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The PETT Series, a New Class of Potent Nonnucleoside Inhibitors of Human Immunodeficiency Virus Type 1 Reverse Transcriptase†

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To identify the minimal structural elements necessary for biological activity, the rigid tricyclic nucleus of the known human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) inhibitor tetrahydroimidazobenzodiazepinthione was subjected to systematic bond disconnection to obtain simpler structures. A rational selection and testing of modeled analogs containing these potential pharmacophoric moieties led to the discovery of a new series of nonnucleoside inhibitors of RT. The lead compound of this new PETT series of nonnucleoside RT inhibitors, *N*-(2-phenylethyl)-*N*′-(2-thiazolyl)thiourea (LY73497), was found to inhibit HIV-1 but not HIV-2 or simian immunodeficiency virus in cell culture at micromolar concentrations. This derivative was also found to inhibit HIV-1 RT. Through an integrated effort involving synthesis and molecular modeling, compounds with nanomolar potency against HIV-1 in cell culture were developed. In these studies, LY300046-HCl was identified as a potent nonnucleoside inhibitor of HIV-1 RT possessing favorable pharmacokinetic properties.

Recently, a number of groups have reported on nonnucleoside inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) that may have potential for therapeutic use in AIDS chemotherapy. These derivatives exhibit high selectivity for HIV-1 RT but do not inhibit HIV-2 or any other nucleic acid polymerase. A number of such agents have been or are being investigated in clinical trials, including tetrahydroimidazobenzodiazepinthiones (TIBO) (11), dipyridodiazepinones (6), pyridinones (2), bisheteroarylpiperazines (13), and α-anilidophenylacetamides (10).

An effort was initiated to identify the minimal structural elements (the pharmacophores) of the TIBO structure necessary for biological activity. The rigid tricyclic nucleus of the TIBO derivative was dismantled by systematically disconnecting bonds in order to generate simpler structures, as shown for one example in Fig. 1. (During the course of this investigation, alternate structures derived from disconnection of the TIBO nucleus were reported [16].) These potential pharmacophoric moieties were then subjected to substructure and similarity searches through the organic compound files of the Lilly Research Laboratories. Some 250 compounds were identified by this approach and submitted for testing against HIV. By process, LY73497 (compound 2; *N*-(2-phenylethyl)-*N*′-(2-thiazolyl)thiourea) was identified as the lead compound in this novel PETT series of nonnucleoside RT inhibitors. Through an integrated effort involving chemical synthesis, molecular modeling, and biological evaluation, modification of the lead structure led to compounds with nanomolar potency against HIV-1 in cell culture. In these studies, LY300046-HCl (Fig. 2; compound 24) was identified as a potent nonnucleoside inhibitor of HIV-1 RT and selected for clinical evaluation.

MATERIALS AND METHODS

Chemistry. Zidovudine (AZT; Wellcome) was purchased from Sigma Chemical Company, St. Louis, Mo. Didanosine (ddI; Bristol Myers Squibb) and Zalcitabine (ddC; Hoffman-LaRoche) were obtained from Raylo Chemicals. The 9-Cl-TIBO derivative (compound 1, R82913; Janssen) was purchased from PharmaTech International Inc. Nevirapine (BI-RG-587; Boehringer Ingelheim) and L-697,661 (Merck) were synthesized in accordance with published procedures (4, 14). The full details of the synthesis and molecular modeling methods used in the preparation and design of thiourea derivatives 2 to 24 in Table 1 will be published elsewhere.

Viruses and cell cultures. The standard laboratory isolate of HIV-1 strain HTLV-III$_B$ was obtained from R. C. Gallo via Professor B. Wahren, SMI, Stockholm, Sweden. Clinical isolates were obtained from J. Albert, SMI, and were from patients in the Stockholm area. These clinical isolates were not characterized with respect to the treatment history of the patients from whom they were obtained. Purified HIV-2 (strain LAV-II) was obtained from R. Kurth, Paul Ehrlich Institute, Langen, Germany. Simian immunodeficiency virus was obtained from H. McClure and P. Fultz, Yerkes Regional Primate Research Center, Atlanta, Georgia and used at the Karolinska Institute (B. Oberg).

Mutant clone 118 contains an Ile instead of a Leu at position 100 of the HIV-1 strain HTLV-III$_B$ RT, and mutant clone 22 contains an Ile instead of a Leu at position 100 and a His instead of a Tyr at position 188 of the HIV-1 strain HTLV-III$_B$ RT. Mutant clone T3-16 was constructed to contain a Cys instead of a Tyr at position 181 in the HIV-1 strain HTLV-III$_B$ RT. The clones have been previously described by Balzarini et al. (1) and Zhang et al. (18, 19).

The MT-4 and MT-2 cell lines (3) were maintained in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. Human peripheral blood lymphocytes (PBL), prepared from HIV-1-seronegative donors by Ficoll-Hypaque gradient centrifugation, were obtained from J. Albert. The PBL were maintained in RPMI 1640 medium containing 10% fetal calf serum and antibiotics.

HIV cell culture antiviral assays. The standard antiviral test utilized an acute infection of MT-4 or MT-2 cells with wild-type HIV-1 strain HTLV-III$_B$ in 96-well microtiter plates using 5 to 10 50% tissue culture-infective doses of virus per well (2×10^4 cells per well) in the presence or absence of potential inhibitors. Antiviral activity was assessed by measuring the protection provided by the inhibitor against the cytopathic effect (CPE) of viral infection. Viability of cells was determined with XTT vital dye. Standard positive controls included AZT and 9-Cl-TIBO (R82913). Initially, test compounds were analyzed in a rapid-

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† This report is dedicated to the memory of Gerald B. Grindey.

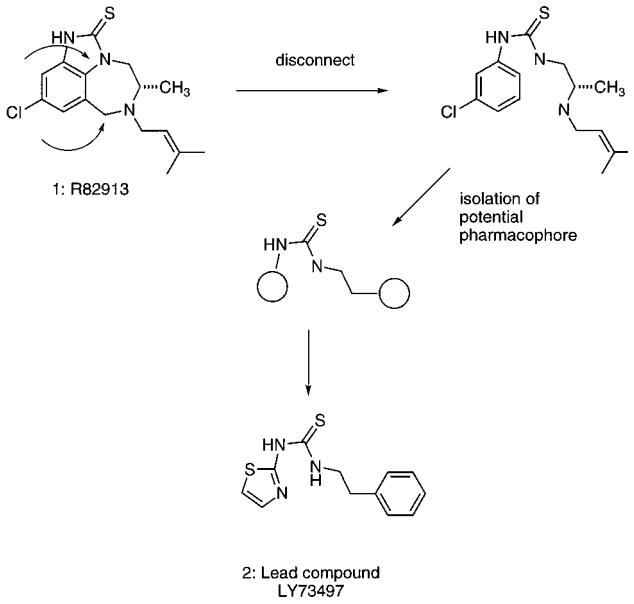


FIG. 1. Dismantling of the rigid tricyclic nucleus of the TIBO derivative to generate a simpler structure.

screen mode that utilized several standard concentrations. Subsequently, the most active derivatives were compared in parallel and a 50% effective dose (ED_{50}) was determined. The ED_{50} and 90% effective dose (ED_{90}) were defined as the inhibitor concentrations at which the viability of the culture was 50 and 90%, respectively, of that obtained in the absence of virus. The 50% cytotoxic dose of an inhibitor (CD_{50}) was assessed in the same experiment by identifying the highest concentration at which the viability of the (protected) infected culture was reduced to 50% of that in the absence of virus. The selectivity index was obtained by dividing the CD_{50} of the inhibitor by the ED_{50}.

PBL were stimulated with 2.5 μg of phytohemagglutinin per ml for 3 days, seeded into 96-well microtiter plates (2×10^4 cells per well), and infected with 10 to 50 50% tissue culture-infective doses of HIV-1 patient isolates per well. Interleukin 2 (10 μg/ml) was present in the medium throughout the assay. On day 6, the medium was removed and replaced with fresh medium. On day 10, the supernatants were assayed for HIV-1 p24 antigen by an antigen capture enzyme-linked immunosorbent assay.

Cell-cell spread assay. MT-2 cells infected with HIV-1 strain $HTLV\text{-}III_B$ or HIV-1 patient isolates showing a >5% CPE were collected, centrifuged, and resuspended in fresh medium (RPMI) to 4×10^2 cells per ml. The infected cells were mixed with fresh MT-2 cells (4×10^5/ml) to give a ratio of 1:1,000 and seeded into microplates (2×10^4/well). Compounds diluted in medium were added, and the cultures were maintained until the infected, untreated control showed a >75% CPE (usually 6 or 7 days). Cell survival was measured with the XTT colorimetric assay described above.

In vitro polymerase assays. Synthetic template-primers $(rA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$, and $(dC)_n \cdot (dG)_{12-18}$ were purchased from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. 16S and 23S rRNA templates were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. A specific 15-base deoxyribonucleotide primer (5′-TAACCTTGCGGCCGT-3′) was synthesized by standard procedures. Calf thymus DNA polymerase alpha and calf liver DNA polymerase gamma were from P-L Biochemicals Inc., Milwaukee, Wis. Activated calf thymus DNA was prepared by treating calf thymus DNA with pancreatic DNase. The assay methods for these cellular enzymes have been described by Parker et al. (9).

Tritium-labeled deoxyribonucleotides dGTP and dTTP were obtained from Amersham. The specific activities were 37 and 110 Ci/mmol, respectively. The four deoxyribonucleoside triphosphates (dATP, dCTP, dTTP, and dGTP) and ddGTP were purchased from Sigma Chemical Company.

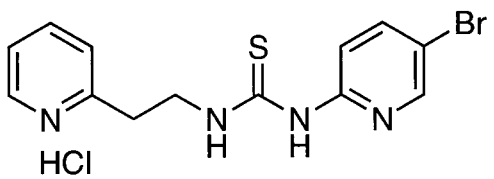


FIG. 2. Structure of LY300046 hydrochloride.

Construction of expression systems and preparation and purification of wild-type HIV-1 RT and mutated recombinant HIV-1 RT from *Escherichia coli* has been described previously (8, 15). The mutant enzymes prepared were HIV-1 RTs with Leu-100→Ile, Tyr-181→Cys, and Tyr-188→His. HIV-2 RT was assayed by using strain LAV-II virus particles disrupted in 50 mM Tris HCl (pH 7.6)–35 mM KCl–4 mM EDTA–1.3% Triton X-100.

The compounds were tested for direct inhibitory activity on recombinant HIV-1 RT in a volume of 100 μl. The RT assay using homopolymeric template-primers $(rA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$, and $(dC)_n \cdot (dG)_{12-18}$ contained 100 mM Tris HCl (pH 7.8), 100 mM KCl, 4 mM dithiothreitol, 4 mM $MgCl_2$, and 250 μg of bovine serum albumin per ml. The template-primer concentrations were saturated at 100 ng/ml, 2.5 μg/ml, and 20 μg/ml, respectively. The dimethyl sulfoxide concentration in the assays, originating from dissolved inhibitors, was 1%.

The assay conditions using the heteropolymeric template-primers, 10 μg of 16S and 23S rRNA (from *E. coli*) per ml, and 125 μg of activated calf thymus DNA per ml were the same as for the homopolymeric template-primers, except that 50 mM KCl and 6 mM $MgCl_2$ were used for the RNA template and 100 mM KCl and 20 mM $MgCl_2$ were used for the activated DNA template. The four deoxynucleoside triphosphates were used as substrates. dATP, dCTP, and dTTP were at saturated concentrations (16 to 17 μM), and tritium-labeled dGTP was used at about the K_m value. All RT-catalyzed reactions were carried out in the linear range.

The reactions were started by addition of enzyme to final concentrations of 250 ng of wild-type RT per ml, 300 ng of Ile-100 RT per ml, 200 ng of Cys-181 RT per ml, and 37.5 ng of His-188 RT per ml, except when using $(rA)_n \cdot (dT)_{12-18}$ as the template-primer, when 100 ng of wild-type RT per ml and 200 ng of each mutant RT per ml were used to achieve significant incorporation.

After 30 min of incubation at 37°C, 45 μl of each reaction mixture was spotted onto filter discs, washed in 5% trichloroacetic acid–ethanol, dried, and counted in scintillation fluid.

Pharmacokinetic studies. (i) Animals and sample collection. All chemicals used for pharmacokinetic studies were reagent grade, and the solvents used were high-pressure liquid chromatography (HPLC) grade (Burdick & Jackson). All glassware and the polycarbonate ultracentrifugation tubes were silylated by gas-phase silylation with hexamethyldisilazane. Water used in drug analysis was purified with a Waters Milli-Q purification system.

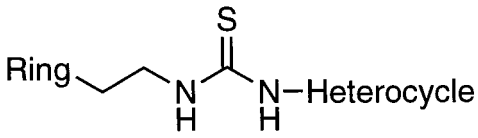
Male Fischer 344 rats weighing 220 ± 20 g were obtained from Harlan Sprague-Dawley. Animals were fasted overnight prior to drug administration. Food was provided approximately 1 h after drug administration, and water was available throughout the study. Animals were administered a single 20-mg/kg oral dose (calculated as free base equivalents) of LY300046-HCl suspended in 10% acacia (4 ml/kg). Brains and whole blood were removed at 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 12 h postadministration (one sample per animal and three animals per time point). Blood samples were collected in heparinized syringes and centrifuged at ca. $3,000 \times g$ for 5 min. Plasma and brain samples were stored at −20°C until the time of analysis.

(ii) Plasma analysis. To a 0.5-ml aliquot of plasma were added 0.5 ml of buffer (ammonium acetate, pH 7, 50 mM) and 30 μl of internal standard solution (10 μg of LY293434 [compound 6] per ml). The mixture was added to 100-mg C_{18} cartridges (Analytichem) conditioned with 1 column volume of methanol, 1 column volume of water, and 1 column volume of buffer. The cartridges were washed with 1 ml each of water and buffer, and the drug was eluted with 0.5 ml of elution solvent (75:25 methanol-buffer ratio with 0.05% trifluoroacetic acid added). Aliquots (100 μl) of the extract were injected into a Varian 5000 HPLC apparatus equipped with a Kratos 783 UV detector and a Zorbax Rx C_8 column (4.6 by 250 mm, 5-μm particle size). A mobile phase of ammonium acetate (50 mM, pH 7)-acetonitrile (50:50) was used with a flow rate of 1.5 ml/min and a column temperature of 30°C. Detection was accomplished by measuring UV absorption at 268 nm. Peak heights were measured with a PE-Nelson Access*Chrom data system, and drug concentrations were determined by linear regression using an eight-point standard curve covering the range of 0.05 to 10 μg/ml.

(iii) Brain analysis. Brain samples (0.5 g) were homogenized with 2.5 ml of acetonitrile containing 0.02% trifluoroacetic acid in a Tekmar Tissumizer. Hexane (2.5 ml) was added to the homogenate, and the contents were mixed and then centrifuged at 2,400 rpm for 5 min. The hexane layer was discarded, and the acetonitrile layer was removed and concentrated to dryness under a gentle stream of nitrogen at ca. 45°C. The residue was redissolved in 50 μl of 50% methanol in water followed by 0.5 ml of water and 0.5 ml of buffer. The extract was further purified by solid-phase extraction as described above for plasma. Aliquots of the extracts (50 μl) were analyzed by reverse-phase HPLC with a Jones Chromatography C8 column (5-μm particle size; 250 by 4.6 mm). The mobile phase and flow rate were as described above for plasma. Concentrations were determined by linear regression with a seven-point standard curve covering the range of 100 to 10,000 ng/g.

Protein-binding studies. Protein binding was assessed in rat and human plasma samples at a concentration of 2 μg/ml by using an ultracentrifugation technique. Briefly, 1 ml of plasma was gently mixed with 2 μg of LY300046-HCl dissolved in 40 μl of methanol. The tubes were gently mixed, and a 200-μl aliquot

TABLE 1. Anti-HIV-1 activities of thiourea derivatives 2 through 24



Compound no.	Ring	Heterocycle	ED_{50} (μM)	CD_{50} (μM)	Selectivity index
2[a]	Phenyl	2-Thiazole	1.33	380	286
3	Phenyl	2-(4-Methylthiazole)	0.50	360	720
4	Phenyl	2-(4-Ethylthiazole)	0.24	343	1,429
5	Phenyl	2-Benzothiazole	3.19	319	100
6	Phenyl	2-Pyridine	0.78	389	499
7	Phenyl	2-(5-Methylpyridine)	0.17	368	2,165
8	Phenyl	2-(5-Chloropyridine)	0.03	51	1,700
9	Phenyl	2-(5-Bromopyridine)	0.03	271	9,033
10	3-Methoxyphenyl	2-Thiazole	0.68	34	50
11	1-Cyclohexenyl	2-Thiazole	0.33	374	1,133
12	2-Fluoro-6-methoxyphenyl	2-Thiazole	0.32	48	150
13	2,5-Dimethoxyphenyl	2-Thiazole	0.28	309	1,104
14	3-Bromo-6-methoxyphenyl	2-Thiazole	0.10	107	1,070
15	2,6-Difluorophenyl	2-Thiazole	0.04	84	2,100
16	2,6-Difluorophenyl	2-(4-Methylthiazole)	0.025	128	5,120
17	2,6-Difluorophenyl	2-(4-Ethylthiazole)	0.030	304	10,133
18	2,6-Difluorophenyl	2-Pyridine	0.01	341	34,100
19	2,6-Difluorophenyl	2-(5-Methylpyridine)	0.33	325	985
20	2,6-Difluorophenyl	2-(5-Chloropyridine)	0.002	305	152,500
21	2,6-Difluorophenyl	2-(5-Bromopyridine)	0.005	269	53,800
22	2-Pyridyl	2-(4-Ethylthiazole)	0.34	103	303
23	2-Pyridyl	2-(5-Chloropyridine)	0.017	68	4,000
24[b]	2-Pyridyl	2-(5-Bromopyridine)	0.016	87	5,438

[a] LY73497.
[b] LY300046.

was transferred to silylated centrifuge tubes, incubated at 37°C for 30 min, and then centrifuged at 436,000 × *g* for 4 h at 37°C in a Beckman TL-100 ultracentrifuge with a TLA-100 rotor. Aliquots of the supernatant (100 μl) were removed and analyzed directly by reverse-phase HPLC with UV absorption detection as described for plasma.

RESULTS

Chemical structure-activity relationships. Following an investigation of the molecular scaffolding of the rigid tricyclic TIBO nucleus, over 250 compounds from the organic files of the Lilly Research Laboratories were identified through substructure and similarity searches as potentially possessing the minimal structural requirements of the TIBO nucleus necessary for biological activity. These derivatives were analyzed by the rapid-screen mode for activity against HIV. One of these compounds, LY73497 (compound 2), was found to inhibit HIV-1 in MT-4 cells with an ED_{50} of 1.33 μM. This derivative was relatively noncytotoxic, with a 50% cytotoxic dose greater than 380 μM, resulting in a selectivity index greater than 286. The anti-HIV-1 activity of the lead compound was confirmed by its 50% inhibition of wild-type HIV-1 RT at 0.95 μM with $(rC)_n \cdot (dG)_{12-18}$ as the template-primer. The compound was not active at 100 μM against HIV-2 or simian immunodeficiency virus (data not shown). In addition, LY73497 was not active at 100 μM against herpes simplex virus, cytomegalovirus, influenza virus, hepatitis B virus, rhinovirus, leukemia virus, or polio virus (data not shown).

An extensive chemical structure-activity relationship study based on this lead structure was performed with over 700 compounds. Representative examples from this study are presented in Table 1. Significant increases in antiviral activity were obtained for a number of derivatives. Compounds 20, 21, 23, and 24 (LY300046-HCl) were 78 to 665 times more active than LY73497 in this assay. The cytotoxic effects of these derivatives on MT-4 cells occurred at significantly higher concentrations, resulting in selectivity indices of 4,000 to 152,500. The effect of LY300046-HCl on the replication of HIV-1 strain $HTLV\text{-}III_B$ in MT-4 cell culture is compared with those of various reference compounds in Table 2. The results show that LY300046-HCl had a lower ED_{50} than the other nonnucleoside compounds (9-Cl-TIBO, L-697,661, and nevirapine), ddC, and ddI. However, in this cell line, AZT was comparable in activity to LY300046-HCl. The concentration of LY300046-HCl required to inhibit the growth of MT-4 cells was about 5,000-fold greater than the concentration that inhibited replication of HIV-1. Consequently, LY300046-HCl had a high selectivity index in this in vitro system.

Activity of LY300046-HCl in cell cultures. Antiviral activity against various clinical isolates of HIV-1 in MT-2 cells is summarized in Table 3. The average ED_{50} of LY300046-HCl against 15 different clinical isolates in MT-2 cells was 0.024 μM, which was considerably lower than that of 9-Cl-TIBO, L-697,661, nevirapine, AZT, ddI, or ddC. The average ED_{50} of AZT against 15 different isolates was only 1.2 μM. However, these isolates were not characterized with respect to the treatment history of the patients from which they were obtained. Therefore, some of the isolates may have been resistant to AZT because of treatment of the patients with this drug.

TABLE 2. Antiviral activities of LY300046-HCl and reference compounds in MT-4 cells infected with HIV-1 strain HTLV-III_B

Compound	Mean ED_{50} (μM) ± SEM	Mean ED_{90} (μM) ± SEM	Mean CD_{50} (μM) ± SEM	Selectivity index
LY300046-HCl	0.016 ± 0.006	0.13 ± 0.05	87 ± 7	5,437
9-Cl-TIBO (R82913)	0.21 ± 0.05	0.65 ± 0.25	47 ± 17	223
L-697,661	0.10 ± 0.03	0.38 ± 0.12	59 ± 31	590
Nevirapine (BI-RG-587)	0.13 ± 0.05	0.57 ± 0.23	156 ± 76	1,200
AZT	0.016 ± 0.01	0.08 ± 0.08	>100	>1,250
ddI	2.6 ± 7.5	29 ± 53	>600	>20
ddC	0.4 ± 0.3	2.3 ± 2.3	202 ± 168	505

The antiviral activity in human PBL infected with clinical isolates of HIV-1 is given in Table 3. The average ED_{50} of LY300046-HCl against 13 different clinical isolates was 0.05 μM, which was similar to those of 9-Cl-TIBO, L-697,661, nevirapine, AZT, and ddC. ddI was approximately 38-fold less active than LY300046-HCl in this assay.

The ability of LY300046-HCl to inhibit the spread of HIV-1 infection in MT-2 cells is also summarized in Table 3. LY300046-HCl had an average ED_{50} of 0.06 μM for the clinical isolates. These values were lower than those of AZT and the other compounds tested.

Activity of LY300046-HCl against resistant isolates. HIV-1 Ile-100, Cys-181, and Ile-100–His-188 RT mutants were used to analyze the antiviral effect of LY300046-HCl in cell culture as shown in Table 4.

Each of the resistant isolates was less susceptible than was the wild-type virus to inhibition by the nonnucleoside compounds. In general, there was cross-resistance between the other nonnucleoside compounds and LY300046-HCl. However, LY300046-HCl was the most active nonnucleoside derivative against these mutants. There was no cross-resistance between the nonnucleoside and nucleoside derivatives, as these viruses were still susceptible to AZT, ddI, or ddC.

Serial passage of HIV-1 strain HTLV-III_B in MT-4 cells in the presence of increasing concentrations of LY300046-HCl selected HIV-1 with the RT mutations Glu-101–Ile-108, Arg-103, Arg-103–Asp-179, and Asp-179–Cys-181 (data not shown).

Inhibition of wild-type and resistant RTs. The 50% inhibitory concentrations (IC_{50}s) of LY300046-HCl and reference compounds were determined for wild-type and mutant RT enzymes that contained the mutations found in viruses with decreased susceptibility to inhibition by LY300046-HCl in cell culture, namely, Ile-100, Cys-181, and His-188. The results of these experiments with the rCdG template-primer pair are shown in Table 5. In general, wild-type RT was considerably more susceptible to inhibition by LY300046-HCl than to inhibition by 9-Cl-TIBO, L-697,661, and nevirapine. The Ile-100, Cys-181, and His-188 mutant enzymes were also more susceptible to inhibition by LY300046-HCl than to inhibition by the reference compounds. However, each of the mutant enzymes was less susceptible to inhibition by the nonnucleoside compounds than was the wild-type enzyme. By contrast, the mutant and wild-type enzymes were equally susceptible to the nucleoside triphosphate ddGTP. These results were consistent with cell culture experiments that demonstrated that HIV-1 stocks containing these mutations were less susceptible to inhibition than was the wild-type virus.

Inhibition of other polymerases. The ability of LY300046-HCl, 9-Cl-TIBO, L697,661, nevirapine, and ddGTP to inhibit HIV-2 RT and cellular DNA polymerases was investigated in vitro. The IC_{50}s of these derivatives were determined for HIV-2; purified human DNA polymerases α, β, and γ; purified calf thymus DNA polymerase α; and calf liver DNA γ. The IC_{50} of LY300046-HCl for each of these polymerases was greater than 270 to 500 μM, which should be compared to the value of 0.027 μM obtained for HIV-1 RT when a DNA-dependent DNA polymerase activity was assayed. A similar pattern was also seen with the other nonnucleoside RT inhibitors (data not shown).

Pharmacokinetics. Male Fischer 344 rats were administered a single 20-mg/kg oral dose of LY300046-HCl. Plasma and brain samples were analyzed by solid-phase extraction followed by reverse-phase HPLC with UV detection. The estimated detection limits of the assays were 25 ng/ml in plasma and 200 ng/g in brain tissue. The peak concentration of LY300046-HCl in plasma was 3.5 μg/ml and occurred at 0.5 h after drug administration. Drug levels in plasma declined rapidly with an overall half-life (calculated from 1 to 8 h) of 1 h, and the area under the concentration-time curve was 6.9 μg/h/ml. The peak concentration of LY300046-HCl in whole brains was 2.9 μg/g, slightly less than that in plasma. Concentrations of LY300046-HCl in the brain declined rapidly after administration, and detectable drug concentrations were observed in only one of

TABLE 3. Antiviral activities of LY300046-HCl and reference compounds in MT-2 cells and human PBL cells infected with clinical isolates

Compound	Mean ED_{50} (μM) ± SEM (no. of isolates)		
	MT-2 cells	PBL cells	Cell-to-cell spread in MT-2 cells
LY300046-HCl	0.02 ± 0.04 (15)	0.05 ± 0.04 (13)	0.06 ± 0.03 (4)
9-Cl-TIBO (R82913)	0.7 ± 0.4 (15)	0.26 ± 0.37 (13)	0.5 ± 0.4 (4)
L-697,661	0.23 ± 0.17 (15)	0.43 ± 0.82 (9)	0.6 ± 0.3 (4)
Nevirapine (BI-RG-587)	0.14 ± 0.17 (15)	0.04 ± 0.02 (4)	
AZT	1.2 ± 1.7 (15)	0.2 ± 0.4 (14)	0.7 ± 0.3 (4)
ddI	6.3 ± 5.9 (15)	1.9 ± 2.5 (11)	32 ± 39 (4)
ddC	0.5 ± 0.3 (15)	0.04 ± 0.03 (15)	0.2 ± 0.1 (4)

TABLE 4. Antiviral activities of LY300046-HCl and reference compounds in MT-4 cells infected with resistant mutants[a]

Compound	ED_{50} (μM) for:			
	Wild-type HIV-1	Clone T3-16 (Cys-181 mutant)	Clone 118 (Ile-100 mutant)	Clone 22 (Ile-100–His-188 mutant)
LY300046-HCl	0.02 ± 0.005	0.7 ± 0.23	0.8 ± 0.9	7.0 ± 2.3
9-Cl-TIBO (R82913)	0.28 ± 0.12	2.8 ± 2.0	>10	>10
L-697,661	0.065 ± 0.045	>10		>10
Nevirapine (BI-RG-587)	0.13 ± 0.05	15.5 ± 8.7	1.1 ± 0.5	14 ± 17
AZT	0.01 ± 0.01	0.006 ± 0.002	0.005 ± 0.002	0.004 ± 0.001
ddI	9.1 ± 4.7	9.8 ± 6.2	7.3 ± 0.8	
ddC	0.4 ± 0.2	0.28 ± 0.06		0.06 ± 0.02

[a] Averages of at least three independent experiments ± the standard deviations are shown.

three animals at 4 and 6 h after drug administration and none thereafter. The area under the concentration-time curve was 5.9 μg/h/ml. Because of the higher LY300046-HCl detection limit in brain tissue than in plasma, an insufficient number of usable datum points were available for accurate estimation of the elimination half-life in the brain. In general, concentrations of LY300046-HCl in the brain were similar to those in plasma at all of the time points examined (Fig. 3), indicating that LY300046-HCl readily crosses the blood-brain barrier in rats.

Several putative metabolite peaks were observed in the plasma chromatograms of rats administered LY300046-HCl and were absent in control plasma samples. The two major metabolites, M1, eluting with a retention time of 2.8 min, and M2, with a retention time of 3.2 min, were generally present in concentrations less than that of the parent drug on the basis of comparison of their peak heights with that of LY300046-HCl. These metabolites were not structurally characterized.

Protein binding. The in vitro protein binding of LY300046-HCl was assessed at a concentration of 2 μg/ml in rat plasma at 37°C by an ultracentrifugation technique followed by HPLC analysis. Initial attempts with ultrafiltration indicated that LY300046-HCl bound to the ultrafiltration membrane. Subsequently, ultracentrifugation was investigated and significant nonspecific binding was also observed with the polycarbonate test tubes used with the ultracentrifugation technique. Silylation of the polycarbonate tubes reduced nonspecific binding to the centrifuge tubes to negligible levels. The mean percentages of binding of LY300046-HCl to proteins were 88.7% in rat plasma and 95.5% in human plasma at the concentration examined.

DISCUSSION

A rational investigation intended to identify the minimum molecular architecture of the TIBO structure necessary for biological activity led to the discovery of LY73497 (compound 2) as the lead compound in the novel PETT series of nonnucleoside inhibitors of HIV-1 RT. Antiviral activity of the PETT compounds was maximized by varying both the thiazole heterocycle and phenyl ring of the parent molecule as shown for some compounds in Table 1. In general, small substituents at position 4 of the 2-thiazole ring increased activity (compounds 3 and 4). Bicyclic substitution with 2-benzothiazole led to a decrease in activity (compound 5). Heterocyclic substitution with 2-pyridine gave approximately a twofold increase in activity over LY73497. Small substituents at position 5 of the 2-pyridine further increased activity by approximately 25-fold (compounds 7 to 9). Mono- and disubstitution with fluoro and methoxy of the phenyl ring of LY73497 led to compounds with improved antiviral activity (compounds 10 and 12 to 15). The 2,6-difluorophenyl ring (compound 15) was preferred, giving an almost 34-fold increase in activity over the lead compound. The activity parameters of the chemical SAR were found to be additive so that combinations of the best phenyl substituents and heterocyclic substituents led to even more potent analogs (compounds 16 to 21). Although a number of these derivatives displayed potent antiviral activity and excellent selectivity, e.g., compounds 20 and 21, they were limited by poor oral bioavailability in rodents. It was found that a 2-pyridyl moiety could be substituted for the phenyl ring with only slight loss of antiviral activity (compound 22 versus compound 4). Introduction of the preferred 5-substituted 2-pyridine heterocycle led to derivatives with potent antiviral activity (compounds 23 and 24). The hydrochloride salts of these derivatives were found to give acceptable levels in blood following oral administration to rats. In these studies, LY300046-HCl (compound 24) was selected for further evaluation.

Cell culture experiments demonstrated that LY300046-HCl was a potent inhibitor of the replication of HIV-1 in human T-cell lines and human PBL, including spread of the virus from infected to uninfected T cells. It was potent against patient isolates as well as laboratory isolates of HIV-1 but was not active against HIV-2 or simian immunodeficiency virus. Overall, it was approximately as active as AZT and more potent than 9-Cl-TIBO, L-697,661, nevirapine, ddC, and ddI. AZT is one of the most active anti-HIV compounds known, and these experiments demonstrate that LY300046-HCl is also a potent anti-HIV agent in vitro.

TABLE 5. Inhibition of wild-type and mutated RTs by LY300046-HCl and reference compounds with a poly(rC) template[a]

Compound	IC_{50} (μM); mutant/wild-type IC_{50} ratio			
	Wild-type HIV-1	Cys-181	Ile-100	His-188
LY300046-HCl	0.017 ± 0.007	2.5 ± 1.10; 147	0.43 ± 0.09; 25	0.20 ± 0.07; 12
9-Cl-TIBO (R82913)	0.20 ± 0.05	13.0 ± 7.40; 65	32.2 ± 2.8; 165	10.3 ± 7.40;51.5
L-697,661	0.06 ± 0.02	7.70 ± 2.80; 128	0.85 ± 0.68; 14	0.60 ± 0.24; 10
Nevirapine (BI-RG-587)	0.15 ± 0.04	201 ± 23.3;1,340	16.2 ± 2.0; 108	0.72 ± 0.04; 4.8

[a] Averages of at least two independent experiments ± the standard deviations are shown.

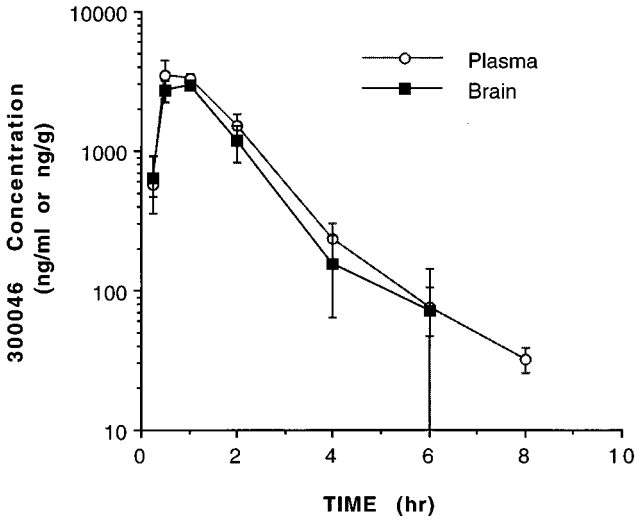


FIG. 3. Drug concentrations in the plasma and brains of male Fischer 344 rats administered a single 20-mg/kg oral dose of LY300046-HCl. Each value shown is the mean ± the standard error of the mean of three samples.

Previous research by other groups has indicated that resistance to TIBO compounds, pyridinones, and nevirapine resulted from mutations in the *pol* gene, which encodes RT (2, 8, 15, 17; for an excellent review, see reference 7). The Cys-181 mutation in clone T3-16 had been previously found to reduce susceptibility to the other nonnucleoside RT inhibitors (8). Likewise, the Leu-100 and His-188 mutations found in clones 118 and 22 have also been reported to decrease susceptibility to a number of nonnucleoside analogs (1). Experiments with these mutant viruses demonstrated that mutations in the Leu-100, Tyr-181, and Tyr-188 codons of HIV-1 RT decreased the susceptibility of the virus to inhibition by LY300046-HCl, 9-Cl-TIBO, L-697,661, and nevirapine. However, LY300046-HCl was more inhibitory to these mutant viruses than were the other nonnucleoside RT inhibitors, and elevated concentrations of LY300046-HCl could inhibit these mutant viruses. Selection of HIV-1 mutants in the presence of LY300046-HCl revealed the mutations Glu-101–Ile 108, Arg-103, Arg-103–Asp-179, and Asp-179–Cys-181. Analysis of these mutants is ongoing.

Analogous experiments were conducted for inhibition of wild-type HIV-1 RT, HIV-2 RT, calf thymus DNA polymerases, and human DNA polymerases. These studies showed that LY300046-HCl is a selective nonnucleoside inhibitor of HIV-1 RT.

The decrease in activity of LY300046-HCl, 9-Cl-TIBO, L-697,661, and nevirapine against purified HIV-1 RT containing the same mutations as found in the resistant viral strains demonstrates that these codon changes do contribute to resistance to these derivatives. These observations, along with the findings of activity against wild-type and resistant viruses, confirm that the mechanism of antiviral activity in cell culture is inhibition of RT.

Preliminary pharmacokinetic studies were also conducted with LY300046-HCl. The compound is rapidly absorbed by rats, with a peak concentration in plasma of 3.5 μg/ml following a single 20-mg/kg oral dose. Concentrations of LY300046-HCl in brain tissue paralleled those in plasma, indicating that LY300046-HCl readily crosses the blood-brain barrier. Penetration of the blood-brain barrier is a desirable property of antiviral agents, considering HIV-associated encephalopathy in AIDS patients (5). The in vitro protein binding of LY300046-HCl was 88.7% in rat plasma and 95.5% in human plasma at a concentration of 2 μg/ml.

On the basis of its simple chemical synthesis, excellent antiviral activity, satisfactory pharmacokinetic profile, and acceptable toxicity, LY300046-HCl is currently in phase I clinical trails for potential use in the treatment of AIDS. (The full details of the preclinical pharmacokinetics and toxicology of LY300046-hydrochloride will be reported elsewhere.) Efforts to discover alternate PETT derivatives with increased activity, particularly against resistant viruses, are currently under way.

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