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Highly potent and selective inhibition of HIV-1 replication by 6-phenylthiouracil derivatives

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HIV-1 replication inhibition; 6-Phenylthiouracil derivative

Introduction

Since the discovery of human immunodeficiency virus type 1 (HIV-1) as the causative agent of the acquired immune deficiency syndrome (AIDS) (Barré-Sinoussi et al., 1983; Gallo et al., 1984), various compounds have been investigated for their chemotherapeutic potential (De Clercq, 1989; Mitsuya et al., 1990). Among these compounds, 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxynucleosides (ddNs) have proved highly potent and selective inhibitors of HIV-1 replication in vitro (Mitsuya et al., 1985; Mitsuya and Broder, 1986). Clinical studies have been initiated with some of the ddNs, i.e. DDC (2′,3′-dideoxycytidine) and DDI (2′,3′-dideoxyinosine). AZT has proven effective in improving the clinical symptoms and prolonging the survival of AIDS patients (Fischl et al., 1987, 1989, 1990). However, the long-term administration of AZT is often associated with its serious side effects such as bone marrow suppression (Richman et al., 1987).

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The ddNs are targeted at the viral RNA-dependent DNA polymerase (reverse transcriptase (RT)) following intracellular conversion, through cellular kinases, to their corresponding 5′-triphosphates. These triphosphate derivatives act as competitive inhibitors/substrates with respect to the natural substrates (dTTP, dCTP, dATP, and dGTP), and following their incorporation in the growing viral DNA chain they lead to chain termination (Furman et al., 1986; St. Clair et al., 1987; Cheng et al., 1987). Furthermore, AZT triphosphate (AZT-TP) and ddN triphosphates (ddN-TPs) exhibit a much higher affinity for the HIV-1 RT and other retroviral RTs than for the cellular DNA polymerases (Vrang et al., 1988; Ono et al., 1989; König et al., 1989). The selectivity of AZT and ddNs as anti-HIV-1 agents is primarily based on this differential affinity for RT and cellular DNA polymerases. Their nonspecific interaction with cellular DNA polymerases, in particular DNA polymerase γ, may contribute to the toxic side effects of this class of compounds.

In the meantime, world-wide efforts have made in search for effective chemotherapeutic agents against AIDS. One such approach is based on the use of HIV protease inhibitors (Meek et al., 1990; McQuade et al., 1990; Roberts et al., 1990; Erickson et al., 1990). In the late stage of the HIV replicative cycle, this virus-encoded enzyme is required for the processing of the *gag-pol* polyprotein to mature *pol* and *gag* proteins (Debouck et al., 1987; Kohl et al., 1988). In fact, a series of peptide derivatives based on the transition-state mimetic concept has been shown to inhibit not only HIV-1 protease activity in a cell-free system but also HIV-1 replication in cell cultures at the nanomolar range (Roberts et al., 1990).

Recently, the benzodiazepine (TIBO) derivatives (Pauwels et al., 1990) and a TIBO-like compound, BI-RG-587 (Merluzzi et al., 1990), have been discovered as new antiviral agents which exhibit a highly potent and specific inhibition of HIV-1. The TIBO compounds inhibit HIV-1 replication in vitro at nanomolar concentrations, whereas they are not inhibitory to HIV-2 at concentrations not toxic to the host cells. TIBO (Debyser et al., 1991) and BI-RG-587 (Merluzzi et al., 1990) have been shown to inhibit HIV-1 replication through an inhibitory effect on HIV-1 RT. Yet, their mode of interaction with RT clearly differs from that of AZT and ddNs.

In 1989, we described a 6-substituted acyclouridine derivative, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT), as a novel lead for specific anti-HIV-1 agents (Baba et al., 1989; Miyasaka et al., 1989). Like TIBO, HEPT is only inhibitory to HIV-1. Other viruses, including HIV-2, are insusceptible to this compound. Although HEPT and TIBO belong to apparently unrelated chemical classes, they share the unique biological property of being specifically inhibitory to HIV-1. Yet, as compared to the TIBO congeners, HEPT has relatively weak activity against HIV-1. Therefore, we have attempted at increasing its potency by introducing various chemical modifications. We have now developed several HEPT derivatives that inhibit HIV-1 replication at about the same (nanomolar) concentration as the TIBO derivatives. In this review, we will focus on the HEPT derivatives as promising candidate drugs for the treatment of HIV-1 infections.

Structure-activity relationship

The HEPT derivatives (with their abbreviations) are listed in Table 1, and their structural formulae are shown in Fig. 1.

The inhibitory effects of the compounds on HIV-1 replication were evaluated in a T4 lymphoblastoid cell line MT-4 (Miyoshi et al., 1982). MT-4 cells were infected with the prototype laboratory strain of HIV-1, and after a 4-day incubation period, the viability of both virus- and mock-infected cells was determined by the tetrazolium dye (MTT) method (Pauwels et al., 1988). This assay method has an advantage over the trypan blue exclusion method in that the antiviral activity of compounds can be monitored in parallel with their cytotoxicity.

Of the HEPT derivatives, HEPT itself was the first compound found to inhibit HIV-1 replication in vitro (Miyasaka et al., 1989; Baba et al., 1989).

TABLE 1

Abbreviations of compounds

Abbreviation	Compound
HEPU	1-[(2-Hydroxyethoxy)methyl]-6-(phenylthio)uracil
HEPT	1-[(2-Hydroxyethoxy)methyl]-6-(phenylthio)thymine
HEPT-M	1-[(2-Hydroxyethoxy)methyl]-6-[(3-methylphenyl)thio]thymine
HEPT-dM	1-[(2-Hydroxyethoxy)methyl]-6-[(3,5-dimethylphenyl)thio]thymine
EPT	1-Ethoxymethyl-6-(phenylthio)thymine
BPT	1-Benzyloxymethyl-6-(phenylthio)thymine
PPT	6-Phenylthio-1-propoxymethylthymine
E-HEPU	5-Ethyl-1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)uracil
E-EPU	1-Ethoxymethyl-5-ethyl-6-(phenylthio)uracil
E-BPU	1-Benzyloxymethyl-5-ethyl-6-(phenylthio)uracil
E-HEPU-dM	5-Ethyl-1-[(2-hydroxyethoxy)methyl]-6-[(3,5-dimethylphenyl)thio]uracil
E-EPU-dM	1-Ethoxymethyl-5-ethyl-6-[(3,5-dimethylphenyl)thio]uracil
E-BPU-dM	1-Benzyloxymethyl-5-ethyl-6-[(3,5-dimethylphenyl)thio]uracil
I-HEPU	1-[(2-Hydroxyethoxy)methyl]-5-isopropyl-6-(phenylthio)uracil
I-EPU	1-Ethoxymethyl-5-isopropyl-6-(phenylthio)uracil
I-BPU	1-Benzyloxymethyl-5-isopropyl-6-(phenylthio)uracil
I-HEPU-dM	1-[(2-Hydroxyethoxy)methyl]-5-isopropyl-6-[(3,5-dimethylphenyl)thio]uracil
P-HEPU	1-[(2-Hydroxyethoxy)methyl]-6-phenylthio-5-propyluracil
HEPT-S	1-[(2-Hydroxyethoxy)methyl]-6-phenylthio-2-thiothymine
E-HEPU-S	5-Ethyl-1-[(2-hydroxyethoxy)methyl]-6-phenylthio-2-thiouracil
E-EPU-S	1-Ethoxymethyl-5-ethyl-6-phenylthio-2-thiouracil
E-BPU-S	1-Benzyloxymethyl-5-ethyl-6-phenylthio-2-thiouracil
E-HEPU-SdM	5-Ethyl-1-[(2-hydroxyethoxy)methyl]-6-[(3,5-dimethylphenyl)thio]-2-thiouracil
E-EPU-SdM	1-Ethoxymethyl-5-ethyl-6-[(3,5-dimethylphenyl)thio]-2-thiouracil
E-BPU-SdM	1-Benzyloxymethyl-5-ethyl-6-[(3,5-dimethylphenyl)thio]-2-thiouracil
I-HEPU-S	1-[(2-Hydroxyethoxy)methyl]-5-isopropyl-6-phenylthio-2-thiouracil
I-EPU-S	1-Ethoxymethyl-5-isopropyl-6-phenylthio-2-thiouracil
I-BPU-S	1-Benzyloxymethyl-5-isopropyl-6-phenylthio-2-thiouracil
I-HEPU-SdM	1-[(2-Hydroxyethoxy)methyl]-5-isopropyl-6-[(3,5-dimethylphenyl)thio]-2-thiouracil
AZT	3′-Azido-3′-deoxythymidine
D4T	2′,3′-Didehydro-3′-deoxythymidine
DDA	2′,3′-Dideoxyadenosine

TABLE 2

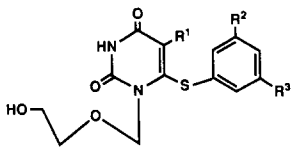
Inhibitory effect of HEPT derivatives on HIV-1 replication in MT-4 cells

Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
HEPU	> 500	> 500	< > 1
HEPT	6.5	> 500	> 77
HEPT-M	2.6	420	162
HEPT-dM	0.26	241	927
EPT	0.33	230	697
BPT	0.093	63	677
PPT	3.0	140	47
E-HEPU	0.12	400	333
E-EPU	0.022	146	6640
E-BPU	0.0049	30	6120
E-HEPU-dM	0.016	155	9700
E-EPU-dM	0.0062	> 100[d]	> 16100
E-BPU-dM	0.0024	> 20[d]	> 8300
I-HEPU	0.072	226	3140
I-EPU	0.0082	99	12000
I-BPU	0.0034	> 20[d]	> 5880
I-HEPU-dM	0.0033	102	30900
P-HEPU	3.6	200	56
HEPT-S	1.6	124	78
E-HEPU-S	0.13	130	1000
E-EPU-S	0.026	66	2540
E-BPU-S	0.0084	> 100[d]	> 11900
E-HEPU-SdM	0.0075	172	23000
E-EPU-SdM	0.0056	> 100[d]	> 17800
E-BPU-SdM	0.0076	> 20[d]	> 2600
I-HEPU-S	0.062	310	5000
I-EPU-S	0.012	> 100[d]	> 8330
I-BPU-S	0.0077	> 20[d]	> 2600
I-HEPU-SdM	0.0048	50	10400
AZT	0.0030	7.8	2600
D4T	0.034	15	441
DDA	6.8	> 500	> 74

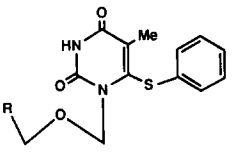
[a]50% Effective concentration, or concentration required to inhibit HIV-1-induced cytopathogenicity in MT-4 cells by 50%; [b]50% cytotoxic concentration, or concentration required to reduce the viability of mock-infected MT-4 cells by 50%; [c]selectivity index, or ratio of CC_{50} to EC_{50}; [d]higher concentrations could not be achieved because of crystallization of the compound in the culture medium. (Data from Baba et al., 1990, 1991a,b.)

HEPT completely protects MT-4 cells against HIV-1-induced destruction at a concentration of 50 μM (Fig. 2). Its 50% effective concentration (EC_{50}) is 6.5 μM (Table 2). HEPT is not toxic to mock-infected MT-4 cells at a concentration of 250 μM (Fig. 2). Thus, HEPT can be considered as a selective inhibitor of HIV-1 replication in vitro. While inhibitory to HIV-1, HEPT does not affect HIV-2 replication even at the highest concentration examined (250 μM) (Fig. 2).

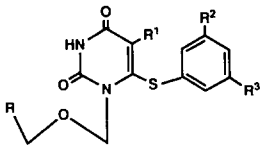
Following the HEPT lead we synthesized the three methylphenylthio analogues of HEPT (*ortho*-methyl, *meta*-methyl, and *para*-methyl HEPT). Of this series, *meta*-methyl HEPT (termed HEPT-M) proved to be the most active



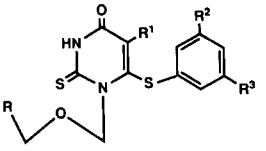
HEPU R^1= H, R^2= R^3= H
HEPT R^1= Me, R^2= R^3= H
HEPT-M R^1= R^2= Me, R^3= H
HEPT-dM R^1= R^2= R^3= Me



EPT R= Me
BPT R= Ph
PPT R= Et



E-HEPU R= CH_2OH, R^1= Et, R^2= R^3= H
E-EPU R= Me, R^1= Et, R^2= R^3= H
E-BPU R= Ph, R^1= Et, R^2= R^3= H
E-HEPU-dM R= CH_2OH, R^1= Et, R^2= R^3= Me
E-EPU-dM R= Me, R^1= Et, R^2= R^3= Me
E-BPU-dM R= Ph, R^1= Et, R^2= R^3= Me
I-HEPU R= CH_2OH, R^1= *i*-Pr, R^2= R^3= H
I-EPU R= Me, R^1= *i*-Pr, R^2= R^3= H
I-BPU R= Ph, R^1= *i*-Pr, R^2= R^3= H
I-HEPU-dM R= CH_2OH, R^1= *i*-Pr, R^2= R^3= Me
P-HEPU R= CH_2OH, R^1= Pr, R^2= R^3= H



HEPT-S R= CH_2OH, R^1= Me, R^2= R^3= H
E-HEPU-S R= CH_2OH, R^1= Et, R^2= R^3= H
E-EPU-S R= Me, R^1= Et, R^2= R^3= H
E-BPU-S R= Ph, R^1= Et, R^2= R^3= H
E-HEPU-SdM R= CH_2OH, R^1= Et, R^2= R^3= Me
E-EPU-SdM R= Me, R^1= Et, R^2= R^3= Me
E-BPU-SdM R= Ph, R^1= Et, R^2= R^3= Me
I-HEPU-S R= CH_2OH, R^1= *i*-Pr, R^2= R^3= H
I-EPU-S R= Me, R^1= *i*-Pr, R^2= R^3= H
I-BPU-S R= Ph, R^1= *i*-Pr, R^2= R^3= H
I-HEPU-SdM R= CH_2OH, R^1= *i*-Pr, R^2= R^3= Me

Fig. 1. Structural formulae of HEPT derivatives.

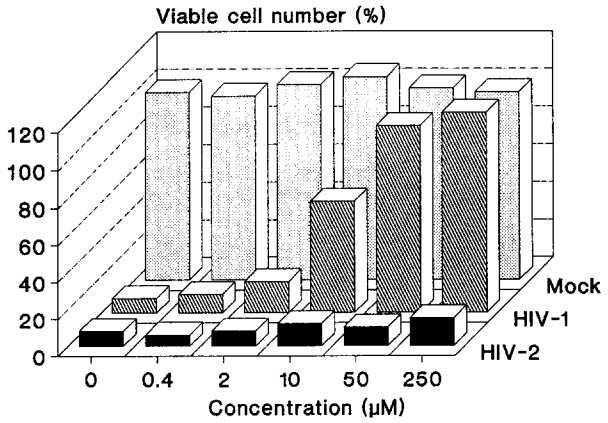


Fig. 2. Inhibitory effect of HEPT on HIV-induced cytopathogenicity in MT-4 cells. MT-4 cells were suspended in culture medium at 1 × 10^5 cells per ml and infected with either HIV-1 ($HTLV\text{-}III_B$) or HIV-2 ($LAV\text{-}2_{ROD}$) at a multiplicity of infection of 0.02. Immediately after virus infection, the cell suspension (100 μl) was added to each well of a microtiter tray containing various concentrations of the test compounds. After a 4-day incubation at 37°C, the number of viable cells was determined by the MTT method (Pauwels et al., 1988).

congener (data not shown); HEPT-M was slightly more potent than the parent compound HEPT (Table 2). The 2-thio analogue of HEPT, termed HEPT-S, was also synthesized and found to be 4-fold more active than HEPT (Table 2).

We also found that substitution of the 2-hydroxyethoxymethyl group at the N-1 position by an ethoxymethyl group markedly increased the anti-HIV-1 activity. In fact, EPT is approximately 20-fold more potent than HEPT (Table 2). With a benzyloxymethyl group as the N-1 side chain, the anti-HIV-1 activity is further increased; yet, BPT is slightly cytotoxic (Table 2).

Another important finding was that substitution of the methyl group at C-5 of the uracil ring by either ethyl or isopropyl (but not *n*-propyl) brought about a marked increase in anti-HIV-1 activity (Table 2). Thus, E-HEPU and I-HEPU are 54- and 92-fold more potent than HEPT.

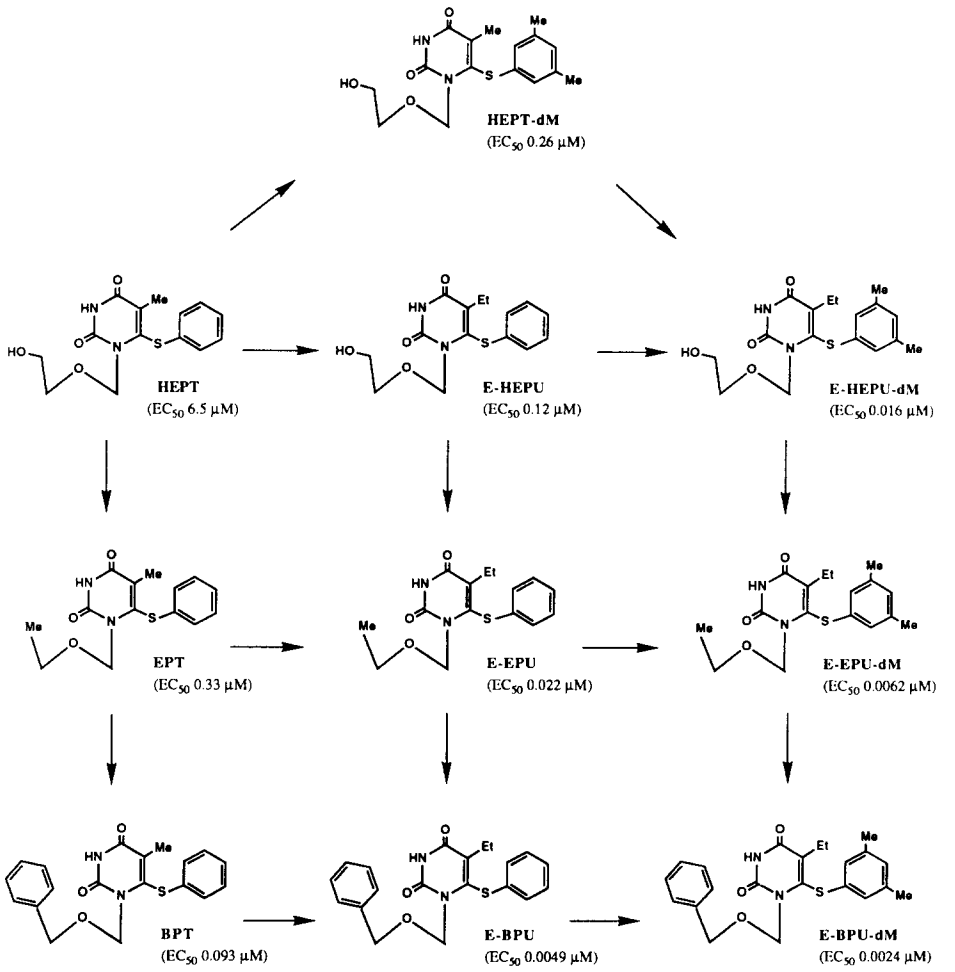


Fig. 3. Structure-activity relationship within the class of HEPT derivatives: chemical modifications leading to increased anti-HIV-1 potency.

When the two modifications, ethoxymethyl or benzyloxymethyl at the N-1 side chain and ethyl or isopropyl at the C-5 position were introduced in the same molecule, the resulting derivatives, E-EPU, E-BPU, I-EPU and I-BPU, achieved remarkable anti-HIV-1 potency, with EC_{50} values below 10 nM (Table 2).

After we had noted that the 3,5-dimethylphenylthio analogue of HEPT (HEPT-dM) was 25-fold more active than HEPT (Table 2), we synthesized the 3,5-dimethylphenylthio analogues of E-HEPU, E-EPU, E-BPU, and I-HEPU. E-HEPU-dM, E-EPU-dM, E-BPU-dM, and I-HEPU-dM proved much more inhibitory to HIV-1 than their counterparts E-HEPU, E-EPU, E-BPU and I-HEPU (Table 2). Furthermore, as originally demonstrated with HEPT-S, the 2-thio analogues of HEPT derivatives are equally inhibitory to HIV-1 replication as the 2-keto analogues (Table 2).

Of the 29 HEPT derivatives listed in Table 1, seven compounds inhibit HIV-1 replication at a concentration that was more than 10 000-fold below the cytotoxicity threshold (selectivity index > 10 000). For nineteen compounds, a selectivity index is greater than 1000 (Table 2).

The structure-activity relationship of the HEPT derivatives is schematically presented in Fig. 3, where the anti-HIV-1 activity increases in the direction of arrows.

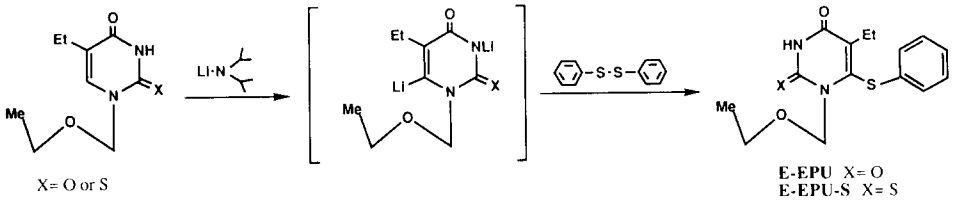
Chemistry

It is well-known that condensation of 6-substituted pyrimidines and appropriately protected sugar derivatives almost always results in the predominant formation of N-3-glycosylated products. Although part of this problem could be solved by nucleophilic substitution at the C-6 position of naturally occurring pyrimidine nucleosides, only few 6-substituted derivatives have been synthesized.

In 1982 Tanaka et al. developed a new method, based on the regiospecific deprotonation of H-6 of uridine with lithium diisopropylamide (LDA). The resulting C-6-lithiated species proved to react with a wide range of electrophiles to provide a general entry to 6-substituted uridines. Most of these 6-substituted uridines were difficult to synthesize by any other method. Later on, this method was successfully applied to 5-substituted uridines and 2′-deoxyuridine (Tanaka et al., 1983a, 1985a). Among the compounds synthesized, 6-iodo- and 6-phenylthiouridines exhibited anti-leukemic activity in cell cultures (Tanaka et al., 1983a).

Since the 6-iodo and 6-phenylthio substituents have a leaving ability, these compounds were highly susceptible to nucleophilic addition-elimination reactions at the C-6 position (Tanaka et al., 1983b, 1985b). Should this chemical property be related to the observed biological activity, some other biological activities might be expected for other uracil nucleoside analogues containing these substituents. In this perspective, the synthesis of the 6-iodo and 6-phenylthio derivatives of 1-[(2-hydroxyethoxy)methyl]uracils was under-

taken. Hence, HEPT and with HEPT a new class of specific anti-HIV-1 agents was born (Miyasaka et al., 1989).



Scheme 1. The synthesis of E-EPU and E-EPU-S.

As described in our recent publications (Tanaka et al., 1991a, 1991b), various C-6-modified HEPT and HEPT-S analogues can be obtained simply by reacting of the corresponding C-6-lithiated species with electrophiles, as illustrated in Scheme 1 for the synthesis of E-EPU and E-EPU-S. Another important feature of this tactic lies in the fact that even a compound bearing an halogen atom or azido group, either in the base moiety or acyclic side chain, could be used as a substrate (Tanaka et al., 1991c). If an appropriate electrophile is not available, the addition-elimination reaction using HEPT or its 6-phenylsulfinyl derivative provides an alternative approach (Tanaka et al., 1983b, 1985b). To introduce C-5 substituents in HEPU, again a lithiation chemistry is applicable. Here, the use of a more basic lithiating agent, lithium 2,2,6,6-tetramethylpiperidine (LTMP), is required (Tanaka et al., 1986).

Antiviral activity spectrum

Immediately after the discovery of its anti-HIV-1 activity, HEPT was examined for its inhibitory effect on the replication of various viruses in various cell systems. HEPT proved inhibitory to HIV-1 in HUT-78 cells (Levy et al., 1984), CEM cells (Foley et al., 1965), MOLT-4 cells (Kikukawa et al., 1986), peripheral blood lymphocyte (PBL) cells and monocyte-macrophage (MP) cells (Baba et al., 1989). Although HEPT is equally inhibitory to several strains of HIV-1, it does not inhibit HIV-2-induced cytopathogenicity in MT-4 cells, irrespective of the HIV-2 strain used. Furthermore, HEPT is inactive against other retroviruses including simian immunodeficiency virus (SIV, strain MAC), simian AIDS-related virus (SRV), and murine Moloney sarcoma virus (MSV) (Baba et al., 1989). Likewise, viruses other than retroviruses (i.e. herpes simplex virus, vaccinia virus, vesicular stomatitis virus, Coxsackie virus type B4, poliovirus type 1, parainfluenza virus type 3, reovirus type 1, Sindbis virus, Semliki forest virus, and human hepatitis virus B) are insensitive to HEPT (Baba et al., 1989, and unpublished data).

Two prototype HEPT derivatives, E-EPU and B-BPU, have also been examined for their inhibitory effects on the replication of different HIV-1 and HIV-2 strains. As shown in Table 3, E-EPU and E-BPU are highly potent inhibitors of HIV-1 replication in MOLT-4 cells and PBL cells, and their EC_{50}

TABLE 3

Inhibitory effect of E-EPU and E-BPU on the replication of HIV-1 and HIV-2 in various cell cultures

Virus	Strain	Cell	E-EPU		E-BPU		AZT	
			EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
HIV-1	HTLV-III_B	MT-4	0.022	146	0.0049	30	0.0030	7.8
		MOLT-4	0.029	127	0.0039	42	0.0041	232
		PBL	0.032	63	0.0072	22	0.0020	54
	HTLV-III_{RF}	MT-4	0.012	–	0.0016	–	0.0028	–
	A012B	MT-4	0.013	–	0.0019	–	0.0030	–
	A012D[a]	MT-4	0.015	–	0.0029	–	0.30	–
	A018A	MT-4	0.027	–	0.0095	–	0.0027	–
	A018C[a]	MT-4	0.032	–	0.010	–	0.40	–
	HIV-$1_{JR\text{-}FL}$[b]	MP	0.013	>20	ND[c]	–	ND[c]	–
HIV-2	LAV-2_{ROD}	MT-4	>146	–	>30	–	0.0028	–
	LAV-2_{EHO}	MT-4	>146	–	>30	–	0.0032	–

Assays of the compounds for HIV-1 (HTLV-III_B and HTLV-III_{RF}) and HIV-2 (LAV-2_{ROD} and LAV-2_{EHO}) replication were performed under the same experimental conditions as described in the legend to Fig. 2. The anti-HIV-1 assay in MOLT-4 cells was performed under similar conditions as described in the footnote to Table 2, except for a 10-fold higher multiplicity of infection and a longer incubation period (8 days). 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, on day 4 after infection of MT-4 cells. The assay procedure for measuring the anti-HIV-1 activity of the compounds in PBL and MP cultures was also based on the quantitative detection of HIV-1 p24 antigen in the culture supernatant. The assays were performed on day 7 in PBL and day 15 in MP. Cytotoxicity of the compounds was evaluated in parallel with their antiviral activity. EC_{50} is based on the inhibition of HIV-1-induced cytopathogenicity or reduction of p24 antigen content in culture supernatant. CC_{50} is based on the reduction of viability of mock-infected cells.

[a]AZT-resistant HIV-1 strains (Larder et al., 1989); [b]PBL- and MP-tropic strain (Koyanagi et al., 1988); [c]Not determined. (Data from Baba et al., 1991a.)

values in PBL cells are quite similar to those obtained in MT-4 cells. However, as noted for HEPT (Baba et al., 1989), E-EPU and E-BPU are not inhibitory to HIV-2-induced cytopathogenicity in MT-4 cells. In contrast, the reference compound AZT is equally inhibitory to HIV-1 and HIV-2. Furthermore, E-EPU is markedly inhibitory to HIV-1 in human monocyte/macrophages. In view of their potential therapeutic use, it would seem important to know whether E-EPU and E-BPU are also inhibitory to AZT-resistant mutants of HIV-1. In our assay system AZT-resistant HIV-1 strains A012D and A018C (Larder et al., 1989) are at least 100-fold less susceptible to AZT than the AZT-sensitive HIV-1 strains A012B and A018A. However, the AZT-resistant HIV-1 strains are equally sensitive to E-EPU and E-BPU as the AZT-sensitive HIV-1 strains.

Mechanism of action

Our studies on the mechanism of action of HEPT have indicated that the

compound does not interfere with an early event (i.e. virus adsorption, penetration, or uncoating) of the HIV-1 replicative cycle. Moreover, HEPT does not suppress virus production in chronically HIV-1-infected MOLT-4 cells (Baba et al., unpublished data), which means that a late event of the virus replicative cycle (i.e. after integration of proviral DNA into host genomic DNA) may also be excluded as a target of the compound.

From 'time of addition' experiments, where HEPT or HEPT-S were added at different times after infection of MT-4 cells with HIV-1 at a high multiplicity of infection, it appeared that HEPT and HEPT-S were still fully inhibitory to HIV-1 replication when added to the cells 7 h after virus infection (Fig. 4). The same result was obtained for the TIBO derivative R 82150 (Fig. 4; see also Fig. 2a in Pauwels et al., 1990). For DDC, AZT and DDI, addition to the cells could be postponed until 4, 5 or 6 h post infection before their inhibitory potency started to decline (Fig. 4). The differences shown by the ddNs may arise from the time taken to phosphorylate these nucleoside analogues to their 5′-triphosphate form, a process that is unnecessary for the TIBO or HEPT derivatives. In marked contrast with the HEPT, TIBO and ddN derivatives, virus adsorption inhibitors such as pentosan polysulfate (Baba et al., 1988a) and dextran sulfate (Baba et al., 1988b) were no longer able to inhibit HIV-1 replication when added 2 h after infection (Fig. 4; see also Fig. 2a in Pauwels et al., 1990). From the 'time of addition' experiments, HEPT may be assumed to act at a stage of the HIV-1 replicative cycle that corresponds to the reverse transcription process.

When HEPT, HEPT 5′-triphosphate (HEPT-TP), E-EPU, and AZT-TP were analyzed for their inhibitory effects on purified HIV-1 recombinant RT (rRT) activity, HEPT and E-EPU were found inhibitory, irrespective of the template-primer/substrate (poly(A)·oligo(dT)/dTTP or poly(C)·oligo(dG)/dGTP) used (Table 4). Interestingly, double-reciprocal plots revealed that the inhibition of HIV-1 rRT by E-EPU was competitive with respect to dTTP but noncompetitive with respect to dGTP (Fig. 5). The K_m values of the HIV-1 rRT for dTTP and dGTP were 28 and 7.7 μM, respectively. The K_i value for E-EPU with dTTP as substrate was 0.42 μM. The compounds were also inhibitory to native HIV-1 RT (HIV-1 nRT). However, the activity of HIV-2 nRT was not affected by E-EPU at concentrations up to 500 μM. In contrast, AZT-TP was equally inhibitory to HIV-1 nRT and HIV-2 nRT (Table 4). Another interesting observation was that HEPT-TP (obtained through chemical synthesis) was totally inactive against HIV-1 RT. This suggests that HEPT does not need to be phosphorylated intracellularly to interact with the HIV-1 RT. In fact, E-EPU, which cannot be phosphorylated, is a potent inhibitor of HIV-1 RT (Table 4).

All HEPT derivatives that were found to be active against HIV-1 replication also proved inhibitory to HIV-1 rRT. A close correlation was observed between their EC_{50} values for inhibition of virus replication and their IC_{50} values for HIV-1 rRT activity (Fig. 6). However, the IC_{50} values for RT activity were approximately 10-fold higher than the EC_{50} values for inhibition of HIV-1

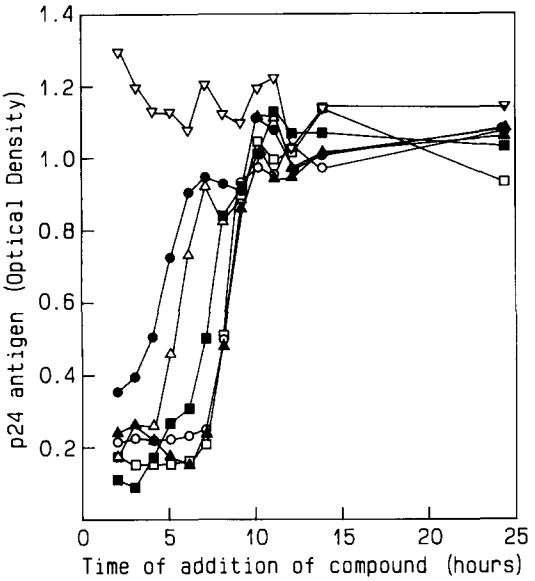


Fig. 4. Time of addition experiment. MT-4 cells were infected at a high multiplicity of infection (> 1) with HIV-1 (HTLV-III_B). After 60 min incubation at 37°C, unadsorbed virus was removed by 4 washing steps. Compounds were added at either 2,3,4,5,6,7,8,9,10,11,12,13 or 24 h after infection. HIV-1 core protein (p24) was determined at 25 h after infection by ELISA. Pentosan polysulfate (50 μg/ml): –▽–; DDC (5 μg/ml): –●–; AZT (0.05 μg/ml): –△–; DDI (100 μg/ml): –■–; R 82150 (0.5 μg/ml): –○–; HEPT (100 μg/ml): –□–; HEPT-S (10 μg/ml): –▲–.

TABLE 4

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

Compound	Enzyme	Template-primer/substrate	IC_{50}[a] (μM)
HEPT	HIV-1 rRT	poly(A)·oligo(dT)/dTTP	53
	HIV-1 rRT	poly(C)·oligo(dG)/dGTP	66
HEPT-TP	HIV-1 rRT	poly(A)·oligo(dT)/dTTP	> 500
E-EPU	HIV-1 rRT	poly(A)·oligo(dT)/dTTP	0.27
	HIV-1 rRT	poly(C)·oligo(dG)/dGTP	0.14
	HIV-1 nRT	poly(A)·oligo(dT)/dTTP	1.1
	HIV-2 nRT	poly(A)·oligo(dT)/dTTP	> 500
AZT-TP	HIV-1 rRT	poly(A)·oligo(dT)/dTTP	0.014
	HIV-1 rRT	poly(C)·oligo(dG)/dGTP	> 100
	HIV-1 nRT	poly(A)·oligo(dT)/dTTP	0.0060
	HIV-2 nRT	poly(A)·oligo(dT)/dTTP	0.0069

HIV-1 rRT was produced in *Escherichia coli* and composed of p66 (46.5%), p55 (32.3%), and p53 (32.3%) (Division of AIDS, NIAID). HIV-1 and HIV-2 nRTs were obtained from disrupted virions in the supernatants of MOLT-4 cells persistently infected with HTLV-III_B and LAV-2_{ROD} respectively. The 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) or [1′,2′-^{3}H]dGTP (42 Ci/mmol), 0.01 OD unit of either poly(A)·oligo(dT) or poly(C)·oligo(dG), test compound, and enzyme (approx. 0.01 U of HIV-1 rRT, or 0.004 U of HIV-1 nRT or HIV-2 nRT). The reaction was stopped with 200 μl of trichloroacetic acid (5%, v/v), and the precipitated materials were analyzed for radioactivity.

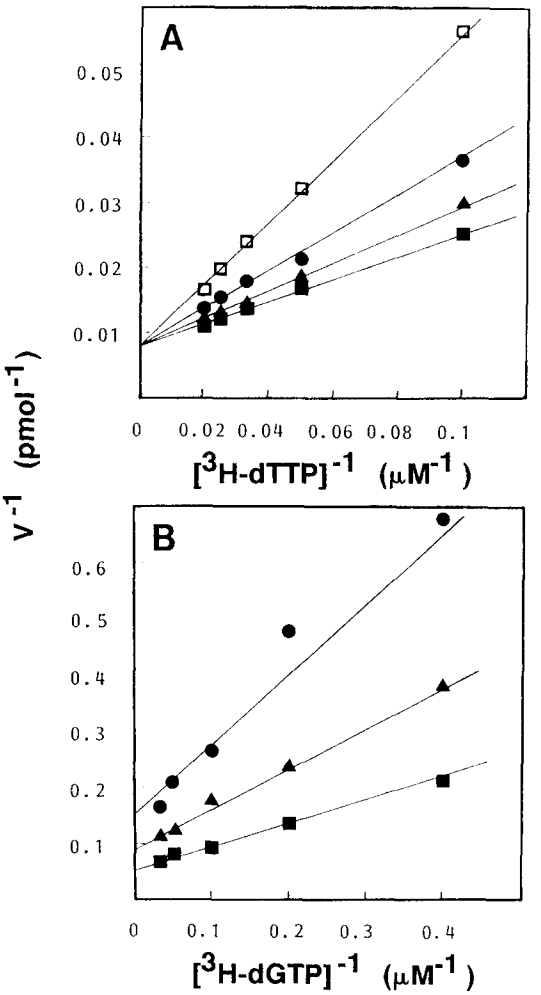


Fig. 5. Double-reciprocal plot analysis for inhibition of recombinant HIV-1 RT by E-EPU. Except for the substrate concentrations, the assay conditions were the same as described in the footnote to Table 4. The reaction was performed in 50 μl containing 0.01 U of HIV-1 rRT, 0.01 OD_{260} unit of either poly(A)·oligo(dT) or poly(C)·oligo(dG), 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. (Data from Baba et al., 1991a.)

replication. A possible explanation for the discrepancy between the IC_{50} and the EC_{50} is the use of artificial homopolymeric template-primers (poly(A)·oligo(dT) and poly(C)·oligo(dG)) in the RT assays. IC_{50} values for the HEPT derivatives in RT assays with endogenous (heteropolymeric) template(s) remain to be determined. Other factors that might play a role in the antiviral activity of the HEPT derivatives, such as penetration into the cells, intracellular metabolism, and possible interactions with viral proteins other than HIV-1 RT, remain also to be determined.

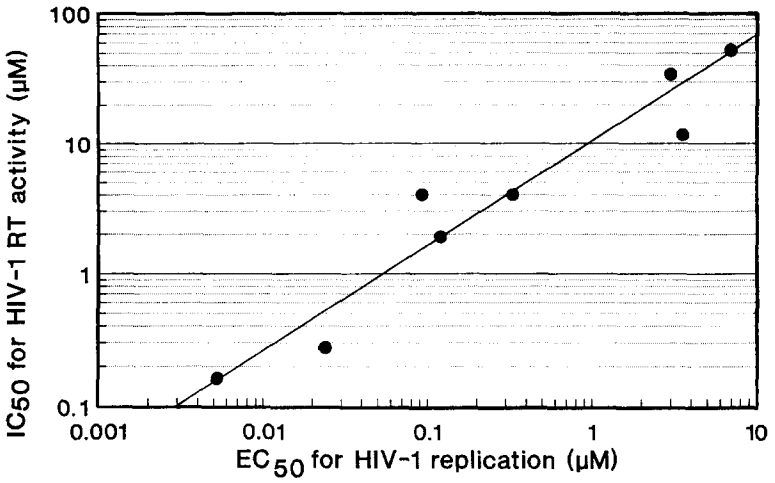


Fig. 6. Correlation between the inhibitory effects of a series of HEPT derivatives on HIV-1 replication in cell cultures and their inhibitory effects on HIV-1 rRT activity. The assay procedures for determination of the EC_{50} for HIV-1 replication in MT-4 cells and the IC_{50} for HIV-1 RT activity are described in the legend to Fig. 2 and footnote to Table 4, respectively. HEPT derivatives: HEPT, EPT, PPT, BPT, E-HEPU, E-EPU, E-BPU and P-HEPU. (Data from Baba et al., 1991a.)

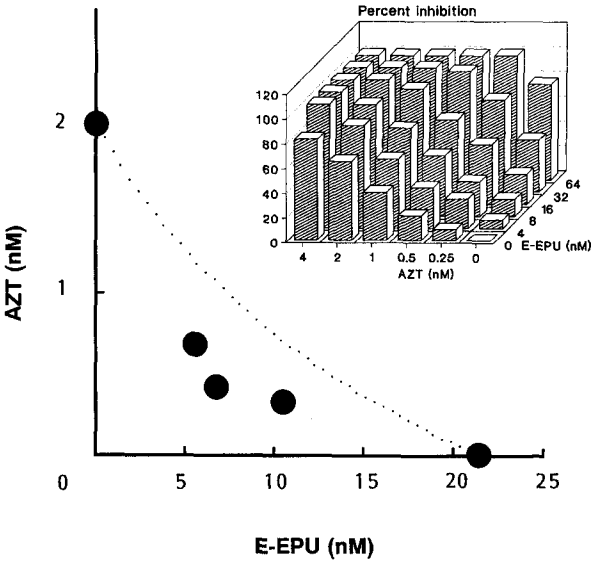


Fig. 7. Isobologram for the combined inhibitory effects of AZT and E-EPU on HIV-1 replication. Percent inhibition of HIV-1-induced cytopathogenicity in MT-4 cells at various concentrations of the drug combination are also illustrated by a 3-dimensional bar graph. Assays were performed under the same experimental conditions as described in the legend to Fig. 2. EC_{50} values were used as the endpoints for calculations. Dotted line represents the unity line for the combination indices equal to 1 (additive effect) under mutually nonexclusive assumptions. (Data from Baba et al., 1991c.)

Combination with other compounds

Combination chemotherapy is an attractive approach that may lead to synergistic activity without toxicity, and, in addition, may prevent the emergence of drug-resistant virus strains. In fact, combinations of various anti-HIV-1 agents have been examined for their inhibitory effects on HIV-1 replication in vitro and found to be synergistic (Hartshorn et al., 1986, 1987; Hammer and Gilles, 1987; Johnson et al., 1989). Combinations of antiviral agents with a different mode of action usually lead to increased activity. Therefore, we examined the combined inhibitory effect of human recombinant interferon α (IFN-α) and HEPT on HIV-1 replication. The combination exerted a synergistic antiviral activity in both MT-4 and PBL cells (Ito et al., 1991).

More recently, the combination of AZT and E-EPU was also examined and analyzed by the isobologram method. As shown in Fig. 7, the two compounds exert a synergistic activity against HIV-1. This combination might be effective in reducing the emergence of drug-resistant mutants, since E-EPU is equally inhibitory to AZT-resistant and AZT-sensitive strains of HIV-1 (Table 3).

Toxicity and pharmacology

Several issues including toxicity and pharmacology of the HEPT derivatives needed to be addressed before they could be further pursued in clinical studies. AZT is known to have myelotoxicity, and this hampers its long-term use in AIDS patients (Richman et al., 1987). A comparative test of AZT and the HEPT derivatives (i.e., E-EPU, E-BPU, E-EPU-S, and E-BPU-S) for their inhibitory effects on colony formation by murine bone marrow progenitor cells in vitro clearly demonstrated that the HEPT derivatives do not suppress the proliferation of murine bone marrow progenitor cells at concentrations up to 10 μM (Table 5). With AZT, however, approx. 50 and 95% inhibition of colony formation is observed at concentrations of 1 and 10 μM, respectively (Table 5). These results indicate that the HEPT derivative, unlike AZT, may not cause bone marrow suppression in vivo.

Acute toxicity of the HEPT derivatives in vivo is very low. Initial toxicological studies in rats have indicated that the 50% lethal doses of E-EPU, E-BPU, E-EPU-S and E-BPU-S after oral administration are higher than 2250 mg/kg. When these compounds were administered orally to rats for 2 weeks, no particular change of their general condition was noted even at a dose of 1000 mg/kg/day. Hematological examinations did not reveal changes in white or red blood cells at a dose of 1000 mg/kg/day, although E-EPU and E-EPU-S decreased the hemoglobin level at this dose. No alterations in the biochemical parameters of the serum were detected, except for a slight increase in the transaminase level following administration of E-EPU at 1000 mg/kg/day. Histological examinations indicated a slight swelling of liver cells following administration of E-EPU, E-BPU or E-EPU-S at 1000 mg/kg/day.

[a]50% Inhibitory concentration. (Data from Baba et al., 1991a.)

TABLE 5

Inhibitory effect of AZT and HEPT derivatives on colony formation of murine bone marrow progenitor cells

Compound	Concentration (μM)	Number of colonies[a]	%
Control		60.7 ± 6.7	100
AZT	10	2.7 ± 0.3	4.4
	1	33.3 ± 1.9	54.9
	0.1	55.3 ± 2.6	91.1
E-EPU	10	61.7 ± 1.2	102
	1	58.3 ± 4.2	96.0
	0.1	56.0 ± 3.1	92.3
E-BPU	10	65.7 ± 2.9	108
	1	61.0 ± 2.3	100
	0.1	59.0 ± 1.5	97.2
E-EPU-S	10	58.3 ± 5.8	96.0
	1	58.0 ± 2.0	95.6
	0.1	62.3 ± 3.5	103
E-BPU-S	10	59.7 ± 5.7	98.4
	1	56.7 ± 4.1	93.4
	0.1	63.0 ± 1.2	104

Murine bone marrow cells (2×10^5 per ml) were suspended in culture medium containing 10% fetal calf serum (FCS), 0.3% agar, and 50 ng/ml of granulocyte-macrophage colony stimulating factor (GM-CSF). The suspension (1 ml) was brought into each well of a plastic tray and overlayed with culture medium containing FCS, agar, GM-CSF, and various concentrations of the test compounds. After a 7-day incubation period at 37°C, the number of colonies was determined.

[a]Values are means ± standard deviations in triplicate experiments.

Yet these compounds did not cause necrosis of the liver cells, a side effect that was seen after administration of AZT at a dose of 500 mg/kg/day.

Pharmacokinetic studies were carried out in rats with HEPT, HEPT-M and HEPT-S given orally at a dose of 20 mg/kg. The plasma concentration of all compounds rose rapidly. The highest concentration in plasma (7.4 μg/ml, 22.8 μM) was achieved by HEPT-S within 30 min. The maximum plasma concentrations (C_{max}) of HEPT and HEPT-M were reached within 5 min following administration, but their C_{max} was considerably lower than that of HEPT-S (Baba et al., 1990). Pharmacokinetic studies were also carried out with E-EPU, E-EPU-S and E-BPU-S in beagle dogs after oral administration of the compounds at a dose of 5 mg/kg. Venous blood samples were collected at different times for up to 6 h after administration. The compounds were rapidly absorbed from the gastrointestinal tract and the highest plasma concentrations were attained within 35 min (E-EPU), 45 min (E-EPU-S) or 25 min (E-BPU-S). The C_{max} of E-EPU, E-EPU-S, and E-BPU-S was 304, 301, and 62 ng/ml, respectively. These values are approximately 45-, 36-, and 19-fold higher than the respective EC_{50} values for HIV-1 replication in MT-4 cells (Table 2).

Conclusion

In the search for novel chemotherapeutic agents effective against HIV infection, we found several 6-phenylthiouracil derivatives to be highly potent and selective inhibitors of HIV-1 in vitro. Of this series, seven compounds inhibited HIV-1 replication at concentrations that were at least 4 orders of magnitude below their cytotoxicity threshold. These compounds interact with HIV-1 reverse transcriptase following a mechanism that is different from that of AZT or the other 2′,3′-dideoxynucleoside analogues. The results of the toxicological tests in rats suggest that the 6-phenylthiouracil derivatives are devoid of acute and subacute toxicity. Plasma drug concentrations achieved following oral administration of the compounds to dogs were well above the virus-inhibitory concentrations. Thus, the 6-phenylthiouracil derivatives can be considered as promising candidate drugs for the treatment of HIV-1 infections.

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References

Baba, M., Nakajima, M., Schols, D., Pauwels, R., Balzarini, J. and De Clercq, E. (1988a) Pentosan polysulfate, a sulfated oligosaccharide, is a potent and selective anti-HIV agent in vitro. Antiviral Res. 9, 335–343.

Baba, M., Pauwels, R., Balzarini, J., Arnout, J., Desmyter, J. and De Clercq, E. (1988b) Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vitro. Proc. Natl. Acad. Sci. USA 85, 6132–6136.

Baba, M., Tanaka, H., De Clercq, E., Pauwels, R., Balzarini, J., Schols, D., Nakashima, H., Perno, C.-F., Walker, R.T. and Miyasaka, T. (1989) Highly specific inhibition of human immunodeficiency virus type 1 by a novel 6-substituted acyclouridine derivative. Biochem. Biophys. Res. Commun. 165, 1375–1381.

Baba, M., De Clercq, E., Iida, S., Tanaka, H., Nitta, I., Ubasawa, M., Takashima, H., Sekiya, K., Umezu, K., Nakashima, H., Shigeta, S., Walker, R.T. and Miyasaka, T. (1990) Anti-HIV-1 activity and pharmacokinetics of novel 6-substituted acyclouridine derivatives. Antimicrob. Agents Chemother. 34, 2358–2363.

Baba, M., De Clercq, E., Tanaka, H., Ubasawa, M., Takashima, H., Sekiya, K., Nitta, I., Umezu, K., Nakashima, H., Mori, S., Shigeta, S., Walker, R.T. and Miyasaka, T. (1991a) Potent and selective inhibition of human immunodeficiency virus type 1 (HIV-1) by 5-ethyl-6-phenylthiouracil derivatives through its interaction with the HIV-1 reverse transcriptase. Proc. Natl. Acad. Sci. USA 88, 2356–2360.

Baba, M., De Clercq, E., Tanaka, H., Ubasawa, M., Takashima, H., Sekiya, K., Nitta, I., Umezu, K., Walker, R.T., Mori, S., Ito, M., Shigeta, S. and Miyasaka, T. (1991b) Highly potent and

selective inhibition of human immunodeficiency virus type 1 (HIV-1) by a novel series of 6-substituted acyclouridine derivatives. Mol. Pharmacol. 39, 806–810.

Baba, M., Ito, M., Shigeta, S., Tanaka, H., Miyasaka, T., Ubasawa, M., Umezu, K., Walker, R.T. and De Clercq, E. (1991c) Synergistic inhibition of human immunodeficiency virus type 1 replication by 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil (E-EPU) and azidothymidine in vitro. Antimicrob. Agents Chemother. 35, 1430–1433.

Barré-Sinoussi, F., Chermann, J.-C., Rey, R., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Brun-Vézinet, F., Rouzioux, C., Rosenbaum, W. and Montagnier, L. (1983) Isolation of a T cell lymphotropic virus from a patients at risk for the acquired immunodeficiency syndrome (AIDS). Science 220, 868–871.

Cheng, Y.-C., Dutchman, G.E., Bastow, K.F., Sarngadharan, M.O. and Ting, R.Y.C. (1987) Human immunodeficiency virus reverse transcriptase: general properties and its interaction with nucleoside triphosphate analogs. J. Biol. Chem. 262, 2187–2189.

De Clercq, E. (1989) New acquisitions in the development of anti-HIV agents. Antiviral Res. 12, 1–20.

Debouck, C., Gorniak, J.G., Strickler, J.E., Meek, T.D., Metcalf, B.W. and Rosenberg, M. (1987) Human immunodeficiency virus protease expressed in *Escherichia coli* exhibits autoprocessing and specific maturation of the gag precursor. Proc. Natl. Acad. Sci. USA 84, 8903–8906.

Debyser, Z., Pauwels, R., Andries, K., Desmyter, J., Kukla, M., Janssen, P.A.J. and De Clercq, E. (1991) An antiviral target on reverse transcriptase of human immunodeficiency virus type 1 revealed by tetrahydroimidazo-[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione derivatives. Proc. Natl. Acad. Sci. USA 88, 1451–1455.

Erickson, J., Neidhart, D.J., VanDrie, J., Kempf, D.J., Wang, X.C., Norbeck, D.W., Plattner, J.J., Rittenhouse, J.W., Turon, M., Wideburg, N., Kohlbrenner, W.E., Simmer, R., Helfrich, R., Paul, D.A. and Knigge, M. (1990) Design, activity, and 2.8 Å crystal structure of a C_2 symmetric inhibitor complexed to HIV-1 protease. Science 249, 527–533.

Fischl, M.A., Richman, D.D., Grieco, M.H., Gottlieb, M.S., Volberding, P.A., Laskin, O.L., Leedom, J.M., Groopman, J.E., Mildvan, D., Schooley, R.T., Jackson, G.G., Durack, D.T., King, D. and the AZT Collaborative Working Group (1987) The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex: a double-blind, placebo-controlled trial. New Engl. J. Med. 317, 185–191.

Fischl, M.A., Richman, D.D., Hansen, N., Collier, A.C., Carey, J.T., Para, M.F., Hardy, W.D., Dolin, R., Powderly, W.G., Allan, J.D., Wong, B., Merigan, T.C., McAuliffe, V.J., Hyslop, N.E., Rhame, F.S., Balfour Jr., H.H., Spector, S.A., Volberding, P.A., Pettinelli, C., Anderson, J. and the AIDS Clinical Trials Group (1989) The safety and efficacy of zidovudine (AZT) in the treatment of subjects with mildly symptomatic human immunodeficiency virus type 1 (HIV) infection: a double-blind, placebo-controlled trial. Ann. Int. Med. 112, 727–737.

Fischl, M.A., Parker, C.B., Pettinelli, C., Wulfsohn, M., Hirsch, M.S., Collier, A.C., Antoniskis, D., Ho, M., Richman, D.D., Fuchs, E., Merigan, T.C., Reichman, R.C., Gold, J., Steigbigel, N., Leoung, G.S., Rasheed, S., Tsiatis, A. and the AIDS Clinical Trials Group (1990) A randomized controlled trial of a reduced daily dose of zidovudine in patients with the acquired immunodeficiency syndrome. New Engl. J. Med. 323, 1009–1014.

Foley, C.E., Lazarus, H., Farber, S., Uzman, B.G., Boone, B.A. and McCarthy R.E. (1965) Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer 18, 522–529.

Furman, P.A., Fyfe, J.A., St. Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Nusinoff-Lehrman, S., Bolognesi, D.P., Broder, S., Mitsuya, H. and Barry, D.W. (1986) Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83, 8333–8337.

Gallo. R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleska, J., Safai, B., White, G., Foster, P. and Markham, P.D. (1984) Frequent detection and isolation of pathogenic retroviruses (HTLV-III) from patients with AIDS and at risk from AIDS. Science 224, 500–503.

Hammer, S.M. and Gillis, J.M. (1987) Synergistic activity of granulocyte-macrophage colony-

stimulating factor and 3′-azido-3′-deoxythymidine against human immunodeficiency virus in vitro. Antimicrob. Agents Chemother. 31, 1046–1050.

Hartshorn, K.L., Sandstrom, E.G., Neumeyer, D., Paradis, T.J., Chou, T.-C., Schooley, R.T. and Hirsch, M.S. (1986) Synergistic inhibition of human T-cell lymphotropic virus type III replication in vitro by phosphonoformate and recombinant alpha-A interferon. Antimicrob. Agents Chemother. 30, 189 191.

Hartshorn, K.L., Vogt, M.W., Chou, T.-C., Blumberg, R.S., Byington, R., Schooley, R.T. and Hirsch, M.S. (1987) Synergistic inhibition of human immunodeficiency virus in vitro by azidothymidine and recombinant alpha A interferon. Antimicrob. Agents Chemother. 31, 168–172.

Ito, M., Baba, M., Shigeta, S., De Clercq, E., Walker, R.T., Tanaka, H. and Miyasaka, T. (1991) Synergistic inhibition of human immunodeficiency virus type 1 (HIV-1) replication in vitro by 1-[(2-hydroxyethoxy)methyl]-6-phenylthiothymine (HEPT) and recombinant alpha interferon. Antiviral Res. 15, 323–330.

Johnson, V.A., Barlow, M.A., Chou, T.-C., Fisher, R.A., Walker, B.D., Hirsch, M.S. and Schooley, R.T. (1989) Synergistic inhibition of human immunodeficiency virus type 1 (HIV-1) replication in vitro by recombinant soluble CD4 and 3′-azido-3′-deoxythymidine. J. Infect. Dis. 159, 837–844.

Kikukawa, R., Koyanagi, Y., Harada, S., Kobayashi, N., Hatanaka, M. and Yamamoto N. (1986) Differential susceptibility to the acquired immunodeficiency syndrome retrovirus in clone cells of human leukemic T-cell line Molt-4. J. Virol. 57, 1159–1162.

Kohl, N., Emini, E., Schleif, W., Davis, L., Heimbach, J., Dixon, R., Scolnick, E. and Sigal, I. (1988) Active human immunodeficiency virus protease is required for virus infectivity. Proc. Natl. Acad. Sci. USA 85, 4686–4690.

König, H., Behr, E., Löwer, J. and Kurth, R. (1989) Azidothymidine triphosphate is an inhibitor of both human immunodeficiency virus type 1 reverse transcriptase and DNA polymerase gamma. Antimicrob. Agents Chemother. 33, 2109–2114.

Koyanagi, Y., O'Brien, W.A., Zhao, J.Q., Golde, D.W., Gasson, J.C. and Chen, I.S.Y. (1988) Cytokines alter production of HIV-1 from primary mononuclear phagocytes. Science 241, 1673–1675.

Larder, B.A., Darby, G. and Richman, D.D. (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 243, 1731–1734.

Levy, J.A., Hoffman, A.D., Kramer, S.M., Landis, J.A., Shimabukuro, J.M. and Oshiro, L.S. (1984) Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 225, 840–842.

McQuade, T.J., Tomasselli, A.G., Liu, L., Karacostas, V., Moss, B., Sawyer, T.K., Heinrikson, R.L. and Tarpley, W.G. A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation. Science 247, 454–456.

Meek, T.D., Lambert, D.M., Dreyer, G.B., Carr, T.J., Tomaszek, T.A., Moore, M.L., Stickler, J.E., Debouck, C., Hyland, L.J., Matthews, T.J., Metcalf, B.W. and Petteway, S.R. (1990) Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature 343, 90–92.

Merluzzi, V.J., Hargrave, K.D., Labadia, M., Grozinger, K., Skoog, M., Wu, J.C., Shih, C.-K., Eckner, K., Hattox, S., Adams, J., Rosehthal, A.S., Faanes, R., Eckner, R.J., Koup, R.A. and Sullivan, J.L. (1990) Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. Science 250, 1411–1413.

Mitsuya, H., Weinhold, K.J., Furman, P.A., St Clair, M.H., Nusinoff-Lehrman, S., Gallo, R.C., Bolognesi, D., Barry, D.W. and Broder, S. (1985) 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA 82, 7096–7100.

Mitsuya, H. and Broder, S. (1986) Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. USA 83, 1911–1915.

Mitsuya, H., Yarchoan, R. and Broder, S. (1990) Molecular targets for AIDS Therapy. Science 249, 1533–1544.

Miyasaka, T., Tanaka, H., Baba, M., Hayakawa, H., Walker, R.T., Balzarini, J. and De Clercq, E. (1989) A novel lead for specific anti-HIV-1 agents: 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine. J. Med. Chem. 32, 2507–2509.

Miyoshi, I., Taguchi, H., Kubonishi, I., Yoshimoto, S., Ohtsuki, Y., Shiraishi, Y. and Akagi, T. (1982) Type C virus-producing cell lines derived from adult T cell leukemia. Gann Monogr. 28, 219–228.

Ono, K., Nakane, H., Herdewijn, P., Balzarini, J. and De Clercq, E. (1989) Differential inhibitory effects of several pyrimidine 2′,3′-dideoxynucleoside 5′-triphosphate on the activities of reverse transcriptase and various cellular DNA polymerases. Mol. Pharmacol. 35, 578–583.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J. and De Clercq, E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20, 309–321.

Pauwels, R., Andries, K., Desmyter, J., Schols, D., Kukla, M.J., Breslin, H.J., Raeymaeckers, A., Van Gelder, J., Woestenborghs, R., Heykants, J., Schellelens, K., Janssen, M.A.C., De Clercq, E. and Janssen, P.A.J. (1990) Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. Nature 343, 470–474.

Richman, D.D., Fischl, M.A., Grieco, M.H., Gottlieb, M.S., Volberding, P.A., Laskin, O.L., Leedom, J.M., Groopman, J.E., Mildvan, D., Hirsch, M.S., Jackson, G.G., Durack, D.T., Nusinoff-Lehrman, S. and the AZT Collaborative Working Group (1987) The toxicity of azidothymidine (AZT) in the treatment patients with AIDS and AIDS-related complex: a double-blind, placebo-controlled trial. New Engl. J. Med. 317, 192–197.

Roberts, N.A., Martin, J.A., Kinchington, D., Broadhurst, A.V., Craig, J.C., Duncan, I.B., Galpin, S.A., Handa, B.K., Kay, J., Kröhn, A., Lambert, R.W., Merrett, J.H., Mills, J.S., Parkes, K.E.B., Redshaw, S., Ritchie, A.J., Taylor, D.L., Thomas, G.J. and Machin, P.J. (1990) Rational Design of Peptide-based HIV-1 protease inhibitors. Science 248, 358–361.

St. Clair, M.H., Richards, C.A., Spector, T., Weinhold, K.J., Miller, W.H., Langlois, A.J. and Furman, P.A. (1987) 3′-Azido-3′-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. Antimicrob. Agents Chemother. 31, 1972–1977.

Tanaka, H., Hayakawa, H. and Miyasaka, T. (1982) Umpolung of reactivity at the C-6 position of uridine: a simple and general method for 6-substituted uridines. Tetrahedron 38, 2635–2642.

Tanaka, H., Matsuda, A., Iijima, S., Hayakawa, H. and Miyasaka, T. (1983a) Synthesis and biological activity of 5-substituted 6-phenylthio- and 6-iodouridines, a new class of antileukemic nucleosides. Chem. Pharm. Bull. 31, 2164–2167.

Tanaka, H., Iijima, S., Matsuda, S., Hayakawa, H., Miyasaka, T. and Ueda, T. (1983b) The reaction of 6-phenylthiouridine with sulfer nucleophiles: a simple and regiospecific preparation of 6-alkylthiouridines and 6-alkylthiouridylic acids. Chem. Pharm. Bull. 31, 1222–1227.

Tanaka, H., Hayakawa, H., Iijima, S., Haraguchi, K. and Miyasaka, T. (1985a) Lithiation of 3′,5′-*O*-(tetraisopropyldisiloxan-1,3-diyl)-2′-deoxyuridine: synthesis of 6-substituted 2′-deoxyuridines. Tetrahedron 41, 861–866.

Tanaka, H., Hayakawa, H., Haraguchi, K. and Miyasaka, T. (1985b) Introduction of an azido group to the C-6 position of uridine by the use of a 6-iodouridine derivative. Nucleosides Nucleotides 4, 607–612.

Tanaka, H., Hayakawa, H., Obi, K. and Miyasaka, T. (1986) Synthetic route to 5-substituted uridines via a new type of desulfurizative stannylation. Tetrahedron 42, 4187–4195.

Tanaka, H., Baba, M., Hayakawa, H., Sakamaki, T., Miyasaka, T., Ubasawa, M., Takashima, H., Sekiya, K., Nitta, I., Shigeta, S., Walker, R.T., Balzarini, J. and De Clercq, E. (1991a) A new class of HIV-1 specific 6-substituted acyclouridine derivatives: synthesis and anti-HIV-1 activity of 5- or 6-substituted analogues of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT). J. Med. Chem. 34, 349–357.

Tanaka, H., Baba, M., Ubasawa, M., Takashima, H., Sekiya, K., Nitta, I., Shigeta, S., Walker, R.T., De Clercq, E. and Miyasaka, T. (1991b) Synthesis and anti-HIV activity of 2-, 3-, and 4-substituted analogues of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT). J. Med. Chem. 34, 1394–1399.

Tanaka, H., Baba, M., Saito, S., Miyasaka, T., Takashima, H., Sekiya, H., Ubasawa, M., Nitta, I., Walker, R.T., Nakashima, H. and De Clercq, E. (1991c) Specific anti-HIV-1 'acyclonucleosides' which cannot be phosphorylated: synthesis of some deoxy analogues of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT). J. Med. Chem. 34, 1508–1511.

Vrang, L., Öberg, B., Löwer, J. and Kurth, R. (1988) Reverse transcriptases from human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV_{MAC}) are susceptible to inhibition by foscarnet and 3′-azido-3′-deoxythymidine triphosphate. Antimicrob. Agents Chemother. 32, 1733–1734.