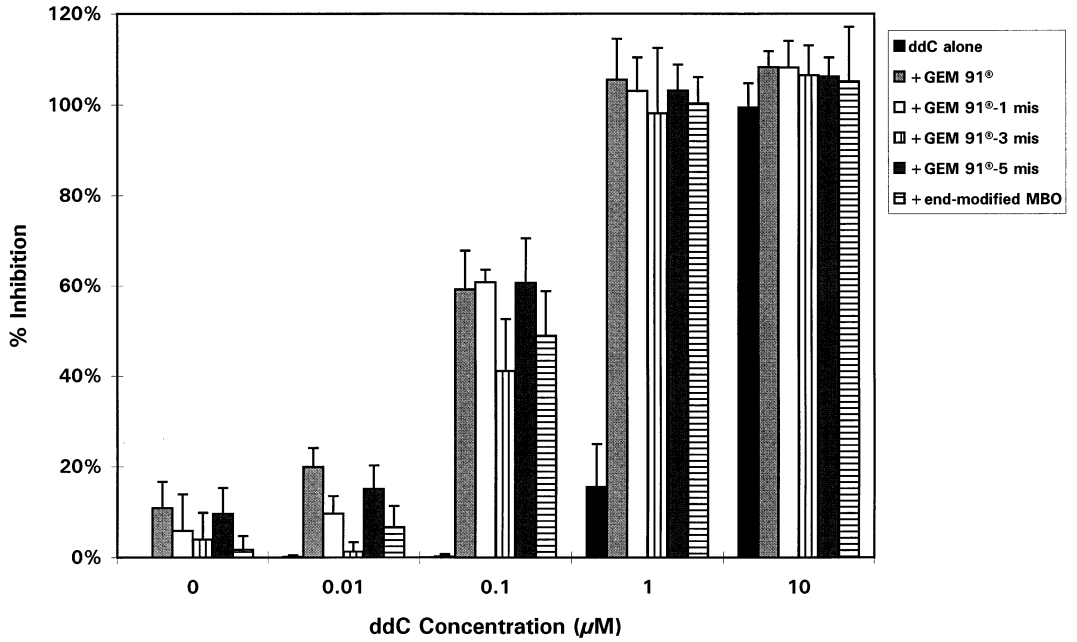

Fig. 2. Effect of ddC in combination with GEM 91®, mismatch antisense controls and end-modified MBO on HIV-1 induced CPE under standard assay conditions. ddC was incubated with MT-4 cells and a $125 \times TCID_{CPE\text{-}90\%}$ concentration of HIV-1 at concentrations of 0.01 to 10 μm either alone or with a fixed concentration (0.05 μM) of GEM 91® or control oligonucleotides. At ddC concentrations of 0.1 and 1.0 μM in combination with all oligonucleotides, a significantly greater antiviral activity was seen than the additive effect of ddC plus oligonucleotide alone (two sample t-test: $P < 0.001$).

3.3. Combined anti-HIV effects of ddC and oligonucleotides: post-adsorption assay

Post-adsorption assays were carried out to investigate whether or not ddC and GEM 91® or its derivatives interacted favorably when cells were pre-incubated with HIV-1 IIIB prior to incubation with drugs, i.e. in an attempt to avoid any virus adsorption inhibition properties exhibited by the oligonucleotides tested. HIV-1 and MT-4 cells were pre-incubated for 4 h and then washed to remove any excess virus prior to incubation with drugs for 6 days. Under these post-adsorption conditions GEM 91® alone exhibited IC_{50} and IC_{90} values of 0.23 and 0.75 μM, respectively following pre-incubation of cells with a $125 \times TCID_{90\%\text{-}CPE}$ concentration of HIV-1 IIIB.

The post-adsorption effects of GEM 91® and control oligonucleotides on the antiviral activity of ddC following incubation with HIV-1 IIIB ($125 \times TCID_{90\%\text{-}CPE}$) are shown in Fig. 3 and Table 1. The IC_{50} and IC_{90} values for ddC were not significantly altered when in combination with any of the GEM 91® derivatives investigated. GEM 91® itself however resulted in a 5.2-fold decrease in ddC IC_{50} value and brought about the largest decrease in ddC IC_{90} value (7.7–4.9 μM). GEM 91® in combination with ddC at concentrations of 0.10 and 1.0 μM, respectively showed significantly greater antiviral activity than the additive effect of ddC alone plus GEM 91® alone ($P < 0.001$) at the same concentrations, as well as an increased antiviral activity over any of the control oligonucleotides in combination with ddC ($P < 0.001$). Each of the phosphorothioate oligonucleotides alone exhibited anti-HIV activities of less than 10% at a concentration of 0.10 μM.

The post-adsorption assay was repeated following incubation of MT-4 cells with a lower concentration of HIV-1 IIIB ($10 \times TCID_{90\%\text{-}CPE}$) and a narrower range of ddC concentrations (Fig. 4). At

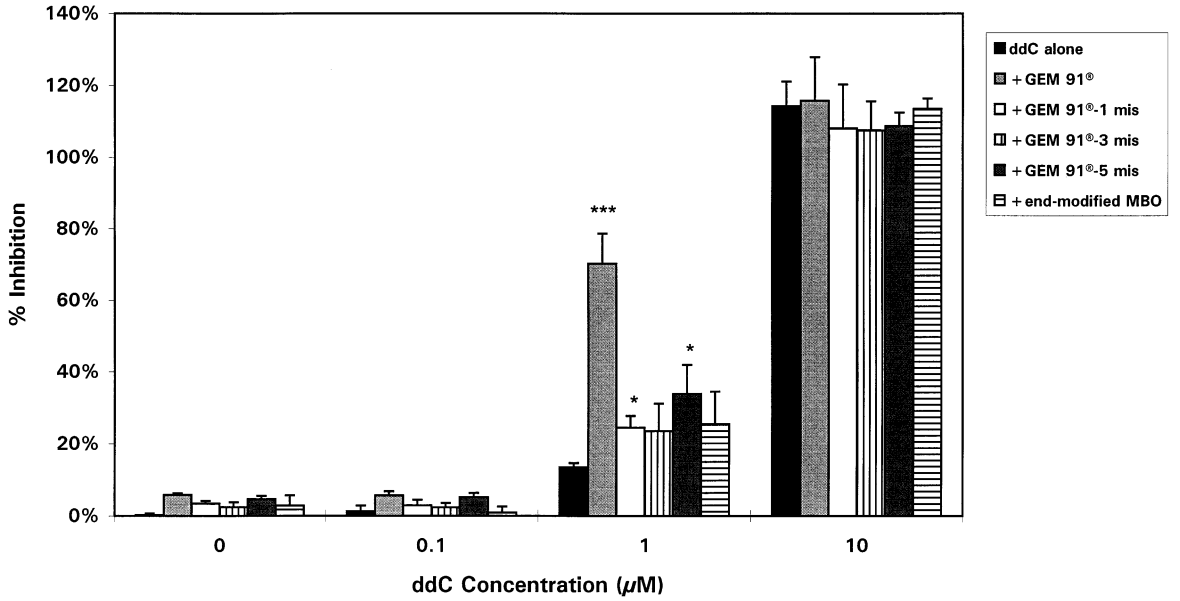

Fig. 3. Post-adsorption effects of ddC in combination with GEM 91®, mismatch antisense controls and end-modified MBO on HIV-1 induced CPE in MT-4 cells. Cells were pre-incubated with 125 × $TCID_{CPE-90\%}$ HIV-1 IIIB for 4 h and then washed to remove any excess virus prior to incubation with ddC (0.1–10 μM) either alone or with a fixed concentration (0.10 μM) of GEM 91® or control oligonucleotides. Drug combinations exhibited significantly greater antiviral activity than the additive effect of ddC alone plus oligonucleotide alone as shown (two sample *t*-test: $*P < 0.05$, $***P < 0.001$).

this lower virus concentration, all the oligonucleotides in combination with ddC showed an advantage over ddC alone at a ddC concentration of 0.4 μM, but GEM 91® again exhibited clearly the strongest interaction, overall resulting in a >6-fold decrease in the IC_{50} value of ddC and a 4.4-fold decrease in IC_{90} value. The control oligonucleotides all showed similar activity in combination with ddC, with decreases in the IC_{50} value of ddC ranging from 1.6- to 3.4-fold and between 1.2- to 1.7-fold changes in the ddC IC_{90} value. GEM 91® at a concentration of 0.10 μM in combination with ddC at concentrations of 0.1 and 0.4 μM exhibited an increased antiviral activity over the GEM 91® derivatives in combination with ddC at the same concentrations ($P < 0.001$).

3.4. GEM 91® in combination with other nucleoside analog drugs

The effect of GEM 91® on the antiviral efficacies of the nucleoside analog reverse transcriptase inhibitors AZT and d4T was also investigated using the same assay conditions as for the studies with ddC. Results from both standard and post-adsorption assays are shown in Table 2. The IC_{50} and IC_{90} values for the nucleoside analogs were decreased to the greatest extent in the standard assay with at least 10-fold changes to these parameters being seen for both AZT and d4T. Post-adsorption data showed 5.8- and 4.6-fold decreases in the IC_{90} values of AZT and d4T respectively at a concentration of GEM 91 which alone gave negligible protection from HIV-induced CPE.

3.5. Drug toxicity

Cytotoxicity assays were carried out with ddC and GEM 91® under the same conditions as were used for the antiviral assays. GEM 91® showed no adverse effects on cell viability at the concentrations used in this study as measured by a decrease in MTT absorbance, whereas ddC exhibited an approximate 25% decrease in cell viability at 160 μM (data not shown), i.e. a concentration signifi-

Table 1
Summary of the post-adsorption effects of GEM 91® and GEM 91® derivatives on the IC_{50} and IC_{90} values of ddC against HIV-induced CPE in MT-4 cells

Combined drug	%Inhibition of drug alone	ddC IC_{50}	ddC IC_{90}	IC_{50} ratio	IC_{90} ratio
ddC Only	—	2.6	7.7	—	—
GEM 91®	5.8	0.5	4.9	5.2	1.6
GEM 91®-1 mis	3.4	2.2	7.8	1.2	1.0
GEM 91®-3 mis	2.4	2.2	7.5	1.2	1.0
GEM 91®-5 mis	4.6	1.8	7.1	1.4	1.1
End-modified MBO	2.8	2.1	7.3	1.2	1.1

All combined drugs were present at a concentration of 0.1 μM, a concentration which resulted in the percentage inhibition shown in column 2 when incubated alone, i.e. with no ddC.
All IC_{50} and IC_{90} values are expressed in μM.

cantly higher than the maximum concentration used in these studies. It should also be noted that for a drug to show a protective effect against HIV in this assay the cells must be viable at the end of the 6-day incubation period. This in vitro cell toxicity data is in agreement with data previously published for these two agents (Blackley et al., 1990; Agrawal and Tang, 1992; Lisziewicz et al., 1994).

4. Discussion

With an ever increasing number of compounds with efficacy against HIV at various stages of clinical and pre-clinical development, it becomes more important that agents from different classes of drugs are screened for potential drug interactions. Results from in vitro studies can indicate possible synergistic or antagonistic drug interactions before drug combinations are assessed in clinical trials and thus, can be used to help in the choosing of drug regimens for the treatment of HIV-infection. This is particularly important for HIV/AIDS as the trend in treatment continues to shift towards multi-drug combinations (Goebel, 1995; Lange, 1995a,b; Gazzard, 1996).

In order to look at possible interactions between the phosphorothioate oligonucleotide GEM 91® and the nucleoside analog ddC, we tested combinations of the two drugs in a HIV-induced cytopathic effect inhibition assay using MT-4 cells. In the standard assay experiments, there was a significant shift in the IC_{50} and IC_{90} values of ddC in the presence of GEM 91®, despite the oligonucleotides being present in the assay at concentrations that exhibited a very limited anti-HIV effect when incubated alone. For example, HIV-induced CPE was not significantly inhibited by ddC at a concentration of 0.1 μM or by GEM 91® at a concentration of 0.05 μM. When these agents were combined at these concentrations however, an approximate 60% decrease in CPE was seen, suggesting a strong synergistic interaction between the two drugs. Results from the same experiment carried out with GEM 91® mismatch control oligonucleotides and end-modified MBO gave similar results suggesting that this synergistic effect is brought about by a sequence non-specific effect of GEM 91®. Comparable data was also obtained from studies looking at the effect of GEM 91® on the activities of AZT and d4T using this standard assay technique.

These results with the standard assay system are consistent with data previously published showing a synergistic interaction between ddA and S-dC14 (Matsukura et al., 1987). The mechanism of that synergistic activity was unclear but seems likely to be predominantly related to non-specific inhibition of HIV-1 adsorption to cells by polyionic oligonucleotides. If the amount of virus inside the cell is significantly reduced by the presence of oligonucleotide, then ddC will exhibit enhanced efficacy at the level of reverse transcription inhibition. The shift in ddC IC_{50} and IC_{90} values that this would bring about may be com-

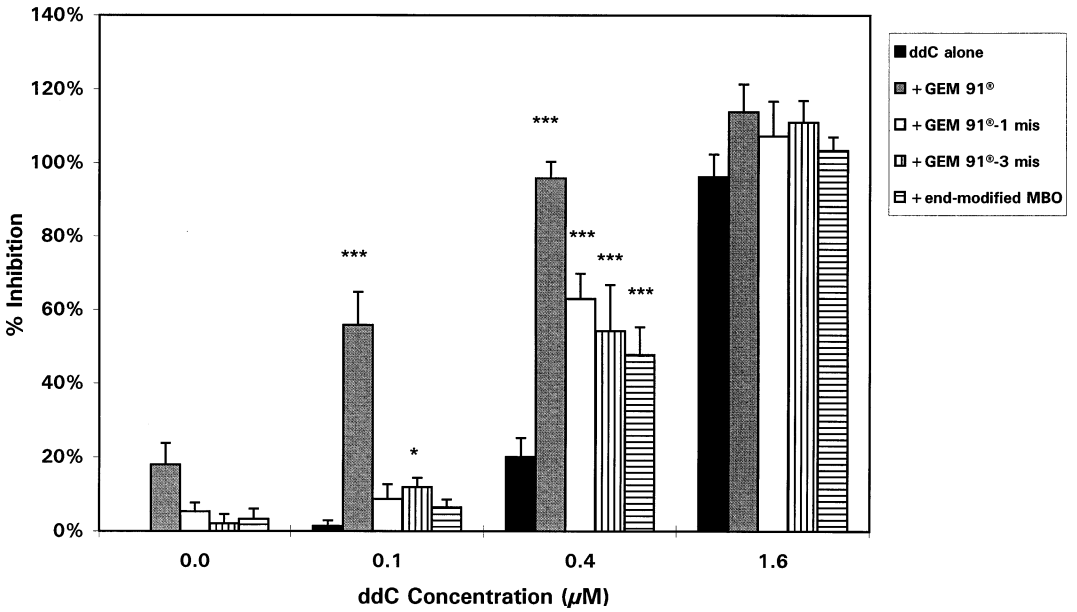

Fig. 4. Post-adsorption effects of ddC in combination with GEM 91®, mismatch antisense controls and end-modified MBO on HIV-1 induced CPE in MT-4 cells. Cells were pre-incubated with $10 \times TCID_{CPE\text{-}90\%}$ HIV-1 IIIB for 4 h and then washed to remove any excess virus prior to incubation with ddC (0.1–1.6 μM) either alone or with a fixed concentration (0.10 μM) of GEM 91® or control oligonucleotides. Drug combinations exhibited significantly greater antiviral activity than the additive effect of ddC alone plus oligonucleotide alone as shown (two sample t-test: $^{*}P < 0.05$, $^{***}P < 0.001$).

parable to that seen in experiments investigating the activity of ddC alone in the presence of high and low concentrations of HIV (Fig. 1).

In further experiments the standard CPE inhibition assay was modified in order to look at post-adsorption drug effects only, i.e. drugs were added only after incubation of HIV-1 IIIB and MT-4 cells and removal of excess virus. GEM 91® alone (0.1 μM) did not exhibit a significant inhibition of HIV-induced CPE but combination with ddC (1 μM) resulted in approximately 70% inhibition. At this concentration ddC alone achieved less than 20% inhibition (Fig. 3). Comparable results were seen in experiments carried out at both high and low concentrations of HIV-1 IIIB. A comparable interaction was not seen with any of the GEM 91® mismatch oligonucleotides or end-modified MBO, suggesting that GEM 91® interacts favorably with ddC through a sequence-specific mechanism. It should be noted that this assay can only determine if an effect is 'sequence-specific', not whether it is a true antisense effect or not. The failure of end-modified MBO to demonstrate enhanced activity over the GEM 91® mismatch controls is consistent with the possibility that GEM 91® reverse transcriptase priming activity may be involved in this synergism with ddC. As with the standard assay results, AZT and d4T again exhibited a similar level of interaction with GEM 91®, suggesting that this synergy may potentially exist between GEM 91® and the nucleoside analog class of drugs as a whole.

With regards to a possible correlation between the data presented and the in vivo situation in HIV-infected patients, a number of points should be made. We have shown in vitro synergy between GEM 91® and ddC, i.e. the antiviral effect of the two drugs combined was significantly greater than the additive effect of the drugs alone. However, this favorable interaction was only seen to be sequence-specific under certain assay conditions that may not be relevant to the true in vivo situation. The high HIV titer used for example may not have clinical relevance in patients receiv-

Table 2
Summary of the effect of GEM 91® on the IC_{50} and IC_{90} values of the nucleoside analog drugs AZT and d4T against HIV-induced CPE in MT-4 cells under standard and post-adsorption incubation conditions

Nucleoside analog	Assay	IC_{50}	IC_{90}	IC_{50} ratio	IC_{90} ratio
AZT	Standard	0.026	0.079	—	—
AZT+GEM 91®	Standard	0.001	0.005	26.0	15.8
d4T	Standard	2.6	7.8	—	—
d4T+GEM 91®	Standard	0.07	0.60	37.1	13.1
AZT	Post-adsorption	0.007	0.058	—	—
AZT+GEM 91®	Post-adsorption	0.002	0.010	3.5	5.8
d4T	Post-adsorption	0.39	3.9	—	—
d4T+GEM 91®	Post-adsorption	0.25	0.85	1.6	4.6

GEM 91® was present at a concentration of 0.05 μM for standard assay experiments and 0.10 μM for post-adsorption studies. All IC_{50} and IC_{90} values are expressed in μM and represent mean values from $n \geq 2$ experiments.

ing current anti-HIV combination therapy. It should also be noted that the synergistic interactions shown were only seen over a relatively narrow concentration range. Although the drug concentrations examined in this study are all within the range of those obtained in vivo, the drug ratios resulting in synergistic effects may not necessarily be achieved in the plasma of HIV-infected patients.

In a previous study the non-sequence specific effects of the homopolymer phosphorothioate S-dC28 were examined in combination with AZT. No synergistic interaction was observed between AZT and this non-complementary oligonucleotide in a post-adsorption assay using acutely infected MT-4 cells (Chou et al., 1991). Interactions were examined using p24 core antigen and reverse transcriptase measurements and data analysed by the median-effect method of Chou and Talalay (Chou and Talalay, 1984), the different assay methods used are unlikely to explain the difference in results from these two studies however. One important difference between that work and the current study, using GEM 91®, is that GEM 91® is complementary to the *gag* initiation site of HIV-1 and has been shown to have sequence specific inhibitory effects on several intracellular stages of the viral replication cycle, as well as the sequence non-specific effects observed with S-dC28 (Yamaguchi et al., 1997).

It is important to note that individual antisense oligonucleotides have properties that are unique to themselves, such as unanticipated interactions with specific enzymes or indirect effects on cell metabolism (Burgess et al., 1995; Bordier et al., 1995; Krieg et al., 1995). Similarly, the nucleoside analog drugs themselves have been shown to exhibit contrasting efficacies in different cell systems and to interact differently with other antiviral agents (Balzarini et al., 1988; Gao et al., 1993, 1994a; Shirasaka et al., 1995). For example, the antiviral drug ribavirin interacts antagonistically with AZT due to a decrease in thymidine kinase activity but does not inhibit the activities of other nucleoside analog drugs (Vogt et al., 1987). Conversely, the ribonucleotide reductase inhibitor hydroxyurea potentiates the activity of ddI to a far greater extent than other dideoxynucleosides, due to its preferential effect in inhibiting dATP synthesis (Gao et al., 1994b).

It is interesting to note that whilst a significant difference in activity between antisense oligonucleotides and mismatch controls is historically difficult to show in this type of cell culture assay (due to the strong non-specific inhibition of HIV-1 adsorption), such a difference in activity may be more clearly seen through its sequence-specific synergy with another drug.

We believe that this is the first report of a synergistic interaction between an antisense phosphorothioate oligonucleotide targeted to HIV and

a nucleoside analog drug. Further studies, including those using different antiviral assays to look at effects on individual stages of virus replication, are required to give us a clearer picture as to the significance of this category of drug synergy.

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