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EFFECT OF HUMAN SERUM ON THE *IN VITRO* ANTI-HIV-1 ACTIVITY OF 1-[(2-HYDROXYETHOXY)METHYL]-6-(PHENYLTHIO)THYMINE (HEPT) DERIVATIVES AS RELATED TO THEIR LIPOPHILICITY AND SERUM PROTEIN BINDING

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Abstract—Several derivatives of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) were examined for their inhibitory effects on the replication of human immunodeficiency virus type 1 (HIV-1) in MT-4 cells in the presence of various concentrations (10–50%) of human serum (HS). Although all HEPT derivatives proved to be highly potent inhibitors of HIV-1 in the presence of 10% fetal bovine serum, some of them were less inhibitory to HIV-1 replication in the presence of HS. The HEPT derivatives were found to be highly bound to HS proteins. Both the anti-HIV-1 activity and HS protein binding of the compounds appeared to be related to their lipophilicity.

A novel series of compounds has been discovered recently to be highly specific inhibitors of human immunodeficiency virus type 1 (HIV-1**) reverse transcriptase (RT). These compounds include 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) [1,2] and its derivatives [3,4], tetrahydroimidazo[4,5,1-*jk*][1,4]-benzoidiazepine-2(1*H*)-one and -thione (TIBO) [5], 6,11-dihydro-11-cyclopropyl-4-methyldipyrido[2,3-*b*:2′,3′-*e*]-[1,4]-diazepin-6-one (Nevirapine) [6], pyridinone derivatives [7], and bis(heteroaryl)piperazines (BHAPs) [8]. More recently, [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)pyrimidines (TSAO) and α-anilinophenylacetamide (α-APA) derivatives have also been identified as specific HIV-1 inhibitors [9, 10]. These compounds interact with HIV-1 RT in a way that is clearly different from the mode of action of the 2′,3′-dideoxynucleoside 5′-triphosphates [11–17].

After the discovery of HEPT as a novel lead for specific anti-HIV-1 agents, we attempted to increase its potency by introducing various chemical modifications and obtained several HEPT derivatives that inhibit HIV-1 replication in the nanomolar concentration range [18]. We also initiated toxicological and pharmacological tests in animals to select the best candidate(s) for clinical trials. The antiviral assay of compounds in the presence of human serum (HS) may be useful for estimation of their clinical efficacy. In this study, we have investigated the effects of different HS concentrations on the *in vitro* anti-HIV-1 activity of selected HEPT derivatives, as related to their lipophilicity and HS protein binding.

MATERIALS AND METHODS

Compounds. Nine HEPT derivatives were used in the experiments. Their names and chemical structures are shown in Fig. 1. The synthesis of these

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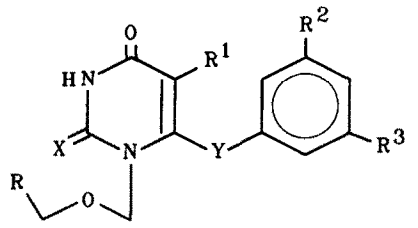
** Abbreviations: HIV-1, human immunodeficiency virus type 1; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; HS, human serum; RT, reverse transcriptase; EC_{50}, 50% antivirally effective concentration; CC_{50}, 50% cytotoxic concentration; E-EPU, 1-ethoxymethyl-5-ethyl-6-(phenylthio)uracil; E-EPU-S, 1-ethoxymethyl-5-ethyl-6-phenylthio-2-thiouracil; E-EPU-dM, 1-ethoxymethyl-5-ethyl-6-[(3,5-dimethylphenyl)thio]-uracil; E-BPU, 1-benzyloxymethyl-5-ethyl-6-(phenylthio)uracil; E-BPU-S 1-benzyloxymethyl-5-ethyl-6-phenylthio-2-thiouracil; E-EBU, 1-ethoxymethyl-5-ethyl-6-benzyluracil; E-EBU-dM, 1-ethoxymethyl-5-ethyl-6-(3,5-dimethylbenzyl)uracil; I-EBU, 1-ethoxymethyl-5-isopropyl-6-benzyluracil; I-EBU-dM, 1-ethoxymethyl-5-isopropyl-6-(3,5-dimethylbenzyl)uracil; AZT, 3′-azido-3′-deoxythymidine; Nevirapine, 6,11-dihydro-11-cyclopropyl-4-methyldipyrido[2,3-*b*:2′,3′-*e*]-[1,4]diazepin-6-one; L-696,229, 3-[2-(benzoxazol-2-yl)ethyl]-5-ethyl-6-methylpyridin-2(1*H*)-one; FBS, fetal bovine serum.



E-EPU	X=O, Y=S, R=Me, R^1=Et, R^2=R^3=H
E-EPU-S	X=S, Y=S, R=Me, R^1=Et, R^2=R^3=H
E-EPU-dM	X=O, Y=S, R=Me, R^1=Et, R^2=R^3=Me
E-BPU	X=O, Y=S, R=Ph, R^1=Et, R^2=R^3=H
E-BPU-S	X=S, Y=S, R=Ph, R^1=Et, R^2=R^3=H
E-EBU	X=O, Y=CH_2, R=Me, R^1=Et, R^2=R^3=H
E-EBU-dM	X=O, Y=CH_2, R=Me, R^1=Et, R^2=R^3=Me
I-EBU	X=O, Y=CH_2, R=Me, R^1=iPr, R^2=R^3=H
I-EBU-dM	X=O, Y=CH_2, R=Me, R^1=iPr, R^2=R^3=Me

Fig. 1. Chemical formulae of HEPT derivatives.

compounds has been described elsewhere [19–22]. 3′-Azido-3′-deoxythymidine (AZT) was purchased from the Sigma Chemical Co. (St. Louis, MO). Nevirapine and a pyridinone derivative, 3-[2-(benzoaxazol-2-yl)ethyl]-5-ethyl-6-methyl-pyridin-2(1*H*)-one (L-696,229) [23], were synthesized at the Mitsubishi Kasei Corp. (MKC) Research Center. All compounds were dissolved in dimethyl sulfoxide at 50 mM (or higher) and stored at −20° until used.

Cells and virus. MT-4 [24] cells were used for the experiments. The cells were grown and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/mL), and gentamicin (20 μg/mL). An HIV-1 strain, HTLV-III_B, was used for the antiviral assays. It was propagated in MT-4 cells. Titers of virus stocks were determined in MT-4 cells, and the virus stocks were stored at −80° until used.

Antiviral assay. Activity of the compounds against the replication of HIV-1 was assessed by the inhibition of virus-induced cytopathicity in MT-4 cells. Briefly, MT-4 cells (1×10^4 cells/well) in microtiter plates were infected with HIV-1 at a multiplicity of infection of 0.02 and cultured in the presence of various concentrations of the test compounds. After a 4-day incubation at 37°, the number of viable cells was assessed by the 3-(4,5-dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT) method [25]. Cytotoxicity of the compounds was evaluated in parallel with their anti-HIV-1 activity. It was based on the viability of mock-infected MT-4 cells as determined by the MTT method. For each compound, the anti-HIV-1 and cytotoxicity assays were carried out in culture medium containing either 10% FBS, 10% HS, 30% HS, or 50% HS. The human AB serum was obtained from Biocell (Rancho Dominguez, CA), and the same lot was used throughout the experiments.

Determination of protein binding. Protein binding of the compounds was determined by the ultrafiltration method using a Centrifree kit (Amicon, MA). After the addition of each compound (at a final concentration of 500 ng/mL) to 100% HS or RPMI-1640 medium containing either 50, 30, or 10% HS, the serum and medium were incubated at 37° for 15 min. The samples (1 mL) were then transferred to sample reservoirs of the kit and ultrafiltrated by centrifugation (1000 *g*) at room temperature for 15 min. The concentration of each compound in the ultrafiltrates was measured by HPLC. Briefly, 1 mL of boric acid-sodium hydroxide buffer (pH 10; 9890 Titrisol, Merck, Germany) was added to 0.3 mL of ultrafiltrates, and the compounds were extracted with 4 mL of diethyl ether by shaking for 10 min. After centrifugation (1800 *g*) for 5 min, the organic layer was evaporated. The resulted residue was dissolved in 150 μL of mobile phase, methanol–acetonitrile–water (30:37:33 for E-EPU-S, 30:32:38 for E-EPU, I-EBU, and I-EBU-dM, and 30:25:45 for E-EBU) containing 0.2% acetic acid. One hundred microliters of the aliquot samples were injected into the HPLC system equipped with an L-6200 pump (Hitachi, Tokyo, Japan), a model 7161 sample injector (Rheodyne CA), a SPD-6A UV detector (Simadzu, Kyoto, Japan), and a TSK gel ODS-80Tm column (150 × 4.6 mm i.d., Tosoh, Tokyo, Japan) maintained at 50°. The flow rate of the mobile phase was 1.0 mL/min. The compounds were detected by ultraviolet spectrometry at a wavelength of 268 nm for E-EBU, I-EBU, and I-EBU-dM, 275 nm for E-EPU, and 284 nm for E-EPU-S. The protein binding of the test compounds was expressed as percent binding calculated according to the following formula: percent binding = $(C_t - C_f)/C_t \times 100$, where C_t is the concentration of the total compound and C_f is the concentration of the free compound (not bound to HS).

Lipophilicity determination. HPLC analysis was performed with a Shimadzu SPD-6A chromatograph equipped with a Pheodyne model 7125 injector and a Shimadzu LC-9A pump (Shimadzu, Kyoto, Japan). Analysis of the compounds was accomplished on an Inertsil ODS-2 column (5 μm, 150 × 4.6 mm i.d., GL Sciences, Tokyo, Japan). The column was maintained at 25°. Ultraviolet spectrometry at a wavelength of 270 nm was used for detection of the compounds. The compounds were eluted with acetonitrile–water (45:55, v/v) at a flow of 1 mL/min. Logarithms of P values were calculated according to the formula previously described by McCall [26]. Benzene (log P = 2.13), bromobenzene (log P = 2.99), and biphenyl (log P = 3.79) were used as reference compounds.

RESULTS

Effect of human serum concentration on the anti-HIV-1 activity of HEPT derivatives in vitro. When we examined the HEPT derivatives (Fig. 1) for their inhibitory effects on HIV-1 replication in MT-4 cells in the presence of 10% FBS, all compounds proved to be highly potent and selective inhibitors of HIV-1 (Table 1). Their 50% antivirally effective concentrations (EC_{50}) ranged from 0.045 to 0.0022 μM. To evaluate the anti-HIV-1 activity of these compounds under more therapeutically oriented conditions, the assays were also performed

Table 1. Effect of serum concentrations on the anti-HIV-1 activity of HEPT derivatives in MT-4 cells

Compound	Serum	EC_{50}* (μM)	CC_{50}† (μM)
E-EPU	10% FBS	0.022 ± 0.006	>100
	10% HS	0.036 ± 0.012	>100
	30% HS	0.066 ± 0.017	>100
	30% HS	0.066 ± 0.017	>100
	50% HS	0.080 ± 0.005	>100
E-EPU-S	10% FBS	0.026 ± 0.001	65 ± 15
	10% HS	0.21 ± 0.014	75 ± 8
	30% HS	0.42 ± 0.10	>100
	50% HS	0.73 ± 0.17	>100
E-EPU-dM	10% FBS	0.0062 ± 0.0006	>100
	10% HS	0.0041 ± 0.0007	72 ± 6
	30% HS	0.0063 ± 0.0013	>100
	50% HS	0.0099 ± 0.0025	>100
E-BPU	10% FBS	0.0049 ± 0.0034	30 ± 8
	10% HS	0.016 ± 0.07	36 ± 5
	30% HS	0.028 ± 0.08	>100
	50% HS	0.070 ± 0.02	>100
E-BPU-S	10% FBS	0.0084 ± 0.0006	>100
	10% HS	0.11 ± 0.03	>100
	30% HS	0.27 ± 0.04	>100
	50% HS	0.61 ± 0.11	>100
E-EBU	10% FBS	0.045 ± 0.004	>100
	10% HS	0.033 ± 0.008	>100
	30% HS	0.048 ± 0.001	>100
	50% HS	0.066 ± 0.002	>100
E-EBU-dM	10% FBS	0.0022 ± 0.0005	>100
	10% HS	0.0027 ± 0.0005	>100
	30% HS	0.0044 ± 0.0009	>100
	50% HS	0.012 ± 0.003	>100
I-EBU	10% FBS	0.014 ± 0.002	>100
	10% HS	0.018 ± 0.001	>100
	30% HS	0.026 ± 0.008	>100
	50% HS	0.063 ± 0.019	>100
I-EBU-dM	10% FBS	0.0022 ± 0.0008	37 ± 9
	10% HS	0.0032 ± 0.0006	51 ± 17
	30% HS	0.0048 ± 0.0011	>100
	50% HS	0.010 ± 0.001	>100
AZT	10% FBS	0.0030 ± 0.0010	7.8 ± 1.0
	10% HS	0.0028 ± 0.0003	7.1 ± 0.5
	30% HS	0.0027 ± 0.0005	5.6 ± 0.6
	50% HS	0.0028 ± 0.0007	4.9 ± 0.4
Nevirapine	10% FBS	0.11 ± 0.01	>100
	10% HS	0.11 ± 0.01	>100
	30% HS	0.087 ± 0.013	>100
	50% HS	0.11 ± 0.01	>100
L-696,229	10% FBS	0.036 ± 0.011	>100
	10% HS	0.044 ± 0.007	>100
	30% HS	0.083 ± 0.001	>100
	50% HS	0.11 ± 0.04	>100

Except for Nevirapine and L-696,229, all data are means ± SD for at least three separate experiments. Data for Nevirapine and L-696,229 represent mean ± range for two separate experiments.

* Fifty percent antivirally effective concentration, or concentration required to inhibit HIV-1-induced cytopathicity in MT-4 cells by 50%.

† Fifty percent cytotoxic concentration, or concentration required to reduce the viability of mock-infected MT-4 cells by 50%.

in the presence of various concentrations (10, 30, and 50%) of HS. Some of the compounds such as E-EPU-S, E-BPU, and E-BPU-S were found to be much less inhibitory to HIV-1 replication in the presence of 10% HS (Table 1). Furthermore, the activity of the HEPT derivatives diminished further when the concentration of HS in the cell culture medium was increased from 10 to 30 and 50% (Table 1). However, the effect of human serum on the anti-HIV-1 activity of the HEPT derivatives differed considerably from one compound to another. Of the HEPT derivatives, E-BPU-S was affected the most and E-EBU the least by the presence of HS (Table 1). In contrast, the anti-HIV-1 activities of AZT and nevirapine were not affected by HS. The EC_{50} of L-696,229 in the presence of 50% HS was 2.5-fold higher than that measured in the presence of 10% HS (Table 1).

Binding of HEPT derivatives to human serum proteins. When the HEPT derivatives E-EPU, E-EPU-S, E-EBU, I-EBU and I-EBU-dM were examined for their binding to HS (10, 30, 50, and 100% in cell culture medium), all compounds were found to be highly bound to serum proteins (Table 2). The binding increased with increasing HS concentration. Of the HEPT derivatives, E-EPU-S showed the highest protein binding: only 0.3 to 0.7% of the total compound remained unbound to serum proteins (Table 2). E-EBU and I-EBU exhibited significantly lower binding to serum proteins as compared with the other HEPT derivatives (E-EPU, E-EPU-S, and I-EBU-dM). The (decreasing) order of protein binding was E-EPU-S > I-EBU-dM = E-EPU > I-EBU > E-EBU.

Lipophilicity of HEPT derivatives. When the HEPT derivatives E-EPU, E-EPU-S, E-BPU, E-BPU-S, E-EBU, E-EBU-dM, I-EBU, and I-EBU-dM were examined for their lipophilicity, all compounds proved to be highly lipophilic. Their log P values ranged from 1.72 to 3.48 (Fig. 2). The highest log P value was recorded for I-EBU-dM, whereas E-EBU had the lowest. The (decreasing)

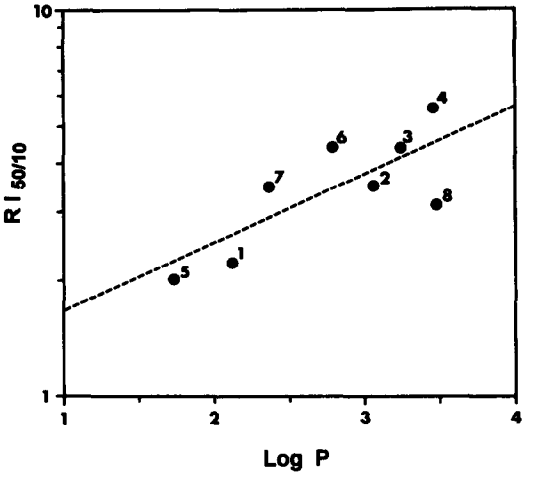


Fig. 2. Correlation between the lipophilicity (log P) of the selected HEPT derivatives and their $RI_{50/10}$ values. The $RI_{50/10}$ corresponds to the ratio of the EC_{50} for HIV-1 replication in the presence of 50% HS to the EC_{50} in the presence of 10% HS (Table 1). HEPT derivatives: (1) E-EPU, (2) E-EPU-S, (3) E-BPU, (4) E-BPU-S, (5) E-EBU, (6) E-EBU-dM, (7) I-EBU, and (8) I-EBU-dM.

Table 2. Binding of HEPT derivatives to human serum proteins

Compound	Binding (%)			
	Concentration of human serum (%)			
	10	30	50	100
E-EPU	96.6 ± 1.2 (3.4)*	98.2 ± 0.6 (1.8)	98.4 ± 0.3 (1.6)	98.9 ± 0.1 (1.1)
E-EPU-S	99.3 ± 0.0 (0.7)	99.7 ± 0.0 (0.3)	99.5 ± 0.3 (0.5)	99.6 ± 0.3 (0.4)
E-EBU	73.3 ± 0.8 (26.7)	85.7 ± 0.8 (14.3)	88.9 ± 0.8 (11.1)	92.6 ± 0.2 (7.4)
I-EBU	78.2 ± 1.5 (21.8)	90.1 ± 0.2 (9.9)	93.0 ± 0.5 (7.0)	95.5 ± 0.3 (4.5)
I-EBU-dM	95.2 ± 0.2 (4.8)	98.4 ± 0.1 (1.6)	99.0 ± 0.1 (1.0)	98.9 ± 0.1 (1.1)

All data are means ± SD for triplicate experiments.
* Values in parentheses represent the percent of the compound that was not bound to HS.

order of lipophilicity expressed as log P was I-EBU-dM ≥ E-BPU-S > E-BPU > E-EPU-S > E-EBU-dM > I-EBU > E-EPU > E-EBU. The relationship between lipophilicity (log P) of the HEPT derivatives and the effect of HS on their anti-HIV-1 activity was assessed. As shown in Fig. 2, a close correlation ($r = 0.824$) was found between their log P and $RI_{50/10}$ (ratio of the EC_{50} for HIV-1 replication in the presence of 50% HS to the EC_{50} in the presence of 10% HS).

DISCUSSION

Since HIV-1 infects and damages the CNS, ideal anti-AIDS drugs should penetrate the blood–brain barrier and suppress the virus replication in the brain. Lipophilicity is an important factor that may affect the entry of compounds into the CNS. The lipophilicity of dideoxynucleosides including AZT is relatively low [27]; therefore, attempts have been made to increase the lipophilicity of dideoxynucleosides [28, 29]. Lipophilicity may also affect protein binding, and this, in turn, may affect the anti-HIV activity, cytotoxicity, and pharmacokinetics of the compounds *in vivo*. To obtain further insight into the interrelationship between these factors, we have investigated the lipophilicity, protein binding and influence of HS on the anti-HIV-1 activity of various HEPT derivatives.

The HEPT derivatives selected for this study were highly potent inhibitors of HIV-1 replication in MT-4 cells under the standard assay conditions using 10% FBS (Table 1). However, some of the HEPT derivatives proved less inhibitory to HIV-1, when the assays were performed in culture medium containing 10% HS. Moreover, their anti-HIV-1 activity diminished further with increasing HS concentration in the cell culture medium (Table 1). The 2-thio analogs (E-EPU-S and E-BPU-S) and 1-benzyloxymethyl analogs (E-BPU and E-BPU-S) were affected more by HS than the other HEPT derivatives. Consequently, E-BPU-S was 73-fold less active in the presence of 50% HS compared with 10% FBS (Table 1). In contrast, the anti-HIV-1 activity of the 6-benzyl analog E-EBU remained virtually unchanged in the presence of increasing concentrations of HS.

In accordance with the results for anti-HIV-1 activity, a marked difference was observed in the protein binding of E-EPU-S and E-EBU (Table 2). Considerable differences were also observed for the lipophilicity of these compounds, suggesting that lipophilicity is an important determinant in the anti-HIV-1 activity of this series of HEPT derivatives through their protein binding. In general, the better the compound is bound to serum proteins the higher the plasma concentration it may be able to achieve *in vivo*. On the other hand, the compound uptake assay suggests that only the molecules unbound to serum proteins could penetrate into the cells and exert their anti-HIV-1 activity (data not shown).

Various issues including efficacy, pharmacokinetics and safety need to be addressed before one (or more) HEPT derivatives can be selected as a candidate(s) for clinical trials. Our present observations provide useful information to estimate the *in vivo* efficacy and clinical dosage of the selected HEPT derivatives.

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REFERENCES

1. Miyasaka T, Tanaka H, Baba M, Hayakawa H, Walker RT, Balzarini J and De Clercq E, A novel lead for specific anti-HIV-1 agents: 1-[(2-Hydroxyethoxy)-methyl]-6-(phenylthio)thymine. *J Med Chem* **32**: 2507–2509, 1989.
2. Baba M, Tanaka H, De Clercq E, Pauwels R, Balzarini J, Schols D, Nakashima H, Perno C-F, Walker RT and Miyasaka T, Highly specific inhibition of human immunodeficiency virus type 1 by a novel 6-substituted acyclouridine derivative. *Biochem Biophys Res Commun* **165**: 1375–1381, 1989.

3. Baba M, De Clercq E, Tanaka H, Ubasawa M, Takashima H, Sekiya K, Nitta I, Umezu K, Nakashima H, Mori S, Shigeta S, Walker RT and Miyasaka T, 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, 1991.
4. Baba M, De Clercq E, Tanaka H, Ubasawa M, Takashima H, Sekiya K, Nitta I, Umezu K, Walker RT, Mori S, Ito M, Shigeta S and Miyasaka T, 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**: 805–810, 1991.
5. Pauwels R, Andries K, Desmyter J, Schols D, Kukla MJ, Breslin HJ, Raeymaeckers A, Van Gelder J, Woestenborghs R, Heykants J, Schellekens K, Janssen MAC, De Clercq E and Janssen PAJ, Potent and selective inhibition of HIV-1 replication *in vitro* by a novel series of TIBO derivatives. *Nature* **343**: 470–474, 1990.
6. Merluzzi VJ, Hargrave KD, Labadia M, Grozinger K, Skoog M, Wu JC, Shih C-K, Eckner K, Hattox S, Adams J, Rosehthal AS, Faanes R, Eckner RJ, Koup RA and Sullivan JL, Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. *Science* **250**: 1411–1413, 1990.
7. Goldman ME, Nunberg JH, O'Brien JA, Quintero JC, Schleif WA, Freund KF, Gaul SL, Saari WS, Wai JS, Hoffman JM, Anderson PS, Hupe DJ, Emini EA and Stern AM, Pyridinone derivatives: Specific human immunodeficiency virus type 1 reverse transcriptase inhibitors with antiviral activity. *Proc Natl Acad Sci USA* **88**: 6863–6867, 1991.
8. Romero DL, Busso M, Tan C-K, Reusser F, Palmer JR, Poppe SM, Aristoff PA, Downey KM, So AG, Resnick L and Tarpley WG, Nonnucleoside reverse transcriptase inhibitors that potently and specifically block human immunodeficiency virus type 1 replication. *Proc Natl Acad Sci USA* **88**: 8806–8810, 1991.
9. Balzarini J, Pérez-Pérez M-J, San-Félix A, Schols D, Perno C-F, Vandamme A-M, Camarasa M-J and De Clercq E, 2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro - 5″ - (4″ - amino - 1″,2″ - oxathiole - 2″,2″-dioxide)pyrimidine (TSAO) nucleoside analogues: Highly selective inhibitors of human immunodeficiency virus type 1 that are targeted at the viral reverse transcriptase. *Proc Natl Acad Sci USA* **89**: 4392–4396, 1992.
10. Pauwels R, Andries K, Debyser Z, Van Daele P, Schols D, Vandamme A-M, Stoffels P, De Vreese K, Woestenborghs R, Janssen CGM, Anné J, Cauwenbergh G, Desmyter J, Heykants J, Janssen MAC, De Clercq E and Janssen PAJ, Potent and highly selective HIV-1 inhibition by a new series of α-anilinophenylacetamide (α-APA) derivatives targeted at HIV-1 reverse transcriptase. *Proc Natl Acad Sci USA* **90**: 1839–1842, 1993.
11. Debyser Z, Pauwels R, Andries K, Desmyter J, Kukla M, Janssen PAJ and De Clercq E, An antiviral target on reverse transcriptase of human immunodeficiency virus type 1 revealed by tetrahydroimidazo[4,5,1-*jk*] [1,4]-benzodiazepine-2(1*H*)-one and -thione derivatives. *Proc Natl Acad Sci USA* **88**: 1451–1455, 1991.
12. Wu JC, Warren TC, Adams J, Proudfoot J, Skiles J, Raghavan P, Perry C, Potocki I, Farina PR and Grob PM, A novel dipyridodiazepinone inhibitor of HIV-1 reverse transcriptase acts through a nonsubstrate binding site. *Biochemistry* **30**: 2022–2026, 1991.
13. Frank KB, Noll GJ, Connell EV and Sim IS, Kinetic interaction of human immunodeficiency virus type 1 reverse transcriptase with the antiviral tetrahydroimidazo[4,5,1-*jk*] [1,4]-benzodiazepine-2(1*H*)-thione compound, R82150. *J Biol Chem* **266**: 14232–14236, 1991.
14. Tramontano E and Cheng Y-C, HIV-1 reverse transcriptase inhibition by a dipyridodiazepinone derivative: BI-RG-587. *Biochem Pharmacol* **43**: 1371–1376, 1992.
15. Debyser Z, Pauwels R, Baba M, Desmyter J and De Clercq E, Common features in the interaction of TIBO and HEPT derivatives with the HIV-1 reverse transcriptase. *Mol Pharmacol* **41**: 963–968, 1992.
16. Balzarini J, Pérez-Pérez M-J, San-Félix A, Camarasa M-J, Bathurst IC, Barr PJ and De Clercq E, Kinetics of inhibition of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase by the novel HIV-1-specific nucleoside analogue [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)thymine (TSAO-T). *J Biol Chem* **267**: 11831–11838, 1992.
17. White EL, Buckheit RW Jr, Ross LJ, Germany JM, Andries K, Pauwels R, Janssen PAJ, Shannon WM and Chirigos MA, A TIBO derivative, R82913, is a potent inhibitor of HIV-1 reverse transcriptase with heteropolymer templates. *Antiviral Res* **16**: 257–266, 1991.
18. Baba M, Shigeta S, Tanaka H, Miyasaka T, Ubasawa M, Umezu K, Walker RT, Pauwels R and De Clercq E, Highly potent and selective inhibition of HIV-1 replication by 6-phenylthiouracil derivatives. *Antiviral Res* **17**: 245–264, 1992.
19. Tanaka H, Baba M, Hayakawa H, Sakamaki T, Miyasaka T, Ubasawa M, Takashima H, Sekiya K, Nitta I, Shigeta S, Walker RT, Balzarini J and De Clercq E, 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, 1991.
20. Tanaka H, Baba M, Ubasawa M, Takashima H, Sekiya K, Nitta I, Shigeta S, Walker RT, De Clercq E and Miyasaka T, 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, 1991.
21. Tanaka H, Takashima H, Ubasawa M, Sekiya K, Nitta I, Baba M, Shigeta S, Walker RT, De Clercq E and Miyasaka T, Structure–activity relationships of 1-[(2-hydroxyethoxy)methyl-6-(phenylthio)thymine (HEPT) analogues: Effect of substitutions at the C-6 phenyl ring and the C-5 position on anti-HIV-1 activity. *J Med Chem* **35**: 337–345, 1992.
22. Tanaka H, Takashima H, Ubasawa M, Sekiya K, Nitta I, Baba M, Shigeta S, Walker RT, De Clercq E and Miyasaka T, Synthesis and antiviral activity of deoxy analogs of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) as potent and selective anti-HIV-1 agents. *J Med Chem* **35**: 4713–4719, 1992.
23. Goldman ME, O'Brien JA, Ruffing TL, Nunberg JH, Schleif WA, Quintero JC, Siegl PKS, Hoffman JM, Smith AM and Emini EA, L-696,229 specifically inhibits human immunodeficiency virus type 1 reverse transcriptase and possesses antiviral activity *in vitro*. *Antimicrob Agents Chemother* **36**: 1019–1023, 1992.
24. Harada S, Koyanagi Y and Yamamoto N, Infection of HTLV-III/LAV in HTLV-I carrying MT-2 and MT-4 cells and application in a plaque assay. *Science* **229**: 563–566, 1985.
25. Pauwels R, Balzarini J, Baba M, Snoeck R, Schols D, Herdewijn P, Desmyter J and De Clercq E, Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods* **20**: 309–321, 1988.

26. McCall JM, Liquid–liquid partition coefficients by high-pressure liquid chromatography. *J Med Chem* **18**: 549–552, 1975.
27. Balzarini J, Cools M and De Clercq E, Estimation of the lipophilicity of anti-HIV nucleoside analogues by determination of the partition coefficient and retention time on a Lichrospher 60 RP-8 HPLC column. *Biochem Biophys Res Commun* **158**: 413–422, 1989.
28. Balzarini J, Van Aerschot A, Pauwels R, Baba M, Herdewijn P and De Clercq E, 5-Halogeno-3′-fluoro-2′,3′-dideoxyuridines as inhibitors of human immunodeficiency virus (HIV): Potent and selective anti-HIV activity of 3′-fluoro-2′,3′-dideoxy-5-chlorouridine. *Mol Pharmacol* **35**: 571–577, 1989.
29. Shirasaka T, Murakami K, Ford H Jr, Kelley JA, Yoshioka H, Kojima E, Aoki S, Border S and Mitsuya H, Lipophilic halogenated congeners of 2′,3′-dideoxypurine nucleosides active against human immunodeficiency virus *in vitro*. *Proc Natl Acad Sci USA* **87**: 9426–9430, 1990.