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Inhibition of human immunodeficiency virus activity by phosphorodithioate oligodeoxycytidine

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ABSTRACT **Phosphorothioate oligodeoxynucleotides exert a sequence-independent cytoprotective effect against human immunodeficiency virus type 1 (HIV-1). We now report that phosphorodithioate-containing oligodeoxycytidines are very potent inhibitors of HIV-1 reverse transcriptase *in vitro*, as they exhibit an increasing inhibitory effect with length and number of phosphorodithioate internucleotide linkages. This inhibitory effect can be at least 30-fold greater with phosphorodithioate oligodeoxycytidine than for the corresponding phosphorothioate analog of similar length. In cell culture, phosphorodithioate oligodeoxycytidines are active inhibitors of syncytia formation and effectively inhibit *de novo* infection of target cells by HIV-1. Moreover, comparative experiments show that a deoxycytidine phosphorodithioate 14-mer is as effective an inhibitor of *de novo* infection as a phosphorothioate-containing 28-mer. Such potent inhibition by oligomers of relatively short length makes dithioate analogs an additional class of potential therapeutic agents against acquired immunodeficiency syndrome.**

Over the past several years, oligodeoxynucleotides and their analogs have been reported to inhibit viral replication and the expression of oncogenes in cell culture (1, 2). Although the pioneering work of Zamecnik and Stephenson (3, 4) used normal oligodeoxynucleotides, recent research has focused on derivatives (Fig. 1) that have certain desirable features, including nuclease resistance and rapid transport into cells (5–8). Generally these oligomers appear to function by inhibiting translation and splicing, but other inactivation mechanisms have also been found (9).

During studies with oligodeoxynucleotides as inhibitors of human immunodeficiency virus type 1 (HIV-1), Matsukura *et al.* discovered that phosphorothioate oligodeoxycytidines exhibited potent antiviral activity (10); in these oligomers, one of the nonbridging oxygens at each linkage in oligodeoxycytidine, $(dC)_n$, has been replaced with sulfur, and individual oligomers are designated $S(dC)_n$. Inhibition of HIV-1 reverse transcriptase (RT) (11) or other steps in its life cycle such as binding, fusion, and entry (5, 10) were suggested as possible sites of action. Subsequent studies corroborated these observations and demonstrated that phosphorothioates are more effective than phosphoramidates and methylphosphonates as sequence-nonspecific inhibitors of HIV-1 replication (12).

These developments prompted us to examine oligodeoxycytidine analogs bearing one or more phosphorodithioate linkages (Fig. 1) as potential inhibitors of HIV-1 activity. This analog (13–20) has the two nonbridging oxygen atoms replaced with sulfur and generates an achiral phosphorus center that is isoelectronic to phosphate; individual analogs are designated $S_2(dC)_n$. Oligodeoxynucleotides with this linkage

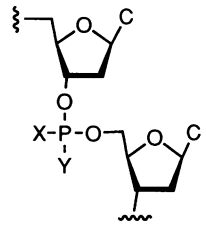

FIG. 1. Schematic representation of phosphodiester and various phosphate-modified oligodeoxynucleotides. X = Y = O, phosphodiester; X = O, Y = S, phosphorothioate; X = O, Y = CH_3, methylphosphonate; X = O, Y = NR_2, phosphoramidate with alkyl or aryl substituents; X = Y = S, phosphorodithioate.

are resistant to nucleases (13, 16, 18) and hybridize to complementary unmodified DNA and RNA (21).

MATERIALS AND METHODS

Oligodeoxycytidines. Phosphorothioate-containing (22), phosphorodithioate-containing (16, 17, 23), and unmodified oligodeoxycytidines (24) were synthesized and characterized by conventional procedures.

Inhibition of Purified HIV-1 RT. Twenty-microliter assay mixtures contained 50 mM Tris (pH 8.3); 10 mM $MgCl_2$; 50 mM KCl; 5 mM dithiothreitol; 1.0 μM preannealed primed template, 5'-^{32}P-labeled d(GpApTpTpCpApGpCpTpApGpTpCpCpA) primer and d(CpApApApCpTpGpTpGpApTpApCpGpApTpGpGpApCpTpApGpCpTpGpApApTpC) template; 250 μM each dNTP; and variable concentrations of the oligodeoxycytidine inhibitor. Reactions were initiated by addition of HIV-1 RT at 10 nM and continued for 15 min at 37°C. Under these conditions, the enzyme was saturated with respect to both substrates. Reaction mixtures were fractionated by denaturing polyacrylamide gel electrophoresis. Results shown in Fig. 2 are of individual experiments. The results reported in Table 1 as ID_{50} (the micromolar concentration of oligomer required to inhibit a reaction to 50% of an uninhibited control) are the average of at least four independent experiments, with a standard deviation being <20%. Purified HIV-1 RT (25, 26) was a gift of S. Hughes (National Cancer Institute).

Inhibition of Other DNA Polymerases. Experimental analyses were essentially identical to those described for HIV-1 RT. Purified calf thymus α polymerase (Pol α) was a gift of R. Kuchta (University of Colorado), avian myeloblastosis virus (AMV) RT was from Life Sciences (St. Petersburg,

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Abbreviations: $(dC)_n$, oligodeoxycytidine of n nucleotides; $S(dC)_n$, phosphorothioate analog of $(dC)_n$; $S_2(dC)_n$, phosphorodithioate analog of $(dC)_n$; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; AMV, avian myeloblastosis virus; Pol I, DNA polymerase I; Pol α, α polymerase.

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FL), and the Klenow fragment of *Escherichia coli* DNA polymerase I (Pol I) was from United States Biochemical. Enzyme concentrations were identical in all cases (10 nM). Primed template was added at concentrations necessary to give polymerizations similar to those observed for HIV-1 RT: 0.05 μM with AMV RT, 5.0 μM with Pol I Klenow fragment, and 2.0 μM with Pol α.

Inhibition of HIV-1 RT in Viral Lysates. Viral lysates from MOLT-3 cells chronically infected with HIV-1 (HTLV-III$_B$ isolate) were incubated for 1 hr at 37°C with variable concentrations of S(dC)$_{12}$ or S$_2$(dC)$_{12}$ in 80 mM Tris (pH 8)/100 mM NaCl/10 mM MgCl$_2$/10 mM dithiothreitol/100 μg of bovine serum albumin per ml, 2.5 μg of poly(rA·dT)$_{12-15}$ and 12 μM [^{3}H]dTTP in a reaction volume of 200 μl. Reaction products were precipitated with trichloroacetic acid, and tritiated thymidine incorporation was monitored by liquid scintillation counting.

Syncytia Formation Assay. Syncytia were measured by mixing 1 × 10^5 CEM target cells with 5 × 10^3 chronically infected MOLT-3/HIV-1 cells in the presence of oligodeoxynucleotides. Samples were incubated for 48 hr at 37°C, and the number of giant cells was quantitated microscopically.

Cell-Free Infection Assay (27). CEM cells (10^5 cells) were infected with HIV-1 [1000 TCID$_{50}$ (tissue culture 50% infective dose) units] in the presence of oligomers in complete RPMI medium [RPMI 1640 medium as described (5) and further modified to include 20 units of recombinant interleukin-2 (Amgen Biologicals) per ml and 15% (vol/vol) of conventional interleukin-2 (Advanced Biotechnology, Silver Spring, MD)] for 24 hr. The cells were washed and plated in the presence of oligomer in 1.5 ml of complete RPMI medium. After 5 days of exposure to the oligodeoxynucleotide, the cells were washed twice with 10 ml of complete RPMI medium and replated in 4 ml of complete RPMI medium without oligomer. RT activity was measured after 2 further days of incubation.

Cytopathic Effect Inhibition Assay (10). Briefly, ATH8 cells (2 × 10^5 cells) were treated with oligodeoxynucleotides and infected with HIV-1 (500 virus particles per cell). Mock infections were identical except that additional complete RPMI medium was added instead of virus. After 6 days, viable cells were counted by using a hemocytometer in conjunction with the trypan blue dye-exclusion method.

RESULTS

Inhibition of HIV-1 RT. One stage of the HIV replicative cycle, which has been a major target for therapeutic intervention, is reverse transcription of viral RNA (28). Our initial investigations focused on the inhibitory effects of phosphorodithioate DNA analogs on the DNA-dependent DNA polymerase activity of recombinant HIV-1 RT (25, 26). A synthetic heteropolymer primer–template system was designed in which a primer anneals to the complementary sequence at the 3′ end of the template. This substrate was recognized by HIV-1 RT with high affinity (K_m = 0.13 μM) and provided an easily quantitated system for measuring DNA polymerase activity in the presence of oligodeoxynucleotide inhibitors.

In a first set of experiments (Fig. 2*a*), (dC)$_n$ analogs of constant length (n = 15) but having variable numbers of phosphorodithioate linkages were tested as inhibitors. In agreement with others (11), we found that the phosphodiester oligomer, (dC)$_{15}$, is a poor inhibitor (ID$_{50}$ = 36 μM, Table 1). A single phosphorodithioate linkage positioned anywhere in the oligomer did not significantly increase its inhibitory effect. However, as more dithioate linkages were added, the inhibitory characteristics increased significantly [compare oligomers S$_2$(dC)$_{15}$(I) and S$_2$(dC)$_{15}$(III) in Table 1]. The most effective inhibitor of similar length was the poly(phosphorodithioate) tetradecamer [S$_2$(dC)$_{14}$, ID$_{50}$ = 62 nM; 13 S$_2$ linkages]. This compound was 30- and 600-fold more inhibitory than S(dC)$_{15}$ and the unmodified pentadecamer, (dC)$_{15}$, respectively.

Examination of ID$_{50}$ values for S$_2$(dC)$_n$ analogs with n from 4 to 28 reveals a correlation between length and inhibition (Fig. 2*b* and Table 1). As the oligomer length approached that of typical primers recognized by RTs (29), inhibition increased significantly. This observation agrees with our initial mechanistic studies, which indicated a competitive inhibition with respect to primed template (W.S.M. and M.H.C., unpublished results). Further length increases gave rise to enhanced inhibition. S$_2$(dC)$_{28}$ has an ID$_{50}$ of 1.5 nM, showing it is 40 times more effective than S$_2$(dC)$_{14}$. A comparative analysis with the phosphorothioate homologs S(dC)$_8$, S(dC)$_{15}$, and S(dC)$_{28}$ reveals an interesting trend. For the two shorter oligomers, the factor by which the phosphorodithioate is more effective than the thioate homolog remained constant, whereas this factor decreased from 30- to 7-fold for the 28-mer homologs. If one plots the increase in inhibition on

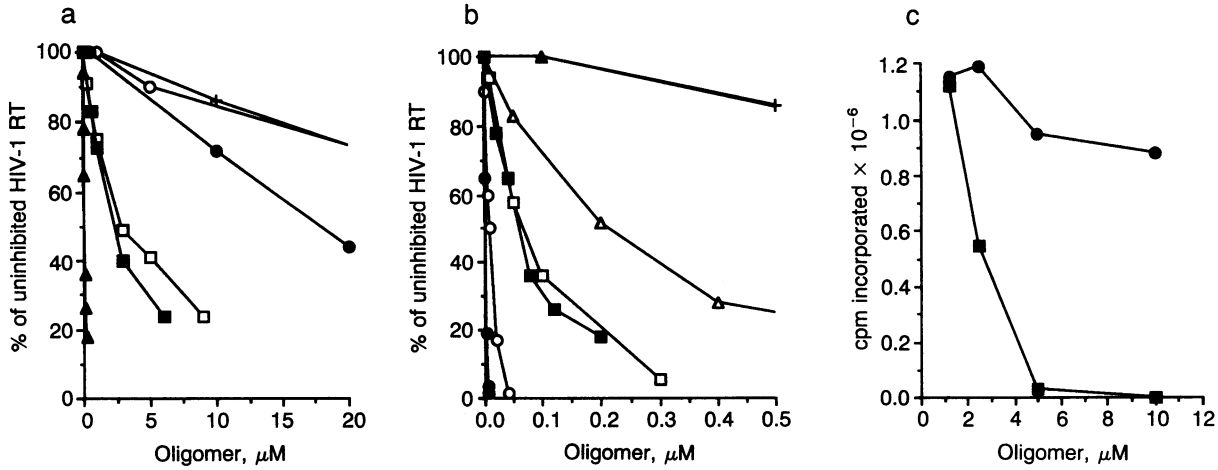

FIG. 2. Inhibition of HIV-1 RT by phosphorothioate and phosphorodithioate oligodeoxycytidine analogs. (*a*) Inhibition by oligomers having a variable number of phosphorodithioate linkages. +, dC$_{15}$; ○, S$_2$(dC)$_{15}$(I); ●, S$_2$(dC)$_{15}$(II); □, S$_2$(dC)$_{15}$(III); ■, S(dC)$_{15}$; ▲, S$_2$(dC)$_{14}$. See the footnote to Table 1 for definitions of (I), (II), and (III). (*b*) Inhibition by phosphorodithioate oligodeoxycytidine analogs of variable length. +, S$_2$(dC)$_4$; ▲, S$_2$(dC)$_8$; △, S$_2$(dC)$_{10}$; □, S$_2$(dC)$_{12}$; ■, S$_2$(dC)$_{14}$; ○, S$_2$(dC)$_{20}$; ●, S$_2$(dC)$_{28}$. For certain oligomers [(dC)$_{15}$ and S$_2$(dC)$_{15}$(I) in *a* and S$_2$(dC)$_4$ and S$_2$(dC)$_8$ in *b*], inhibition was minimal, and only the initial data points are plotted. (*c*) Inhibition by S(dC)$_{12}$ and S$_2$(dC)$_{12}$ of HIV-1 RT in crude viral lysates. ●, S(dC)$_{12}$; ■, S$_2$(dC)$_{12}$.

Table 1. ID_{50} values obtained for HIV-1 RT with phosphorodithioate- and phosphorothioate-containing oligodeoxycytidine analogs

Oligodeoxycytidine	ID_{50}, μM
$(dC)_{15}$	36
$S_2(dC)_{15}$(I)*	33
$S_2(dC)_{15}$(II)†	18
$S_2(dC)_{15}$(III)‡	2.8
$S_2(dC)_4$	20
$S_2(dC)_8$	1.2
$S_2(dC)_{10}$	0.22
$S_2(dC)_{12}$	0.062
$S_2(dC)_{14}$	0.062
$S_2(dC)_{20}$	0.010
$S_2(dC)_{28}$	0.0015
$S(dC)_8$	25
$S(dC)_{15}$	1.8
$S(dC)_{28}$	0.010

In sequences of oligomers I, II, and III below, p represents phosphodiester and x represents phosphorodithioate.
*d(CpCpCpCpCpCpCxCpCpCpCpCpCpCpC).
†d(CpCxCpCpCpCpCpCpCpCpCpCpCxCpC).
‡d(CxCpCxCpCxCpCxCpCxCpCxCpCxCpC).

a per residue basis vs. phosphorodithioate oligomer length, the curve fits an exponential decay (data not shown). This indicates a law of diminishing return for dithioate oligomers beyond a certain length (12–14 nucleotides). In contrast, the largest increment in inhibition for thioate oligomers occurred in going from a 15-mer to a 28-mer. So whereas phosphorothioate oligomers of greater length are required for potent inhibition, relatively short phosphorodithioate oligomers are effective inhibitors.

Our attention turned to viral lysates to ensure that phosphorodithioate analogs had inhibitory properties against the RNA-dependent DNA polymerase activity of the p66–p51 heterodimer found in virusoids. The results (Fig. 2*c*) reveal that the phosphorodithioate dodecamer, $S_2(dC)_{12}$, is an effective inhibitor of HIV-1 RT in these lysates under conditions where the thioate analog of the same length is unable to alter activity even at the highest oligomer concentration tested. These observations mimic results with recombinant HIV-1 RT as reported in Fig. 2*a* and *b* and confirm the conclusion that relatively short phosphorodithioate oligomers are potent inhibitors of this enzyme.

Inhibition of Other DNA Polymerases. To complete the RT inhibition analysis, the effect of these oligodeoxycytidines on other DNA polymerases was studied (Table 2). AMV RT was strongly inhibited by $S_2(dC)_{14}$ but not $S(dC)_{15}$. Inhibition of the Klenow Pol I fragment was also examined. Here $S_2(dC)_{14}$ was less effective at inhibiting the Klenow polymerase than the HIV-1 RT by a factor of about 20, but for $S(dC)_{15}$, this factor (the selectivity index) was only 8. Finally, the inhibitory properties against calf thymus Pol α were examined. Both the phosphorothioate and phosphorodithioate analogs of oligodeoxycytidine are good inhibitors with selectivity indices of only 2. These results are in agreement with reports that the K_i for $S(dC)_{28}$ is about the same for calf thymus Pol α and HIV-1 RT (11). While cytotoxicity may appear to be a likely possibility from these studies, cellular compartmentalization and the preexistence of the cellular polymerases as part of large protein–DNA complexes may protect these enzymes from inhibition by oligodeoxynucleotides. So far there is little evidence to suggest that phosphorothioate or phosphorodithioate oligodeoxynucleotide analogs are strongly cytotoxic to cells in culture (see results of the cytopathic effect inhibition assay below and refs. 5 and 10).

Table 2. Inhibitory effect of phosphorothioate and phosphorodithioate oligodeoxycytidine analogs on various DNA polymerases

	$S_2(dC)_{14}$		$S(dC)_{15}$	
Enzyme	ID_{50}, μM	S.I.*	ID_{50}, μM	S.I.*
HIV-1 RT	0.062		1.8	
AMV RT	0.250		41	
Pol I (Klenow)	1.20	20	13	8
Pol α	0.16	2.6	3	2

*The selectivity index (S.I.) is the ratio of the ID_{50} for Pol I or calf thymus Pol α divided by the ID_{50} for HIV-1 RT.

Inhibition of Syncytia Formation. A characteristic feature of HIV-1 infection of susceptible cells *in vitro* is the formation of syncytia (30, 31), which are supracellular aggregates caused by interactions between viral gp120 and CD4. By mixing chronically HIV-1-infected cells expressing gp120 with target cells bearing CD4 receptors, we were able to assess the effect of oligodeoxycytidine analogs on potential cell-surface interactions. A comparative analysis with the $S(dC)_{12}$ and $S_2(dC)_{12}$ homologs was simultaneously conducted. Oligomer length was set at 12 nucleotides to test whether our observations of potent RT inhibition at reduced length for dithioate oligomers would carry over to this analysis. The phosphorodithioate dodecamer, $S_2(dC)_{12}$, was indeed an effective inhibitor of syncytia formation (Fig. 3*a*). Comparison with the thioate dodecamer shows that the dithioate is the more potent inhibitor. If one notes either the concentration at which 50% of the syncytia are inhibited or at which nearly all syncytia are eliminated, the dithioate oligomer is about 4-fold more effective.

Inhibition of HIV-1 Replication. The effect of phosphorothioate and phosphorodithioate oligodeoxycytidine dodecamers, $S(dC)_{12}$ and $S_2(dC)_{12}$, on infection of target cells by HIV-1 was also examined. In the first experiments, target CEM cells were infected with HIV-1 in the presence of oligomer. After infection the cells were washed to remove excess oligomer and replated without oligomer to avoid fortuitous RT inhibitory effects by the dithioate oligomer. S_2dC_{12} was found to protect cells against infection by HIV-1 (Fig. 3*b*), as a strong antiviral effect was observed at 1.25 μM; by 2.5 μM, this oligomer appeared to suppress infection of target cells. In contrast, $S(dC)_{12}$ exhibited little inhibitory effect even at 10 μM, which suggests that phosphorothioate oligomers of greater length are required to observe antiviral effects in the 1–2 μM range (10, 32). To insure that these assays were reflective of viral infection, we examined other HIV-1 proteins and found that $S_2(dC)_{12}$ inhibits the production of all immunoprecipitable HIV-1 proteins in the concentration range tested (C.A.S., W.S.M., and M.H.C., unpublished results).

Other experiments measured the ability of dithioate oligomers to rescue viable cells under conditions where HIV-1 infection caused cell death (Fig. 4). As this assay examined the effect of oligonucleotides on uninfected cells in culture, cytotoxicity could also be evaluated. ATH8 cells were infected with HIV-1 in the presence of $S_2(dC)_{12}$ or $S_2(dC)_{14}$. A simultaneous comparison with a phosphorothioate oligomer was again conducted, but because $S(dC)_{12}$ and $S(dC)_{14}$ lacked efficacy in a similar concentration range, a comparison was made with $S(dC)_{28}$, the most effective phosphorothioate oligomer previously examined (10).

Both $S_2(dC)_{12}$ and $S_2(dC)_{14}$ appeared to exert a cytoprotective effect on cells infected *de novo* with HIV-1. $S_2(dC)_{12}$ substantially blocked HIV-1 infection at 3 μM (Fig. 4*a*), but at 0.3 μM the cytoprotective effect was marginal. For S_2dC_{14} (Fig. 4*b*), strong anti-HIV activity was observed from 0.5 μM to 1 μM. As expected (10), the $S(dC)_{28}$ was also cytoprotective in this concentration range (0.5–3 μM). These results are encouraging as they demonstrate that $S_2(dC)_{14}$ is as cytoprotective against HIV-1 as is $S(dC)_{28}$. Phosphorodithioate and

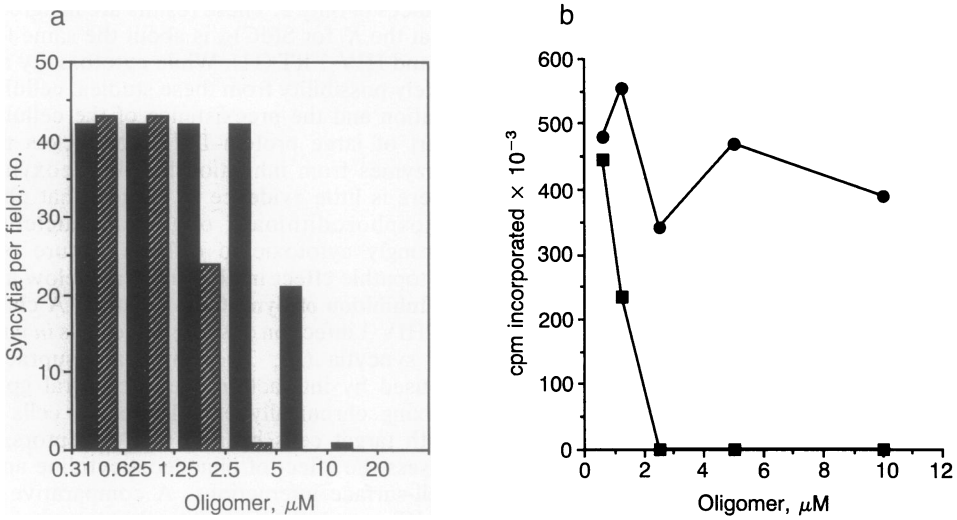

FIG. 3. (*a*) Inhibition of syncytia formation by $S_2(dC)_{12}$ (hatched bars) and $S(dC)_{12}$ (solid bars). (*b*) Inhibition of HIV-1 replication by $S_2(dC)_{12}$ (■) and $S(dC)_{12}$ (●) as measured by the *de novo* synthesis of HIV-1 RT.

phosphorothioate oligomers also exert their cytoprotective effect at concentrations well below levels at which significant cytotoxicity occurs. Even at the highest concentrations tested (10 μM), the cytotoxicity of $S_2(dC)_{12}$ and $S_2(dC)_{14}$ was similar to that of $S(dC)_{28}$, and in all cases the effect was marginal (25–35% loss in cell number).

DISCUSSION

These results suggest that phosphorodithioate DNA analogs should be further investigated as a potential therapeutic agent against HIV-1. *In vitro* experiments demonstrate that phosphorodithioate oligodeoxycytidine inhibits both the DNA-dependent and RNA-dependent DNA polymerase activity of HIV-1 RT. This inhibition is dramatic even for oligomers containing 12 and 14 nucleotides and also exceeds the inhibitory potential of the corresponding phosphorothioates by at least 30-fold. Cell culture studies demonstrate that the phosphorodithioate 14-mer, $S_2(dC)_{14}$, has the same cytoprotective effect against HIV-1 infection as the phosphorothioate 28-mer $S(dC)_{28}$. However, the cell-culture experiments do not necessarily demonstrate that the cytoprotective effect of phosphorodithioate oligomers is due to inhibition of reverse transcriptase. The results of the syncytia formation assays indicate that these oligomers may exert an inhibitory effect at the cell surface. However, since the relative inhibitory activity of dithioate oligomer compared with its thioate homolog in the cell-free infection assay is greater than that observed in the syncytia formation assay (Fig. 3), dithioate oligomers may exert antiretroviral effects at several stages in the infection process. These results indicate that dithioate DNA analogs may protect cells against HIV infection by multiple mechanisms—a possibility that could enhance their potential therapeutic value.

The enhanced antiviral activity of relatively short phosphorodithioate oligomers is particularly exciting for two reasons. First, shorter oligomers are more efficiently transported across cell membranes (33). Second, as one needs to perform fewer synthetic couplings to produce a potent inhibitor, phosphorodithioate analogs of reduced length represent a more feasible class of potential therapeutic oligonucleotides.

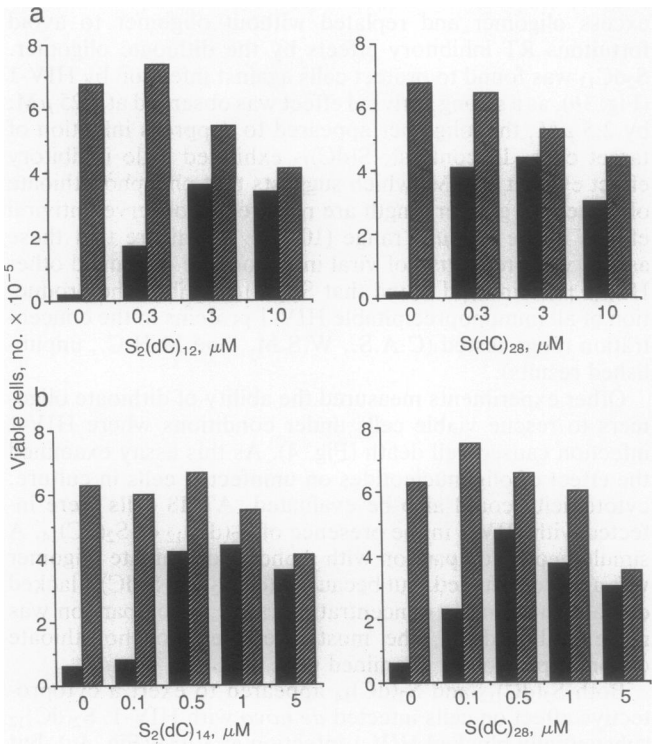

FIG. 4. Inhibition of HIV-1 replication by phosphorothioate and phosphorodithioate oligodeoxycytidine analogs as measured in the cytopathic effect inhibition assay. (*a*) Inhibition of the cytopathic effect of HIV-1 by $S_2(dC)_{12}$ (*Left*) and $S(dC)_{28}$ (*Right*). (*b*) Inhibition of cytopathic effect by $S_2(dC)_{14}$ (*Left*) and $S(dC)_{28}$ (*Right*). Solid bars, with virus; hatched bars, without virus.

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