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Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelope-mediated cell fusion

(phosphorothioate/oligonucleotide/gp120/V3 loop/random libraries)

JACQUELINE R. WYATT*, TIMOTHY A. VICKERS*, JOSEPH L. ROBERSON†, ROBERT W. BUCKHEIT, JR.†, THOMAS KLIMKAIT‡, ELIZABETH DEBAETS*, PETER W. DAVIS*, BERNARD RAYNER§, J. L. IMBACH§, AND DAVID J. ECKER*

*ISIS Pharmaceuticals, 2292 Faraday Avenue, Carlsbad, CA 92008; †Southern Research Institute, 431 Aviation Way, Frederick, MD 21701; ‡CIBA-Geigy, Pharmaceutical Research, Basel, Switzerland; and §Universite Montepellier II, Laboratoire de Chimie Bio Organique, Place Eugene Bataillon, 34095 Montpellier, Cedex 5, France

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ABSTRACT **The phosphorothioate oligonucleotide $T_2G_4T_2$ was identified as an inhibitor of HIV infection *in vitro* by combinatorial screening of a library of phosphorothioate oligonucleotides that contained all possible octanucleotide sequences. The oligonucleotide forms a parallel-stranded tetrameric guanosine-quartet structure. Tetramer formation and the phosphorothioate backbone are essential for antiviral activity. The tetramer binds to the human immunodeficiency virus envelope protein gp120 at the V3 loop and inhibits both cell-to-cell and virus-to-cell infection.**

Specific binding of nucleic acids to proteins regulates cellular functions at many levels. Recently the notion of using synthetic nucleic acids as ligands to modulate the activities of proteins for therapeutic purposes has received considerable attention. Several *in vitro* strategies have been developed to selectively screen for nucleic acid sequences that bind to specific proteins (1, 2), including proteins that are not naturally bound by nucleic acids (3).

The selection strategy known as SURF (synthetic unrandomization of randomized fragments) (4) was employed to screen libraries of oligonucleotides for anti-human immunodeficiency virus (HIV) activity in cell culture. This selection technique involves iterative synthesis and screening of increasingly simplified sets of oligonucleotide sequences. Iterative techniques have been used with peptides (5–7). Oligonucleotide analogs can be used because this technique does not require enzymes either to synthesize the randomized oligonucleotides or to amplify the active molecular species. The use of nuclease-resistant oligonucleotide analogs allows screening for functional activities in cell culture systems in which unmodified RNA or DNA is rapidly degraded. Assay of libraries in the presence of whole viruses and cells provides the opportunity for inhibition at any step in the viral life cycle. Since functional inhibition of viral infection rather than nucleic acid binding is the selection criterion, the chances are enhanced that a useful compound will be identified.

When the SURF strategy was used in a functional screen for an inhibitor of HIV infection, phosphorothioate oligonucleotides with four consecutive guanosines were found to have activity. Of the active compounds, $T_2G_4T_2$ was chosen for further studies. Physical characterization of this oligonucleotide demonstrated that it formed a tetramer stabilized by G quartets. G-quartet structures are intra- or intermolecular four-stranded helices stabilized by planar Hoogsteen-paired quartets of guanosine (8, 9). The structures are stabilized by monovalent ions bound between two planes of G quartets and coordinated to the carbonyl oxygens. The G-quartet structure and a phosphorothioate backbone were shown to be required for antiviral activity.

Cell culture experiments revealed that the G-quartet structure blocks binding of HIV virions to cells and virus-mediated cell fusion. Virus and cell membrane fusion is initiated by binding of gp120, the major envelope protein of HIV, to its cellular receptor. The mechanism by which membrane attachment occurs is not well understood, but a particular region of gp120, the V3 loop, is essential. The V3 loop maintains a high percentage of positively charged amino acids across all HIV strains (10), and the extent of virus-mediated cell fusion and rapid viral replication have been correlated with increased cationic composition of the V3 loop (11). *In vitro* studies showed that the G-quartet structure bound directly to the V3 loop of gp120. We hypothesize that the G-quartet structure provides a scaffold to present the phosphorothioate groups in a favorable orientation for binding to the highly cationic V3 loop.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Phosphorothioate oligonucleotides were synthesized by standard protocols (12). For oligonucleotides with randomized positions, the proportions of the individual amidites in a mixture were adjusted until equal amounts of the four dimers were obtained when coupled to dT on controlled-pore glass, as judged by reversed-phase HPLC. Oligonucleotides were purified by reversed-phase HPLC with a gradient of methanol in water. Several purified oligonucleotides were analyzed for base composition by total digestion with nuclease followed by reversed-phase HPLC (Len Cummins, personal communication) and yielded the expected ratio of each base.

Oligonucleotide 5320 ($T_2G_4T_2$) was purified by reverse-phase chromatography and then by size-exclusion chromatography to purify the tetramer (see below). Prior to antiviral screening, oligonucleotides were diluted to 1 mM strand concentration in 40 mM sodium phosphate, pH 7.2/100 mM KCl and incubated at room temperature overnight. Extinction coefficients were determined as described (13). Samples were sterilized by filtration through 0.2-μm cellulose acetate filters.

Characterization of Tetramer. Monomeric and tetrameric forms of oligonucleotides were separated on a Pharmacia Superdex 75 HR 10/30 size-exclusion column in 25 mM

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Abbreviations: HIV, human immunodeficiency virus; SURF, synthetic unrandomization of randomized fragments.

sodium phosphate, pH 7.2/0.2 mM EDTA at a flow rate of 0.5 ml/min with detection at 260 nm. For purification, a Pharmacia Superdex 75 HiLoad 26/60 column was used with 10 mM sodium phosphate (pH 7.2) at a flow rate of 2 ml/min.

Dissociation of the tetramer at 37°C was followed by size-exclusion chromatography after dilution of a 1 mM solution of oligonucleotide to 10 μM with phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8 mM Na_2HPO_4). Oligonucleotide 5320 in K^+ and the phosphodiester $T_2G_4T_2$ were diluted from solutions in 40 mM sodium phosphate, pH 7.2/100 mM KCl. Oligonucleotide 5320 in Na^+ was diluted from a solution in 40 mM sodium phosphate, pH 7.2/100 mM NaCl.

The tetramer formed was parallel-stranded as determined by analysis of the complexes formed by the phosphorothioate oligonucleotides $T_2G_4T_2$ and 5′-$T_{13}G_4T_4$-3′. Each sample contained 125 μM unlabeled and 15 pM radioactively labeled amounts of one or both of the oligonucleotides. The samples were heated in 50 mM sodium phosphate, pH 7.2/200 mM KCl in a boiling water bath for 15 min then incubated for 48 hr at 4°C. Samples were analyzed by autoradiography of a nondenaturing 20% polyacrylamide (19:1 acrylamide/*N,N′*-methylenebisacrylamide weight ratio) gel run at 4°C in 1× TBE running buffer (89 mM Tris/89 mM boric acid/2 mM EDTA).

Antiviral Assays. Oligonucleotides were screened in an acute HIV-1 infection assay which measures protection from HIV-induced cytopathic effects (14). Assay of virus-induced cell fusion used chronically HIV-1-infected Hut-78 cells (Hut/4-3) and $CD4^+$ HeLa cells harboring a bacterial *lacZ* gene driven by a retroviral long terminal repeat. Cells were cocultured for 20 hr in the presence or absence of oligonucleotide and then fixed with 1% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline. A standard *o*-nitrophenyl β-D-galactopyranoside assay was used to quantitate β-galactosidase expression.

Binding of 5320 to gp120. Biotinylated oligonucleotides were incubated with gp120-coated microtiter plates. After 1 hr, streptavidin-alkaline phosphatase was added. After 1 hr, *p*-nitrophenyl phosphate substrate was added and absorbance at 405 nm was measured. The site of 5320 binding to gp120 was determined by competition with antisera specific for various regions of the protein (15–17). Oligonucleotide (25 μM) was incubated in gp120-coated microtiter plates for 1 hr. Antiserum was added and incubated for 40 min. After washing, antibody bound was quantitated by using protein A/G-alkaline phosphatase.

Extent of binding of 5320 to gp120 and CD44 on HeLa cells harboring an HIV-1 *env* c gene (18, 19) was quantitated. Binding to gp120 was detected by using a fluorescein-conjugated murine mouse IgG monoclonal antibody (mAb) specific for gp120 of HIV-1_{IIIB} (AGMED, Bedford, MA). CD44 binding was detected with fluorescein-conjugated mouse anti-CD44 IgG mAb (Becton Dickinson). Cells were incubated 15 min at room temperature with oligonucleotide before antibody was added and incubated for 1 hr at 4°C. After washing, immunofluorescence was measured on a Becton Dickinson FACScan. Extent of binding to CD4 on CEM-T4 cells (20) was determined by using Q425, a mouse anti-CD4 IgG mAb (21) and goat $F(ab')_2$ anti-mouse IgG as above.

RESULTS AND DISCUSSION

Selection and Characterization of $T_2G_4T_2$. A phosphorothioate oligonucleotide library containing all possible sequences of eight nucleotides divided into 16 sets, each consisting of 4,096 sequences, was screened for inhibition of HIV infection (Table 1). The *in vitro* assay measured protection of cells from HIV-induced cytopathic effects (14). In the initial rounds of selection, antiviral activity was observed only in the set containing guanosine in two fixed positions. Subsequent rounds of selection showed that four consecutive guanosines were required for maximum antiviral activity. No strong selection preference was observed for nucleotides flanking the guanosine core. The sequence $T_2G_4T_2$ (oligonucleotide 5320) was chosen for further study because structures of similar sequences have been well characterized (22–25). The concentration of 5320 required for 50% inhibition of virus-induced cell killing (IC_{50}) was 0.3 μM. The antiviral activity of this oligonucleotide was not a result of inhibition of cell metabolism; cytotoxic effects were not observed until cells were incubated with ≈100 μM 5320.

Although the oligonucleotide 5320 has a phosphorothioate backbone, evidence suggests that it adopts a four-stranded, parallel helix as do phosphodiester oligonucleotides of similar sequence (22–25). The oligonucleotides in the combinatorial library pools that show antiviral activity (Table 1) and oligonucleotide 5320 form multimeric complexes as shown by size-exclusion chromatography. The retention time of the complex was that expected for a tetrameric species, as judged from plots of retention time versus the logarithm of the molecular weight of phosphorothioate oligonucleotide standards (data not shown). The circular dichroism spectrum of the multimeric form of oligonucleotide 5320 is characterized

Table 1. Activity of phosphorothioate oligonucleotide pools in acute HIV assay

Round	Combinatorial pool	IC_{50}, μM			
		X = A	X = G	X = C	X = T
1	NNANXNNN	Inactive	Inactive	Inactive	Inactive
	NNGNXNNN	Inactive	19.5 (5%)	Inactive	Inactive
	NNCNXNNN	Inactive	Inactive (0%)	Inactive	Inactive
	NNTNXNNN	Inactive	Inactive	Inactive (0%)	Inactive
2	NNGXGNNN	60.7	1.8 (36%)	55.6	56.2 (3%*)
3	NNGGGXNN	8.0	0.5 (94%)	3.1 (19%*)	8.6
4	NAGGGGXN	0.5	0.5	0.5	0.5 (87%)
	NGGGGGXN	0.5	0.6 (99%*)	0.4	0.5
	NCGGGGXN	0.7	0.6	0.5 (91%)	0.4
	NTGGGGXN	0.4 (82%)	0.5	0.4	0.5
5	XTGGGGTN	0.2 (94%)	0.6 (89%*)	0.3 (94%)	0.3 (94%)
6	TTGGGGTX	0.6 (90%)	0.6	0.5	0.3 (93%)

Random positions, N, are an equimolar mixture of each base. Antiviral data are reported as the quantity of drug (μM oligonucleotide strand) required to inhibit 50% of virus-induced cell killing (IC_{50}). Error in the IC_{50} is ±0.1 μM. "Inactive" pools showed no antiviral activity at 100 μM strand concentration. The percent tetramer is given in parentheses for selected pools.
*Multiple aggregate species.

by a peak at 265 nm and a trough at 242 nm (data not shown), similar to the spectra reported by others for oligodeoxynucleotide tetramers (24, 26–28). It has been reported that when two phosphodiester oligonucleotides of dissimilar size, but each containing four or five guanosines in a row, are incubated together, five distinct aggregate species are formed in a nondenaturing gel (29, 30). In principle, only a tetramer of parallel strands can explain this pattern. When this experiment was performed with two phosphorothioate oligonucleotides, the antiviral oligonucleotide 5320 and a 21-residue oligonucleotide containing four guanosines near the 3′ end (5′-$T_{13}G_4T_4$-3′), the five aggregate species expected for a parallel-stranded tetramer were observed in a nondenaturing gel (Fig. 1*A*).

The Tetramer Is Active Against HIV. Samples of 5320 screened for antiviral activity were diluted from a 1 mM stock solution that was at least 98% tetramer. The tetramer is stable indefinitely at 1 mM strand concentration; no decrease in tetramer was observed over 5 months in a 1 mM sample in buffer containing 100 mM KCl at room temperature. Upon dilution to concentrations used in antiviral assays (<25 μM) dissociation of the tetramer begins; however, kinetics of the dissociation are very slow (Fig. 1*B*). Slow kinetics for association and dissociation of intermolecular G-quartet complexes have been reported (27, 29). The half-life for the dissociation of the potassium form of 5320 is about 45 days. During the 6-day period of the acute antiviral assay, at least 70% of the sample remained in the tetramer form whether the sample was prepared in Na^+ or K^+. Both Na^+ and K^+ forms have the same IC_{50} values in the acute antiviral assay, even though K^+ preferentially stabilized the tetramer.

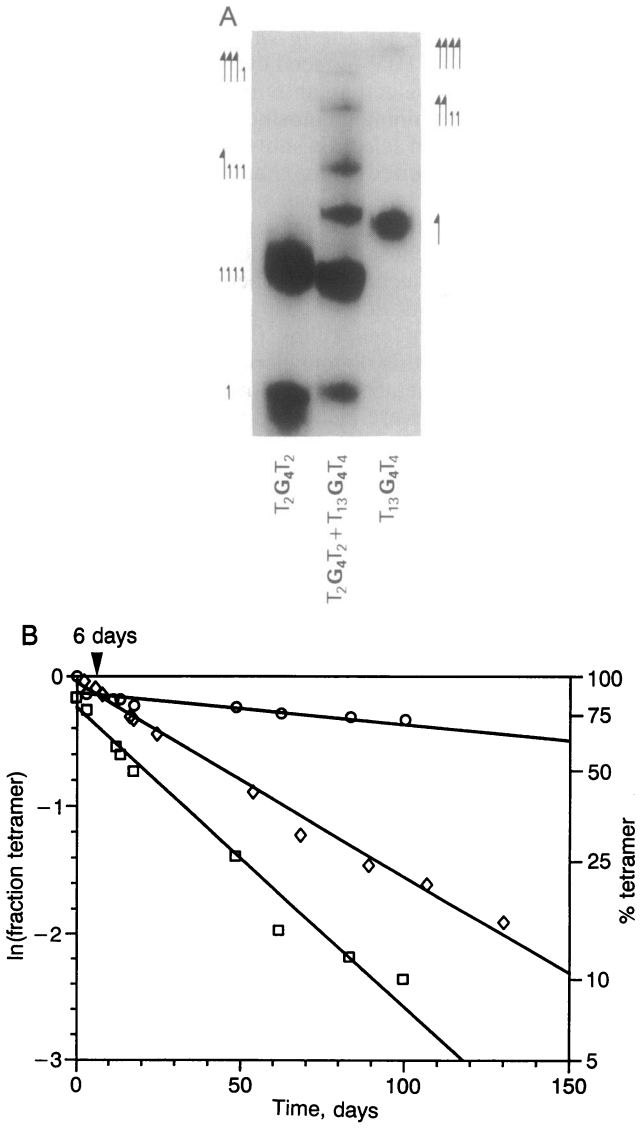

FIG. 1. (*A*) Autoradiogram of a gel electrophoresis experiment showing a pattern characteristic of a parallel-stranded tetramer. Lane 1, $T_2G_4T_2$ (5320); lane 2, $T_2G_4T_2$ incubated with phosphorothioate 5′-$T_{13}G_4T_4$-3′; lane 3, 5′-$T_{13}G_4T_4$-3′. (*B*) Dissociation of tetramers formed by 5320 in Na^+ (□), 5320 in K^+ (◇), and the phosphodiester analog (○) is slow, with half-lives on the order of months. The natural logarithm of the fraction tetramer as determined from size-exclusion chromatography is plotted as a function of incubation time at 37°C.

Heat denaturation of the tetrameric complex formed by 5320 before addition to the antiviral assay resulted in loss of activity; antiviral activity was recovered upon renaturation (data not shown). The striking difference in antiviral activity among the initial 16 sets of oligonucleotides used for combinatorial screening can be explained by the presence or absence of the G-core and therefore the tetramer structure (Table 1). In the initial round of screening, ≈12% of the molecules in the active 5′-NNGNGNNN-3′ pool contained at least four sequential guanosines, and size-exclusion chromatography showed that 5% of the oligonucleotides formed tetramers (Table 1). In contrast, in the other three round 1 pools where X = G only 0.4% of the molecules contained at least four sequential guanosines and no tetramer was observed. In other pools, there were no molecules with four consecutive guanosines.

The sequence $T_2G_4T_2$ with a phosphodiester backbone was inactive in the anti-HIV assay, even though the phosphodiester tetramer appears to be kinetically more stable than that formed by the phosphorothioate 5320 (Fig. 1*C*). Thus, the G-quartet structure was not sufficient for antiviral activity. Two hypotheses were considered: the phosphorothioate backbone may be mechanistically required or the modified backbone may prevent nuclease-mediated degradation of the oligonucleotide. Oligonucleotide analogs with the glycosidic bond oriented in the α position are resistant to nuclease degradation (31). Both the phosphorothioate α-oligonucleotide and the phosphodiester α-oligonucleotide formed tetramers as indicated by size-exclusion chromatography. Only the phosphorothioate analog was active against HIV, with an IC_{50} of 0.5 μM. Thus, the phosphorothioate backbone is mechanistically required for antiviral activity of this oligonucleotide.

Tetramer Inhibits Viral Binding or Fusion to $CD4^+$ Cells. Oligonucleotide 5320 directly inhibited viral binding or fusion in a cellular system (Fig. 2). In this assay, chronically HIV-1-infected cells served as donor cells for the viral proteins envelope and Tat and the $CD4^+$ recipient cells harbored a reporter gene (*lacZ*) controlled by the Tat-transactivatable HIV long terminal repeat. Upon envelope-mediated fusion of the two cell types, the Tat protein provided by the donor cell induced production of β-galactosidase from the reporter gene within the recipient (T.K., unpublished data). The presence of 1 μM 5320 effectively blocked fusion-induced β-galactosidase expression in this assay. The control phosphorothioate oligonucleotide 5′-TGTGTGTG-3′ did not reduce cell fusion significantly. Consistent with the result that no activity was observed in any chronic infection model tested, neither oligonucleotide interfered with HIV gene induction or transfection efficiency when the recipient cells were transfected with proviral DNA of a fusion-incompetent HIV-1 mutant (32).

Tetramer Binds to the V3 Domain of gp120. Cellular experiments indicated that 5320 blocks viral binding or fusion; therefore, the affinities of the 5320 tetramer for CD4 and gp120 were determined. Biotinylated 5320 bound to immobilized gp120 with a dissociation constant (K_d) of <1 μM. In contrast, a control phosphorothioate, 5′-$T_2A_4T_2$-biotin-3′,

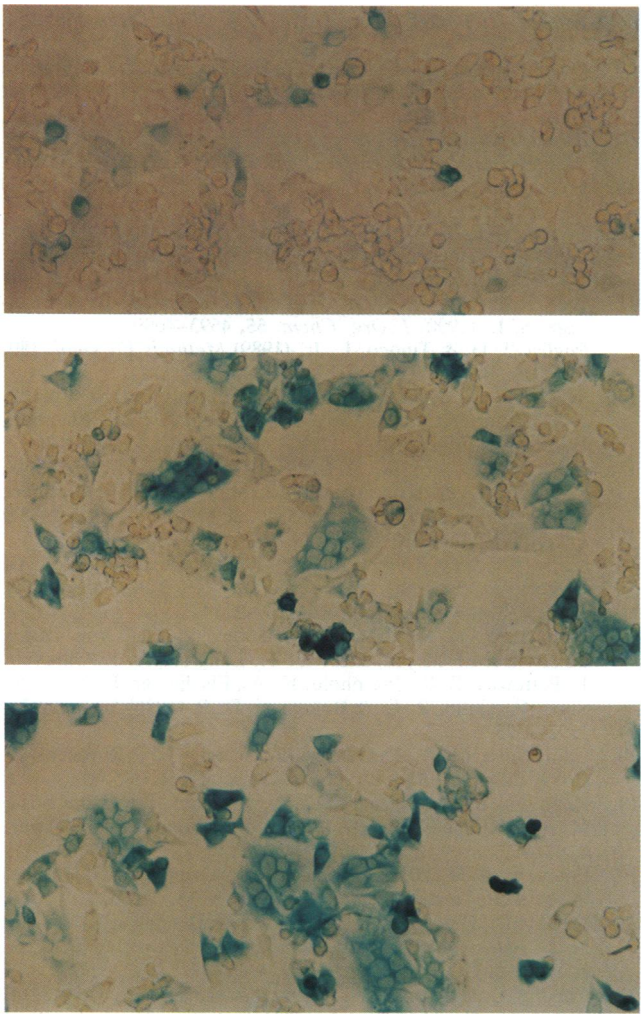

FIG. 2. 5320 inhibits syncytium formation between chronically infected cells and CD4$^+$ cells (harboring an HIV promoter-driven *lacZ* gene). Cells were cultured in the presence of 1 μM 5320 (*Top*), in the presence of 1 μM control 5′-TGTGTGTG-3′ (*Middle*), or in the absence of oligonucleotide (*Bottom*).

bound weakly to gp120 with an estimated K_d of 260 μM. Similar experiments using CD4-coated microtiter plates showed that biotinylated 5320 also associated with CD4; however, the K_d of $\approx$25 μM was considerably weaker than that for gp120. The control bound CD4 with an $\approx K_d$ of 240 μM. Qualitative gel shift assays (33) were performed to determine the affinity of 5320 for other HIV proteins (Tat, p24, reverse transcriptase, Vif, protease, gp41), soluble CD4, and nonrelated proteins (bovine serum albumin, transferrin, RNase V_1). Both monomeric and tetrameric forms of 5320 bound to albumin and reverse transcriptase. Tetramer-specific binding was observed only to gp120 and CD4.

The V3 loop of gp120 (amino acids 303–338) is considered the principal neutralizing domain of the protein; peptides derived from this region elicit type-specific neutralizing antibodies that block viral infection by blocking fusion (34). The V3 loop of gp120 is also the site of action of anionic polysaccharides, such as dextran sulfate, that inhibit viral binding, replication, and syncytium formation (10). Dextran sulfate is a competitive inhibitor of binding of biotinylated 5320 to gp120 immobilized on a microtiter plate. About 50% of the tetramer binding was inhibited at a dextran sulfate concentration between 10 and 50 μg/ml. Dextran sulfate has been shown to inhibit binding of gp120-specific antibodies to gp120 in this concentration range (10). The oligonucleotide 5320 also interferes with binding of antibodies directed against the V3 loop region of gp120, but not with binding of antibodies specific for another region of the protein (15–17) (Fig. 3*A*). The control oligonucleotide had no effect on antibody binding.

The tetramer also binds to the V3 loop of gp120 expressed on cells. Binding of a monoclonal antibody specific for the V3 loop of gp120 was inhibited by 5320 at a concentration of $\approx$0.5 μM (K_i) as determined by immunofluorescence flow cytometry (Fig. 3*B*). The control oligonucleotide had little effect on binding at concentrations up to 50 μM. Neither oligonucleotide significantly decreased binding of antibodies directed to human CD44 on the same cells or to CD4 (21) on CEM-T4 cells (20).

ELISAs were performed to determine whether 5320 could block the interaction between CD4 and gp120 (data not shown). About 50% of the binding of CD4 to immobilized

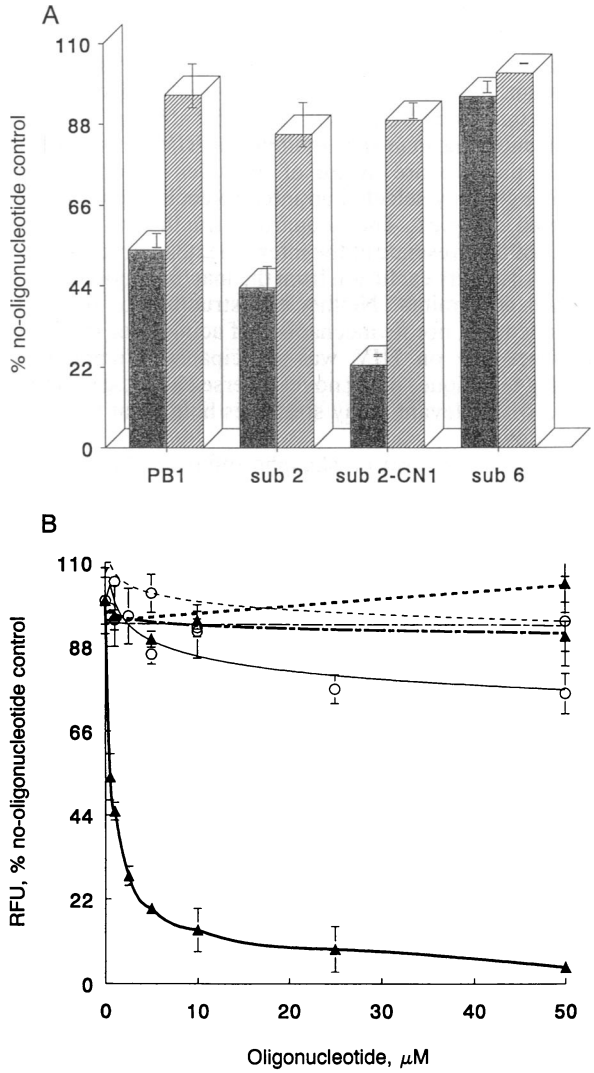

FIG. 3. (*A*) Oligonucleotide 5320 (shaded bars) blocks binding of antibodies directed to the gp120 V3 loop (amino acids 303–338). Binding of polyclonal antibodies PB1 (specific for amino acids 292–474), PB1-sub2 (amino acids 295–404), and PB1 sub2-CN1 (amino acids 295–333) are inhibited by 5320. Binding of PB1-sub6 (amino acids 350–474) was not inhibited by 5320. The control oligonucleotide (hatched bars) had no effect. (*B*) 5320 blocks binding of an antibody specific for the V3 loop of gp120 (solid line), but not antibodies specific for CD44 (even dashes) or CD4 (uneven dashes), as determined by immunofluorescence flow cytometry. The percent relative fluorescence units (RFU) is plotted against concentration of 5320 (▲) and control (○). A concentration of 0.5 μM 5320 inhibited 50% of the binding of the anti-gp120 antibody.

gp120 was inhibited by 5320 at ≈2.5 μM. The control oligonucleotide had no effect. Compounds that bind to the V3 loop of gp120 can inhibit fusion without completely blocking the interaction between CD4 and gp120 (10). Unlike 5320, dextran sulfate does not prevent the gp120/CD4 interaction in an ELISA, even at concentrations 10,000-fold above its IC_{50} (10).

Phosphorothioate oligonucleotides of at least 15 nucleotides are known to be non-sequence-specific inhibitors of HIV (35). In the acute assay system used here, previously tested phosphorothioate oligonucleotides of 18–28 nucleotides have IC_{50} values of 0.2–4 μM (36). Stein and coworkers have shown that phosphorothioate oligonucleotides of at least 18 nucleotides bind to the V3 loop of gp120 (37) and to the CD4 receptor and other cell surface antigens (35). Variation in the binding and antiviral activities of long mixed-sequence oligonucleotides most likely results from folding into unknown structures with varying affinities for membrane surface proteins. In contrast, 5320 adopts a defined tetrameric structure. The antiviral activity is 2- to 25-fold better, on a weight basis, than that of longer oligonucleotides.

Conclusions. The SURF combinatorial strategy was effective in identifying an *in vitro* inhibitor of HIV. This is the first example that we are aware of in which a combinatorial screening strategy selected an intermolecular noncovalently assembled complex. The G-quartet structure was selected because of the presence of the intermolecular structure in the active pool and its exclusion from the inactive pools based on sequence constraints. Neither the structure of the active oligonucleotide, nor its mechanism of action, nor the molecular target site on HIV was anticipated. Combinatorial screening techniques using novel diverse chemical structures coupled with relevant assay strategies hold great promise in drug discovery.

The tetrameric form of phosphorothioate $T_2G_4T_2$ blocks cell-to-cell and virion-to-cell spread of HIV infection by binding to the gp120 V3 loop. The tetramer provides a rigid, compact structure with a high thio-anionic charge density that may be the basis for its strong interaction with the cationic V3 loop. Although the V3 loop is a hypervariable region, the functional requirement for cationic residues in the V3 loop may limit the virus's ability to become resistant to dense polyanionic inhibitors. The oligonucleotide has been screened against a panel of clinical isolates and drug-resistant strains of HIV. The IC_{50} values range from 0.2 to 2 μM for all strains tested (R.W.B. and J.L.R., unpublished data). Compounds derived from the G-quartet structural motif are potential candidates for use in anti-HIV chemotherapy.

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