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Potent and selective inhibition of human immunodeficiency virus type 1 (HIV-1) by 5-ethyl-6-phenylthiouracil derivatives through their interaction with the HIV-1 reverse transcriptase

(acquired immune deficiency syndrome/antiviral chemotherapy)

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ABSTRACT **In the search for 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio)thymine (HEPT) derivatives, we have found several 5-ethyl-6-(phenylthio)uracil analogues to be highly potent and selective inhibitors of human immunodeficiency virus (HIV) type 1. 1-Benzyloxymethyl-5-ethyl-6-phenylthiouracil, the most potent congener of the series, inhibits HIV-1 replication in a variety of cell systems, including peripheral blood lymphocytes, at a concentration of 1.5–7.0 nM, which is lower by a factor of 10^3 than the 50% antivirally effective concentration of the parent compound HEPT. The 5-ethyl-6-(phenylthio)uracil analogues, like HEPT itself, do not inhibit HIV-2 replication but do inhibit replication of 3′-azido-3′-deoxythymidine-resistant mutants of HIV-1. 1-Benzyloxymethyl-5-ethyl-6-phenylthiouracil and its congeners are targeted at the HIV-1 reverse transcriptase (RT). They do not inhibit HIV-2 RT. They do not need to be metabolized to exert their inhibitory effect on HIV-1 RT. Yet this inhibitory effect is competitive with the natural substrate dTTP. The HEPT derivatives represent a group of RT inhibitors with a unique mode of interaction with HIV-1 RT.**

Human immunodeficiency virus (HIV) type 1 is the causative agent of AIDS, which is characterized as a systemic and life-threatening disorder. A number of compounds have been reported as active anti-HIV-1 agents *in vitro* (1, 2). Among these compounds, 3′-azido-3′-deoxythymidine (AZT) is the only drug that has so far been approved for clinical use in patients. Although AZT does prolong survival in AIDS patients (3, 4), its use is compounded by serious side effects such as bone marrow suppression (5), resumption of virus replication during therapy (6), and emergence of AZT-resistant HIV-1 mutants (7–9). Several other dideoxynucleoside analogues such as 2′,3′-dideoxycytidine and 2′,3′-dideoxyinosine have been the subject of clinical studies. These agents including AZT are assumed to be targeted at the viral RNA-dependent DNA polymerase (reverse transcriptase; RT) after their intracellular conversion to their 5′-triphosphate (TP) form (10, 11). Such TP derivatives interact not only with HIV-1 RT but also other retroviral RTs (12) and even cellular DNA polymerases (13). This nonspecific action may contribute to the toxic side effects of this class of compounds.

Although the toxicity of AZT seems tolerable to some extent, it is highly desirable to find other compounds with reduced toxicity. One such approach is based on acyclic nucleosides and, in fact, several acyclic nucleoside analogues as well as phosphonomethyl derivatives have been shown to inhibit the replication of HIV-1 *in vitro* (14, 15). In this regard, we have reported on a 6-substituted acyclouridine derivative 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) (16). This compound is a potent and selective inhibitor of HIV-1, yet it is totally inactive against HIV-2 or any other retrovirus (17). This unique feature, which makes HEPT fundamentally different from any other anti-HIV nucleoside analogue, has prompted us to synthesize a wide variety of HEPT derivatives. We have examined these analogues for their inhibitory effects on HIV-1 replication *in vitro* and found that the 5-ethyl derivatives by far exceeded HEPT in both anti-HIV-1 potency and selectivity. The compounds inhibit HIV-1 RT and do so in a competitive fashion with respect to the natural substrate dTTP.

MATERIALS AND METHODS

Compounds. HEPT and its derivatives, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)uracil (HEPU), 1-ethoxymethyl-6-(phenylthio)thymine (EPT), 1-propoxymethyl-6-(phenylthio)-thymine, 1-benzyloxymethyl-6-(phenylthio)thymine (BPT), 5-ethyl-1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)uracil (E-HEPU), 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil (E-EPU), 5-ethyl-1-benzyloxymethyl-6-(phenylthio)uracil (E-BPU), and 5-propyl-1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)uracil (Table 1) were prepared according to a procedure described elsewhere (refs. 16 and 18 and unpublished data). Purity of these compounds was examined by TLC on silica gel. For identification of the compounds, ^{1}H NMR spectra were recorded at 250 MHz on an AC-250 Bruker NMR spectrometer, and elemental analyses were carried out to get analytical results within 0.4% of the theoretical values (16, 18). The compounds were dissolved in dimethyl sulfoxide at 50 mM or higher and stored at −20°C until used. Solubility of each compound in culture medium was much greater than its cytotoxic concentration. AZT and 2′,3′-didehydro-3′-deoxythymidine (D4T) were synthesized at the Rega Institute by P. Herdewijn and his col-

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Abbreviations: HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; HIV, human immunodeficiency virus; AZT, 3′-azido-3′-deoxythymidine; RT, reverse transcriptase; PBL, peripheral blood lymphocyte; MP, monocyte–macrophage; HEPU, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)uracil; EPT, 1-ethoxymethyl-6-(phenylthio)thymine; BPT, 1-benzyloxymethyl-6-(phenylthio)thymine; E-HEPU, 5-ethyl-1-[(2-hydroxyethoxy]methyl]-6-(phenylthio)uracil; E-EPU, 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil; E-BPU, 5-ethyl-1-benzyloxymethyl-6-(phenylthio)uracil; D4T, 2′,3′-didehydro-3′-deoxythymidine; TP, triphosphate; CC_{50}, 50% cytotoxic concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; r, recombinant; n, native.

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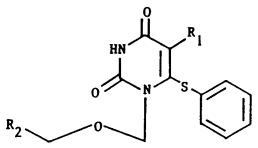
Table 1. Inhibitory effect of HEPT derivatives on HIV-1 replication, cell viability, and HIV-1 RT activity

Compound	R_1*	R_2*	Virus replication EC_{50}, μM	Virus replication CC_{50}, μM	RT activity IC_{50}, μM
HEPU	H	CH_2OH	>500	>500	>500
HEPT	CH_3	CH_2OH	6.5	>500	53
EPT	CH_3	CH_3	0.33	230	4.1
PPT	CH_3	C_2H_5	3.0	140	34
BPT	CH_3	C_6H_5	0.093	63	4.0
E-HEPU	C_2H_5	CH_2OH	0.12	400	1.9
E-EPU	C_2H_5	CH_3	0.022	146	0.27
E-BPU	C_2H_5	C_6H_5	0.0049	30	0.16
P-HEPU	C_3H_7	CH_2OH	3.6	200	11
AZT			0.0030	7.8	0.014†
D4T			0.034	15	ND

EC_{50} is based on the inhibition of HIV-1-induced cytopathogenicity in MT-4 cells. CC_{50} (50% cytotoxic concentration) is based on the reduction of viability of mock-infected MT-4 cells. IC_{50} is for purified recombinant HIV-1 RT activity using poly(rA)·oligo(dT) as the template–primer. ND, not determined; PPT, 1-propoxymethyl-6-(phenylthio)thymine; P-HEPU, 5-propyl-1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)uracil. All data represent mean values for more than two experiments.

*R_1 and R_2 refer to the following structure.



†AZT-TP was used.

leagues, and HEPT-TP and AZT-TP were prepared in the Mitsubishi Kasei Corporation Research Center (Yokohama, Japan).

Cells. MT-4 cells (19), MOLT-4 clone 8 cells (20), peripheral blood lymphocytes (PBLs), and monocyte–macrophages (MPs) were used in the anti-HIV assays. The cells were mycoplasma-negative. MT-4 and MOLT-4 cells were grown and maintained in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, penicillin G (100 units/ml), and gentamicin (20 μg/ml). PBLs and MPs were obtained from healthy donors. PBLs were stimulated with phytohemagglutinin and cultured with RPMI 1640 medium containing 20% fetal calf serum, antibiotics, and interleukin 2. Isolation and cultivation of MPs were carried out as described by Perno *et al.* (21).

Viruses. Seven strains of HIV-1 (HTLV-III_B, HTLV-III_{RF}, HIV-$1_{JR\text{-}FL}$, A012B, A012D, A018A, and A018C) and two strains of HIV-2 (LAV-2_{ROD} and LAV-2_{EHO}) were used in the anti-HIV assays. HIV-$1_{JR\text{-}FL}$ is a PBL- and MP-tropic strain (22), and A012D and A018C are AZT-resistant HIV-1 mutants (7). Except for HIV-$1_{JR\text{-}FL}$, these viruses were obtained from the culture supernatants of MOLT-4 or CEM cells persistently infected with virus. Titers of virus stocks were determined in MT-4 cells. HIV-$1_{JR\text{-}FL}$ was propagated in PBLs.

Antiviral Assays. Activity of the compounds against HIV-1 (HTLV-III_B and HTLV-III_{RF}) and HIV-2 (LAV-2_{ROD} and LAV-2_{EHO}) replication was based on the inhibition of virus-induced cytopathogenicity in MT-4 cells or MOLT-4 cells. Briefly, MT-4 and MOLT-4 cells were suspended in culture medium at 1×10^5 cells per ml and infected with HIV at a multiplicity of infection of 0.02 and 0.2, respectively. Immediately after virus infection, the cell suspension (100 μl) was added to each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. After a 4-day incubation at 37°C, MOLT-4 cells were subcultured at a ratio of 1:5 with fresh culture medium containing appropriate concentrations of the compounds and further incubated. The number of viable MT-4 and MOLT-4 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method on days 4 and 8 after virus infection, respectively (23). Activity of the compounds against the clinical isolates of HIV-1 (A012B, A012D, A018A, and A018C) was determined by the amount of HIV-1 p24 antigen in culture supernatant [using a sandwich ELISA kit (Abbott)] on day 4 after infection of MT-4 cells.

The assay procedure for measuring the anti-HIV-1 activity of the compounds in PBLs and MPs was also based on the quantitative detection of HIV-1 p24 antigen in the culture supernatant. Phytohemagglutinin-stimulated PBLs (1×10^6 cells per ml) were infected with HIV-1 (HTLV-III_B) at a multiplicity of infection of 0.2. After virus adsorption for 2 hr, the cells were extensively washed to remove unadsorbed virus particles and cultured at 37°C in the presence of various concentrations of the test compounds. On day 4 after virus infection, the cells were subcultured at a ratio of 1:5 with fresh culture medium containing appropriate concentrations of the compounds. MPs (1×10^6 cells per well) were cultured in a 24-well tray for 5 days and infected with 1 ng of HIV-$1_{JR\text{-}FL}$ p24. After a 2-day incubation period, the cells were washed and cultured in the absence or presence of the test compound. The cells were given fresh culture medium every 4 days. The assay was performed on day 7 in PBLs and on day 15 in MPs after virus infection.

Cytotoxicity of the compounds was evaluated in parallel with their antiviral activity. It was based on the viability of mock-infected cells as determined by the MTT method.

RT Assay. The effect of the various compounds on HIV RT activity was evaluated with three enzymes, purified recombinant HIV-1 RT (HIV-1 rRT), native HIV-1 RT (HIV-1 nRT), and native HIV-2 RT (HIV-2 nRT). HIV-1 rRT (BH-10) was produced in *Escherichia coli* and composed of three proteins of 66 kDa (46.5%), 55 kDa (32.3%), and 53 kDa (21.2%). Specific activity of the enzyme was 2 units/μg (Division of AIDS, National Institute of Allergy and Infectious Diseases). HIV-1 and HIV-2 nRTs were obtained from disrupted virions that had been partially purified and concentrated from the supernatants of MOLT-4 cells persistently infected with HTLV-III_B and LAV-2_{ROD}, respectively. The standard assay was performed at 37°C for 30 min in a 50-μl reaction mixture containing 50 mM Tris·HCl (pH 8.4), 2 mM dithiothreitol, 100 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 μCi of either [*methyl*-^{3}H]dTTP (46 Ci/mmol, Amersham; 1 Ci = 37 GBq) or [1′,2′-^{3}H]dGTP (42 Ci/mmol, Amersham), 0.01 OD 260 unit of either poly(rA)·$(dT)_{12\text{-}18}$ (16.2 units/mg, Pharmacia) or poly(rC)·$(dG)_{12\text{-}18}$ (15.0 units/mg, Pharmacia), test compound, and enzyme (≈0.01 unit of HIV-1 rRT or 0.004 unit of HIV-1 or HIV-2 nRT). The reaction was stopped with 200 μl of 5% (vol/vol) trichloroacetic acid, and the precipitated materials were analyzed for radioactivity.

RESULTS

Antiviral Activity. When we evaluated the HEPT derivatives for their inhibitory effects on HIV-1 (HTLV-III_B) replication in MT-4 cells, all compounds except for HEPU proved inhibitory to HIV-1 (Table 1). In particular, the 5-ethyl derivatives E-HEPU, E-EPU, and E-BPU proved remarkably active, their EC_{50} values being 0.12, 0.022, and 0.0049 μM, respectively. These values are approximately 55, 300, and 1300 times lower than the EC_{50} of HEPT (6.5 μM). Fig. 1 *A* and *B* shows the number of viable MT-4 cells either HIV-1-infected or mock-infected in the presence of various concentrations of E-EPU and E-BPU, respectively. E-EPU and E-BPU completely protected the cells against virus-

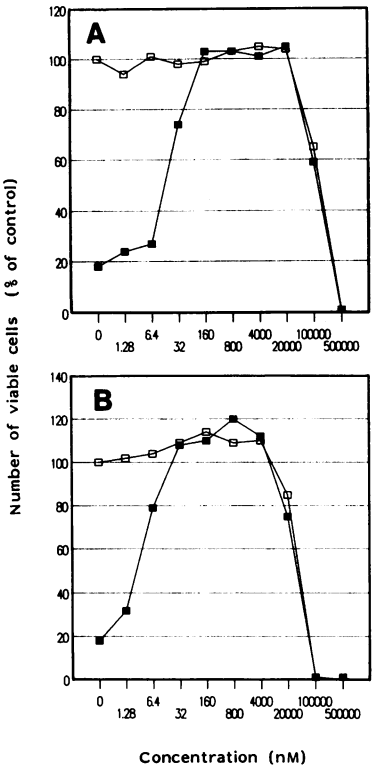


FIG. 1. Inhibitory effect of E-EPU (*A*) and E-BPU (*B*) on HIV-1-induced cytopathogenicity in MT-4 cells. MT-4 cells were infected with HIV-1 (HTLV-III_B) and incubated with various concentrations of the test compounds for 4 days. The viability of virus-infected cells (■) and mock-infected cells (□) was assessed by the MTT method. The number of viable cells is expressed as percent of the mock-infected control.

induced cell destruction at 0.16 and 0.032 μM, respectively. On the other hand, these compounds did not markedly reduce the viability of mock-infected MT-4 cells at concentrations up to 100 and 20 μM, respectively. The CC_{50} values of E-HEPU, E-EPU, and E-BPU for mock-infected MT-4 cells were 400, 146, and 30 μM, respectively (Table 1). Thus, their selectivity indexes, based on the ratio of CC_{50} to EC_{50}, were 3300 (E-HEPU), 6600 (E-EPU), and 6100 (E-BPU). When evaluated under the same conditions, AZT achieved a selectivity index of 2600.

In the next set of experiments, E-HEPU, E-EPU, and E-BPU were examined for their inhibitory effects on HIV-1 (HTLV-III_B) replication in a variety of cell systems including PBLs. Their inhibitory effects on another HIV-1 strain (HTLV-III_{RF}) and two HIV-2 strains (LAV-2_{ROD} and LAV-2_{EHO}) were also evaluated in MT-4 cells. As shown in Table 2, all compounds proved effective against HIV-1 replication in MOLT-4 and PBL cells. Furthermore, their EC_{50} values in these cell cultures were quite similar to those obtained in MT-4 cells. E-HEPU, E-EPU, and E-BPU were as active against HTLV-III_{RF} as HTLV-III_B (Table 2). However, as noted for HEPT (17), they did not inhibit HIV-2-induced cytopathogenicity in MT-4 cells. In contrast, the reference compounds AZT and D4T were equally inhibitory to the replication of HIV-1 and HIV-2 (Table 2). As MPs may play an important role in the pathogenesis of AIDS (24), E-EPU was examined for its inhibitory effect on the replication of HIV-1 (HIV-1_{JR-FL}) (22) in human MP cultures. E-EPU proved markedly inhibitory to HIV-1, and its EC_{50} value for this strain was 0.013 μM (Table 2).

In view of their therapeutic use, we investigated whether the compounds were also inhibitory to AZT-resistant mutants of HIV-1. Specifically, we examined the effects of

Table 2. Inhibitory effect of HEPT derivatives on the replication of HIV-1 and HIV-2 in various cell cultures

Compound	Virus	Strain	Cell	EC_{50}, μM	CC_{50}, μM
E-HEPU	HIV-1	HTLV-III_B	MT-4	0.12	400
	HIV-1	HTLV-III_B	MOLT-4	0.19	400
	HIV-1	HTLV-III_B	PBL	0.19	108
	HIV-1	HTLV-III_{RF}	MT-4	0.049	—
	HIV-2	LAV-2_{ROD}	MT-4	>400	—
	HIV-2	LAV-2_{EHO}	MT-4	>400	—
E-EPU	HIV-1	HTLV-III_B	MT-4	0.022	146
	HIV-1	HTLV-III_B	MOLT-4	0.029	127
	HIV-1	HTLV-III_B	PBL	0.032	63
	HIV-1	HTLV-III_{RF}	MT-4	0.012	—
	HIV-1	HIV-1_{JR-FL}	MP	0.013	>20
	HIV-2	LAV-2_{ROD}	MT-4	>146	—
	HIV-2	LAV-2_{EHO}	MT-4	>146	—
E-BPU	HIV-1	HTLV-III_B	MT-4	0.0049	30
	HIV-1	HTLV-III_B	MOLT-4	0.0039	42
	HIV-1	HTLV-III_B	PBL	0.0072	22
	HIV-1	HTLV-III_{RF}	MT-4	0.0016	—
	HIV-2	LAV-2_{ROD}	MT-4	>30	—
	HIV-2	LAV-2_{EHO}	MT-4	>30	—
AZT	HIV-1	HTLV-III_B	MT-4	0.0030	7.8
	HIV-1	HTLV-III_B	MOLT-4	0.0041	232
	HIV-1	HTLV-III_B	PBL	0.0020	54
	HIV-1	HTLV-III_{RF}	MT-4	0.0028	—
	HIV-2	LAV-2_{ROD}	MT-4	0.0028	—
	HIV-2	LAV-2_{EHO}	MT-4	0.0032	—
D4T	HIV-1	HTLV-III_B	MT-4	0.034	15
	HIV-1	HTLV-III_B	MOLT-4	0.045	122
	HIV-1	HTLV-III_B	PBL	0.038	48
	HIV-1	HTLV-III_{RF}	MT-4	0.038	—
	HIV-2	LAV-2_{ROD}	MT-4	0.078	—
	HIV-2	LAV-2_{EHO}	MT-4	0.12	—

EC_{50} is based on the inhibition of HIV-induced cytopathogenicity in MT-4 and MOLT-4 cells or the reduction of p24 antigen in culture supernatant of PBLs and MPs. CC_{50} is based on the reduction of viability of mock-infected cells. All data represent mean values for at least two experiments.

E-EPU and E-BPU on the replication of two AZT-resistant HIV-1 mutants (A012D and A018C) and the corresponding AZT-sensitive strains (A012B and A018A) in MT-4 cells. When the EC_{50} (or EC_{90}) values were determined based on a 50% (or 90%) reduction in the amount of p24 antigen in the culture supernatant, it appeared that the AZT-resistant HIV-1 strains were at least 100-fold less susceptible to inhibition by AZT than the AZT-sensitive strains (Table 3). Both E-EPU and E-BPU were found to be equally active against the AZT-resistant and AZT-sensitive HIV-1 strains (Table 3).

Table 3. Inhibitory effect of HEPT derivatives on the replication of clinical isolates of HIV-1 including two AZT-resistant mutants

Compound	EC_{50} (EC_{90}), μM			
	A012B	A012D*	A018A	A018C*
AZT	0.0030 (0.0097)	0.30 (>4.0)	0.0027 (0.012)	0.40 (>4.0)
E-EPU	0.013 (0.029)	0.015 (0.065)	0.027 (0.11)	0.032 (0.12)
E-BPU	0.0019 (0.0058)	0.0029 (0.015)	0.0095 (0.026)	0.010 (0.028)

EC_{50} and EC_{90} (90% antiviral effective concentration) are based on the reduction of p24 antigen in culture supernatant of MT-4 cells. All data represent mean values for two experiments.
*AZT-resistant HIV-1 strains.

Inhibition of HIV-1 RT. We have demonstrated (17) that HEPT suppresses HIV-1 proviral DNA synthesis but not earlier steps in the virus replicative cycle (i.e., virus adsorption, penetration, or uncoating). According to a "time of addition" experiment, whereby the compound was added at various times subsequent to virus infection and then followed for its inhibitory effect on virus replication, the RT reaction was considered to be a likely target for the anti-HIV-1 activity of HEPT (unpublished data). In fact, all the HEPT derivatives that were active against HIV-1 replication also proved inhibitory to HIV-1 rRT (Table 1). Furthermore, a close correlation was observed between their EC_{50} for inhibition of virus replication and the IC_{50} required to achieve 50% inhibition of RT activity, although the IC_{50} values for inhibition of RT activity appeared to be 10–40 times higher than the EC_{50} values for inhibition of HIV-1 replication (Table 1).

When the effects of HEPT derivatives on HIV-1 rRT were examined with poly(rC)·oligo(dG) as the template–primer, HEPT and E-EPU exhibited similar IC_{50} values for the enzyme (Table 4). These compounds also inhibited HIV-1 nRT. However, the activity of HIV-2 nRT was not affected by E-EPU up to 500 μM. In contrast, AZT-TP was equally inhibitory to both HIV-1 nRT and HIV-2 nRT (Table 4).

The mode of inhibition of HIV rRT by E-EPU was then analyzed under various assay conditions. Double-reciprocal plots revealed that the inhibition of the enzyme by E-EPU was competitive with respect to dTTP [with poly(rA)·oligo(dT) as template–primer] and noncompetitive with respect to dGTP [with poly(rC)·oligo(dG) as template–primer] (Fig. 2). The K_m values for dTTP and dGTP were 28 and 7.7 μM, respectively. The K_i value of HIV-1 rRT for E-EPU with dTTP as substrate was 0.42 μM. The kinetics of inhibition were also studied with various concentrations of poly(rA)·oligo(dT) and found to be noncompetitive (data not shown).

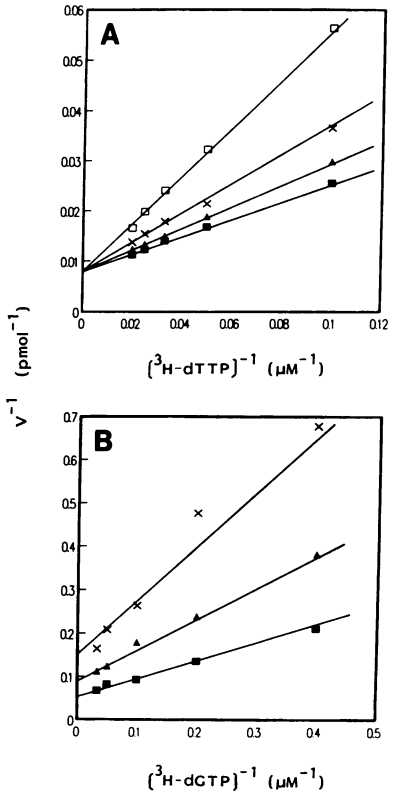


FIG. 2. Double-reciprocal plot analysis for inhibition of purified recombinant HIV-1 RT by E-EPU. Reactions were performed in 50 μl containing 0.01 unit of HIV-1 rRT, 0.01 OD 260 unit of either poly(rA)·oligo(dT) (12.3 μg/ml) (*A*) or poly(rC)·oligo(dG) (13.3 μg/ml) (*B*), various concentrations of either [^{3}H]dTTP (*A*) or [^{3}H]dGTP (*B*), and E-EPU at 0 (■), 0.2 (▲), 0.4 (×), and 0.8 (□) μM.

DISCUSSION

Until the discovery of HEPT as a specific inhibitor of HIV-1 (16, 17), no other compound had ever been reported to discriminate between HIV-1 and HIV-2 in terms of antiviral activity. HEPT indeed specifically inhibits the replication of various strains of HIV-1 in various cell systems; however, its potency is not very high (17). We have attempted to increase the potency and selectivity of HEPT by introducing structural modifications in both the heterocyclic ring and acyclic side chain. In fact, we have synthesized more than a hundred derivatives of HEPT and examined them for anti-HIV-1 activity. Among these derivatives, E-EPU and E-BPU proved to be highly potent inhibitors of HIV-1 with selectivity indexes >6000 (Table 1). Despite their high potency against HIV-1, these compounds did not inhibit HIV-2 replication (Table 2).

Table 4. Inhibitory effect of HEPT, HEPT-TP, E-EPU, and AZT-TP on HIV RT activity

Compound	Enzyme	Template–primer	Substrate	IC_{50}, μM
HEPT	HIV-1 rRT	poly(rA)·oligo(dT)	dTTP	53
	HIV-1 rRT	poly(rC)·oligo(dG)	dGTP	66
HEPT-TP	HIV-1 rRT	poly(rA)·oligo(dT)	dTTP	>500
E-EPU	HIV-1 rRT	poly(rA)·oligo(dT)	dTTP	0.27
	HIV-1 rRT	poly(rC)·oligo(dG)	dGTP	0.14
	HIV-1 nRT	poly(rA)·oligo(dT)	dTTP	1.1
	HIV-2 nRT	poly(rA)·oligo(dT)	dTTP	>500
AZT-TP	HIV-1 rRT	poly(rA)·oligo(dT)	dTTP	0.014
	HIV-1 rRT	poly(rC)·oligo(dG)	dGTP	>100
	HIV-1 nRT	poly(rA)·oligo(dT)	dTTP	0.0060
	HIV-2 nRT	poly(rA)·oligo(dT)	dTTP	0.0069

All data represent mean values for at least two experiments.

Studies on the structure–activity relationship of these compounds revealed that substitution of the methyl group at C-5 by an ethyl group markedly enhanced the anti-HIV-1 activity (Table 1). In contrast, replacement of the methyl group with a propyl group (P-HEPU) did not potentiate the anti-HIV-1 activity of HEPT, and replacement of the methyl group with hydrogen (HEPU) completely abolished the activity. The compounds with an ethoxymethyl side chain at N-1, such as EPT and E-EPU, proved more inhibitory to both HIV-1 replication and HIV-1 RT than the 1-(2-hydroxyethoxymethyl) derivatives HEPT and E-HEPU (Table 1). With a benzyloxymethyl group as the N-1 side chain, the anti-HIV-1 activity was further improved; yet these compounds (BPT and E-BPU) were slightly cytotoxic. These results indicate that a hydroxyl group at the end of N-1 side chain is not necessary to impart anti-HIV-1 activity. Thus, the compounds do not need to be phosphorylated intracellularly to interact with the HIV-1 RT. Even if HEPT would be phosphorylated intracellularly (for which there is no evidence), HEPT-TP could not interfere with the activity of HIV-1 RT because chemically synthesized HEPT-TP was totally inactive against HIV-1 RT (Table 4).

In keeping with its lack of activity against HIV-2 replication in cell culture, E-EPU did not prove inhibitory to HIV-2 RT (Table 4). Why does the compound interact with HIV-1 and not HIV-2 RT? Although HIV-2 RT shares ≈60% of its amino acid sequences with HIV-1 RT (25), there may be distinct differences in their functional domains such as substrate-binding sites, template-binding sites, and allosteric sites. One possibility is that HIV-1 RT but not HIV-2 RT has the specific portion called a "hydrophobic pocket" in nucleotide-binding sites. E-EPU may interact with this portion; thereby, the activity of HIV-1 RT is suppressed. In fact,

DNA polymerase α is inhibited by the compounds that bind to the hydrophobic pockets (26).

Analysis of the mode of inhibition of HIV-1 RT by E-EPU revealed that, like AZT-TP, E-EPU inhibits the enzyme competitively with dTTP (Fig. 2). In contrast with AZT-TP, however, E-EPU was also inhibitory to HIV-1 RT in a noncompetitive fashion, when the enzymatic activity was measured with dGTP as substrate (Table 4 and Fig. 2). The potential for such a multifaceted kinetic picture with nucleotide inhibitors of HIV-1 RT has been observed with AZT-TP and 3'-amino-dTTP as a competitive and a noncompetitive inhibitor against dTTP, respectively (27). For E-EPU, the compound may combine with different forms of the enzyme depending on the template–primer/substrate system used in the assay. As shown with AZT-TP and dTTP (27), E-EPU and dTTP may combine with the same form of the enzyme–primer complex, yet E-EPU and dGTP may not do so. Substitution of the phenylthio group at C-6 position by hydrogen, halogens, or linear carbon chains completely abolished the anti-HIV-1 activity of HEPT and its analogues (data not shown), suggesting that this part of the molecule may be an important determinant in the mode of interaction of these compounds with the HIV-1 RT.

It has recently been reported that some benzodiazepin (TIBO) derivatives are highly specific and potent inhibitors of HIV-1 replication (28). They do not suppress the replication of HIV-2 or other retroviruses. They are also assumed to inhibit HIV-1 replication through an inhibitory effect on the RT (28). The anti-HIV-1 potency of E-EPU and E-BPU is similar to that of the TIBO derivatives. Given their unique specificity for HIV-1, the HEPT and TIBO derivatives may work by a similar mechanism of action. However, the TIBO derivatives apparently differ from the HEPT analogues in that they do not act as competitive inhibitors of HIV-1 RT with respect to dTTP. Further studies seem warranted to resolve the precise mechanism by which these compounds interact with the RT.

Several issues including toxicology and pharmacokinetics need to be addressed before clinical studies can be envisaged with the HEPT derivatives. However, the emergence of AZT-resistant HIV-1 mutants after long-term administration (>6 months) with AZT (7–9) points to the importance of alternative drugs for the treatment of AIDS. The compounds described herein (E-EPU and E-BPU) offer interesting perspectives as candidate drugs for AIDS chemotherapy.

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