The stereoselective targeting of a specific enzyme-substrate complex is the molecular mechanism for the synergic inhibition of HIV-1 reverse transcriptase by (R)-(−)-PPO464: a novel generation of nonnucleoside inhibitors

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Abstract

The human immunodeficiency virus type 1 (HIV-1) nonnucleoside reverse transcriptase (RT) inhibitor pyrrolopyridooxazepinone (PPO) derivative, (+/−)-PPO294, was shown to be active toward wild type and mutated HIV-1 RT and to act synergistically in combination with 3′-azido-3′-deoxythymidine (Campiani, G., Morelli, E., Fabbrini, M., Nacci, V., Greco, G., Novellino, E., Ramunno, A., Maga, G., Spadari, S., Caliendo, G., Bergamini, A., Faggioli, E., Uccella, I., Bolacchi, F., Marini, S., (1999) J. Med. Chem. 42, 4462-4470). The (+/−)-PPO294 racemate was resolved into its pure enantiomers, and the absolute configuration was determined by x-ray analysis. Only one enantiomer, (R)-(−)-PPO464, displayed antiviral activity against both the wild type and the K103N mutant HIV-1 RT and was found to interact exclusively with the reaction intermediate formed by RT complexed with both the DNA and the nucleotide substrates. Being the first compound of its class to display this behavior, (R)-(−)-PPO464 is the representative of a novel generation of nonnucleoside inhibitors. (R)-(−)-PPO464 showed significant synergism when tested in combination with other RT inhibitors and efficiently inhibited viral replication when tested against the laboratory strain HIV-1 IIIB or against either wild type or multidrug-resistant clinical isolates. Pharmacokinetic studies in mice and rats showed a more favorable profile for (R)-(−)-PPO464 than for the corresponding racemate. (R)-(−)-PPO464 was also found to easily cross the blood-brain barrier. The coadministration of the HIV-1 protease inhibitor ritonavir increased the bioavailability of (R)-(−)-PPO464, having little effect on its plasma and brain elimination rates.
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1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; PPO, pyrrolopyridoaxazepinone; TP, template-primer; xRTT, 3′-azido-3′-deoxythymidine 5′-triphosphate; HPLC, high pressure liquid chromatography; AUC, area under the concentration-time curve; SSE, sum(s) of squares of errors.
significant immune system function restoration, and clinical improvement (10). In particular, the results of clinical trials employing NNRTIs as components of highly active antiretroviral therapy in combination with NRTIs and/or PIs showed that NNRTIs often act synergistically with NRTIs and, due to positive pharmacokinetic properties, allow simplified administration schedules with a substantial improvement in patients’ adherence to treatment (11). Moreover, NNRTIs are characterized by less severe adverse effects than NRTIs or PIs (12). Several factors may restrict the selection of antiviral drugs to be used in the combination therapy, such as drug compatibilities, adverse side effects, and cross-resistance (13, 14). Therefore, the development of novel NNRTIs with improved pharmacological, pharmacokinetic, and drug resistance mutation profiles, is critical for a more successful application of NNRTIs in combination therapy. In an effort aimed to identify new lead molecules for the development of NNRTIs with high inhibitory activity against wild type and mutated RT, we have selected the PPO derivative (+)-PPO294 (1) as a promising lead compound (15). In the present work, we have characterized the mechanism of action of both (+)- and (-)-enantioomers of (+)-PPO294 against HIV-1 recombinant RT enzymes and viral isolates, either wild type or bearing the K103N mutation. Our results show that only the (-)-enantionier (R)-(-)-PPO464 (Fig. 1) retained full antizymeic and antiviral activity, acting through a distinct mechanism with respect to other NNRTIs. These novel properties make (R)-(-)-PPO464 a promising lead for the synthesis of more active third generation compounds.

MATERIALS AND METHODS

Chemistry

Melting points were determined using a Buchi melting point B-540 apparatus and were uncorrected. IR spectra were taken with PerkinElmer Life Sciences 200 and FT 1600 spectrophotometers. 1H NMR spectra were recorded on a Bruker 200-MHz spectrometer or on a Gemini Varian 200-MHz spectrometer with TMS as internal standard; the values of the chemical shifts (δ) are given in ppm, and coupling constant (J) is given in Hz.

The reactions were carried out in an argon atmosphere. TLCs were performed on silica gel plates (Riedel-de-Haën; Art.37341). Merck silica mesh) column. Yields refer to purified products and are not optimized. Organic extracts were dried over MgSO4, and the solvents were removed under reduced pressure. Optical rotations were measured at room temperature on a PerkinElmer Life Sciences 200 and FT 1600 spectrophotometers. 1H NMR spectra were recorded on a Bruker 200-MHz spectrometer or on a Gemini Varian 200-MHz spectrometer with TMS as internal standard; the values of the chemical shifts (δ) are given in ppm, and coupling constant (J) is given in Hz.

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Racemate Resolution

A solution of the racemic compound (+)-PPO294 (20 mg, 0.066 mmol) in 1 ml of hexanes/ethanol/triethylamine (95:5:0.1), after filtration on a 0.45-μm Millipore Corp. filter, was resolved at 0 °C on a semipreparative Chiralpak-AD (10 μm, 20 × 250 mm) using hexanes/ethanol/triethylamine (95:5:0.1) as eluent (5 ml/min). The effluent was monitored at λ = 304 nm, and the enantiomers (+)-PPO463 and (-)-PPO464 were eluted with retention times of 33.7 and 38.4 min, respectively. The fractions were collected and amplified to provide (+)-PPO463 (8.7 mg, 87%, 100% enantiomeric excess) and (-)-PPO464 (8.9 mg, 89%, 99.3% enantiomeric excess) as colorless liquids. The purity of the enantiomers was evaluated by analytical HPLC using the same mobile phase (1 ml/min) (retention times: (+)-PPO463 peak, 8.0 min; (-)-PPO464 peak, 8.7 min). Solutions collected were dried with a vacuum pump, since an oily residue was obtained. (+)-PPO463 (-)-PPO464. 1H NMR (CDCl3) δ 1.10 (t, 3H, J = 7.0 Hz), 2.45 (q, 2H, J = 7.1 Hz), 6.49 (m, 1H, 7.03 (m, 1H), 7.10–7.30 (m, 5H), 7.37 (m, 1H), 7.51 (m, 1H), 7.94 (m, 1H), 8.07 (m, 1H). MS (EI) m/z: 304 (M+). (+)-PPO463: [α]22° = +56.7 (c = 0.56 (CHCl3)); (-)-PPO464: [α]22° = −56.5 (c = 0.57 (CHCl3)). Anal. Calcd. for C21H19N2O2: C, 74.98; H, 5.30; N, 9.20. Found: for (+)-PPO464, C, 75.40; H, 6.65; N, 8.96; for (-)-PPO464, C, 75.07; H, 6.05; N, 9.07.

Synthesis of (−)-9-Bromo-6-ethyl-6-phenylpyrrolo[1,2-d]pyrido[3,2,1-f,1]4-oxazepin-7(6H)-one, (−)-PPO600

To a solution of (+)-PPO463 (65 mg, 0.214 mmol) in anhydrous tetrahydrofuran (5 ml) cooled at −70 °C was added N-bromosuccinimide (38 mg, 0.214 mmol). The reaction mixture was stirred vigorously for 1.5 h at −70 °C and then was gradually warmed to room temperature. After 16 h at room temperature, the solvent was removed in vacuo, and the residue was taken up in dichloromethane; the organic layer was washed with brine, dried, and concentrated. The crude product was purified by flash chromatography using dichloromethane/hexane (8:2) as eluent. Recrystallization from hexane gave 31 mg (38%) of pure (−)-PPO600 as colorless prisms (mp 155–156 °C). IR (CHCl3) 1654 cm−1; 1H NMR (CDCl3) δ 1.08 (t, 3H, J = 7.0 Hz), 2.42 (q, 2H, J = 7.1 Hz), 7.04 (dd, 1H, J = 7.5, 4.8 Hz), 7.19 (m, 5H), 7.35–7.43 (m, 2H), 7.94 (m, 1H), 8.94 (m, 1H). MS (EI) m/z (%): 382/384 (M+), 282/284 (100). [α]22° = −17 (c = 3.2 (CHCl3)). Anal. Calcd. for C15H12N2BrO2: C, 59.55; H, 3.95; N, 7.31. Found: C, 59.37; H, 3.72; N, 7.18.

X-ray Crystallography

Single crystals of (−)-PPO600 were obtained by dissolving 20 mg in methanol and allowing the solution to concentrate at room temperature. A colorless single crystal of (−)-PPO600 was used for X-ray data collection on a Siemens P4-four-circle diffractometer with graphite monochromated Mo-Kα radiation (λ = 0.71069 Å). Lattice parameters were determined by least-squares refinement on 47 randomly selected and automatically centered reflections. The a2θ scan technique was used in the data collection in the 4 ≤ 2θ ≤ 50° scan range.

Crystal system: orthorhombic; space group: P212121; a = 8.11(1), b = 13.965(2), c = 15.085(2) Å, V = 1708.9(4) Å3, Z = 4, Dc = 1.490 g/cm3. 3469 reflections were collected at 22 °C, of which 3004 are unique (Rint = 0.07). Absorption correction obtained by a2θ scans was applied. The structure was solved by direct methods implemented in the SHELX-97 program (39). The refinement was carried out by full matrix anisotropic least-squares on F2 for all reflections for nonhydrogen atoms by using the SHELX-97 program (39).

The absolute configuration of the chiral center at the pyridooxazine nucleus was made by the refined Flack parameter (16). It resulted as S.

The final refinement converged to R1 = 0.078, wR2 = 0.129 for I > 2σ(I), goodness of fit = 1.02. Minimum and maximum height in last Δρ map of −0.58 and 0.47 eÅ−3. On the basis of the stereochemistry of (−)-PPO600 was assigned the stereochemistry of (−)-PPO463 and of (−)-PPO464 (i.e. (S)- and (R)-, respectively).

Chemicals

[1H]dTMP (40 Ci/mmol) was from Amersham Pharmacia Biotech, and unlabeled dNTPs were from Roche Molecular Biochemicals. Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and purchased from Merck or Fluka.

Nucleic Acid Substrates

The homopolymer poly(a) (Amersham Pharmacia Biotech) was mixed at weight ratios in nucleotides of 10:1, to the oligomer oligo(dT)12-18 (Amersham Pharmacia Biotech) in 20 mM Tris-HCl (pH
Expression and Purification of Recombinant HIV-1 RT Forms

Recombinant RT was expressed and purified to >95% purity as described (21) and had a specific activity on polynucleotide polymerase activity corresponding to the incorporation of 1 nmol of dNMP into acid-precipitable material in 60 min at 37 °C.

HIV-1 RT RNA-dependent DNA Polymerase Activity Assay

RNA-dependent DNA polymerase activity was assayed as follows; a final volume of 25 μl contained buffer A (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.2% bovine serum albumin, 4% glycerol), 10 mM MgCl₂, 0.5 μg of polynucleotide polymerase activity (TP) (corresponding to 0.3 μM 3′-OH ends), 10 μM [3H]dTPP (1 Ci/mmol), and 2–4 nm RT. Reactions were incubated for 10 min at 37 °C. 20-μl aliquots were then spotted on GF/C glass fiber filters, which were immediately immersed in 5% ice-cold trichloroacetic acid. Filters were washed twice in 5% ice-cold trichloroacetic acid and once in ethanol for 5 min and dried, and acid-precipitable radioactivity was quantitated by scintillation counting.

Inhibition Assays

Reactions were performed under the conditions described for the HIV-1 RT RNA-dependent DNA polymerase activity assay. Incorporation of radioactive dTPP into polynucleotide polymerase activity at different concentrations of DNA or dNTP was monitored in the presence of increasing amounts of inhibitor as indicated in the figure legends. Data were then plotted according to Dixon.

Kinetics of (R)-(−)-PPO464 Binding

HIV-1 RT (20–40 nm), either wild type or mutated, was incubated for 2 min at 37 °C in a final volume of 4 μl in the presence of polynucleotide polymerase activity, corresponding to 15 nm 3′-OH ends, Buffer A, and 10 mM MgCl₂. (−)-PPO464 was then added to a final volume of 5 μl, at a concentration at which [E][I]/[E]o = (1 - 1/[Kd(E)]) > 0.9. Then 145 μl of a mix containing Buffer A, 10 mM MgCl₂, and 10 μM [3H]dTPP (1 Ci/mmol) were added at different time points. After an additional 10 min of incubation at 37 °C, 50-μl aliquots were spotted on GF/C filters, and acid-precipitable radioactivity was measured as described for the HIV-1 RT RNA-dependent DNA polymerase activity assay. The quantity (vobs − v1) of vobs, representing the normalized difference between the amount of dTPP incorporated at the zero time point and at the different time points, was then plotted against time.

Thermodynamic Parameter Determination

Reactions were carried out at different temperatures under the conditions described for the inhibition assays. Separate incubations were performed for each temperature in a HAAZE E9 thermostatted water bath. Kd values for nevirapine inhibition at the different temperatures were then calculated as described (see below).

Steady-state Kinetic Model for the Inhibition of the RT Reaction by (R)-(−)-PPO464

A simplified reaction pathway was assumed for the RT-catalyzed reaction. As shown in Scheme 1A, RT follows an ordered bimolecular mechanism, where TP binds first with an equilibrium dissociation constant K(TP), followed by dNTP binding with the corresponding equilibrium constant Kd(dNTP), to form the catalytically competent ternary complex that reacts with the substrate to give products RT-P with an overall reaction rate kcat. NNRTI binding was assumed to be possible in principle to each reaction intermediate: free enzyme (RT), only two enzymatic forms exist at the steady state: the free enzyme (RT) and the ternary complex. Inhibitor binding is driven by the corresponding equilibrium constants K(TP) and K(ter). When K(TP) = K(ter), the reaction is fully noncompetitive; with K(TP) > K(ter), inhibition is competitive at low [I], since only the RT-TP-dNTP1 complex can be formed, becoming mixed-noncompetitive at higher [I], when also the RT1 complex is formed. When K(TP) ≫ K(ter), inhibition is fully competitive, since inhibitor binding is determined exclusively by the degree of saturation of RT with its substrates. Similarly, with saturating TP and variable dNTP (Scheme 1B, left part), only the binary RT-TP and the ternary RT-TP-dNTP complexes exist at the steady state. Inhibitor binding can be described by the equilibrium dissociation constants K(bin) and K(ter) for the RT-TP-I and RT-TP-dNTP-I complexes, respectively. Also, under these conditions the following relationships hold: K(bin) = K(ter), noncompetitive inhibition; K(bin) > K(ter), mixed noncompetitive inhibition; K(bin) >> K(ter), fully competitive inhibition.

Kinetic Parameter Calculation

All values were calculated by nonlinear least squares computer fitting of the experimental data to the appropriate rate equations. Kd values were calculated according to the equation for uncompetitive inhibition.

\[ v = \frac{V_{max} [I]}{[K_{d(I)}] + ([I]/[K_{d(I)}])} \]  

(Eq. 1)

where Kd(I) is the apparent equilibrium dissociation constant of the inhibitor from the ternary complex as shown in Scheme 1, measured at different substrate concentrations.

Dependence of the Kd(I) on the substrate concentrations was fitted to the equation,

\[ K_{d(I)} = (1 + [I]/[K_{d(I)}]) \]  

(Eq. 2)

where Kd(ter) is the true equilibrium dissociation constant of the inhibitor from the ternary complex at infinite substrate concentration.

Dose-dependent inhibition at different substrate concentrations was fitted to the equation,

\[ E = \frac{E_{max}[I]/[K_{d(I)}]}{1 + ([I]/[K_{d(I)}])} \]  

(Eq. 3)

where E is the observed inhibition (percentage), Emax is the inhibition at infinite inhibitor concentration, and Kd(I) is the apparent equilibrium dissociation constant of the inhibitor from the ternary complex as shown in Scheme 1, measured at different substrate concentrations.
Steroselective Inhibition of HIV-1 RT by (R)-(−)-PPO464

For determination of inhibitor binding kinetics, $k_{on}$ values were determined by fitting the experimental data to the single exponential equation,

$$v_0 = v t = A(1 - e^{-kt}) \tag{Eq. 4}$$

where $A$ is a constant and $t$ represents time.

For the thermodynamic parameter calculations, $\Delta H^{\text{ter}}$ and $\Delta S^{\text{ter}}$ values were determined according to the van't Hoff equation,

$$\ln (1/K_{\text{obs}}) = -\Delta H^{\text{ter}}/RT + \Delta S^{\text{ter}}/R \tag{Eq. 5}$$

where $R$ is the gas constant, and $T$ is the absolute temperature (Kelvin). $\Delta G^{\text{ter}}$ was calculated from the equation,

$$\Delta G^{\text{ter}} = R T \ln [E] \tag{Eq. 6}$$

where $E$ is the observed effect (percentage of activity). $E_{\text{act}}$ is the control effect (activity in the absence of the inhibitor). $D_{\text{50}}$ is the 50% inhibitory concentration, and $m$ is a sigmoidicity parameter.

I was then calculated according to Berenbaum by the equation,

$$I = \frac{D_{\text{1}}/(D_{\text{501}} + D_{\text{2}}/D_{\text{502}})}{D_{\text{2}}/(D_{\text{502}} + D_{\text{1}}/D_{\text{501}})} \tag{Eq. 8}$$

where $D_{\text{1}}$ and $D_{\text{2}}$ are the concentrations of the drugs giving 50% inhibition when tested in the combination $D_{\text{1}} + D_{\text{2}}$, and $D_{\text{501}}$ and $D_{\text{502}}$ are the concentrations of each drug giving 50% inhibition when tested individually. A value for $I < 1$ indicates synergy, $I > 1$ indicates antagonism, and $I = 1$ indicates additivity, according to the Lowe additivity model.

For combination of two drugs at a fixed molar ratio ($R = ([\text{drug 1}]/[\text{drug 2}])$, $D_{\text{1}}$ and $D_{\text{2}}$ values were calculated from the $D_{\text{50}}$ value derived from Equation 7, with ($D_{\text{1}} + D_{\text{2}} = D_{\text{1}} + D_{\text{2}} = RD$).

Expected $D_{\text{1}}$, $D_{\text{2}}$, and $D_{\text{50}}$ values for the combination of $I$ drugs under the null reference hypothesis of no interaction were derived by inserting estimated $D_{\text{50}}$ and $m$ values for each drug in the combination in the specific form of the Lowe additivity equation, which assumes that Equation 7 is appropriate for each drug individually.

$$1 = \frac{D_{\text{1}}}{D_{\text{501}}(1 - E_{\text{act}}/E_{\text{max}})} + \frac{D_{\text{2}}}{D_{\text{502}}(1 - E_{\text{act}}/E_{\text{max}})} + \ldots + \frac{D_{I-1}}{D_{I}(1 - E_{\text{act}}/E_{\text{max}})} \tag{Eq. 9}$$

The null reference hypothesis of no interaction (Equation 9) corresponded to $I = 1$.

All of the analysis was based on the results of three independent experiments for each drug combination, and the S.D. values for each parameter estimate are indicated.

The second approach was based on the multiple drug effect analysis of Chou and Talalay (18, 19). This method involves plotting dose-effect curves for each agent and for one or more multiply diluted, fixed combination of the agents using the “median effect” equation: $f_{\text{act}} = (D_{\text{EDm}})^m$. In this equation, $D$ represents the dose, $D_{\text{EDm}}$ is the dose required for 50% effect (e.g. 50% inhibition of viral replication), $m$ is the fraction affected by the dose $D$, $f_{\text{act}}$ is the fraction unaffected, and $m$ is a coefficient signifying the sigmoidicity of the dose-effect curve. The dose-effect curve is plotted using a logarithmic conversion of this equation that determines the $m$ and $D_{\text{50}}$ values. Based on the slope of the dose-effect curves, it can be decided whether the agents have mutually exclusive effects (e.g. a similar mode of action) or mutually nonexclusive effects (e.g. an independent mode of action). A “combination index” (CI) is then determined using the equation,

$$CI = (D_{\text{1}}/(D_{\text{501}})) + (D_{\text{2}}/(D_{\text{502}})) + \ldots + (D_{I}/(D_{\text{50I}})) + (D_{\text{502}})/(D_{\text{502}}) \tag{Eq. 10}$$

where ($D_{\text{501}}$) represents the dose of agent 1 required to produce 50% effect alone, and ($D_{\text{2}}$) is the dose of that agent required to produce the same 50% effect in combination with ($D_{\text{1}}$). Similarly, ($D_{\text{502}}$) is the dose of agent 2 required to produce the same 50% effect alone, and ($D_{\text{2}}$) is the dose required to produce the same effect in combination. If the agents are mutually exclusive, then $m = 0$ (i.e. CI is the sum of two terms); if mutually nonexclusive, then $m = 1$ (i.e. CI is the sum of three terms). If it is uncertain whether the agents act in a similar or independent manner, the formula may be solved in both ways. Values of CI less than 1 indicate synergy, values greater than 1 indicate antagonism, and values equal to 1 indicate additive effect.

Viral Isolation and Drug Susceptibility Assay with Clinical Isolates

The infectivity titer of HIV isolates was determined by end point titration of peripheral blood mononuclear cell culture supernatants and expressed as 50% tissue culture infective dose (TCID50) (37). In vitro drug susceptibility assays were performed using a modified ACTG/AIDS consensus method (38). Briefly, on day 0, 2000 TCID50 of virus stock suspension, each inoculated on 2 x 10^6 peripheral mononuclear cells growing in each well of a 96-well plate. After 4 h at 37 °C, half of the supernatant was discarded and replaced with medium containing conditions to achieve the required drug concentration. Plates were then incubated at 37 °C in a 5% CO2 atmosphere. On day 4, half of the supernatant was replaced with fresh medium containing the appropriate drug concentration. On day 7, the supernatant of each well was assayed for p24 antigen production. The 50% inhibitory dose (ID50) was determined for each drug tested through dose-effect analysis of the p24 value in the control wells (without drug) and the corresponding wells in the presence of the drugs. In parallel, drug toxicity controls and back virus titration were performed.

Pharmacokinetic Studies

Drug Administration and Plasma and Brain Sampling—Disposition studies were done in male CD1 mice and CD-COBs rats weighing about 30 and 300 g, respectively (Charles River). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., Suppl. 40, 18 February 1992, Circolare No. 8, G.U., 14 July 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996). A chronic jugular catheter (PE-50 tubing) was implanted in each rat under chloral hydrate anesthesia 24 h before the study started. Rats were given compound (−)-PPO294 subcutaneously or orally in PEG-400/ethanol/ Tween 80 (60:30:10, v/v/v), and serial blood samples were drawn at various times. Samples were centrifuged, and the plasma was stored at −20 °C. At the end of the study (4 h after dosing), rats were killed by decapitation under deep anesthesia, and brains were rapidly removed, blotted with paper to remove excess surface blood, and stored at −20 °C until analysis. Mice were given the active isomer of compound (−)-PPO294 only orally (20 mg/kg, dissolved as above). Some received 20 mg/kg ritonavir (dissolved in PEG-400/ethanol/water, 40:10:50 v/v/v) 30 min before the test compound. The animals were killed by decapitation under deep anesthesia 15, 30, 60, 120, and 180 min after dosing. Plasma and brain samples were processed as described for the rat. Rats tolerated the (−)-PPO294 dose with no observable discomfort. Mice given the (R)-(−)-PPO464 were slightly ataxic within the first 30–60 min of dosing, and the effect was enhanced and prolonged by ritonavir co-administration.

Drug Analysis—The test compound was extracted from plasma and brain homogenates (methanol: 1 g/5 ml) and measured by high performance liquid chromatography with UV detection (300 nm) as previously described (15). Separation was done on a Spheri-5 RP 18 Brownlee column (25 cm × 4.6 mm ID, 5-μm particle size), with a New Guard RP-18 5 μm precolumn, at room temperature. The mobile phase was CH3OH, 0.025 M CH3COONa, n-propyl alcohol (63:36:1, v/v/v), buffered at pH 5 with acetic acid. It was delivered isocratically at a flow rate of 1.2 ml/min. The retention times were 20.2 min for compound (−)-PPO294 and 22.8 min for the structurally related internal standard (15). The sensitivity limit was 0.15 nmol/ml or g, using 0.2 ml of plasma or ~200 mg of brain tissue. At this concentration, the coefficient of variation was between 15 and 20%, and higher concentrations generally gave a coefficient of variation less than 10% in both tissues. Over the sampling interval, the plasma and brain area under the concentration-time curve (AUC) were determined using the linear trapezoidal rule. Then the value was extrapolated to infinity (AUC), when possible, using the terminal slope and the last plasma or brain concentration. The elimination half-life ($t_{1/2}$) was determined by the usual equation. The maximum concentration ($C_{max}$) and the time ($t_{max}$) of its occurrence were read directly from the plasma and brain concentration-time data. Plasma and brain concentrations of compound (−)-PPO294 and its...
RESULTS

(R)-(-)-PPO464 Is a Stereoselective, Uncompetitive Inhibitor of Wild Type HIV-1 Reverse Transcriptase—The two (+) and (-) enantiomers of (±)-PPO294 were isolated in pure form from a racemic preparation as described under “Materials and Methods.” The configuration of the chiral center was assessed on the 9-bromo derivative (±)-PPO600 (Fig. 1). After determination of the absolute configuration, the enantiomers were then indicated as (S)(+)-PPO463 and (R)(-)-PPO464, respectively. When tested as inhibitors of WT HIV-1 RT, as shown in Fig. 2A, only the (R)(-)-PPO464 was effective as an inhibitor of the viral enzyme. To determine its mode of action, the dependence of the inhibition on both the 3’-OH TP (poly(rA)/oligo(dT)) and the dTTP substrates was studied. In both cases, the kinetic parameters $V_m$ and $K_m$ were reduced in an inhibitor concentration-dependent manner (Fig. 2, B and C, for poly(rA)/oligo(dT); data not shown for dTTP), indicating a purely uncompetitive mechanism of inhibition with respect to both substrates of the reaction.

(R)(-)-PPO464 Binds Specifically to the Ternary Complex of HIV-1 Reverse Transcriptase with both the 3’-OH and dNTP Substrates—As outlined under “Materials and Methods,” HIV-1 RT follows an obligatory ordered ternary complex-reac-

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Structures of the clinically used NNRTIs nevirapine, efavirenz, and delavirdine and of the PPO series of compounds used for this study. In the drawing of the structure of the compound (S)(-)-PPO600, the nonhydrogen atom ellipsoids enclose 50% probability.

active isomer were expressed as mean ± S.D. Differences between vehicle- and ritonavir-treated animals were assessed using Student’s $t$ test.

![Figure 2](https://example.com/fig2.png)

**Fig. 2.** Inhibition of wild type HIV-1 RT by (R)-(−)-PPO464. Assays were performed under the conditions described under “Materials and Methods.” A, inhibition of HIV-1 RT activity by the two enantiomers (R)-(−)-PPO464 (triangles) and (S)-(−)-PPO463 (circles). B, plot of the dependence of HIV-1 RT inhibition by (R)-(−)-PPO464 on the 3’-OH concentration. When points were fitted to the uncompetitive model of Equation 1, the sums of squares of errors (SSE) were 5.6, 21.2, 9.2, 0.22, and 2.7, respectively, for the curves obtained at (R)-(−)-PPO464 concentrations of 0 (squares), 5 nM (rhombics), 100 nM (triangles), and 200 nM (closed circles). The SSE for fitting to the noncompetitive model were 5.5, 35.2, 19.8, 1.2, and 4.6, respectively, whereas for the competitive model, SSE were 5.8, 58.7, 63.6, 3.1, and 8.3, respectively. C, plot of the dependence on the (R)-(−)-PPO464 concentration of HIV-1 RT $K_m$ (ns) and $V_m$ (pmol × 30 min$^{-1}$) for the poly(rA)/oligo(dT) substrate. Curves were fitted to the following equations: $K_m^{(obs)} = K_m/(1 + [I]/K_i)$ and $V_m^{(obs)} = V_m/(1 + [I]/K_i)$.
Stereoselective Inhibition of HIV-1 RT by (R)-(−)-PPO464

**Fig. 3.** Dependence of (R)-(−)-PPO464 inhibition potency on the substrate concentration. Assays were performed under the conditions described under "Materials and Methods." A, (R)-(−)-PPO464 inhibitory activity (Effect, E) on HIV-1 RT at increasing 3'-OH concentrations. 3'-OH concentrations tested were 5, 10, 20, 100, and 200 mM. B, dependence of (R)-(−)-PPO464 inhibition constants ($K_a$) on 3'-OH concentrations. C, (R)-(−)-PPO464 inhibitory activity (Effect, E) on HIV-1 RT at increasing dTTP concentrations. dTTP concentrations tested were 0.5, 2, 10, and 20 μM. D, dependence of (R)-(−)-PPO464 inhibition constants ($K_a$) on dTTP concentrations.

**Table I**

<table>
<thead>
<tr>
<th>RT</th>
<th>$K_d$ (μM)</th>
<th>$k_{on}^a$ (s$^{-1}$)</th>
<th>$k_{on}^b$ (μM$^{-1}$s$^{-1}$)</th>
<th>Viral isolate</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.025 ± 0.005</td>
<td>1.8 ± 0.2 × 10$^{-3}$</td>
<td>4.5 ± 0.5 × 10$^4$</td>
<td>Wild type</td>
<td>0.04 ± 0.005</td>
</tr>
<tr>
<td>K103N</td>
<td>0.2 ± 0.01</td>
<td>1.2 ± 0.1 × 10$^{-3}$</td>
<td>0.6 ± 0.01 × 10$^4$</td>
<td>HIV-1 IIIB</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MDR</td>
<td>3.5 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$ Calculated from $K_d = k_{on}/k_{off}$.

$^b$ Calculated from $k_{on} = k_{on}(K_d + I)$.

$^c$ Clinical isolate. The multidrug resistance (MDR) isolate carried the RT mutations M41L/K103N/T215Y.

$^d$ Laboratory lymphotropic strain.

$^e$ Values are the means of three independent experiments and calculated at $T = 300$ K. Significance of the differences between the mean values obtained between wild type and K103N RT was $p < 0.05$ according to a Student’s t test under the null hypothesis that the mean values were equal.

and C). The potency of inhibition was increased by increasing concentrations of the dNTP substrate (data not shown). According to Scheme 1, in the presence of saturating 3'-OH TP concentrations, this reflects the exclusive binding of the inhibitor to the ternary complex (RT-TP-dNTP). As shown in Fig. 4D, the dependence of the $K_d$ (obs) on the dNTP concentration allowed an estimation of the inhibition constant $K_d$. Table 1 summarizes the calculated kinetic parameters for the interaction of WT RT and the K103N mutant with the 3'-OH TP and dNTP substrates, and the (R)-(−)-PPO464 inhibitor. (R)-(−)-PPO464 Effectively Inhibits Viral Replication of either Wild Type or Multidrug-resistant Viral Isolates—When the (R)-(−)-PPO464 inhibitor was tested against a WT HIV-1 IIIB laboratory strain, it effectively suppressed viral replication with an ID$_{50}$ of 3.4 μM, which accounted for a 10-fold resistance level with respect to the laboratory reference WT virus HIV-1 IIIB and about 50-fold with respect to the wild type clinical isolate (Table I). This difference could be due to genetic polymorphisms between the genomic sequence of the reference strain used either to generate the recombinant RT through site-directed mutagenesis (HIV-1 HXB2) or in the antiproliferative assay (HIV-1 IIIB) and the genome of the clinical isolate. However, the level of resistance to (R)-(−)-PPO464 of the multidrug-resistant mutant virus was comparable with efavirenz and 4-fold lower than that of nevirapine and delavirdine (Table II). (R)-(−)-PPO464 also effectively suppressed virus replication in different HIV-1-infected cell lines with favorable toxicity profiles (Table III).

**Kinetic and Thermodynamic Characterization of (R)-(−)-PPO464 Binding to HIV-1 Reverse Transcriptase**—The observed differences between the $K_d$ values of (R)-(−)-PPO464 for
the different values showed that resistance arose mainly from energetic (the following equations: the TTP substrate. Curves were fitted to bics tant HIV-1 RT by (12.7, respectively, for the curves obtained tions were performed under the conditions described under “Materials and Methods.” A, inhibition of HIV-1 RT activity by the two enantiomers (R)-(−)-PPO464 (triangles) and (S)-(−)-PPO463 (circles). B, plot of the dependence of HIV-1 RT inhibition by (R)-(−)-PPO464 on the 3′-OH concentration. When points were fitted to the uncompetitive model of Equation 1, the SSE were 45.6, 87.2, 13.2, 18, and 12.7, respectively, for the curves obtained at (R)-(−)-PPO464 concentrations of 0 (circles), 20 nM (squares), 100 nM (rhombics), 200 nM (triangles), and 400 nM (closed circles). The SSE for fitting to the noncompetitive model were 45.5, 135.2, 39.8, 41.2, and 44.6, respectively, whereas for the competitive model, SSE were 45.8, 153.6, 83.1, and 78.3, respectively. C, plot of the dependence on the (R)-(−)-PPO464 concentration of HIV-1 RT Km (nM) and Vmax (pmol × 30 min−1) values for the TTP substrate. Curves were fitted to the following equations: Km(obs) = Kf/Kd (1 + [I]) and Vmax (obs) = Vmax/1 + [I]/Km. D, dependence of (R)-(−)-PPO464 inhibition constant (Km(obs)) on dTTP concentrations.

Fig. 4. Inhibition of the K103N mutant HIV-1 RT by (R)-(−)-PPO464. Assays were performed under the conditions described under “Materials and Methods.” A, inhibition of HIV-1 RT activity by the two enantiomers (R)-(−)-PPO464 (triangles) and (S)-(−)-PPO463 (circles). B, plot of the dependence of HIV-1 RT inhibition by (R)-(−)-PPO464 on the 3′-OH concentration. When points were fitted to the uncompetitive model of Equation 1, the SSE were 45.6, 87.2, 13.2, 18, and 12.7, respectively, for the curves obtained at (R)-(−)-PPO464 concentrations of 0 (circles), 20 nM (squares), 100 nM (rhombics), 200 nM (triangles), and 400 nM (closed circles). The SSE for fitting to the noncompetitive model were 45.5, 135.2, 39.8, 41.2, and 44.6, respectively, whereas for the competitive model, SSE were 45.8, 153.6, 83.1, and 78.3, respectively. C, plot of the dependence on the (R)-(−)-PPO464 concentration of HIV-1 RT Km (nM) and Vmax (pmol × 30 min−1) values for the TTP substrate. Curves were fitted to the following equations: Km(obs) = Kf/Kd (1 + [I]) and Vmax (obs) = Vmax/1 + [I]/Km. D, dependence of (R)-(−)-PPO464 inhibition constant (Km(obs)) on dTTP concentrations.

The peculiar mechanism of resistance, the apparent association rates (kapp) for (R)-(−)-PPO464 to the wild type and the K103N enzymes were determined. The corresponding association and dissociation rates were derived from the relationships kapp = km(Kd + [I]) and Kd = koff/kon and are shown in Table I. The comparison between the different values showed that resistance arose mainly from a reduced association rate (konWT/Kon(mut) = 7.5) of the drug to the K103N mutant, whereas the dissociation rates were comparable (koffWT/Koff(mut) = 1.5). These results suggested that the K103N mutation imposed a thermodynamic barrier to the binding of the inhibitor. To directly verify this hypothesis, the thermodynamic parameters for the formation of the RT-TTP-dNTP-I complex were determined from the temperature dependence of the Kd values. As can be seen from computed values listed in Table I, the ΔH° [k(J,kl)] value of the heat change for complex formation with the K103N mutant was higher than the one for the wild type enzyme. This translated into a less negative ΔG° [k(J,kl)] value for the reaction involving the K103N RT with respect to WT RT. These results are consistent with the hypothesis that the K103N mutation imposes a thermodynamic barrier to inhibitor binding, as reflected by the different kon values (Table I). This barrier increased the activation energy for complex formation (ΔG°[k(J,kl)]) and shifted the free energy (ΔG°[k(J,kl)]) of the system toward lesser negative values.

(R)-(−)-PPO464 Displays Synergistic Inhibitory Activity toward WT and K103N HIV-1 Reverse Transcriptase When Combined with NNRTIs and NRTIs—The peculiar mechanism of action displayed by the (R)-(−)-PPO464 suggested a possible synergistic action in combination with other RT inhibitors targeted to different reaction intermediates. We have tested this hypothesis by challenging recombinant RT, either WT or bearing the K103N mutation, with different double or triple combinations of (R)-(−)-PPO464 with nevirapine and AZT. Nevirapine is an NNRTI that acts as a purely noncompetitive inhibitor of RT, thus interacting with all of the mechanistic forms of the enzyme (free RT, binary RT-TP, and ternary RT-TP-dNTP complexes). AZT is a NRTI and, as such, exclusively targets the binary RT-TP complex. As shown by the combination index values listed in Table IV, (R)-(−)-PPO464 was found to be significantly synergistic when tested in double combination with nevirapine, both against WT RT and K103N. Synergism was also observed toward both enzymes in combi-
nation with AZTTP. A triple combination of \((R)-(\rightarrow)-\text{PPO464}\), AZTTP, and nevirapine was also synergistic toward both enzymes. The derived 50% inhibitory concentrations for each drug in the combination were compared with the corresponding values expected under the null reference hypothesis of no interaction. This allowed us to determine the relative contribution of each drug to the observed synergy. Combination of \((R)-(\rightarrow)-\text{PPO464}\) with nevirapine resulted in a 3–4-fold potentiation of the effect of both drugs in the combination. When the double \((R)-(\rightarrow)-\text{PPO464}/\text{AZTTP}\) and the triple \((R)-(\rightarrow)-\text{PPO464}/\text{AZTTP}/\text{nevirapine}\) associations were tested, a 2–3-fold potentiation of all drugs was observed, as can be seen from the comparison of the observed \(D_{50}\) values with the expected ones under the hypothesis of no interaction.

The significance of the observed synergism was also tested in drug susceptibility assays using either the wild type laboratory strain HIV-1 IIIB or the multidrug-resistant (M41L/K103N/T215Y) clinical isolate. The compound showed a strong synergistic anti-HIV-1 activity when tested in combination with AZTTP, and nevirapine was also synergistic toward both en-...
form of RT thermodynamically disfavored for NNRTI binding. From the recently solved crystallographic structure of the K103N mutant complexed with efavirenz, it can be seen that substitution of an Asn leads to the formation of a hydrogen bond between Asn and Lys, which is not present in the wild type enzyme (21). These two amino acids line the proposed entry site for the NNRTIs; thus, the presence of an additional hydrogen bond can probably confer greater stability to the uninhibited form of the enzyme, disfavoring the structural rearrangements that are a consequence of NNRTIs binding. The structural basis for the absolute preference of (R)-(−)-PPO464 for the ternary complex is less clear. By comparing the structures of the unliganded enzyme with those of the enzyme-DNA and enzyme-DNA-dNTP complexes, the major observed differences were movements of the fingers and thumb subdomains (22–25). Since NNRTI binding involves a rearrangement of the structural elements surrounding the binding pocket, it is possible that local differences might facilitate the access of (R)-(−)-PPO464 to the binding site, partially explaining the observed mechanism of inhibition. Other second generation NNRTIs have been shown to bind with variable affinities to different reaction intermediates (28, 27); moreover, in a previous work, we have shown that the second generation NNRTI efavirenz displays preferential affinity for either the binary or the ternary complex of RT with its substrates, with respect to the unliganded enzyme (28). Thus, it is conceivable to propose an induced fit mechanism for binding of some classes of NNRTIs, which is triggered by complex formation among RT and the TP and dNTP substrates. This specificity is absolute in the case of (R)-(−)-PPO464, which binds exclusively the ternary complex. The selective targeting of NNRTIs to different mechanistic forms of the enzyme can provide the rationale for a new approach in combination chemotherapy. Associations of NNRTIs and NNRTIs able to specifically inhibit different enzymatic forms within the reaction pathway are expected to avoid antitargetic effects, due to competition among the inhibitors for the same form of the enzyme, and to enhance synergistic activity. The results of combinations of (R)-(−)-PPO464 with NNRTIs and NNRTIs presented in this study support this view. The observed antagonistic effect of (R)-(−)-PPO464 in combination with delavirdine or nevirapine in infected cell-based assays (Table IV) might suggest a possible competition of (R)-(−)-PPO464 and these NNRTIs for a common metabolic pathway (e.g., intracellular import), since they have been found to act synergistically at the level of their molecular target (Table III).

The pharmacokinetic results of the present study confirm and extend previous findings on the disposition of compound (−)-PPO294 in rodents, evidencing differences and similarities between species and between the racemate and its active isomer (R)-(−)-PPO464 (15). The mean relative bioavailability amounted to only about 12% in rats compared with about 30% in mice (15). However, presystemic metabolism may substantially differ from one species to another (29). In mice, the mean oral bioavailability, in terms of Cmax and AUC, was slightly higher for the (R)-(−)-PPO464 (present data) than for the corresponding racemate (15). This suggests that the pharmacokinetics of (−)-PPO294 may be governed by stereoselectivity, the (+)-isomer undergoing more marked presystemic metabolism than the active (−)-isomer. Interestingly, ritonavir increased the Cmax and AUC of the (R)-(−)-PPO464 while having little effect on its plasma and brain elimination t1/2 after oral dosing. This metabolic interaction is likely to be mainly due to inhibition of P450 enzymes, particularly members of the CYP3A subfamily (30). Because HIV-1 replicates in the central nervous system, the antiretroviral therapy needs to consider whether drugs adequately cross the blood-brain barrier. The present study shows that (R)-(−)-PPO464 easily crosses the blood-brain barrier in rodents, approaching micromolar concentrations at a site of infection and viral replication for HIV-1 after a 20 mg/kg dose. Transport of (R)-(−)-PPO464 across the blood-brain barrier may occur by passive diffusion (31). This is supported by the observation that ritonavir did not alter the brain-to-plasma distribution ratio of (R)-(−)-PPO464 in mice. This PI was an excellent substrate and potent inhibitor of P-glycoprotein (30, 32, 33), a membrane-localized drug transport mechanism expressed at the blood-brain barrier (34–36). The detailed knowl-

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**TABLE V**

**Synergy determination of (R)-(−)-PPO464 activity in combination with AZT, NVP, and DLV against HIV IIIB wild type laboratory strain and a multidrug-resistant (MDR) clinical isolate**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>ID_{50} of compounds alone (μM)</th>
<th>ID_{50} of compounds in combination with PPO464 (μM)</th>
<th>ID_{50} of PPO464 in combination with compounds (μM)</th>
<th>CI (nonmutually exclusive assumption)</th>
<th>CI (mutually exclusive assumption)</th>
<th>Interaction index (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>0.56</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>2</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>AZT</td>
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<td>0.016</td>
<td>0.20</td>
<td>0.71</td>
<td>0.61</td>
<td>0.3</td>
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<td>Delavirdine</td>
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<td>0.026</td>
<td>0.99</td>
<td>3.6</td>
<td>2.4</td>
<td>1.9</td>
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<tr>
<td>Nevirapine</td>
<td>7.5</td>
<td>7.4</td>
<td>1.85</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* CI > 1, CI = 1, and CI < 1 indicate antagonistic, additive, and synergistic activities, respectively. The CI values were calculated at 50% antiviral activity using both the mutually nonexclusive and the mutually exclusive form of the equation of Chou and Talalay (18, 19), as described under “Material and Methods.”

* NA, not applicable.

* Against the HIV IIIB wild type strain.

**Fig. 5.** Mean plasma concentration-time curves of compound (−)-PPO294 after a subcutaneous (open squares; n = 3) and oral (closed squares; n = 4) dose of 20 mg/kg in rats.
edge of the mechanism of inhibition of (R)-(−)-PPO464, its pharmacokinetic properties, and its interaction with other anti-RT drugs provides the necessary background for the development of novel third generation NNRTIs.

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REFERENCES