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ACCELERATED COMMUNICATION

Human Immunodeficiency Virus Type 1 Drug-Resistance Patterns with Different 1-[(2-Hydroxyethoxy)methyl]-6-(phenylthio)thymine Derivatives

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SUMMARY

Of the class of the 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)-thymine (HEPT) derivatives, several congeners were found to inhibit (at 50% effective concentrations ranging from 0.02 to 0.6 μg/ml) the replication of mutant human immunodeficiency virus type 1 (HIV-1) strains that had been selected for resistance against bis(heteroaryl)piperazine, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thiones (TIBO), nevirapine, [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO), or pyridinone and showed amino acid substitutions at positions 100, 103, 106, 138, and 181, respectively. When HIV-1 strains were selected for resistance against three different HEPT derivatives [i.e., HEPT and its derivatives 5-ethyl-1-ethoxymethyl-6-benzyluracil (E-EBU) and 5-ethyl-1-ethoxymethyl-6-(3,5-dimethylbenzyl)uracil (EBU-dM)], HEPT selected for the mutation 188-Tyr→His, E-EBU for 181-Tyr→Cys, and E-EBU-dM for 106-Val→Ala, in the reverse transcriptase of the mutant viruses. These virus strains showed markedly decreased sensitivity to HEPT derivatives. Moreover, the HEPT-resistant virus strains also proved cross-resistant to virtually all other HIV-1-specific inhibitors, including TIBO, nevirapine, and TSAO.

Several classes of structurally different compounds have been credited with a highly specific activity against HIV-1. These compounds are not inhibitory to any DNA or RNA viruses other than HIV-1. The first compounds shown to be specific HIV-1 inhibitors were the HEPT derivatives (1–3) and TIBO R82150 and R82913 (4, 5). Later, other compounds were found to exhibit an antiviral activity spectrum similar to that of HEPT and TIBO, i.e., nevirapine (BI-RG-587) (6, 7), pyridinone L-697,661 (8), BHAP (U-87201E) (9), TSAO-T (10–14), and α-anilinophenylacetamide (15). All these compounds inhibit the HIV-1 RT activity and are targeted at a non-substrate binding site of the HIV-1 RT (6, 8–10, 15–23).

A potential drawback of these compounds may be the rapid selection for drug-resistant HIV-1 strains, as has been shown to occur *in vitro* in cell culture and *in vivo* in patients infected with HIV-1 (24–35). The molecular basis for the rapid emergence of drug-resistant virus is the appearance of mutations at well defined sites of the RT gene. To date, several authors have reported on the selection of HIV-1 mutants resistant to pyridinone, nevirapine, TIBO, TSAO, or BHAP. HIV-1 mutant strains that were selected in the presence of pyridinone or nevirapine showed cross-resistance to other HIV-1-specific RT inhibitors, such as TIBO and HEPT (24, 25, 28). The molecular

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ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thiones; BHAP, bis(heteroaryl)piperazine; TSAO-T, 1-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)thymine; RT, reverse transcriptase(s); TSAO, [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide); TSAO-m^{3}T, 1-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)-*N*^{3}-methylthymine; TSAO-e^{3}T, 1-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)-*N*^{3}-ethylthymine; E-EBU, 5-ethyl-1-ethoxymethyl-6-(benzyl)uracil; E-EPU, 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil; E-EBU-dM, 5-ethyl-1-ethoxymethyl-6-(3,5-dimethylbenzyl)uracil; E-EPU-S, 5-ethyl-1-ethoxymethyl-6-(phenylthio)-2-thiouracil; EC_{50}, 50% effective concentration; $CCID_{50}$, 50% cell culture infective dose; PCR, polymerase chain reaction.

basis for the decreased sensitivity of these HIV-1 mutant strains to the various classes of HIV-1-specific inhibitors seemed to be a single mutation in the RT gene at position 181 (tyrosine to cysteine). For the TSAO-resistant HIV-1 strains we found an amino acid substitution at position 138 (glutamate to lysine), which has not been reported for HIV-1 mutant strains selected for resistance to the other HIV-1-specific inhibitors (30–33). Also, Dueweke *et al.* (35) recently reported that BHAP-resistant HIV-1 contained the mutation proline to leucine at position 236, which has not been observed in other drug-resistant HIV-1 strains. Both mutant virus strains remained highly sensitive (138-Glu→Lys) or even became hypersensitive (236-Pro→Leu) to the other HIV-1-specific RT inhibitors (i.e., nevirapine, TIBO, and pyridinone). These observations should stimulate the search for simultaneous or alternating drug combination strategies with compounds that among themselves do not select for cross-resistant mutant HIV-1 strains.

The HEPT derivatives have not been analyzed for their resistance to HIV-1 mutant strains that were selected for resistance to the other classes of HIV-1-specific inhibitors and contain a well defined amino acid substitution in their RT genome. Nor has the emergence of drug-resistant virus under HEPT therapy been investigated. In the present study, we found that HEPT derivatives (Fig. 1) in general remain inhibitory to HIV-1 strains selected for resistance to various other classes of HIV-1-specific inhibitors. In contrast, HIV-1 mutant strains selected for resistance in the presence of the HEPT derivatives show cross-resistance to virtually all other HIV-1-specific inhibitors. In addition, different HEPT derivatives were found to select for different mutations in the HIV-1 RT gene.

Materials and Methods

Test compounds. The synthesis of the TSAO derivatives of thymine (TSAO-T), N^3-methylthymine (TSAO-m^{3}T), and N^3-ethylthymine (TSAO-e^{3}T) has been described elsewhere (12–14). TIBO R82913 was kindly provided by Dr. Zhang-Hao (National Institutes of Health, Bethesda, MD) and was also obtained from Pharmatech International Inc. (West Orange, NJ). Nevirapine (BI-RG-587), pyridinone L-697,661, and BHAP (U-87201E) were kindly provided by Dr. P. Ganong (Boehringer Ingelheim, Ridgefield, CT), Dr. M. Goldman (Merck, Sharp & Dohme, West Point, PA), and Dr. G. W. Tarpley (Upjohn Company, Kalamazoo, MI), respectively. HEPT and its derivatives E-EPU, E-EBU, E-EBU-dM, and E-EPU-S were synthesized as described previously (3, 36–38) and were kindly provided by Dr. M. Baba (Fukushima Medical College, Fukushima, Japan).

Cells and viruses. CEM cells were obtained from the American Type Culture Collection (Rockville, MD). HIV-1(III_B) was originally obtained from the culture supernatant of persistently HIV-1-infected H9 cells and was kindly provided by Dr. R. C. Gallo and Dr. M. Popovic (National Institutes of Health, Bethesda, MD).

Selection of HIV-1(III_B) mutant strains. HIV-1(III_B) was subjected to two or three passages in 5–6-ml CEM cell cultures (3–4 × 10^5 cells/ml) in the presence of 2–3 times the EC_{50} value of TSAO-m^{3}T, nevirapine, pyridinone L-697,661, TIBO R82913, or BHAP, as described previously (31), or the EC_{50} value of HEPT, E-EBU, or E-EBU-dM, in 25-cm^2 culture flasks (Falcon; Becton Dickinson). The culture medium consisted of RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, and 0.075% $NaHCO_3$. The multiplicity of the initial infection was 200 times the $CCID_{50}$. Passages were performed every 3–4 days by addition of 0.5–1.0 ml of the infected culture supernatant to 4–5 ml of a suspension containing 3–4 × 10^5 uninfected CEM cells/ml and increasing (5-fold) concentrations of the inhibitor. The virus that was recovered from the passage in the presence of the highest concentration of the inhibitors was subjected to at least

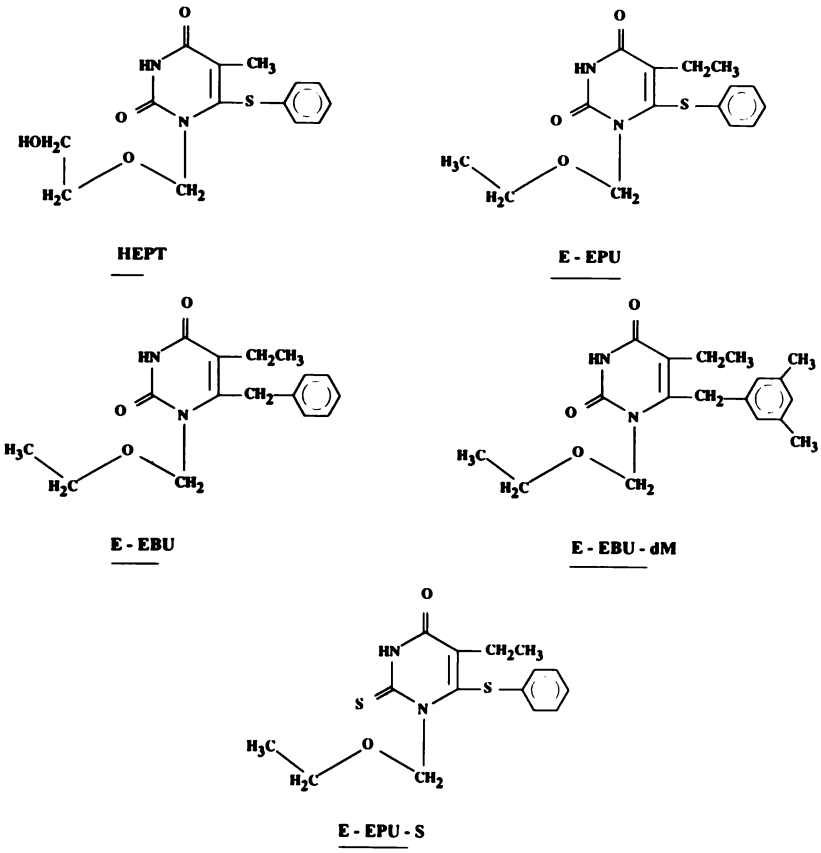

Fig. 1. Formulae of HEPT and its derivatives E-EPU, E-EBU, E-EBU-dM, and E-EPU-S.

two additional passages in cell culture before being titrated and evaluated for its sensitivity to the various test compounds.

Activity of the various test compounds against replication of several mutant HIV-1 strains in CEM cells. CEM cells were suspended at 250,000 cells/ml of culture medium and infected with HIV-1(III_B), the TSAO-m^3T-resistant HIV-1 strain (HIV-1/TSAO-m^3T), the nevirapine-resistant HIV-1 strain (HIV-1/Nev), the pyridinone-resistant HIV-1 strain (HIV-1/Pyr), the TIBO R82913-resistant HIV-1 strain (HIV-1/TIBO), or the BHAP-resistant HIV-1 strain (HIV-1/BHAP) at 100 $CCID_{50}$/ml. Then, 100 μl of the infected cell suspensions were added to 200-μl microtiter plate wells containing 100 μl of an appropriate dilution of the test compounds. After 4–5 days of incubation at 37°, the cell cultures were examined for syncytium formation. The EC_{50} was determined as the compound concentration required to inhibit syncytium formation by 50%.

Preparation of HIV-1-infected MT-4 cell cultures for PCR analysis and sequencing of the RT gene. CEM cells (3×10^5 cells/ml) were infected with different HIV-1 strains at 200 times the $CCID_{50}$ value and were incubated in RPMI 1640 culture medium for 3 days at 37°. Then, cells were centrifuged and washed twice with phosphate-buffered saline in 1.5-ml Eppendorf tubes. To 10^6 CEM cells were added 100 μl containing 10 μl of PCR buffer [10× concentrated: 100 mM Tris·HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin (Cetus-Vanderheyden, Brussels, Belgium)], 8 μl of $MgCl_2$ (25 mM), 72 μl of Milli-Q water, and 10 μl of proteinase K (10 μg; Calbiochem) in 0.5% Tween 20 and 0.5% Nonidet P-40 in H_2O. The cell suspension was then incubated at 56° for 1 hr and subsequently heated at 95° for 10 min. The samples were stored at −20° before PCR analysis.

Amplification of proviral DNA was performed with an extract from 1×10^5 cells in 10 mM Tris·HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 2.5 units of thermostable DNA polymerase (Dyna Zyme; Finnzymes Inc.), and 0.15 μM concentrations of each primer, in a final volume of 100 μl. Oligonucleotides were chosen (sense primer, 5′-CCTGAAAATCCATACAATACTCCAGTATTTG-3′; reverse complement primer, 5′-AGTGCTTTGGTTCCTCTAAGGAGTTTAC-3′) to give a 727-base pair fragment covering amino acids 50–270. The PCR product was purified from a 1% low-melting point agarose gel by MagicPCR Preps (Promega), directly sequenced with a Taq Dye Deoxy Terminator sequencing kit (Applied Biosystems), and analyzed on a model 373A DNA sequencer (Applied Biosystems).

RT assay. The reaction mixture (50 μl) contained 50 mM Tris·HCl, pH 7.8, 5 mM dithiothreitol, 300 mM glutathione, 500 μM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 μg of bovine serum albumin, a fixed concentration of the labeled substrate [^{3}H]dGTP (2.5 μM, 2 μCi), a fixed concentration of the template/primer poly(C)·oligo(dG) (0.1 mM), 0.06% Triton X-100, 5 μl of inhibitor solution [containing various concentrations (5-fold dilutions) of the compounds], and 5 μl of the RT preparation. The reaction mixtures were incubated at 37° for 30 min, at which time 100 μl of calf thymus DNA (150 μg/ml), 2 ml of $Na_4P_2O_7$ (0.1 M in 1 M HCl), and 2 ml of trichloroacetic acid (10%, v/v) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analyzed for radioactivity. The IC_{50} of the test compounds was determined as the compound concentration that inhibited the virus particle-derived RT activity by 50%.

Results

Selection of mutant HIV-1 strains resistant to HIV-1-specific inhibitors. HIV-1(III_B)-infected CEM cells were cultured in the presence of TSAO-m^3T, nevirapine, pyridinone L697,661, TIBO R82913, and BHAP at 2–3-fold the EC_{50} of the test compound. Generally, virus-induced syncytia appeared after one to three subcultivations of the HIV-1-infected CEM cells in the presence of low concentrations of the HIV-1-specific inhibitors (other than the HEPT derivatives), and giant cell formation became abundant after one to three additional subcultivations in the presence of fixed concentrations of the test compounds. Further stepwise (~5-fold) increases of the compound concentrations did not result in any marked inhibition of syncytium formation. This suggests that the virus strains that emerged after low-dose therapy must have become resistant to the inhibitor used (31). The RT gene of the virus strains that emerged under treatment with escalating concentrations of the HIV-1-specific inhibitors, i.e., HIV-1/BHAP (2.5 μg/ml), HIV-1/Nev (2.5 μg/ml), HIV-1/Pyr (2.5 μg/ml), HIV-1/TIBO (2.5 μg/ml), and HIV-1/TSAO-m^3T (10 μg/ml), was sequenced and found to contain a single amino acid mutation, i.e., 100-Leu→Ile, 106-Val→Ala, 181-Tyr→Cys, 103-Lys→Asn, and 138-Glu→Lys, respectively.

In contrast to the other HIV-1-specific inhibitors, HEPT and its derivatives did not easily select for resistant HIV-1 mutant strains (Fig. 2). For the HEPT derivatives a greater number of subcultivations were required, compared with the other compounds, before syncytium formation became evident. Moreover, increasing the initial HEPT derivative concentration by 4–5-fold frequently resulted in a decrease of the virus

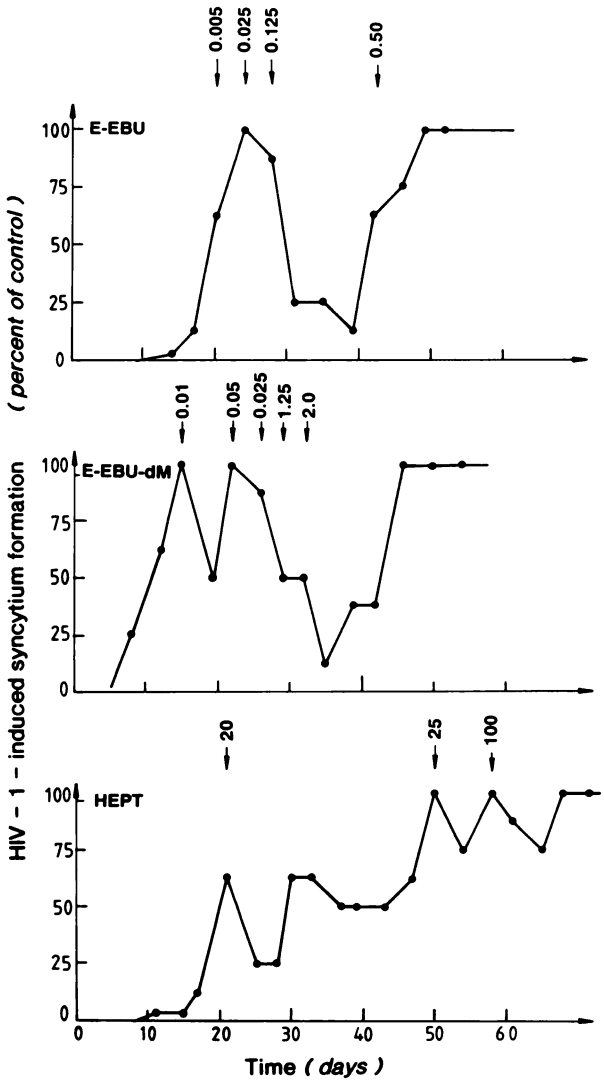

Fig. 2. Selection of mutant HIV-1 strains resistant to HEPT and its derivatives E-EBU and E-EBU-dM. *Arrows*, time points during the selection procedure where the compound concentration (μg/ml) was increased.

titer, requiring again several additional subcultivations before abundant syncytium formation occurred. In contrast to the other compounds, which led to the emergence of drug-resistant virus within ~20–30 days (31) (data not shown), HEPT and its derivatives gave rise to resistance against the highest concentrations of the test compounds only after ~50–60 days (Fig. 2).

Anti-HIV-1 activity of HEPT derivatives. Several HEPT derivatives were evaluated for their inhibitory effect on HIV-1(III_B) replication in human T4 lymphocyte CEM cells (Table 1). The prototype compound HEPT inhibited HIV-1-induced cytopathicity in CEM cells at an EC_{50} of 5 μg/ml. However, a number of HEPT derivatives in which the base part and the aliphatic side chain were modified (Fig. 1) proved to be active against HIV-1 at concentrations that were at least 1000-fold lower (EC_{50}, 0.003–0.005 μg/ml) than that of HEPT. The HEPT derivatives were 2–10-fold more active against HIV-1 *in vitro* than were the other HIV-1-specific inhibitors (i.e., TIBO R82913, nevirapine, pyridinone, BHAP, and TSAO).

Inhibitory effect of HEPT derivatives on HIV-1 mutant strains selected for resistance against other HIV-1-specific inhibitors. A number of mutant HIV-1 strains containing an amino acid change in their RT at position 100 (HIV-1/BHAP), 103 (HIV-1/TIBO), 106 (HIV-1/Nev), 138 (HIV-1/TSAO-m^3T), or 181 (HIV-1/Pyr) were examined for their sensitivity to the HEPT derivatives and other HIV-1-specific inhibitors. The HEPT derivatives were 6–20-fold less active against HIV-1/BHAP, 7–190-fold less active against HIV-1/TIBO, 10–30-fold less active against HIV-1/Nev, 3–40-fold less active against HIV-1/TSAO-m^3T, and 30–130-fold less active against HIV-1/Pyr (Table 1). Given the high potency of the HEPT derivatives against HIV-1(III_B), the HEPT derivatives remained markedly active against the mutant HIV-1 strains, with their EC_{50} values being well below 0.5 μg/ml in virtually all cases.

Striking differences were noted in the inhibitory effects of the other HIV-1-specific inhibitors on the HIV-1 mutant strains. For example, the BHAP-resistant HIV-1 strains (containing the 100-Leu→Ile mutation) were highly sensitive to nevirapine and TSAO (EC_{50}, 0.04–0.08 μg/ml) but only partially sensitive to TIBO R82913 and pyridinone (~14-fold reduction in sensitivity). The nevirapine-resistant HIV-1 strain (containing the 106-Val→Ala mutation) was co-resistant to TSAO (EC_{50}, 1.9–27 μg/ml) and partially sensitive to the other HIV-1-specific inhibitors (25–35-fold reduction in sensitivity). The TIBO-resistant HIV-1 strain (containing the 103-Lys→Asn mutation) was resistant to nevirapine and BHAP (EC_{50}, >1.5 μg/ml) and less sensitive to TSAO (4–7-fold) and pyridinone (70-fold). The TSAO-m^3T-resistant HIV-1 strain (containing the 138-Glu→Lys mutation) retained full sensitivity to BHAP and nevirapine but showed a 15–25-fold decreased sensitivity to TIBO and pyridinone. Finally, the pyridinone-resistant HIV-1 strain (containing the 181-Tyr→Cys mutation) proved resistant to TIBO, nevirapine, and TSAO (EC_{50}, 2.0 to >50 μg/ml) and partially sensitive to BHAP (EC_{50}, 0.4 μg/ml) (Table 1). Thus, the HIV-1-specific inhibitors show differential activity against the HIV-1 mutant strains, depending on the nature of the amino acid substitutions in the RT.

Drug sensitivity of HIV-1 mutant strains selected for resistance to HEPT, E-EBU, and E-EBU-dM. HIV-1 mutant strains were selected for resistance to three different HEPT derivatives (i.e., HEPT, E-EBU, and E-EBU-dM). As a rule, the three HIV-1 mutant strains (designated HIV-1/HEPT, HIV-1/E-EBU, and HIV-1/E-EBU-dM, respectively) proved to be highly resistant to all HEPT derivatives tested (Table 2). E-EBU-dM was inhibitory to the HIV-1 mutant strains at an EC_{50} of 0.8–1.4 μg/ml, that is, at a 270–360-fold higher concentration than required to inhibit wild-type HIV-1(III_B) replication. E-EPU, E-EBU, and E-EPU-S even had 500–5000-fold decreased antiviral activity against the HIV-1 mutant strains.

When the other HIV-1-specific inhibitors were evaluated for their activity against the HIV-1/HEPT, HIV-1/E-EBU, and HIV-1/E-EBU-dM strains, marked cross-resistance was observed to virtually all compounds (EC_{50}, 2.0 to >50 μg/ml). Only BHAP showed some inhibitory activity against the HIV-1/HEPT strain (EC_{50}, 0.5 μg/ml), and BHAP and pyridinone were inhibitory to HIV-1/E-EBU and HIV-1/E-EBU-dM at an EC_{50} of 0.1 μg/ml (Table 2).

Determination of the nucleotide sequence of the RT gene of the HIV-1 mutant strains selected for resistance to the HEPT derivatives. The specific nucleotide sequence of the first part of the RT gene (nucleotides 150–810) of HIV-

TABLE 1

Antiviral activity of HIV-1-specific inhibitors against mutant HIV-1 strains selected for resistance against several HIV-1-specific inhibitors

Data are the mean ± standard deviation of two to five independent experimental determinations.

Compound	EC_{50}					
	HIV-1 (III_B)	HIV-1/BHAP (100-Leu→Ile)[a]	HIV-1/Nev (106-Val→Ala)	HIV-1/TSAO-m^3T (138-Glu→Lys)	HIV-1/Pyr (181-Tyr→Cys)	HIV-1/TIBO R82913 (103-Lys→Asn)
	μg/ml					
E-EBU-dM	0.003 ± 0.001	0.03 ± 0.01	0.03 ± 0.01	0.14 ± 0.08	0.37 ± 0.5	0.02 ± 0.01
E-EPU	0.005 ± 0.002	0.03 ± 0.0	0.08 ± 0.01	0.015 ± 0.0	0.26 ± 0.16	0.63 ± 0.15
E-EBU	0.004 ± 0.002	0.09 ± 0.02	0.12 ± 0.06	0.08 ± 0.0	0.11 ± 0.04	0.24 ± 0.14
E-EPU-S	0.004 ± 0.0	0.07 ± 0.0	0.09 ± 0.02	0.08 ± 0.0	0.53 ± 0.25	0.77 ± 0.21
HEPT	7.5 ± 0.0	40 ± 14	90 ± 85	53 ± 46		
TIBO R82913	0.02 ± 0.01	0.27 ± 0.21	0.50 ± 0.30	0.3 ± 0.0	2.0 ± 0.0	4.3 ± 1.2
Nevirapine	0.03 ± 0.03	0.04 ± 0.01	2.3 ± 0.6	0.03 ± 0.02	2.5 ± 0.58	1.5 ± 0.87
Pyridinone	0.007 ± 0.003	0.10 ± 0.09	0.08 ± 0.0	0.17 ± 0.06	≥5	0.50 ± 0.44
BHAP	0.02 ± 0.0	2.5 ± 2.3	0.40 ± 0.0		0.40 ± 0.14	1.7 ± 0.58
TSAO-m^3T	0.03 ± 0.005	0.04 ± 0.01	2.5 ± 2.3	>50	2.2 ± 1.9	0.15 ± 0.13
TSAO-e^3T	0.06 ± 0.06	0.08 ± 0.0	27 ± 21	>50	>50	0.23 ± 0.17
TSAO-T	0.03 ± 0.005	0.07 ± 0.0	1.9 ± 1.1	3.0 ± 1.7	2.0 ± 0.0	0.23 ± 0.06

[a] Amino acid mutation in the RT of the HIV-1 mutant strains.

TABLE 2

Antiviral activity of HIV-1-specific inhibitors against mutant HIV-1 strains selected for resistance against HEPT derivatives

Data are the mean ± standard deviation of two to five independent experimental determinations.

Compound	EC_{50}			
	HIV-1 (III_B)	HIV-1/HEPT (188-Tyr→His)[a]	HIV-1/E-EBU (181-Tyr→Cys)	HIV-1/E-EBU-dM (106-Val→Ala)
		μg/ml		
E-EBU-dM	0.003 ± 0.001	0.65 ± 0.21	1.1 ± 0.79	1.4 ± 0.85
E-EPU	0.005 ± 0.002	≥20	≥20	10 ± 0.0
E-EBU	0.004 ± 0.002	7.3 ± 4.6	≥20	2.0 ± 0.0
E-EPU-S	0.004 ± 0.0	≥20	≥20	10 ± 0.0
HEPT	7.5 ± 0.0	≥250	>100	≥100
TIBO R82913	0.02 ± 0.01	≥5	≥5	≥5
Nevirapine	0.03 ± 0.03	5	>50	≥50
Pyridinone	0.007 ± 0.003	1.5 ± 0.71	>10	0.10 ± 0.09
BHAP	0.02 ± 0.0	0.35 ± 0.21	9.0 ± 1.4	0.11 ± 0.08
TSAO-m^3T	0.03 ± 0.005	≥50	>50	>50
TSAO-e^3T	0.06 ± 0.06	>50	>50	>50
TSAO-T	0.03 ± 0.005	3.3 ± 0.3	6.5 ± 2.1	2.3 ± 1.8

[a] Amino acid substitution in the RT of the HIV-1 mutant strains.

1/HEPT, HIV-1/E-EBU, and HIV-1/E-EBU-dM was determined and compared with that of the corresponding wild-type gene (III_B). Different mutations were detected in the RT of the mutant virus strains (Table 2). In the HEPT-resistant HIV-1 strain, a single transition mutation of the first base (thymine to cytosine) of codon 188 was observed, leading to the amino acid change tyrosine (TAT) to histidine (CAT). The E-EBU-resistant HIV-1 strain had a transition mutation in the second base (adenine to guanine) of codon 181 of the RT gene, leading to the amino acid change tyrosine (TAT) to cysteine (TGT). The E-EBU-dM-resistant HIV-1 strain had a single transition mutation in the second base of codon 106 of the RT gene, leading to the replacement of valine (GTA) by alanine (GCA) (Table 2). No other mutations were seen in the part of the RT enzyme covering amino acids 50–270.

Inhibitory effects of test compounds on mutant HIV-1 particle-derived RT in the presence of poly(C)·oligo(dG) as the template/primer. The inhibitory effects of a number of HIV-1-specific inhibitors (i.e., the HEPT derivatives E-EBU-dM and E-EBU, nevirapine, pyridinone, BHAP, and TSAO-m^3T) on virus particle-derived RT of the mutant HIV-1/Nev, HIV-1/Pyr, HIV-1/E-EBU-dM, and HIV-1/E-EBU strains were determined and compared with wild-type HIV-1(III_B) RT (Fig. 3). Irrespective of the nature of the mutant HIV-1 strains, a close correlation was found between inhibition of RT activity by the HIV-1-specific inhibitors and their corresponding antiviral activities in cell culture against the particular mutant virus strains. The correlation coefficients for RT inhibition and antiviral activity in CEM cell cultures were 0.947, 0.849, 0.840, 0.828, and 0.971 for HIV-1/Nev (containing the 106-Val→Ala substitution), HIV-1/Pyr (containing the 181-Tyr→Cys substitution), HIV-1/E-EBU-dM (containing the 106-Val→Ala substitution), HIV-1/E-EBU (containing the 181-Tyr→Cys substitution), and wild-type HIV-1(III_B), respectively (Fig. 3). As a rule, inhibition of the HIV-1 mutant strains in cell culture by the test compounds was at least 1–2 orders of magnitude more potent than inhibition of the RT of the particular viruses.

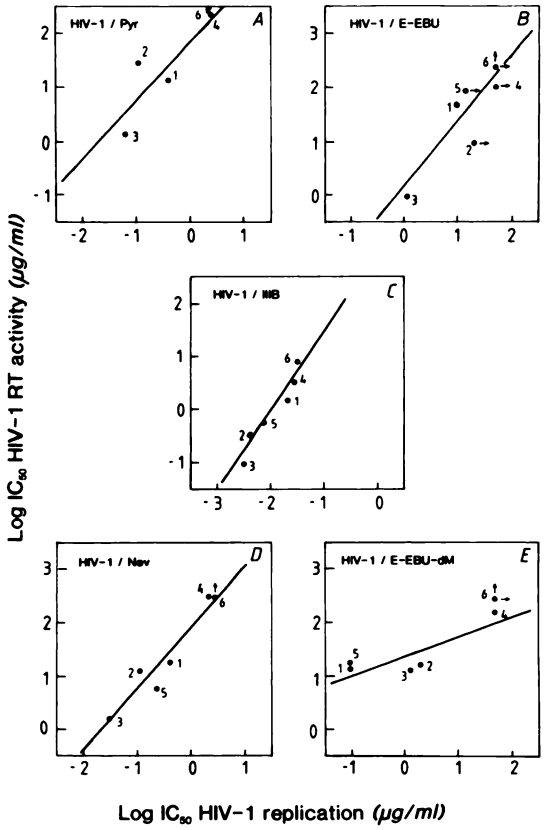

Fig. 3. Correlation between the antiviral activity of the test compounds against mutant HIV-1 replication in CEM cell cultures and their inhibitory effects on mutant HIV-1 particle-derived RT. *1*, BHAP; *2*, E-EBU; *3*, E-EBU-dM; *4*, nevirapine; *5*, pyridinone; *6*, TSAO-m^3T. A, HIV-1/Pyr; B, HIV-1/E-EBU (both mutant viruses containing the 181-Tyr→Cys change in their RT); C, HIV-1(III_B); D, HIV-1/Nev; E, HIV-1/E-EBU-dM (both mutant viruses containing the 106-Val→Ala change in their RT). The correlation coefficients in A, C, D, and E were calculated with those data points that had a well defined value for both parameters (IC_{50} for HIV-1 replication and IC_{50} for HIV-1 RT activity). The correlation coefficient for the data presented in B was calculated with all data points shown. Correct inhibition values for inhibition of HIV-1/E-EBU replication could not be obtained, due to insolubility of the test compounds.

Discussion

The HEPT, TIBO, nevirapine, pyridinone, BHAP, TSAO, and α-anilinophenylacetamide derivatives belong to structur-

ally different classes of HIV-1-specific RT inhibitors. Minor modifications of HEPT render this molecule 1000-fold more inhibitory to HIV-1-induced syncytium formation in CEM cells. The high antiviral activities of the HEPT derivatives were initially reported in human T4 lymphocyte cell lines other than CEM (i.e., MT-4) (19, 39).

The nature of the mutation present in the RT of the different HIV-1 mutant strains that were selected for resistance against several HIV-1-specific inhibitors by treatment of HIV-1-infected cell cultures with escalating drug concentrations clearly determines the magnitude of the decreased virus sensitivity to the HEPT derivatives. The 100-Leu→Ile, 106-Val→Ala, and 138-Glu→Lys mutations afford a relatively minor decrease in sensitivity, whereas the 103-Lys→Asn and 181-Tyr→Cys mutations result in a more pronounced decrease in sensitivity to the HEPT derivatives. However, all HEPT derivatives remain active against the mutant viruses at an EC_{50} ($\leq$ 0.5 μg/ml) that is easily attainable in human serum. The HIV-1-specific inhibitors (including the HEPT derivatives) possess a low toxicity profile, as also revealed by phase I/II studies in patients (40, 41). Thus, treatment of HIV-1-infected individuals with the HEPT derivatives at doses that exceed those required to inhibit the HIV-1 mutant strains *in vitro* may be a feasible and achievable goal.

Depending on the nature of the amino acid mutation in the RT, the HIV-1 strains selected in the presence of one or another of the HIV-1-specific inhibitors may show sensitivity to the other HIV-1-specific inhibitors (Table 1). These observations thus reveal different preferences of HIV-1-specific inhibitors for different amino acids in the RT enzyme. However, the 181-Tyr→Cys mutation is the only mutation that markedly affects virus sensitivity to all HIV-1-specific inhibitors. This is in agreement with previous observations (24, 25, 31).

It is striking that the HIV-1 mutant strains selected for resistance against the HEPT derivatives are cross-resistant to virtually all other HIV-1-specific inhibitors. The HEPT-, E-EBU-, and E-EBU-dM-resistant HIV-1 mutant strains showed an amino acid substitution at position 188, 181, or 106, respectively. These substitutions have been seen in other mutant HIV-1 strains selected for resistance to TIBO, pyridinone, and nevirapine, respectively. Although no other amino acid mutations were observed within the amino acid 50–270 region of the RT of the mutant viruses, the HIV-1/Nev and HIV-1/E-EBU-dM strains (both containing a single 106-Val→Ala change) and the HIV-1/Pyr and HIV-1/E-EBU strains (both containing a single 181-Tyr→Cys change) differ considerably from one another in terms of their sensitivity to the HIV-1-specific inhibitors (including the HEPT derivatives). Indeed, the HEPT derivatives were 20–100-fold less inhibitory to HIV-1/E-EBU-dM than to HIV-1/Nev and 40–200-fold less inhibitory to HIV-1/E-EBU than to HIV-1/Pyr (except for E-EBU-dM, which was only 3-fold less inhibitory). Also, BHAP and TSAO-m^3T were much more inhibitory to HIV-1/Pyr than to HIV-1/E-EBU, and TIBO, nevirapine, and TSAO-m^3T were much more inhibitory to HIV-1/Nev than to HIV-1/E-EBU-dM. It is noteworthy that a close correlation has been found between inhibition of mutant HIV-1 particle-derived RT by the different test compounds and their inhibitory effect on mutant HIV-1 replication in cell culture (Fig. 3). However, it is puzzling why apparently identical single mutations differentially affect virus sensitivity to the HIV-1-specific inhibitors. These observations might be explained by another mutation, either in the RT at a location outside the nucleotide 150–810 region or in a viral protein other than the RT. However, it is unlikely that a second mutation occurred in the RT nucleotide regions outside the nucleotide 150–810 domain, because these regions are not conserved but are highly variable in their nucleotide sequences and it has been suggested that they are not endowed with a crucial role in the catalytic RT activity. Further studies are required to clarify this issue.

The fact that different HEPT derivatives select for different mutations in the RT gene points to multiple interaction sites for this class of compounds within the RT. Direct interaction of the HEPT derivatives with the amino acids at positions 106, 181, and 188 is suggested by the present studies. In this respect, the HEPT derivatives closely mimic nevirapine, which has been reported to interact with amino acids 106, 181, and 188 (25, 26, 42, 43). This contrasts with the behavior of the TSAO derivatives, which consistently select for an amino acid change (Glu→Lys at position 138) (31–33) that is not selected for by any of the other HIV-1-specific inhibitors.

In conclusion, the HEPT derivatives retain marked activity against HIV-1 mutant strains that are resistant to other HIV-1-specific inhibitors. When directly selected for resistance to the HEPT derivatives, the HIV-1 mutant strains are resistant not only to the HEPT derivatives but also to the other HIV-1-specific RT inhibitors. From a clinical viewpoint, our observations suggest that the HEPT derivatives may be beneficial in the treatment of those HIV-1 infections that have become resistant to other HIV-1-specific inhibitors.

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