ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1995, p. 2426–2435 Vol. 39, No. 11
0066-4804/95/$04.00+0

T30177, an Oligonucleotide Stabilized by an Intramolecular Guanosine Octet, Is a Potent Inhibitor of Laboratory Strains and Clinical Isolates of Human Immunodeficiency Virus Type 1

JOSHUA O. OJWANG,[1] ROBERT W. BUCKHEIT,[2] YVES POMMIER,[3] ABHIJIT MAZUMDER,[3] KAREN DE VREESE,[4] JOSÉ A. ESTÉ,[4] DIANE REYMEN,[4] LUKE A. PALLANSCH,[2] CAROL LACKMAN-SMITH,[2] THOMAS L. WALLACE,[1] ERIK DE CLERCQ,[4] MICHAEL S. MCGRATH,[5] AND ROBERT F. RANDO[1]*

Triplex Pharmaceutical Corporation, The Woodlands, Texas 77380[1]; Frederick Research Center, Frederick, Maryland 21701[2]; Laboratory of Molecular Pharmacology, National Cancer Institute, Bethesda, Maryland 20892-4255[3]; Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium[4]; and Department of Laboratory Medicine, San Francisco General Hospital, University of California at San Francisco, San Francisco, California, 94110[5]

Received 22 June 1995/Returned for modification 1 August 1995/Accepted 21 August 1995

T30177, an oligonucleotide composed of only deoxyguanosine and thymidine, is 17 nucleotides in length and contains single phosphorothioate internucleoside linkages at its 5′ and 3′ ends for stability. This oligonucleotide does not share significant primary sequence homology with or possess any complementary (antisense) sequence motifs to the human immunodeficiency virus type 1 (HIV-1) genome. T30177 inhibited replication of multiple laboratory strains of HIV-1 in human T-cell lines, peripheral blood lymphocytes, and macrophages. T30177 was also found to be capable of inhibiting multiple clinical isolates of HIV-1 and preventing the cytopathic effect of HIV-1 in primary $CD4^+$ T lymphocytes. In assays with human peripheral blood lymphocytes there was no observable toxicity associated with T30177 at the highest concentration tested (100 μM), while the median inhibitory concentration was determined to be in the range of 0.1 to 1.0 μM for the clinical isolates tested, resulting in a high therapeutic index for this drug. In temporal studies, the kinetics of addition of T30177 to infected cell cultures indicated that, like the known viral adsorption blocking agents dextran sulfate and Chicago sky blue, T30177 needed to be added to cells during or very soon after viral infection. However, analysis of nucleic acids extracted at 12 h postinfection from cells treated with T30177 at the time of virus infection established the presence of unintegrated viral cDNA, including circular proviral DNA, in the treated cells. In vitro analysis of viral enzymes revealed that T30177 was a potent inhibitor of HIV-1 integrase, reducing enzymatic activity by 50% at concentrations in the range of 0.050 to 0.09 μM. T30177 was also able to inhibit viral reverse transcriptase activity; however, the 50% inhibitory value obtained was in the range of 1 to 10 μM, depending on the template used in the enzymatic assay. No observable inhibition of viral protease was detected at the highest concentration of T30177 used (10 μM). In experiments in which T30177 was removed from infected cell cultures at 4 days post-HIV-1 infection, total suppression of virus production was observed for more than 27 days. PCR analysis of DNA extracted from cells treated in this fashion was unable to detect the presence of viral DNA 11 days after removal of the drug from the infected cell cultures. The ability of T30177 to inhibit both laboratory and clinical isolates of HIV-1 and the experimental data which suggest that T30177 represents a novel class of integrase inhibitors indicate that this compound is a viable candidate for evaluation as a therapeutic agent against HIV-1 in humans.

One relatively new approach used in the development of antiviral therapeutics for human immunodeficiency virus type 1 (HIV-1) is the use of oligonucleotides designed as antisense agents (24, 26, 31). While much effort is being spent on rationally designed oligonucleotides such as antisense agents, there have also been recent findings of multiple alternative mechanisms by which oligonucleotides can inhibit viral infections (16, 27, 33, 35). For example, Stein et al. (37) have characterized the interaction of oligo(dC), which contains a phosphorothioate (PT) backbone [poly(SdC)] with the v3 loop of HIV-1 gp120. It was determined that $poly(SdC)_{28}$ specifically interacted with the positively charged v3 loop with a K_d of approximately 5×10^{-7} M. Stein et al. (37) then postulated that the interaction of poly(SdC) with the HIV-1 v3 loop may be a mechanism by which poly(SdC) could inhibit HIV-1 in vivo. More recently, Wyatt et al. (42) have described the interaction of a short dG-rich oligonucleotide, synthesized with a total PT backbone, which also interacts with the v3 loop of HIV-1 gp120. In addition, we have previously reported that oligonucleotides containing only deoxyguanosine (dG) and thymidine (T) synthesized with natural phosphodiester (PD) internucleoside linkages were capable of inhibiting HIV-1 in culture (33). The most efficacious member of this dG-rich class of oligonucleotides, I100-15, was found to be capable of folding upon itself to form a structure stabilized by the formation of two stacked guanosine tetrads which yielded a guanosine octet (35). Furthermore, it was observed that the positions of the guanosine bases in the I100-15 sequence, found in both the

* Corresponding author. Mailing address: Triplex Pharmaceutical Corporation, 9391 Grogan's Mill Rd., The Woodlands, TX 77380. Phone: (713) 363-8761. Fax: (713) 363-1168.

tetrads and the connecting loops in that structure, were extremely important to the overall anti-HIV-1 activity of the oligonucleotide (35).

We have recently synthesized T30177, a modified variant of I100-15, which has the same sequence as I100-15 but which contains an hydroxyl moiety at its 3′ terminus and a single PT internucleoside linkage at both the 5′ and the 3′ ends. We have substituted T30177 for I100-15 solely for manufacturing reasons in that T30177 is easier to characterize than I100-15. Here we report on the mechanism by which T30177 is able to inhibit multiple HIV-1 laboratory strains in acute and long-term suppression assays. Our data indicate that T30177 is a potent and selective inhibitor of HIV-1 via at least two mechanisms. One mechanism involves interfering with CD4- and gp120-mediated cell fusion events. We also show that T30177 is a potent inhibitor of the HIV-1 integrase enzyme in vitro and that by blocking these events in the viral life cycle T30177 is able to suppress virus production for prolonged periods after an initial short treatment regimen with the drug.

MATERIALS AND METHODS

Oligonucleotides. The deoxyguanosine-rich and other oligodeoxynucleotides used in the study were synthesized, purified, and characterized as reported previously (33, 35). (See Table 4 for the sequences and PT patterns of the oligonucleotides used in the antiviral assays.)

Materials. Zidovudine (3′-azido-3′-deoxythymidine; AZT) and the nucleoside analogs 2′,3′-dideoxyinosine (ddI) and 2′,3′-dideoxycytidine (ddC) were obtained from the AIDS Research and Reference Reagents Program, National Institute of Allergy and Infectious Diseases. Dextran sulfate (DS5000) was purchased from Sigma, and the bicyclam derivatives JM2763 and JM3100 (14) were obtained from Johnson Matthey (West Chester, Pa.). Chicago sky blue (CSB) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute.

Cytotoxicity analysis. The cytotoxicity of T30177 was assessed by using CEM-SS, MT4, or CEMx174 cells (32) as well as MT2, AA5, Vero, U937, or NIH3T3 cells and the CellTiter 96 Aqueous Non-Radioactivity Cell Proliferation Assay (Promega) as described previously (33, 35).

In other experiments the effect of T30177 on the viability of primary human peripheral blood mononuclear cells (PBMCs), peripheral blood lymphocytes (PBLs), and macrophages was determined by the trypan blue dye exclusion technique (20) or by measuring the degree of [^{3}H]thymidine or [^{3}H]leucine uptake in these cells (29).

Antiviral assays. (i) HIV-1 infection assays with cell lines. Laboratory strains of HIV-1, HIV-2, simian immunodeficiency virus, or the low-passage isolate HIV-1_{DV} (33) were used to infect established cell lines by using the indicated multiplicity of infection (MOI) of virus for 1 h at 37°C prior to washing and resuspension in medium containing increasing concentrations of drug. At 4 to 6 days postinfection, drug-treated and control wells were analyzed for an HIV-1-induced cytopathic effects and for the presence of viral reverse transcriptase (RT) or viral p24 antigen in the culture medium (7, 33, 35). The AZT-resistant strain of HIV-1 (ADP/141) was kindly provided by Brendan Larder and the AIDS Directed Programme Reagent Project, Medical Research Council, England.

(ii) HIV-1 infection of PBMCs and PBLs. PBMCs and PBLs were isolated from the blood of HIV-1-negative and hepatitis B virus-negative (healthy) donors by using the Ficoll-Hypaque density gradient centrifugation technique and were then cultured as described by Gartner and Popovic (18).

PBMCs (2×10^5 cells per well) were infected with various isolates of HIV-1 at an MOI of 0.01. After 2 h at 37°C the cells were washed and treated with various concentrations of T30177 or AZT as described by Buckheit and Swanstrom (7). At 7 days after infection, HIV-1 replication was analyzed by the Coulter p24 antigen-capture assay.

PBLs (5×10^5 cells per well) were infected with HIV-1 isolates at an MOI of 0.2. This level of infection yielded satisfactory RT activity for the virus control at day 7 postinfection (7). The degree of HIV-1 replication was analyzed by either the RT or the p24 assay system.

(iii) Inhibition of acute infection of primary human macrophages. Human macrophage cultures were established as described by Crowe et al. (12). Macrophages were cultivated in RPMI 1640 supplemented with 10% human serum. After incubation overnight at 37°C the macrophages were infected with HIV-1_{DV} at an MOI of 0.1 for 24 h at 37°C in the presence of the indicated amount of drug (29). On day 7 postinfection the adherent macrophages were washed extensively with phosphate-buffered saline (PBS) and were lysed with detergent. Cytoplasmic HIV-1 p24 levels were then quantitated, and percent inhibition was calculated and was compared with that for control infected but untreated cells.

(iv) Long-term suppression studies. Long-term suppression assays were performed as described by Rando et al. (35), with minor modifications. MT-4 cells were infected with HIV-1_{IIIB} (MOI, 0.01) by using drug concentrations representing 1-, 10-, or 100-fold the median inhibitory concentration (IC_{50}) of each compound. At 4 days postinfection, the cells were washed twice with PBS and were resuspended in complete medium without drug (day 0). Viral breakthrough was monitored at several time points by measurement of the levels of viral p24 antigen production in the culture medium (35) or the amount of intracellular viral DNA.

(v) Other viral assays. Respiratory syncytial virus (strain A2) and influenza virus A (strain H3N2) assays were performed as described by Wyde et al. (43), while herpes simplex virus type 1 and 2 plaque reduction assays were performed as described previously (25). Vesicular stomatitis virus, vaccinia virus, Sindbis virus, coxsackievirus B4, poliovirus type 1, and Semliki forest virus assays were performed as described by De Clercq (13). The arenaviridae assays (Junin and Tacaribe viruses) were performed as described by Andrei and De Clercq (2). Punta Toro virus (ATCC VR-559) and yellow fever virus (vaccine strain 17D) assays were performed with Vero cells.

Flow cytometric analysis of HIV-1-infected lymphocytes. At 7 days post-HIV-1 infection of PBMCs cells from both the drug-treated and control wells were washed and treated with fluorochrome-labeled monoclonal antibodies to CD4 or CD8 (Becton Dickinson). The cells were then washed again and fixed with 2% paraformaldehyde before cytofluorometric analysis (11, 12).

Single-cycle analysis of HIV-1 cDNA. CEM-SS cells (2×10^6 cells per well) in 0.5 ml of complete medium were infected with HIV-1_{SKI} at an MOI of 1.0 for 45 min on ice, at which time complete culture medium (10 ml) was added to the cells. The infected cells were then pelleted (1,000 rpm for 10 min at 4°C), washed twice, and dispensed into a 24-well flat-bottom plate (2×10^5 cells per well). The indicated amount of drug was added to the infected cell cultures at various times during or after infection. The cells were harvested at 12 h postinfection, at which time the cell pellets were lysed and intracellular DNA was analyzed for the presence of viral cDNA by the PCR technique described previously (35). The primers used were 5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′ and 5′-TTTGGTCCTTGTCTTATGTCCAGAATCG-3′, which amplifies a 115-bp segment of the HIV-1 genome. The cycle conditions used were 95°C for 10 min to denature the DNA; this was followed by 30 cycles of 95°C for 75 s, 60°C for 75 s, and a final extension step at 60°C for 10 min. Thirty microliters of the amplification reaction mixture was mixed with 10 μl of γ-^{32}P-labeled internal probe (5′-ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC-3′), and this mixture was placed at 95°C for 7.5 min and then annealed at 55°C for 15 min. The resultant products were separated by electrophoresis on a 10% polyacrylamide gel.

Analysis of viral replication. Low- and high-molecular-weight DNAs were prepared from 2×10^7 HIV-1_{SKI}-infected (MOI, 1) CEM-SS cells (untreated or treated with increasing concentrations of drug) by the protocol originally described by Hirt (21) and modified by Gowda et al. (19).

DNA (300 ng) obtained from the low-molecular-weight Hirt fractions was used as the template in the PCR analysis, undergoing a 30-cycle amplification reaction under the conditions described by Steinkasserer et al. (38). PCR primer sets included control primers for the amplification of mitochondrial DNA (sense, 5′-GAATGTCTGCACAGCCACTTT-3′; antisense, 5′-ATAGAAAGGCTAGGACCAAAC-3′; amplified product, 427 bp), primers for the detection of early viral transcription events (M667 and AA55 primers as described by Zack et al. [44]; amplified product, 142 bp); primers for the detection of the viral *gag* gene (sense, 5′-agtgggggacatcaagcagccatgcaaat-3′; antisense, 5′-tttggtccttgtcttatgtccagaatg-3′; amplified product, 300 bp), and primers for the detection of circular proviral DNA (sense, 5′-ccttttagtcagtgtggaaaatctctagca-3′; antisense, 5′-cagtgggttccctagttagc-3′; amplified product, 536 bp). PCR products were separated by agarose gel electrophoresis and were visualized by ethidium bromide staining.

RT enzyme inhibition assays. Purified recombinant RT (HIV-1_{BH10}) was obtained from the Center for AIDS Research, University of Alabama. Three different template-primer systems, primed rRNA, gapped duplex DNA, and poly(rA) · $p(dT)_{12-18}$ were used in the enzyme assays to evaluate the inhibition of HIV-1 RT as described by White et al. (41) and Parker et al. (34).

Integrase enzyme assays. Purified recombinant HIV-1 integrase enzyme (wild type) was a generous gift from R. Craigie, Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases. All 3′-processing and strand-transfer reactions were performed as described previously by Fresen et al. (15) and Mazumder et al. (28).

Protease assays. HIV-1 protease enzyme (Bachem) was diluted to 116 μg/ml in 50 mM sodium acetate, 5 mM dithiothreitol, 2 mM EDTA, and 10% glycerol (pH 5.0), and the mixture was stored as 10-μl aliquots at −20°C. HIV-1 protease substrate I (Molecular Probes) was diluted to a working concentration of 0.32 nmol/μl. Enzyme (20 μl), substrate (20 μl), and drug (20 μl) were added to each well of a microtiter plate. Positive and negative controls were evaluated in parallel. Fluorescence was quantitated on a Labsystems Fluoroskan II instrument by using wavelengths of 355 nm for excitation and 460 nm for emission at 37°C at time zero and at 30-min intervals for 2 h.

HeLa–CD4–β-galactosidase cell assays. Two different assays with genetically engineered HeLa cells were performed as described previously (6). These assays used the HeLa–CD4–LTR–β-galactosidase cell line (23). The assays use a *tat* protein-induced transactivation of the β-galactosidase gene driven by the HIV-1

TABLE 1. Cytotoxicity of T30177 in established cell lines and primary cells

Cell line and type	Cytotoxicity (μM)[a]		
	TC_{25}	TC_{50}	TC_{95}
Cell lines[b]			
CEM-SS	50.8 ± 3.2	92.0 ± 3.0	>100
MT4	34 ± 4.0	70 ± 7.1	>100
CEMx174	10 ± 2.5	50 ± 5.2	>100
MT2	27 ± 3.5	61.2 ± 5.5	>100
AA5	45.66 ± 2.0	94.2 ± 3.1	>100
U937	>100	>100	>100
Vero	>100	>100	>100
NIH3T3	>100	>100	>100
Primary human cells[c]			
PBLs	>100	>100	>100
PBMCs	>100	>100	>100
Macrophages	>100	>100	>100

[a] TC_{25}s, TC_{50}s, and TC_{95}s, concentrations of T30177 required to inhibit 25, 50, and 95% of growth (cell lines) or cell survival (primary human cells), respectively.
[b] The cytotoxicity of T30177 in human cell lines was determined by using log-phase, growing cells.
[c] The cytotoxicity of T30177 in primary human cells was determined by the trypan blue exclusion technique or by measuring the uptake of [^{3}H]thymidine or [^{3}H]leucine by slowly growing primary cells.

long terminal repeat (LTR). One assay involved infecting the HeLa–CD4–LTR–β-galactosidase cells with HIV-1, while the second assay monitored the expression of β-galactosidase after incubation of the HeLa–CD4–LTR–β-galactosidase cell with HL2/3 cells (6, 9). The HL2/3 cells express both the HIV-1 envelope glycoprotein and the *tat* gene product so that cocultivation of these cells with the HeLa–CD4–LTR–β-galactosidase cells would allow for gp120- and CD4-mediated cell fusion. The extent of cell fusion can then be monitored by the degree of *tat* transactivation of LTR-driven β-galactosidase expression (6, 9).

RESULTS

Cytotoxicity assays. The cytotoxicity of T30177 was determined by using several different cell lines and primary human cells (Table 1). The cytotoxicity profile obtained by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay for log-phase, growing cells was variable, depending on the cell line used, while the more slowly growing PBMCs, PBLs, and macrophages all tolerated the compound at concentrations exceeding 100 μM, as monitored by the trypan blue exclusion, [^{3}H]thymidine uptake, or [^{3}H]leucine uptake technique.

Inhibition of viral replication in cell lines. CEM-SS cells were infected with HIV-1_{RF} at an MOI of 0.01 and were treated with T30177, AZT, or ddC for 6 days. In this assay system T30177 inhibited HIV-1_{RF} replication in a dose-dependent manner, with an IC_{50} of 0.075 μM, while the control drugs AZT and ddC had IC_{50}s of 0.007 and 0.057 μM, respectively. When T30177 was assayed against additional strains of HIV-1 in a variety of different cell lines, the degree of inhibition observed for each strain of HIV-1 analyzed was greatly influenced by the cell line used (Table 2). In addition, as observed for DS5000, T30177 was inhibitory for an AZT-resistant strain of HIV-1 tested (ADP/141), which has four mutations in its RT gene (67N, 70R, 215F, and 219Q). T30177 was more active against the strains of HIV-1 and simian immunodeficiency virus tested than against the two strains of HIV-2 tested (ROD and EHO; Table 2). In addition, T30177 was found to be inactive against a variety of enveloped (herpes simplex virus types 1 and 2, Sindbis virus, Semliki forest virus, vesicular stomatitis virus, vaccinia virus, Punta Toro virus, yellow fever virus, respiratory syncytial virus, influenza virus A, and Junin and Tacaribe viruses) and nonenveloped (coxsackievirus and poliovirus) viruses tested, with the IC_{50}s found to be greater than the highest concentration of drug tested (200 μg/ml or 37

TABLE 2. Inhibitory effects of T30177, AZT, and DS5000 on viral replication

Virus and strain	Cell line	IC_{50} (μM)[a]		
		T30177	AZT	DS5000[b]
HIV-1[c]				
SKI	CEM-SS	0.025 ± 0.0006	0.022 ± 0.0001	
	MT2	0.06 ± 0.001	0.66 ± 0.005	
RF	CEM-SS	0.075 ± 0.0002	0.007 ± 0.0002	
	MT2	0.270 ± 0.04	0.20 ± 0.03	
	MT4	0.037 ± 0.03		0.018 ± 0.02
DV	SUP T1	0.06 ± 0.004	0.03 ± 0.005	
IIIB	CEM-SS	2.83 ± 0.17	0.002 ± 0.0003	
	MT2	1.94 ± 0.12	0.01 ± 0.004	
	MT4	0.15 ± 0.02		0.034 ± 0.016
	SUP T1	0.6 ± 0.06	0.03 ± 0.006	
	AA5	<0.32	<0.003	
ADP/141	MT4	0.27 ± 0.05		0.032 ± 0.008
HIV-2 or SIV[c]				
$HIV\text{-}2_{ROD}$	MT4	27.5 ± 11.6		0.082 ± 0.088
$HIV\text{-}2_{EHO}$	MT4	5.98 ± 1.05		0.084 ± 0.086
SIV_{MAC251}	MT4	1.5 ± 1.2		0.548 ± 0.48

[a] The IC_{50} results are averages of three or more experiments.
[b] For DS5000 the results (in micromolar) are an approximation based on the average molecular weight (5,000) of the material used in the present studies.
[c] The MOI used for all HIV-1, HIV-2 and simian immunodeficiency virus (SIV) strains tested was 0.01.

TABLE 3. HIV-1 replication in primary human cells treated with T30177, ddI, or AZT

Virus strain (cell)	HIV-1 isolate	IC_{50} (μM)[a]		
		T30177	ddI	AZT
Laboratory isolates (PBMCs)[b]	IIIB	0.12 ± 0.06	0.74 ± 0.05	0.003 ± 0.0002
	JR_{CSF}	0.28 ± 0.04	2.0 ± 0.5	0.0025 ± 0.001
	RF	0.75 ± 0.13	ND[c]	0.272 ± 0.003
	MN	1.35 ± 0.10	ND	0.053 ± 0.001
Clinical isolates (PBLs)[b]	WEJO (SI)	0.30 ± 0.001	2.18 ± 0.026	0.017 ± 0.0001
	BAKI (SI)	0.23 ± 0.005	2.61 ± 0.003	0.020 ± 0.006
	WOME (SI)	0.71 ± 0.002	0.41 ± 0.008	0.025 ± 0.0003
	ROJO (SI)	3.9 ± 0.02	0.87 ± 0.001	0.052 ± 0.0004
	JOGA (NSI)	0.33 ± 0.004	ND	>1.0
	BLCH (NSI)	3.08 ± 0.006	ND	0.022 ± 0.0008
	VIHU (NSI)	1.3 ± 0.02	1.21 ± 0.009	0.036 ± 0.0007
	Southeast Asia	0.58 ± 0.003	ND	0.06 ± 0.005
	North American 1	0.25 ± 0.003	ND	0.01 ± 0.004
	North American 2	2.92 ± 0.005	ND	1.65 ± 0.007
	942716	0.86 ± 0.006	ND	0.002 ± 0.0003
	942751	0.38 ± 0.003	2.2 ± 0.02	0.028 ± 0.0025

[a] The IC_{50} was determined by the Coulter p24 antigen capture or RT assay.
[b] Antiviral assays were performed with laboratory strains of HIV-1 in PBMCs or with syncytium-inducing (SI) or non-syncytium-inducing (NSI) clinical isolates of HIV-1 in PBLs.
[c] ND, the value was not determined.

μM). This is in contrast to DS5000, which was found to be a potent inhibitor of all of the enveloped viruses tested (with IC_{50}s ranging between 1 and 15 μg/ml) except for vaccinia and Semliki forest viruses.

Inhibition of HIV-1 replication in peripheral blood cells. Activated PBMCs were infected with HIV-1 and were cultured in the presence of T30177, AZT, or ddI. Treatment of infected PBMCs with T30177 inhibited the replication of all four HIV-1 isolates tested, with IC_{50}s ranging from 0.28 to 1.35 μM and IC_{90}s ranging from 0.98 to 5.52 μM (Table 3). In this assay AZT was more efficacious on a molar scale than T30177 against all HIV-1 isolates tested, while at the same time T30177 was more potent than ddI against the two HIV-1 strains tested. It is also noteworthy that HIV-1_{IIIB} was more susceptible to T30177 in assays performed with PBMCs than in assays performed with T-cell lines (Tables 2 and 3).

We also evaluated the ability of T30177 to inhibit the infection of PBLs using a variety of clinical isolates of HIV-1 which were both syncytium-inducing and non-syncytium-inducing strains of HIV-1 from different geographic regions. T30177 inhibited the viral replication of all of the HIV-1 isolates tested, with IC_{50}s ranging from 0.23 to 3.08 μM (Table 3). It is noteworthy that T30177 was active against both non-syncytium-inducing and syncytium-inducing isolates and was very active against the JOGA isolate, which was obtained from a pediatric patient. The JOGA isolate was observed to be relatively resistant to AZT treatment (Table 3).

Fully differentiated macrophages were infected with HIV-1_{DV} and were treated with T30177 or AZT. T30177 significantly inhibited HIV-1 replication in macrophages (data not shown). However, because of the long exposure times of the cells to virus (24 h), T30177 and AZT worked best when they were administered at concentrations greater than the IC_{50}s obtained for these drugs in assays performed in established cell lines.

Variations in viral MOI. To investigate the effects of variations in the MOI on the anti-HIV-1 activity of T30177, CEM-SS or MT4 cells were infected with HIV-1_{RF} or HIV-1_{IIIB} at various MOIs. Unlike AZT, T30177 was much less sensitive to changes in the viral MOI. For example, in these assays, when the MOI of HIV-1_{RF} was changed from 0.01 to 1.28, only a 14-fold increase in the IC_{50} of T30177 was found, while at the same time the IC_{50} of AZT increased more than 1,000-fold (data not shown).

Effect of T30177 on CD4 and CD8 T-cell subsets. To determine the effect of T30177 treatment on the CD4/CD8 ratio in vitro, CD4 and CD8 antigen expression was analyzed on the surface of cultured PBMCs at 7 days postinfection with either laboratory strains or clinical isolates of HIV-1. In these experiments treatment with either AZT or T30177 increased the number of $CD4^+$ T cells in the cell culture relative to the number in untreated infected cultures. The results obtained with the southeast Asia isolate (AZT susceptible; Table 3) and North American isolate 2 (AZT resistant; Table 3) of HIV-1 are presented in Fig. 1. The observed increase in the number of $CD4^+$ cells was dependent on the drug concentration used and was inversely correlated with the level of virus production (Tables 2 and 3). These results suggest that the blockage of HIV-1 replication parallels the suppression of the cytopathic effects of the virus in primary human lymphocytes.

In vitro some HIV-1 isolates infect $CD4^+$ lymphocytes, shed infectious virus into the culture medium, but do not cause destruction of the infected cells (17). This may explain the results obtained when we used North American isolate 1 (data not shown). When this virus was used to infect PBMCs, in the absence of drug, a CD4/CD8 ratio of 0.35 was observed at 7 days postinfection. At the same time analysis of the culture medium for cells infected with this isolate revealed the presence of viral p24 antigen (Table 3), which suggested that a productive viral infection had occurred.

Time of drug addition studies. T30177, DS5000, or AZT was added to MT-4 cells infected with HIV-1_{IIIB} (MOI, 1) at various times postinfection. Test compounds were added at a concentration 100-fold higher than the determined IC_{50} of each drug in the standard assay performed with MT-4 cells and the IIIB strain of HIV-1 (Table 2). Postponing the addition of T30177 for 1 h was enough to dramatically reduce the inhibitory effects of this compound in a fashion similar to that of DS5000 and clearly different from that of AZT, which lost its protective capacity when it was added to the cell culture me-

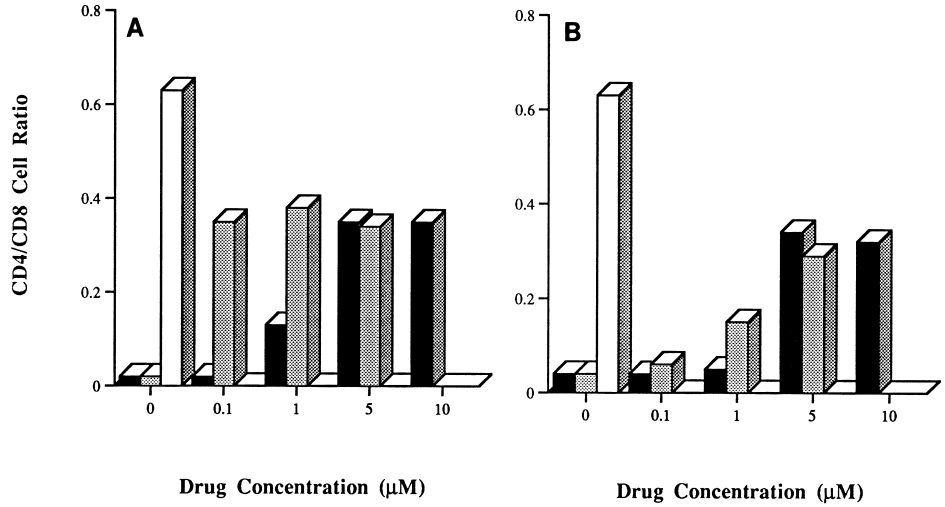

FIG. 1. Effect of T30177 (■) or AZT (▨) on the ratio of CD4 to CD8 antigen-bearing cells in a population of HIV-1-infected PBMCs. (A) PBMCs were infected with the southeast Asia isolate of HIV-1. (B) Cells were infected with the North American isolate 2 of HIV-1. In all assays the levels of CD4 and CD8 antigen expression on the surfaces of cultured PBMCs were analyzed by flow cytometry at 7 days postinfection and after treatment with the indicated amount of drug. The CD4/CD8 ratio obtained after culturing uninfected and untreated PBMCs for 7 days is shown by the white bars (no virus).

dium 3 or 4 h postinfection (Fig. 2). A similar result was obtained when comparing T30177 with CSB, a known inhibitor of both virus binding to cells and fusion-related events (10), in that the antiviral activities of both T30177 and CSB were greatly reduced if they were added to the infected cell cultures 1 h after virus infection (data not shown).

HeLa–CD4–β-galactosidase cell studies. To differentiate the effects of T30177 on early events in the viral life cycle through integration and subsequent production of the *tat* gene product from the inhibition of HIV-1 gp120-mediated cell fusion, two experimental protocols were used. The first protocol monitored the effects of drug on the ability of HIV-1_{RF} to infect or replicate within HeLa–CD4–LTR–β-galactosidase cells and was performed as described in Materials and Methods. In this experiment drug interdiction at any step in the viral life cycle through the production of the *tat* gene product would cause a decrease in the level of expression of the β-galactosidase gene, the transcription of which is regulated by the HIV-1 LTR. The results show that T30177 is a potent inhibitor of β-galactosidase production in this assay, with an IC_{50} of 0.009 μM, while the IC_{50} of CSB obtained in the same experiment was 0.26 μM (Fig. 3A). In control experiments T30177 had no observable direct effect on β-galactosidase enzyme activity at concentrations up to 10 μM (data not shown).

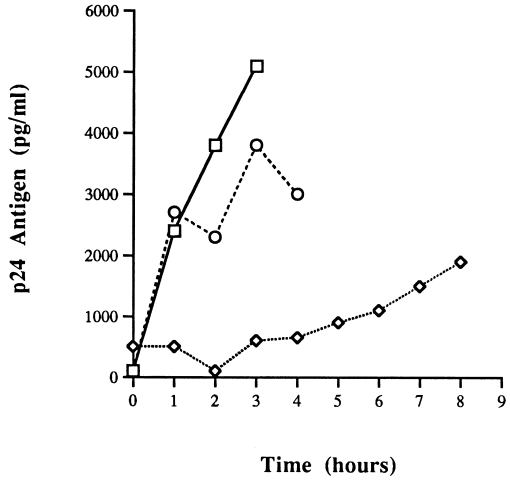

FIG. 2. Effect of time of drug addition on the inhibition profiles of T30177 (○), AZT (◇), and DS5000 (□). MT4 cells infected with HIV-1_{IIIB} at an MOI of 1 were treated at various times during (time zero) or after virus infection with the test compounds at a concentration 100-fold greater than their respective IC_{50}s. Viral p24 levels in the culture medium were monitored at 29 h postinfection. The results are averages of three or more experiments.

The second protocol used was a virus-free assay designed to monitor gp120- and CD4-mediated cell fusion events. In this assay T30177 was able to interfere with the fusion process (Fig. 3B). However, the observed IC_{50} (1 μM) was approximately 100-fold higher than that needed to interfere with β-galactosidase production in the virus infection assay (Fig. 3A). In the same assay system the IC_{50} of CSB increased approximately threefold, to 0.8 μM, over the concentration needed to interrupt β-galactosidase production in the virus infection assay (Fig. 3).

The three-dimensional structure of an oligonucleotide with the sequence of T30177 is stabilized by the formation of an intramolecular G octet (35). Previously, we have reported how the replacement of one of the dG residues involved with tetrad formation with a deoxyadenosine (dA) reduced the anti-HIV-1 activity of the resultant molecule (35). To determine the effects of intramolecular tetrad formation in T30177 on the observed inhibition of β-galactosidase production in the two assays for which the results are presented in Fig. 3, we tested T30526, an oligonucleotide in which a dA residue has been substituted for a dG at a position that would interrupt the formation of one of the two tetrads involved in the G octet. T30526 has the same partial PT pattern as T30177 (Table 4). T30526 was found to be approximately 100-fold less potent than T30177 in inhibiting HIV-1_{RF} production in culture assays (Table 4) and 10- to 15-fold less potent at inhibiting virus-infected cell β-galactosidase production (Fig. 3A), and it did not inhibit cell fusion at the highest concentration of drug tested (20 μM; Fig. 3B).

Long-term suppression of HIV-1. In separate experiments, HIV-1_{IIIB}-infected MT-4 cells were treated with T30177, AZT, DS5000, or the bicyclam compounds JM2763 or JM3100 for 4

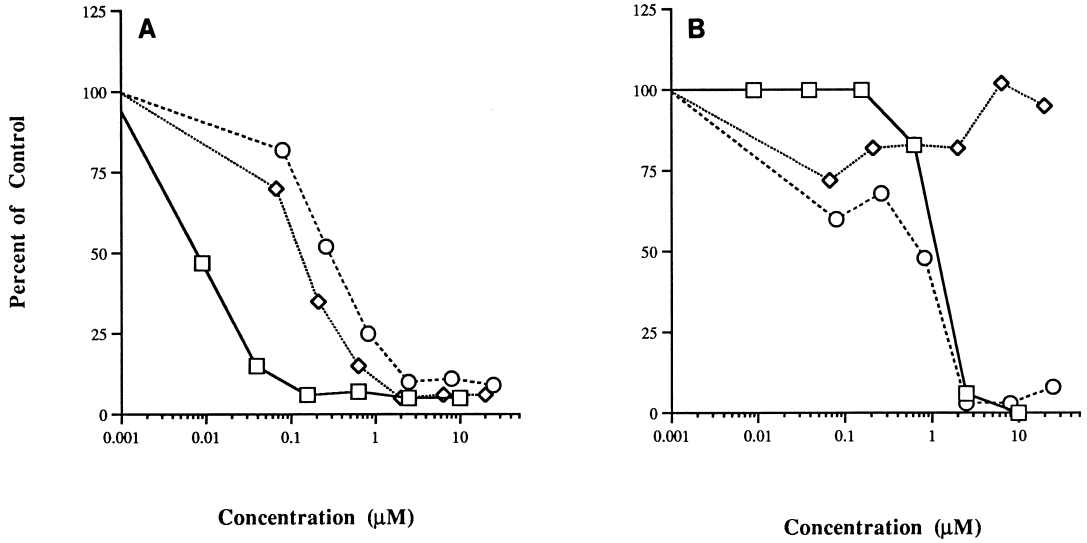

FIG. 3. HeLa–CD4–β-galactosidase cell assays. □, T30177; ◇, T30526; ○, CSB. (A) HeLa–CD4–β-galactosidase cells were incubated in medium containing drug for 1 h before virus was added to the culture medium. At 1 h after the addition of virus the cells were washed extensively to remove unbound virus and extracellular test material. At 48 h postinfection the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Blue multinuclear cells were then counted under an inverted microscope (6). (B) HeLa–CD4–β-galactosidase cells were incubated for 1 h in the presence of test compound, at which time equal numbers of HL2/3 cells were added to each well. Cells were incubated for 48 h, at which time they were fixed, stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and counted under an inverted microscope.

days by using drug concentrations equivalent to 1-, 10-, or 100-fold their respective IC_{50}s (Table 4). After 4 days in culture the cells were washed and then further cultured in complete medium without drug. The cells were monitored daily for the appearance of virus-induced syncytium formation and every second or third day for viral p24 antigen in the culture medium. In cells treated with T30177 at 100-fold the IC_{50} (approximately 10 μM), suppression of virus p24 production was observed for at least 27 days after removal of drug from the infected cell culture (Fig. 4). Furthermore, there was no detectable viral cDNA (by PCR analysis) in cells examined for up to 11 days after the removal of T30177 from the infected cell culture (data not shown). For cells treated in the same fashion with AZT, DS5000, JM2763, or JM3100, measurable levels of viral p24 antigen were found in the culture medium within 3 days after removal of the drug (Fig. 4). The degree of continued suppression was contingent upon the concentration of T30177 used in the assay and the duration of the drug treatment regimen (data not shown). The concentration and duration of treatment regimen data are consistent with those reported previously for I100-15 (35).

To determine if exposure of cells to T30177 protects them from subsequent infection with HIV-1, cultures of HIV-1-infected MT-4 cells treated for 4 days with T30177 (100-fold the IC_{50}) were washed and then reinfected with HIV-1_{IIIB} before resuspension in fresh culture medium without drug. In these assays there was no protection of cells from the second round of viral infection (data not shown).

Single cycle analysis of viral cDNA. Total DNA from HIV-1_{SKI}-infected CEM-SS cells was isolated at 12 h postinfection and was analyzed for the presence of viral cDNA as described in Materials and Methods. In this experiment viral cDNA was detected in cells treated with 1 or 10 μM T30177 (approximately 10- to 100-fold the IC_{50}) even when the drug was added to the cell cultures at the time of virus infection (Fig. 5). This is in contrast to the results obtained when the adsorption-blocking drug CSB (10 μM), the nucleoside RT inhibitor ddC (10 μM), or the nonnucleoside RT inhibitor UC38 (1 μM) were used as control drugs. UC38 is an analog of oxathiin carboxanilide (3, 30). As expected, there was no detectable viral DNA in cells treated during or very soon after virus infection with any of the three control drugs when the drugs were used at concentrations 10- to 100-fold their reported IC_{50}s (Table 4; Fig. 5).

Analysis of replicated viral DNA. We have previously reported on the presence of viral cDNA in T30177-treated SUP T1 cells at 36 h postinfection when the cells were infected with HIV-1_{DV} at a low MOI (35). As described above, viral cDNA

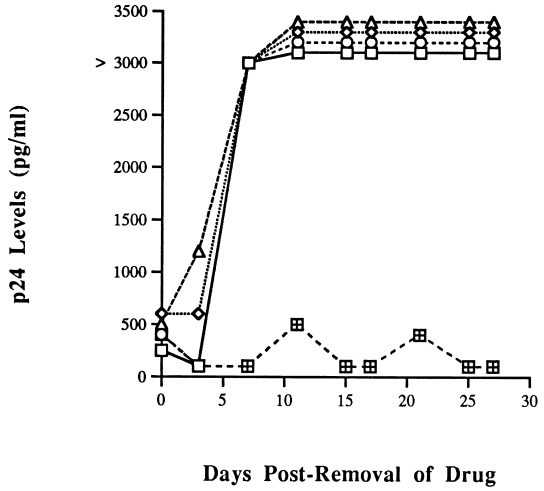

FIG. 4. Long-term suppression of HIV-1_{IIIB} after treatment of infected cell cultures with T30177. (A) MT4 cells were infected with HIV-1_{IIIB} at an MOI of 0.01 and were then cultured for 4 days in the presence of T30177 (⊞), AZT (□), DS5000 (◇), JM2763 (○), or JM3100 (△) by using concentrations of drugs equivalent to 100-fold the respective IC_{50}s. After 4 days the cells were washed extensively and were further incubated in drug-free medium. The level of viral p24 antigen in the culture medium was monitored at various times after removal of drug from the infected cell cultures. The values given are the averages of three or more experiments.

TABLE 4. Inhibition of various HIV-1 strains in culture assays and the HIV-1 enzymes in vitro

Drug group and compound	Nucleotide sequence	Backbone[a]	IC_{50} (μM)						
			Antiviral assay			Anti-integrase assay		Anti-RT assay	
			RF	SKI	IIIB	3′-Proc.	Transfer	rA · dT	rRNA
G Octet, anti-HIV-1									
T30175	5′-GTGGTGGGTGGGTGGGT-3′	PD	6.58			0.17	0.125		
T30177	5′-GTGGTGGGTGGGTGGGT-3′	pPT	0.075	0.025	2.83	0.092	0.046	7.5	0.78
T30038	5′-GTGGTGGGTGGGTGGGT-3′	PT	0.30			0.09	0.07		
T30526	5′-GTGATGGGTGGGTGGGT-3′	pPT	11.7			0.2	0.123		
G Octet, thrombin binding									
T30340	5′-GGTTGGTGTGGTTGG-3′	PD	>100.0			>0.50	>0.50		
T30659	5′-GGTTGGTGTGGTTGG-3′	pPT	>20.0	2.81	>20.0	>0.50	>0.50		
T30341	5′-GGTTGGTGTGGTTGG-3′	PT	4.76			0.042	0.023		
Antisense, anti-HIV-1									
T30658	5′-TCTTCCTCTCTCTACCACGCTCTC-3′	PD	>20.0	>20.0	>20.0	>0.50	>0.50		
T30662	5′-TCTTCCTCTCTCTACCACGCTCTC-3′	pPT	>20.0	>20.0	>20.0	>0.50	>0.50		
T30531	5′-TCTTCCTCTCTCTACCACGCTCTC-3′	PT	0.17			0.030	0.036		
Controls									
AZT			0.007	0.022	0.002	>1.0	>1.0	0.6	0.0135
DS5000[c]			0.016		0.031	0.07	0.06		
ddC			0.057	0.26	0.078	>1.0	>1.0		
UC38			0.02	0.09	0.09				
CSB			1.7	1.6	0.6				

[a] Oligonucleotides were synthesized with a PD backbone, a PT backbone, or a partial phosphorothioate (pPT) backbone in which only the terminal 3′ and 5′ internucleoside linkages were phosphorothioate.

[b] Antiviral results were obtained with CEM-SS or MT-4 cells. Anti-integrase results were obtained by monitoring the 3′-processing (3′-Proc.) or strand-transfer (transfer) activity of the enzyme. RT assay results were obtained by using the poly(rA) · $p(dT)_{12–18}$ (rA · dT) template or the primed rRNA template. The data are averages of three or more experiments.

[c] For DS5000 the results (in micromolar) are an approximation based on the average molecular weight (5,000) of the material used in the present studies.

was also detected in T30177-treated cells at 12 h postinfection with virus at a high MOI (Fig. 5). To determine the extent of viral replication within these cells, we used PCR primers which would differentiate between the different stages of viral replication through the production of circular proviral DNA (2-LTR circles). The results of these experiments indicate that viral replication has occurred in the T30177-treated cells up to and including the time of production of 2-LTR circles (Fig. 6).

Inhibition of viral enzymes. Oligonucleotides with PT backbones have been reported to be much more potent inhibitors of HIV-1 RT than the same molecules with PD backbones (33). T30177 was able to inhibit HIV-1 RT; however, the IC_{50} for the enzyme was greater than 5 μM when gapped duplex DNA templates (Table 4) or RNA-DNA templates (data not shown) were used. It is noteworthy that when the primed rRNA template was used the IC_{50} of T30177 was in the range of 1 μM (Table 4).

T30177 was also tested for its ability to inhibit HIV-1 protease and integrase enzymes. When concentrations of T30177 up to 10 μM were used in protease inhibition assays, no effect on the viral enzyme was observed (data not shown). However, when it was assayed for its effect on HIV-1 integrase, T30177 was able to reduce both the 3′-processing and strand transfer activities of the integrase enzyme, with IC_{50}s of 0.092 and 0.046 μM, respectively (Table 4).

To determine if the sequence, three-dimensional structure, chemical composition of the backbone, or a combination of these parameters contributed to the observed anti-integrase activity of T30177, we synthesized and tested the oligonucleotides listed in Table 4 for their enzyme inhibitory activities. T30038, T30175, and T30526 are variations of T30177. T30340, T30341, and T30659 are variations of the thrombin-binding aptamer sequence reported by Bock et al. (5). Both the G-rich sequence of the anti-HIV-1 oligonucleotide T30177 and the thrombin-binding aptamer have been shown to fold upon themselves to form structures stabilized by intramolecular G octets (35, 36, 39). Oligonucleotides T30531, T30658, and T30662 are variations of the antisense compound GEM91, which has been reported to be a potent inhibitor of HIV-1 (1).

Any of the sequence motifs tested were potent inhibitors of the HIV-1 integrase enzyme when the oligonucleotides were synthesized with a PT backbone (Table 4). When the number of PT linkages in the backbone was reduced to one linkage at each end of the molecule (pPT), the thrombin-binding aptamer (T30659) and the antisense sequence (T30662) no longer displayed anti-integrase activity, while the level of inhibition observed with T30177 was relatively the same as that observed with the total PT version of this molecule (T30038). For compounds with total PD backbones, only the anti-HIV-1 sequence motif was able to inhibit viral integrase, with IC_{50}s of 170 and 125 nm for the 3′-processing and strand transfer enzyme activities, respectively. T30526, the tetrad-disrupted mutant version of T30177, was still able to inhibit viral integrase protein in this assay, albeit at a concentration two- to three-fold higher than that observed with T30177.

DISCUSSION

In this report, we have expanded upon our earlier observations (33, 35) on the anti-HIV-1 activities of G-rich oligonucleotides by demonstrating the efficacy of T30177 against multiple laboratory strains and clinical isolates of HIV-1. Using the

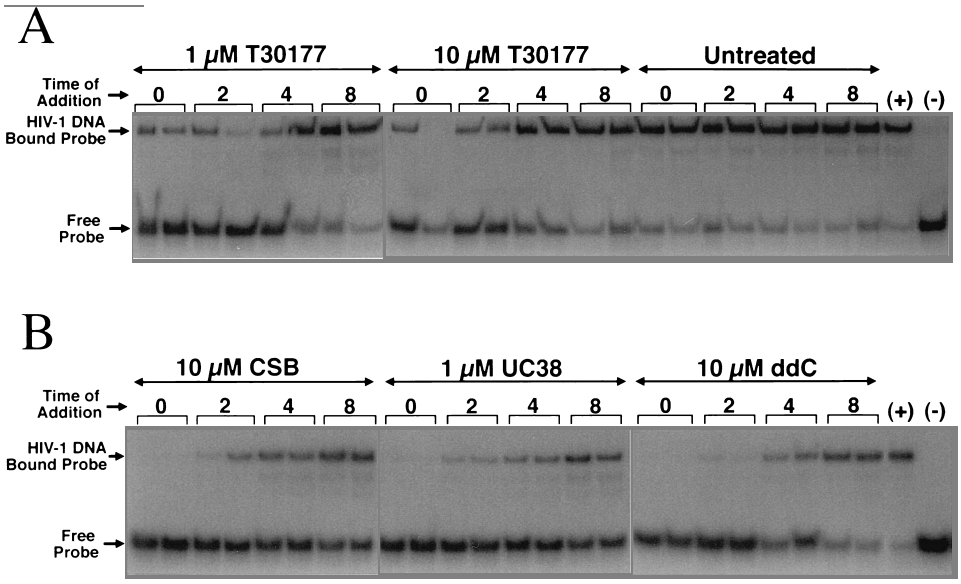

FIG. 5. Single-cycle analysis of viral DNA. CEM-SS cells infected with HIV-1_{SKI} at an MOI of 1 were treated with T30177, UC38, CSB, or ddC at the indicated times after viral infection. Time zero indicates that cells were treated with drug during virus infection. After 12 h the DNA was extracted from the infected cells and was used as a template for PCR. The concentrations of drug used in each assay are equivalent to 10- to 100-fold their respective IC_{50}s.

cytotoxicity (Table 1) and the efficacy data (Tables 2 and 3), we found that T30177 has a wide range of therapeutic indices, depending on the viral strain and the cell line used in a given assay. For example, when T30177 was used to inhibit HIV-1_{SKI} in CEM-SS cells a therapeutic index of 3,680 was obtained. However, when measuring the effect on HIV-1_{RF} in MT2 cells, the therapeutic index of T30177 was only 226.

The variability in efficacy of T30177 in PBMCs and PBLs, which depended on the clinical isolate tested, was very similar to the variation in activity observed for the nucleoside analogs AZT and ddI. It is noteworthy that an approximately 20-fold variation in the IC_{50} was observed for T30177 when it was used to inhibit HIV-1_{IIIB} in CEM-SS cells (2.8 μM) compared with that when it was used to inhibit HIV_{IIIB} in PBMCs (0.12 μM) (Tables 2 and 3). An explanation for this observation may be that when viruses are propagated continuously in homogeneous cell lines they "adapt" to those cells and begin to display phenotypes different from those of low-passage clinical isolates. Therefore, the results obtained by using clinical isolates to infect heterogeneous populations of primary cells (PBMCs or PBLs) may be more predictive of in vivo efficacy than the data generated with laboratory strains of HIV-1 in established cell lines. It is unlikely that HIV-1_{IIIB} is a resistant strain of HIV-1 since T30177 was more effective against this virus in PBMCs than in cell lines. However, given the well-documented ability of HIV-1 to mutate and thus develop resistance to known therapies, efforts are under way to determine if resistant mutants can arise after treatment of HIV-1-infected cells with T30177.

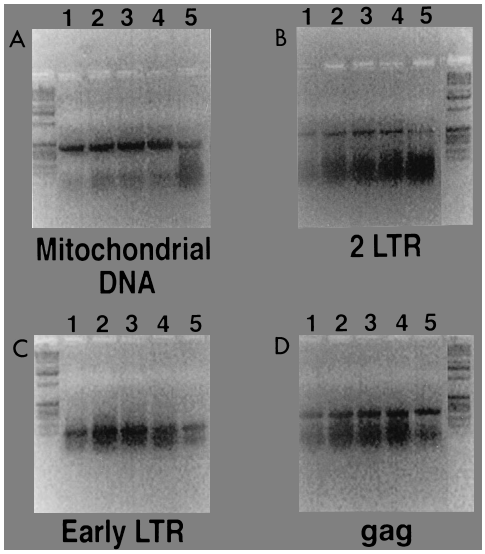

FIG. 6. Analysis of replicated viral DNA. CEM-SS cells were infected with HIV-1_{SKI} at an MOI of 1 and were then treated with T30177. At 18 to 20 h postinfection the low-molecular-weight Hirt DNA was analyzed with PCR primers which amplify mitochondrial DNA, early virus-synthesized cDNA, viral *gag* cDNA, and viral 2-LTR circles. Viral 2-LTR circles represent the point in the replicative cycle when the provirus forms a circle and the LTRs at each end of the genome become juxtaposed. The drug concentrations used were 0.0, 0.01, 0.1, 1, and 10 μM (lanes 1 to 5, respectively).

In mechanism of action studies it was found that T30177 displayed some antiviral properties which indicated a mechanism of action similar to those of the known blockers of virus adsorption or virus-mediated cell fusion such as dextran sulfate and CSB (Fig. 2 and 3). Like CSB and DS5000, T30177 needed to be added to cells at the time of or soon after virus infection. However, T30177 is 100-fold less effective in inhibiting gp120-induced cell fusion events than it is at inhibiting early events in the viral life cycle, suggesting a specific point of interdiction with virus distinct at least from that of CSB. In addition, the antiviral profile of T30177 also displayed other characteristics which distinguished T30177 from DS5000 and CSB. For example, while DS5000 is active against a wide range of enveloped viruses, T30177 appears to be a more selective inhibitor of retroviruses, with maximum efficacy displayed when it was used to inhibit strains of HIV-1.

The experimental results presented in Fig. 5 show that unlike the control drugs CSB, AZT, and UC38, when T30177 was added to cell cultures during virus infection it was unable to

completely block viral infection even when it was used at concentrations 100-fold the IC_{50} (~10 μM). Furthermore, analysis of viral DNA demonstrated that viral replicative intermediates including circular proviral DNA were present in infected cells treated with T30177 (Fig. 5 and 6). These data, coupled with the ability of T30177 to completely suppress viral outbreak (Fig. 4) and possibly to clear virus from infected cell cultures after removal of the drug from infected cells (a profile not observed for AZT, DS5000, JM2763, or JM3100), suggest that a second mechanism of action distinct from the inhibition of virus binding or inhibition of cell fusion events is at work. One possible alternative mechanism is that T30177 interferes with the viral integration process. A combination of activities including inhibition of viral attachment or internalization, virus-mediated cell fusion events, and viral integration could explain the loss of virus from infected cell cultures. This hypothesis is supported by the observation that T30177 is a potent inhibitor of HIV-1 integrase function in vitro (Table 4), by the observed accumulation of circularized proviral DNA in the low-molecular-weight Hirt DNA fractions (Fig. 6), by the lack of activity against HIV-1 protease in vitro, and by an inability to inhibit HIV-1 production when it is used in the low micromolar range in chronically infected cells (data not shown).

It is clear that highly charged molecules such as DS5000 and oligonucleotides with total PT backbones are excellent inhibitors of the integrase enzyme in vitro. However, since of all the pPT molecules tested T30177 maintained its level of enzyme inhibitory activity (Table 4), it is unlikely that the mechanism of inhibition is totally based on a polyanion effect, as is seen for compounds such as DS5000 and suramin (8). It is unclear at this time whether the G octet structure with the two-base-long T/dG loops found in T30177 is of paramount importance for inhibition of viral integrase since the G octet sequence found in T30659 did not inhibit integrase activity while T30526 (tetrad disrupting mutant) was able to inhibit enzyme activity, albeit at a reduced level.

While the time of drug addition studies would suggest interference with virus internalization as a key mechanism of action for T30177, it is also clear that readily detectable virus does enter the cells. It is quite possible that T30177 inhibits HIV-1 via several different mechanisms of action. Another possibility is that T30177 is carried into the cell along with the infecting virus or is slow to accumulate within cells, hence the need to add drug during virus infection. Experiments designed to address these possibilities are under way.

The recently reported rate of emergence of drug-resistant virus to currently approved therapies against HIV-1 (half-life of approximately 2 days) suggests that single-drug therapy for this virus cannot succeed (22, 40), and therefore, a likely treatment regimen for any new drug candidate would be in combination with one or more other drugs which have different antiviral mechanisms of action. Further experimentation might determine that the actual mechanism of action of T30177 may not be via either inhibition of virus binding or internalization or inhibition of viral integration; however, it is unlikely that this oligonucleotide acts via the same mechanism as drugs currently in use against HIV-1. In additional studies we have determined that T30177 is stable in serum and within cells, with a half-life measured in days (4). This information, taken together with the ability of T30177 to suppress HIV-1 for more than 4 weeks after an initial treatment regimen in culture, makes this class of compounds an attractive candidate for use in the development of oligonucleotide-based therapeutic agents for HIV-1.

ACKNOWLEDGMENTS

We thank A. Elbaggari, M. Hogan, and N. Chaudhary for technical assistance and helpful, thought-provoking discussions. We also thank R. Unger for editorial assistance.

REFERENCES

1. **Agrawal, S., and J. Y. Tang.** 1992. GEM*91—an antisense oligonucleotide phosphorothioate as a therapeutic agent for AIDS. Antisense Res. Dev. **2:**261–266.
2. **Andrei, G., and E. De Clercq.** 1990. Inhibitory effects of selected antiviral compounds on arenavirus replication in vitro. Antiviral Res. **14:**287–299.
3. **Bader, J. P., J. B. McMahon, R. J. Schultz, V. L. Narayanan, J. B. Pierce, W. A. Harrison, O. S. Weislow, C. F. Midelfort, S. F. Stinson, and M. R. Boyd.** 1991. Oxathiin carboxanilide, a potent inhibitor of human immunodeficiency virus reproduction. Proc. Natl. Acad. Sci. USA **88:**6740–6744.
4. **Bishop, J. S., J. K. Guy-Caffey, J. O. Ojwang, S. R. Smith, M. E. Hogan, R. F. Rando, and N. Chaudhary.** Intramolecular G-tetrad motifs confer nuclease resistance to a potent anti-HIV oligonucleotide, submitted for publication.
5. **Bock, L. C., L. C. Griffin, J. A. Latham, E. H. Vermaas, and J. J. Toole.** 1992. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature (London) **355:**564–566.
6. **Buckheit, R. W., J. L. Roberson, C. Lackman-Smith, J. R. Wyatt, T. A. Vickers, and D. J. Ecker.** 1994. Potent and specific inhibition of HIV envelope-mediated cell fusion and virus binding by G-quartet-forming oligonucleotide (ISIS 5320). AIDS Res. Hum. Retroviruses **10:**1497–1506.
7. **Buckheit, R. W., Jr. and R. I. Swanstrom.** 1991. HIV-heterogeneity: characterization of HIV-1 isolates displaying an apparent absence of virion-associated reverse transcriptase activity. AIDS Res. Hum. Retroviruses **7:**295–302.
8. **Carteau, S., J.-F. Mouscadet, H. Goulaouic, F. Subra, and C. Auclair.** 1993. Inhibitory effect of the polyanionic drug suramin on the *in vitro* HIV DNA integration reaction. Arch. Biochem. Biophys. **305:**606–610.
9. **Ciminale, V., B. K. Felber, M. Campbell, and G. N. Pavlakis.** 1990. A bioassay for HIV-1 based on Env-CD4 interaction. AIDS Res. Hum. Retroviruses **6:**1281–1287.
10. **Clanton, D. J., R. A. Moran, J. B. McMahon, O. S. Weislow, R. W. Buckheit, Jr., M. G. Hollingshead, V. Ciminale, B. K. Felber, G. N. Pavlakis, and J. P. Bader.** 1992. Sulfonic acid dyes: inhibition of the human immunodeficiency virus and mechanism of action. J. Acquired Immune Defic. Syndr. **5:**771–781.
11. **Crissman, H. A., and J. A. Steinkamp.** 1990. Cytochemical technique for multivariant analysis of DNA and other cellular constituents, ch. 13, p. 229–230. *In* M. R. Melamed, T. Lindmo, and M. L. Mendelsohm (ed.), Flow cytometry and sorting. John Wiley & Sons, Inc., New York.
12. **Crowe, S. M., J. Mills, and M. S. McGrath.** 1987. Quantitative immunocytofluorographic analysis of CD4 surface antigen expression and HIV infection of human peripheral blood monocyte/macrophages. AIDS Res. Hum. Retroviruses **3:**135–145.
13. **De Clercq, E.** 1985. Antiviral and antimetabolic activities of neplanocins. Antimicrob. Agents Chemother. **28:**84–89.
14. **De Clercq, E., N. Yamamoto, R. Pauwels, J. Balzarini, M. Witvrouw, K. De Vreese, Z. Debyser, B. Rosenwirth, P. Peichl, R. Datema, D. Thornton, R. Skerlj, F. Gaul, S. Padmanabhan, G. Bridger, G. Henson, and M. Abrams.** 1994. Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. Antimicrob. Agents Chemother. **38:**668–674.
15. **Fresen, M., K. W. Kohn, F. Leteurtre, and Y. Pommier.** 1993. Inhibitors of human immunodeficiency virus integrase. Proc. Natl. Acad. Sci. USA **90:**2399–2403.
16. **Gao, W., C. A. Stein, J. S. Cohen, G. E. Dutschman, and Y. C. Cheng.** 1989. Effect of phosphorothioate homo-oligodeoxynucleotides on herpes simplex virus type 2-induced DNA polymerase. J. Biol. Chem. **264:**11521–11526.
17. **Garry, R. F.** 1989. Potential mechanisms for the cytopathic properties of HIV. AIDS **3:**683–694.
18. **Gartner, S., and M. Popovic.** 1990. Virus isolation and production, p. 59–63. *In* A. Aldovini and B. Walker (ed.), Techniques in HIV research. Stockton Press, New York.
19. **Gowda, S. D., B. S. Stein, N. Mohagheghpour, C. J. Benike, and E. G. Engleman.** 1989. Evidence that T cell activation is required for HIV-1 entry in $CD4^+$ lymphocytes. J. Immunol. **142:**773–780.
20. **Griffiths, B.** 1992. Animal cell culture; a practical approach, p. 48. IRL Press, Oxford.
21. **Hirt, B. J.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26:**365–369.
22. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
23. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J. Virol. **66:**2232–2239.

24. **Letsinger, R. L., G. Zhang, D. K. Sun, T. Ikeuchi, and P. S. Sarin.** 1989. Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. Proc. Natl. Acad. Sci. USA **86:**6553–6556.
25. **Lewis, A. F., J. C. Drach, S. M. Fennewald, J. H. Huffman, R. G. Ptak, J. P. Sommadossi, G. R. Revankar, and R. F. Rando.** 1994. Inhibition of human cytomegalovirus in culture by alkenyl gaunine analogs of the thiazolo[4,5-*d*]pyrimidine ring system. Antimicrob. Agents Chemother. **38:**2889–2895.
26. **Lisziewicz, J., D. Sun, V. Metelev, P. Zamecnik, R. C. Gallo, and S. Agrawal.** 1993. Long-term treatment of human immunodeficiency virus-infected cells with antisense oligonucleotide phosphorothioates. Proc. Natl. Acad. Sci. USA **90:**3860–3864.
27. **Marshall, W. S., G. Beaton, C. Y. Stein, M. Matsukura, and M. H. Caruthers.** 1992. Inhibition of human immunodeficiency virus activity by phosphorothioate oligodeoxycytidine. Proc. Natl. Acad. Sci. USA **89:**6265–6269.
28. **Mazumder, A., D. Cooney, R. Agbaria, M. Gupta, and Y. Pommier.** 1994. Inhibition of human immunodeficiency virus type 1 integrase by 3′-azido-3′-deoxythymidylate. Proc. Natl. Acad. Sci. USA **91:**5771–5775.
29. **McGrath, M. S., K. M. Hwang, S. E. Caldwell, I. Gaston, K.-C. Luk, P. Wu, V. L. Ng, S. Crowe, J. Daniels, J. Marsh, T. Deinhart, P. V. Lekas, J. C. Vennari, H.-W. Yeung, and J. D. Lifson.** 1989. GLQ223: an inhibitor of human immunodeficiency virus replication in acutely and chronically infected cells of lymphocyte and mononuclear phagocyte lineage. Proc. Natl. Acad. Sci. USA **86:**2844–2848.
30. **McMahon, J. B., R. W. Buckheit, Jr., R. J. Gulakowski, M. J. Currens, D. T. Vistica, R. H. Shoemaker, S. F. Stinson, J. D. Russell, J. P. Bader, V. L. Narayanan, R. J. Shultz, W. G. Brouwer, E. E. Felauer, and M. R. Boyd.** Biological and biochemical anti-HIV activity of U-38, a new nonnucleoside reverse transcriptase inhibitor. J. Pharmacol. Exp. Ther., in press.
31. **Milligan, J. F., M. D. Matteucci, and J. C. Martin.** 1993. Current concepts in antisense drug design. J. Med. Chem. **36:**1923–1937.
32. **Nara, P. L., and P. J. Fischinger.** 1988. Quantitative infectivity assay for HIV-1 and HIV-2. Nature **332:**469–470.
33. **Ojwang, J., A. Elbaggari, H. B. Marshall, K. Jayaraman, M. S. McGrath, and R. F. Rando.** 1994. Inhibition of human immunodeficiency virus type 1 activity *in vitro* by oligonucleotides composed entirely of guanosine and thymidine (GTOs). J. Acquired Immune Defic. Syndr. **7:**560–570.
34. **Parker, W. B., E. L. White, S. C. Shaddix, L. J. Ross, R. W. Buckheit, Jr., J. M. Germany, J. A. Secrist III, R. Vince, and W. M. Shannon.** 1991. Mechanism of inhibition of human immunodeficiency virus type 1 reverse transcriptase and human DNA polymerases α, β, and γ by the 5′-triphosphates of carbovir, 3′-azido-3′-deoxythymidine, 2′,3′-dideoxyguanosine, and 3′-deoxythymidine. J. Biol. Chem. **266:**1754–1762.
35. **Rando, R. F., J. Ojwang, A. Elbaggari, G. R. Reyes, R. Tinder, M. S. McGrath, and M. E. Hogan.** 1995. Suppression of human immunodeficiency virus type 1 activity in vitro by oligonucleotides which form intramolecular tetrads. J. Biol. Chem. **270:**1754–1760.
36. **Schultze, P., R. F. Macaya, and J. Feigon.** 1994. Three-dimensional solution structure of the thrombin-binding DNA aptamer d(GGTTGGTGTGGT TGG). J. Mol. Biol. **235:**1532–1547.
37. **Stein, C. A., A. M. Cleary, L. Yakubov, and S. Lederman.** 1993. Phosphorothioate oligodeoxynucleotides bind to the third variable loop domain (v3) of human immunodeficiency virus type 1 gp120. Antisense Res. Dev. **3:**19–31.
38. **Steinkasserer, A., R. Harrison, A. Billich, F. Hammerschmid, G. Werner, B. Wolff, P. Peichl, G. Palfi, W. Schnitzel, E. Mlynar, and B. Rosenwirth.** 1995. Mode of action of SDZ NIM811, a nonimmunosuppressive cyclosporin A analog with activity against human immunodeficiency virus type 1 (HIV-1): interference with early and late events in HIV-1 replication. J. Virol. **69:** 814–824.
39. **Wang, K. Y., S. McCurdy, R. G. Shea, S. Swaminathan, and P. H. Bolton.** 1993. A DNA aptamer which binds to and inhibits thrombin exhibits a new structural motif for DNA. Biochemistry **32:**1899–1904.
40. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) **373:**117–122.
41. **White, E. L., R. W. Buckheit, Jr., L. J. Ross, J. M. Germany, K. Andries, R. Pauwels, P. A. Janssen, W. M. Shannon, and M. A. Chirigos.** 1991. A TIBO derivative, R82913, is a potent inhibitor of HIV-1 reverse transcriptase with heteropolymer templates. Antiviral Res. **16:**257–266.
42. **Wyatt, J. R., T. A. Vickers, J. L. Roberson, R. W. Buckheit, Jr., T. Klimkait, E. DeBaets, P. W. Davis, B. Rayner, J. L. Imbach, and D. J. Ecker.** 1994. Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelop-mediated cell fusion. Proc. Natl. Acad. Sci. USA **91:**1356–1360.
43. **Wyde, P. R., L. R. Meyerson, and B. E. Gilbert.** 1993. An in vitro evaluation of the antiviral activity of SP-303, an Euphorbiaceae shrub extract, against a panel of respiratory viruses. Drug Dev. Res. **28:**467–472.
44. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell **61:**213–222.