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Highly Potent and Selective Inhibition of Human Immunodeficiency Virus Type 1 By a Novel Series of 6-Substituted Acyclouridine Derivatives

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SUMMARY

In the search for novel derivatives of 1-[2-(hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT), we have found that several 5-ethyl-6-(3,5-dimethylphenylthio)uracil and 5-ethyl-6-(3,5-dimethylbenzyl)uracil analogues are exquisitely potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) replication in a variety of cell culture systems. Of this series, 5-ethyl-1-ethoxymethyl-6-(3,5-dimethylbenzyl)uracil (E-EBU-dM) emerged as the most active congener. Its 50% inhibitory concentration for HIV-1 (HTLV-III$_B$) in MT-4 cells and peripheral blood lymphocytes is 2.2 and 0.45 nM, respectively. These concentrations are more than 10^5-fold lower than the 50% cytotoxic concentrations of E-EBU-dM for the host cells. All compounds proved equally inhibitory to a number of clinical HIV-1 isolates, including a 3′-azido-2′,3′-dideoxythymidine-resistant variant. However, as previously noted for HEPT, they do not inhibit human immunodeficiency virus type 2 replication. Reverse transcriptase assays have revealed that these HEPT derivatives act specifically on HIV-1 reverse transcriptase, according to a mechanism that is different from that of the dideoxynucleosides.

Continuous efforts are being made to find effective chemotherapeutic agents against HIV-1, the causative agent of AIDS. Since the discovery of AZT as a potent and selective anti-HIV-1 agent (1), a number of compounds have been shown to inhibit HIV-1 replication *in vitro* (2, 3). AZT is still the only drug that has been licensed for clinical use in the treatment of AIDS or AIDS-related complex. Although AZT treatment leads to an improvement of the clinical symptoms and prolongation of the survival of patients with AIDS (4, 5), the usefulness of AZT is limited by serious side effects such as bone marrow suppression (6). Furthermore, long term treatment with AZT leads to the emergence of AZT-resistant HIV-1 variants (7-9). Therefore, it is mandatory to develop new compounds with potent activity, reduced toxicity, and, preferably, a different mechanism of action.

We have recently demonstrated the 6-substituted acyclouridine derivative HEPT as a new lead for anti-HIV-1 agents (10). HEPT is an unusual compound, in that it is only inhibitory to HIV-1 (11). HIV-2 and other animal retroviruses are insensitive to HEPT (11). In this respect, HEPT behaves dissimilarly from other anti-HIV agents such as AZT and other 2′,3′-dideoxyn-

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ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; HEPT, 1-[2-(hydroxyethoxy)methyl]-6-(phenylthio)thymine; E-EBU-dM, 5-ethyl-1-ethoxymethyl-6-(3,5-dimethylbenzyl)uracil; PBL, peripheral blood lymphocytes; AIDS, acquired immune deficiency syndrome; MP, monocyte-macrophages; AZT, 3′-azido-2′,3′-dideoxythymidine; E-HEPU-dM, 5-ethyl-1-[(2-hydroxyethoxy)methyl]-6-(3,5-dimethylphenylthio)uracil; E-EPU-dM, 5-ethyl-1-ethoxymethyl-6-(3,5-dimethylphenylthio)uracil; E-BPU-dM, 1-benzyloxymethyl-5-ethyl-6-(3,5-dimethylphenylthio)uracil; E-HEPU-SdM, 5-ethyl-1-[(2-hydroxyethoxy)methyl]-6-(3,5-dimethylphenylthio)-2-thiouracil; E-EPU-SdM, 5-ethyl-1-ethoxymethyl-6-(3,5-dimethylphenylthio)-2-thiouracil; E-BPU-SdM, 1-benzyloxymethyl-5-ethyl-6-(3,5-dimethylphenylthio)-2-thiouracil; E-HEBU-dM, 5-ethyl-1-[(2-hydroxyethoxy)methyl]-6-(3,5-dimethylbenzyl)uracil; D4T, 2′,3′-dideoxy-2′,3′-didehydrothymidine; AZT-TP, 3′-azido-2′,3′-dideoxythymidine 5′-triphosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcriptase; EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; HEPT-M, 1-[(2-hydroxyethoxy)methyl]-6-(3-methylphenylthio)thymine; HEPT-dM, 1-[(2-hydroxyethoxy)methyl]-6-(3,5-dimethylphenylthio)thymine; HEPT-S, 1-[(2-hydroxyethoxy)methyl]-6-phenylthio-2-thiothymine; IC_{50}, 50% inhibitory concentration; rRT, recombinant reverse transcriptase; nRT, native reverse transcriptase; TIBO, tetrahydro-imidazo [4,5,1-jk][1,4]benzodiazepin-2(1H)-one and -thione.

ucleoside analogues (12), which are active against HIV-1, HIV-2, and various other animal retroviruses (13, 14). Following the HEPT lead, we have designed new 6-substituted acyclouridine derivatives and found that they inhibit HIV-1 replication within the nanomolar concentration range. This anti-HIV-1 activity may be mediated by an inhibitory effect on the HIV-1 RT.

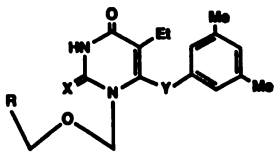
Materials and Methods

Compounds. HEPT and its derivatives (Fig. 1), i.e., E-HEPU-dM, E-EPU-dM, E-BPU-dM, E-HEPU-SdM, E-EPU-SdM, E-BPU-SdM, E-HEBU-dM, and E-EBU-dM, were prepared according to the procedures that had been described elsewhere (10, 15, 16).[1] Purity of the compounds was checked by thin layer chromatography on silica gel, and ^{1}H NMR spectra were recorded for their identification. AZT and D4T (17–19) were synthesized at the Rega Institute, whereas AZT-TP was prepared in the Mitsubishi Kasei Corporation Research Center.

Cells. MT-4 cells (20), MOLT-4 clone 8 cells (21), PBL, and MP were used in the anti-HIV assays. The cell lines were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, and 20 μg/ml gentamicin. PBL and MP were obtained from healthy donors. PBL were stimulated with phytohemagglutinin and cultured with RPMI 1640 medium containing 20% fetal bovine serum, antibiotics, and interleukin-2. Isolation and cultivation of MP were carried out according to the procedure described by Perno *et al.* (22).

Viruses. Five strains of HIV-1 (HTLV-III$_B$, HTLV-III$_{RF}$, HIV-1$_{HE}$, HIV-1$_{JR\text{-}FL}$, and A012D) and two strains of HIV-2 (LAV-2$_{ROD}$ and LAV-2$_{EHO}$) were used in the anti-HIV assays. HIV-1$_{HE}$ is a clinical isolate from a Belgian patient with AIDS (23), and A012D is an AZT-resistant HIV-1 variant (7, 8). HIV-1$_{JR\text{-}FL}$ is a PBL- and MP-tropic strain (24). Except for HIV-1$_{JR\text{-}FL}$, these viruses were obtained from the culture supernatants from MOLT-4 or CEM cells persistently infected with virus. Titers of HIV stocks were determined in MT-4 cells, and virus stocks were stored at −80° until use.

Antiviral assays. Activity of the compounds against the replication of HIV-1 (HTLV-III$_B$, HTLV-III$_{RF}$, and HIV-1$_{HE}$) and HIV-2 (LAV-2$_{LOD}$ and LAV-2$_{EHO}$) was based on the inhibition of virus-induced cytopathogenicity in MT-4 or MOLT-4 cells. Briefly, MT-4 or MOLT-4 cells were suspended in culture medium at 10^5 cells/ml and infected with HIV at a multiplicity of infection of 0.02 or 0.2, respectively. Immediately after virus infection, the cell suspension (100 μl) was brought into each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. After a 4-day incubation at 37°, MOLT-4 cells were subcultured at a ratio of 1:5 with fresh culture medium, containing appropriate concentrations of the test compounds, and further incubated. The number of viable MT-4 and MOLT-4 cells was determined by the MTT method on days 4 and 7 after virus infection, respectively (25). Activity of the compounds against the AZT-resistant HIV-1 variant (A012D) was based on the amount of HIV-1 p24 antigen in the culture supernatant and was determined by a sandwich enzyme-linked immunosorbent assay kit (Abbott) on day 4 after infection of MT-4 cells.



E-HEPU-dM X= O, Y= S, R= CH_2OH
E-EPU-dM X= O, Y= S, R= CH_3
E-BPU-dM X= O, Y= S, R= Ph
E-HEPU-SdM X= S, Y= S, R= CH_2OH
E-EPU-SdM X= S, Y= S, R= CH_3
E-BPU-SdM X= S, Y= S, R= Ph
E-HEBU-dM X= O, Y= CH_2, R= CH_2OH
E-EBU-dM X= O, Y= CH_2, R= CH_3

Fig. 1. Structural formulae of novel HEPT derivatives.

The assay procedure for measuring the anti-HIV-1 activity of the compounds in PBL and MP was also based on the quantitative detection of HIV-1 p24 antigen in the culture supernatant. Phytohemagglutinin-stimulated PBL (10^6/ml) were infected with HIV-1 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° 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. MP (10^5/ml) were cultured in a 24-well tray for 5 days and infected with HIV-1$_{JR\text{-}FL}$ (1 ng of p 24). After a 2-day incubation period, the cells were washed and cultured in the absence or presence of the test compounds. MP were fed with fresh culture medium every 5 days. The assay was performed on day 7 after virus infection for PBL and on day 15 for MP.

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

RT assay. The effect of the compounds on HIV RT activity was evaluated with four enzymes from different sources, i.e., two HIV-1 rRT samples obtained from NIAID and MicroGeneSys, HIV-1 nRT, and HIV-2 nRT. The NIAID HIV-1 rRT was produced in *Escherichia coli* and consisted of p66 (46.5%), p55 (32.3%), and p53 (21.2%). Specific activity of the enzyme was 2 units/μg.[2] The MicroGeneSys HIV-1 rRT (p66) was produced in an insect cell/baculovirus expression system. Its specific activity was 1 unit/μg. 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 assays were performed at 37° 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, 0.01 absorbance unit of either poly(A)·oligo(dT)$_{12\text{-}18}$ (16.2 absorbance units/mg; Pharmacia) or poly(C)·oligo(dG)$^{12\text{-}18}$ (15.0 absorbance units/mg; Pharmacia), either 0.43 μM [*methyl*-^{3}H] dTTP (46 Ci/mmol; Amersham) or 0.48 μM [1′,2′-^{3}H]dGTP (42 Ci/mmol; Amersham), test compound, and enzyme (approximately 0.01

TABLE 1

Inhibitory effect of HEPT derivatives on HIV-1 (HTLV-III$_B$) replication in MT-4 cells

All data represent mean values ± standard deviations for at least three separate experiments.

Compound	EC$_{50}$[a]	CC$_{50}$[b]	SI[c]
	μM	μM	
E-HEPU-dM	0.016 ± 0.0021	155 ± 27	9,700
E-EPU-dM	0.0062 ± 0.0008	>100[d]	>16,100
E-BPU-dM	0.0024 ± 0.0006	>20[d]	>8,300
E-HEPU-SdM	0.0075 ± 0.0005	172 ± 75	23,000
E-EPU-SdM	0.0056 ± 0.0020	>100[d]	>17,800
E-BPU-SdM	0.0076 ± 0.0025	>20[d]	>2,600
E-HEBU-dM	0.015 ± 0.002	256 ± 22	17,100
E-EBU-dM	0.0022 ± 0.0005	249 ± 48	113,000
HEPT[e]	6.5 ± 1.1	>500	77
AZT[e]	0.0030 ± 0.0010	7.8 ± 1.0	2,600
D4T[e]	0.034 ± 0.016	15 ± 4.0	440

[a] Concentration required to inhibit HIV-1-induced cytopathogenicity in MT-4 cells by 50%.
[b] Concentration required to reduce the viability of mock-infected MT-4 cells by 50%.
[c] Selectivity index, ratio of CC$_{50}$ to EC$_{50}$.
[d] Higher concentrations could not be achieved because of crystallization of the compound in the culture medium.
[e] Data taken from Ref. 26.

[1] Manuscript in preparation.

[2] AIDS Division, NIAID, unpublished data.

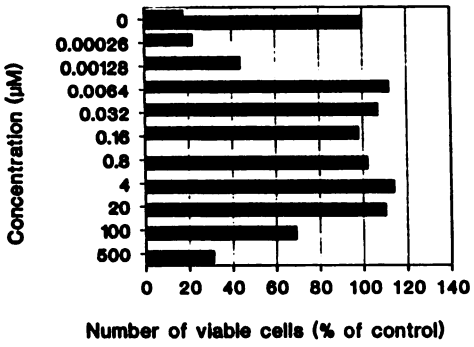


Fig. 2. Inhibitory effect of E-EBU-dM on HIV-1-induced cytopathogenicity in MT-4 cells. MT-4 cells were infected with HIV-1 (HTLV-III_B) and incubated in the presence of various concentrations of the compound for 4 days. The viability of virus-infected cells (■) and mock-infected cells (▨) was assessed by the MTT method. The number of cells is expressed as percentage of the mock-infected control.

unit of HIV-1 rRT, 0.004 unit of HIV-1 nRT, or 0.004 unit of HIV-2 nRT). The reaction was stopped with 200 μl of 5% trichloroacetic acid, and the precipitated materials were analyzed for radioactivity.

Results

Antiviral activity. Among the various new HEPT derivatives that had been synthesized, the 5-ethyl-6-(3,5-dimethylphenylthio)uracil and 5-ethyl-6-(3,5-dimethylbenzyl)uracil analogues (Fig. 1) proved to be highly potent and selective inhibitors of HIV-1 replication. These compounds inhibited the cytopathogenicity of HIV-1 (HTLV-III_B) in MT-4 cells at a concentration ranging from 2 to 16 nM (Table 1). E-BPU-dM and E-EBU-dM were the most active, with their EC_{50} being 2.4 and 2.2 nM, respectively (Table 1). These values are approximately 2700 and 3000 times lower than the EC_{50} of the parent compound HEPT (6.5 μM).

Viability of the HIV-1-infected or mock-infected MT-4 cells after exposure to varying concentrations of E-EBU-dM is presented in Fig. 2. E-EBU-dM completely protected the cells against virus-induced cell destruction at a concentration of 6.4 nM. The compound did not markedly reduce the viability of mock-infected MT-4 cells at concentrations up to 100 μM. Even at 500 μM, MT-4 cells survived, although cell proliferation was inhibited at this concentration (Fig. 2). The CC_{50} of E-EBU-dM for mock-infected MT-4 cells was 249 μM (Table 1). Thus, the selectivity index of E-EBU-dM, based on the CC_{50} to EC_{50} ratio, was 113,000.

In the next set of experiments, E-EPU-dM, E-EPU-SdM, and E-EBU-dM were examined for their inhibitory effects on the replication of HIV-1 (HTLV-III_B) in several cell systems. AZT was included as a reference compound. The compounds proved to be highly effective against HIV-1 (HTLV-III_B), irrespective of the cell system used (MT-4, MOLT-4, or PBL). In fact, the greatest effect (lowest EC_{50}) was recorded in PBL (Table 2). The lowest EC_{50} (0.45 nM) was noted with E-EBU-dM in PBL. In addition to the HTLV-III_B, other HIV-1 strains, including a clinical isolate (HIV-1_{HE}) and an AZT-resistant variant (A012D), were also highly susceptible to the inhibitory effects of E-EPU-dM, E-EPU-SdM, and E-EPU-dM (Table 2). The susceptibility of A012D to AZT was at least 100-fold lower than that of AZT-sensitive strains. However, E-EPU-dM, E-EPU-SdM, and E-EBU-dM inhibited the replication of A012D with an EC_{50} similar to that for the other HIV-1 strains (Table 2). Furthermore, E-EBU-dM proved to be markedly inhibitory to the replication of HIV-1 (HIV-$1_{JR\text{-}FL}$) in MP (EC_{50}, 0.74 nM) (Table 2). As noted previously for HEPT (11), the new HEPT congeners did not inhibit HIV-2 replication. In contrast, AZT was equally inhibitory to the replication of HIV-1 and HIV-2 (Table 2).

Inhibition of RT. Our previous studies with HEPT suggested that it interacts with the reverse transcription process without being converted to its triphosphate (11).[3] In fact, E-EBU-dM was found to be a potent inhibitor of HIV-1 RT, irrespective of the source of the enzyme (Table 3). The compound was more inhibitory to HIV-1 RT with poly(C)·oligo(dG) as template-primer and dGTP as substrate than when poly(A)·oligo(dT) and dTTP were used as the template-primer and substrate, respectively. E-EBU-dM did not affect the activity of HIV-2 RT at concentrations up to 500 μM, regardless of the template-primer/substrate system used (Table 3). In contrast, AZT-TP was equally inhibitory to HIV-1 RT and HIV-2 RT.

The inhibition of HIV-1 rRT (obtained from National Institute of Allergy and Infectious Diseases) by E-EBU-dM was further analyzed with varying substrate and compound concentrations. Double-reciprocal plots revealed that inhibition of the enzyme by the compound is competitive with respect to dTTP [with poly(A)·oligo(dT) as template-primer] and noncompetitive with respect to dGTP [with poly(C)·oligo(dG) as template-primer] (Fig. 3). The K_m of HIV-1 rRT for dTTP and dGTP was 27 and 7.7 μM, respectively. The K_i of the enzyme for E-EBU-dM with dTTP as substrate was 0.42 μM.

Discussion

In a previous study on the structure-activity relationship of the HEPT derivatives, we found that HEPT-M was slightly (2.5-fold) more active than its parent compound HEPT (26). We intended, therefore, to synthesize the dimethyl analogue HEPT-dM. This compound proved to be 30-fold more inhibitory to HIV-1 replication than HEPT (data not shown). We also found that substitution of the methyl group at C-5 of the uracil ring by an ethyl group and substitution of the 2-(hydroxyethoxy)methyl group at the N-1 position by an ethoxymethyl group markedly increased the anti-HIV-1 activity (27).

Based on these observations, we have synthesized E-HEPU-dM, E-EPU-dM, and E-BPU-dM (Fig. 1) and examined the compounds for their anti-HIV-1 activity. These derivatives, and in particular E-EPU-dM and E-BPU-dM, exhibited remarkable anti-HIV-1 activity, with EC_{50} values below 10 nM (Table 1). As previously demonstrated with HEPT-S (26), the 2-thio analogues E-HEPU-SdM, E-EPU-SdM, and E-BPU-SdM were equally inhibitory to HIV-1 replication as their 2-keto counterparts (Table 1). Substitution of the dimethylphenylthio group at the C-6 position of E-EPU-dM by a dimethylbenzyl group further increased the anti-HIV-1 activity, such that a selectivity index of more than 100,000 was achieved (Table 1). The resulting product, E-EBU-dM, may be considered one of the most potent and selective inhibitors of HIV-1 that has been described so far.

It is noteworthy that even these exquisitely selective inhibitors of HIV-1 have no effect on the replication of HIV-2 (Table 2). Previously, we ascertained that HEPT does not interfere

[3] M. Baba, unpublished data.

TABLE 2

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

All data represent mean values for at least two separate experiments.

Compound	Virus	Strain	Cell	EC_{50}[a]	CC_{50}[b]
				μM	μM
E-EPU-dM	HIV-1	$HTLV\text{-}III_B$	MT-4	0.0062[c]	>100[c]
			MOLT-4	0.0034	>100
			PBL	0.0018	56
		$HTLV\text{-}III_{RF}$	MT-4	0.0045	
		$HIV\text{-}1_{HE}$	MT-4	0.016	
		A012D	MT-4	0.0027	
	HIV-2	$LAV\text{-}2_{ROD}$	MT-4	>100	
		$LAV\text{-}2_{EHO}$	MT-4	>100	
E-EPU-SdM	HIV-1	$HTLV\text{-}III_B$	MT-4	0.0056[c]	>100[c]
			MOLT-4	0.0031	>100
			PBL	0.0022	33
		$HTLV\text{-}III_{RF}$	MT-4	0.0026	
		$HIV\text{-}1_{HE}$	MT-4	0.011	
		A012D	MT-4	0.0027	
	HIV-2	$LAV\text{-}2_{ROD}$	MT-4	>100	
		$LAV\text{-}2_{EHO}$	MT-4	>100	
E-EBU-dM	HIV-1	$HTLV\text{-}III_B$	MT-4	0.0022[c]	249[c]
			MOLT-4	0.0012	168
			PBL	0.00045	73
		$HTLV\text{-}III_{RF}$	MT-4	0.0013	
		$HIV\text{-}1_{HE}$	MT-4	0.0076	
		A012D	MT-4	0.0012	
		$HIV\text{-}1_{JR\text{-}FL}$	MP	0.00074	>20
	HIV-2	$LAV\text{-}2_{ROD}$	MT-4	>249	
		$LAV\text{-}2_{EHO}$	MT-4	>249	
AZT	HIV-1	$HTLV\text{-}III_B$	MT-4	0.0030[d]	7.8[d]
			MOLT-4	0.0023	90
			PBL	0.0014	26
		$HTLV\text{-}III_{RF}$	MT-4	0.0020	
		$HIV\text{-}1_{HE}$	MT-4	0.0035	
		A012D	MT-4	0.33	
	HIV-2	$LAV\text{-}2_{ROD}$	MT-4	0.0028[d]	
		$LAV\text{-}2_{EHO}$	MT-4	0.0020	

[a] Concentration required to inhibit by 50% HIV-induced cytopathogenicity or p24 antigen production.
[b] Concentration required to reduce the viability of mock-infected cells by 50%.
[c] Data taken from Table 1.
[d] Data taken from Ref. 26.

TABLE 3

Inhibitory effect of E-EBU-dM and AZT-TP on HIV RT activity

All data represent mean values for at least two separate experiments.

Compound	Enzyme	Template-primer	Substrate	IC_{50}
				μM
E-EBU-dM	HIV-1 rRT (A)[a]	Poly(A)·oligo(dT)	dTTP	0.11
		Poly(C)·oligo(dG)	dGTP	0.044
	HIV-1 rRT (B)[a]	Poly(A)·oligo(dT)	dTTP	0.16
		Poly(C)·oligo(dG)	dGTP	0.036
	HIV-1 nRT	Poly(A)·oligo(dT)	dTTP	0.36
	HIV-2 nRT	Poly(A)·oligo(dT)	dTTP	>500
		Poly(C)·oligo(dG)	dGTP	>500
AZT-TP	HIV-1 rRT (A)[a]	Poly(A)·oligo(dT)	dTTP	0.0082
		Poly(C)·oligo(dG)	dGTP	>100
	HIV-1 rRT (B)[a]	Poly(A)·oligo(dT)	dTTP	0.0032
		Poly(C)·oligo(dG)	dGTP	>100
	HIV-1 nRT	Poly(A)·oligo(dT)	dTTP	0.0048
	HIV-2 nRT	Poly(A)·oligo(dT)	dTTP	0.0081
		Poly(C)·oligo(dG)	dGTP	>100

[a] HIV-1 rRTs were obtained from National Institute of Allergy and Infectious Diseases (A) and MicroGeneSys (B).

with an early event (i.e., adsorption, penetration, or uncoating) of the HIV-1 replicative cycle (11) and does not suppress virus production in MOLT-4 cells chronically infected with HIV-1 (26). HEPT itself, but not HEPT triphosphate, is inhibitory to HIV-1 RT (27). This suggests that HEPT interacts with the reverse transcription process without being converted to its triphosphate form. Also, E-EBU-dM, which has no hydroxyl group to be phosphorylated, is a potent inhibitor of HIV-1 RT (Table 3). In keeping with its inactivity against HIV-2 replication in cell culture, E-EBU-dM did not show any inhibition of HIV-2 RT (Table 3).

Analysis of the mode of inhibition of HIV-1 RT by E-EBU-

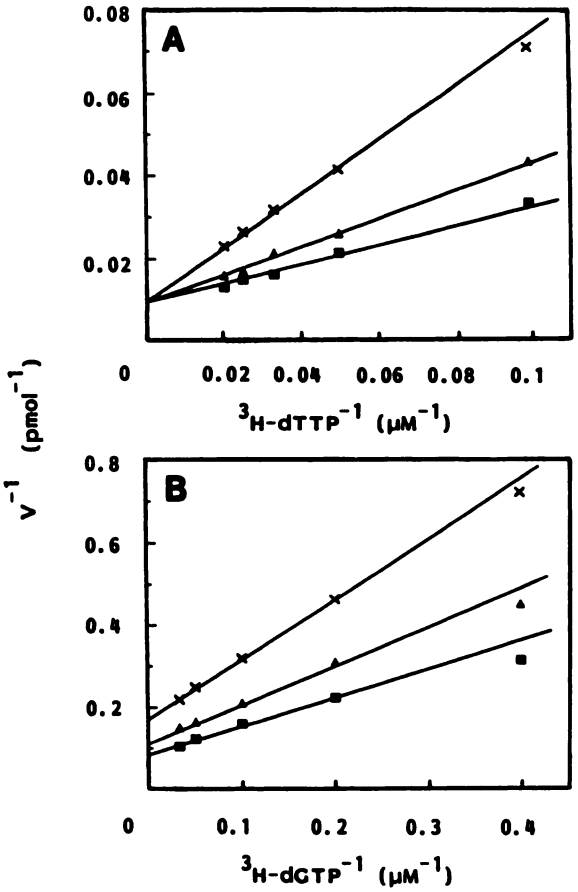


Fig. 3. Double-reciprocal plot analysis of inhibition of HIV-1 rRT by E-EBU-dM. Except for the incubation time and substrate concentrations, the assay conditions described in Materials and Methods were used. Reactions were performed for 15 min in a 50-μl reaction mixture containing 0.01 unit of the enzyme and either 0.01 unit of poly(A)·oligo(dT), varying concentrations of [^{3}H]dTTP (as indicated on the *x*-axis), and E-EBU-dM [0 (■), 0.2 (▲), or 0.4 (×) μM] (A) or 0.01 unit of poly(C)·oligo(dG), varying concentrations of [^{3}H]dGTP (as indicated on the *x*-axis), and E-EBU-dM [0 (■), 0.1 (▲), or 0.2 (×) μM] (B).

dM revealed that E-EBU-dM, like AZT-TP, inhibits the enzyme competitively with dTTP (Fig. 3). Unlike AZT-TP, however, E-EBU-dM is also inhibitory to HIV-1 RT in a noncompetitive fashion if dGTP is used as substrate (Table 3 and Fig. 3). E-EBU-dM may combine with different forms of HIV-1 RT, depending on the template-primer/substrate system used in the assay, as has been shown with AZT-TP and 3′-amino-dTTP (28). We do not know which mechanism, competitive or noncompetitive inhibition, is responsible for the inhibition of HIV-1 replication in cell cultures. Furthermore, E-EBU-dM may have another target than HIV-1 RT, because there is some discrepancy between the IC_{50} values of E-EBU-dM for HIV-1 RT and the EC_{50} values for HIV-1 replication in cell cultures (Tables 2 and 3). The exact mechanism of interaction between the HEPT derivatives and HIV-1 RT in an endogenous (heteropolymeric) template system remains to be clarified.

The HEPT derivatives behave remarkably similarly to the benzodiazepine (TIBO) derivatives, in that they are highly specific and potent inhibitors of HIV-1 but do not interfere with the replication of HIV-2 or other retroviruses (23). The TIBO derivatives are also assumed to interact with HIV-1 RT, and it is quite possible that the HEPT and TIBO derivatives share a similar mechanism of action. It would now seem imperative to elucidate in molecular terms how these apparently unrelated classes of anti-HIV-1 compounds interact with the RT.

Studies on pharmacokinetics, metabolic disposition, and toxicology are indispensable before the novel 6-substituted acyclouridine derivatives can be advocated for clinical use in the prophylaxis or therapy of AIDS (or AIDS-related complex). However, considering the severity of the AIDS pandemic and the urgent need for an effective therapy, the *in vitro* anti-HIV-1 potency and selectivity of the new HEPT derivatives (i.e., E-EBU-dM) make them particularly attractive for further development as candidate anti-AIDS drugs.

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