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New Tetrahydroimidazo[4,5,1-*jk*][1,4]-Benzodiazepin-2(1*H*)-One and -Thione Derivatives Are Potent Inhibitors of Human Immunodeficiency Virus Type 1 Replication and Are Synergistic with 2′,3′-Dideoxynucleoside Analogs

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Tetrahydro-imidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives were shown to specifically block human immunodeficiency virus type 1 (HIV-1) replication through a unique interaction with the HIV-1 reverse transcriptase (RT). Through further modification of the lead compounds and structure-activity relationship analysis several new TIBO derivatives that show high potency, selectivity, and specificity against HIV-1 have been obtained. A new TIBO derivative, R86183, inhibits the replication of HIV-1, but not HIV-2, in a variety of CD4$^+$ T-cell lines and peripheral blood lymphocytes, at a concentration of 0.3 to 30 nM, which is at least 4 orders of magnitude lower than the 50% cytotoxic concentration. Whereas an HIV-1 strain containing the Leu-100→Ile mutation in the RT gene is about 400-fold less susceptible, R86183 still inhibits the replication of an HIV-1 strain containing the Tyr-181→Cys RT mutation by 50% at a concentration of 130 nM. R86183 inhibits the poly(C) · oligo(dG)$_{12-18}$-directed HIV-1 RT reaction by 50% at a concentration of 57 nM. The antiviral activity of 22 TIBO derivatives in cell culture correlated well with their activity against HIV-1 RT. No such correlation was found for their cytotoxicity. The combination of R86183 with either zidovudine or didanosine resulted in a synergistic inhibition of HIV-1 (strain III$_B$) replication. Combination of R86183 with the protease inhibitor Ro31-8959 was found to be additive. Also described is a dilution protocol circumventing overestimation and underestimation of antiviral activity due to adherence to plastic surfaces.

We previously described a new class of highly selective and specific inhibitors of human immunodeficiency virus type 1 (HIV-1) replication, the tetrahydro-imidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives (20, 25). This HIV-1-specific inhibition is due to a stereospecific interaction of TIBO with HIV-1 reverse transcriptase (RT) template-primer complex (12, 25). In contrast, no inhibition of cellular DNA polymerases α, β, and γ has been observed (12). The HIV-1 RT inhibition is template dependent with a preference for the RNA-dependent DNA polymerization step (12). Independently, we have observed a similar activity profile for a group of acyclic uridine analogs related to HEPT ([1-(2-hydroxyethoxy)methyl]6-(phenyl)thymine) (3) and more recently for a group of α-anilinophenyl acetamide (α-APA) derivatives (24). A TIBO-like activity profile has also been found for dipyridodiazepinones (22), pyridinones (16), bis(heteroaryl)piperazines (33) and [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO) derivatives (6). It has been demonstrated that in vitro passage of HIV-1 in the presence of these compounds can lead to the emergence of drug resistance (14, 23, 32, 33). Sequence analysis and site-directed mutagenesis showed that this resistance is most often caused by a tyrosine-to-cysteine mutation at RT residue 181, for which most of the compounds in this class are thought to be cross-resistant. Other mutations conferring resistance have been mapped to the regions containing residues 98 to 110 and 179 to 190, which constitute the drug-binding pocket on the RT heterodimer located in the immediate vicinity of the catalytic center (1, 10, 17, 18, 34).

We now describe the methodology and results in the development of a new series of TIBO derivatives including the antiviral profile of R86183, an 8-chloro TIBO derivative with good activity against HIV-1 strains containing a Tyr-181→Cys mutation which is synergistic with the dideoxynucleoside analogs zidovudine (AZT; 3′-azido-3′-deoxythymidine; Retrovir) and didanosine (DDI; 2′,3′-dideoxyinosine; Videx).

MATERIALS AND METHODS

Compounds. 6-substituted 4,5,6,7-tetrahydro-5-methylimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-ones and their synthesis have been described previously (20). The synthesis of TIBO compounds in which the urea oxygen is replaced by sulfur has also been described (19). AZT was obtained from Wellcome (Aalst, Belgium). DDI was purchased from Sigma Chemical Co. (St. Louis, Mo.). Ro31-8959 (QC.AsnPhe[CH(OH)CH2N] DIQ.NHtBu; QC:quinoline-2-carbonyl; SIQ:(4as, 8as)-decahydro-3(S)-isoquinolinecarbonyl) was kindly provided by N. Roberts (Roche Products Limited, Welwyn Garden City, United Kingdom). Stock solutions (20 mg/ml) were prepared in dimethyl sulfoxide, aliquoted, and stored at −20°C under light-protective conditions. Immediately prior to their dilution in microtiter plates, intermediate 5- to 10-fold dilutions were

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prepared in RPMI 1640 DM medium (Flow Laboratories, Irvine, United Kingdom; E. Merck, Darmstadt, Germany).

Cells. The $CD4^+$ T-cell lines MT-4, MOLT-4, and CEM were used in the anti-HIV assays. The cells were grown and maintained in RPMI 1640 DM medium supplemented with 10% (vol/vol) fetal calf serum and 20 μg of gentamicin per ml (complete medium). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured every 3 to 4 days. Peripheral blood lymphocytes (PBL) from healthy HIV-negative donors were obtained by the Ficoll-Hypaque technique.

Viruses. The viruses used in these studies (HIV-1 IIIB [36], NDK [35], HE [25], 2749M [38], and 2750M [38]; HIV-2 ROD [9] and EHO [30]; and simian immunodeficiency virus [SIV] MAC_{251} [15], agm3 [4, 11], and mndGB1 [37]) have been described in detail previously. The HIV-1 mutant strains 13MB1 (Leu-100→Ile), 13CN1 (Tyr-181→Cys), and 39MH1 (Val-106→Ala) were isolated in our laboratory after serial passage of the III_B(LAI) strain (MT-4 cells), the NDK strain (CEM cells), and the HE strain (MT-4 cells), respectively, in the presence of TIBO R82913 (13MB1 and 13CN1) or α-APA R89439 (39MH1) (unpublished data). All virus isolates were titrated in MT-4 cells by determination of the virus stock dilution causing cytopathic changes in 50% of the cultures.

Antiviral assay with MT-4 cells and MOLT-4 cells. Different dilution protocols for the compounds were programmed as indicated by using a Biomek 1000 Workstation (Beckman Instruments, Inc., Palo Alto, Calif.). In the standard protocol, the eight-channel pipetting tool was used to transfer 25-μl volumes to the next series of wells with mixing volumes of 8 × 75 μl, divided over the starting and destination wells, with no tip touch and with blowout. Tips (Beckman) were changed every three dilution steps, i.e., two tip changes for a series of 9 dilutions. Antiviral assays with MT-4, MOLT-4, and CEM cells and PBL were subsequently performed as previously reported (26, 29). The experiments were conducted at least two times, and final data are given as median values.

Combination studies. The combined effect of two compounds on HIV-1 [strain III_B(LAI)] replication in MT-4 cells was studied under the experimental conditions described for the 50% effective concentration (EC_{50}) determinations. A dilution matrix (8 by 6) with fivefold drug dilutions was prepared in three or four individual 96-well plates. On the basis of the three-dimensional surface diagrams, volumes of synergy and antagonism at 95% confidence limits were calculated by using the MacSynergy II program 1.0 (C. Shipman, University of Michigan, Ann Arbor) (29). According to the authors of this program, synergy and antagonism volumes under 25 $(\mu g/ml)^2\%$ at 95% confidence should be regarded as insignificant. Values between 25 and 50 $(\mu g/ml)^2\%$ indicate minor but significant synergy. Values over 50 or 100 $(\mu g/ml)^2\%$ indicate moderate or intense synergy or antagonism, respectively.

High-pressure liquid chromatography (HPLC) analysis. To 1-ml aliquots of medium, pipetted into 10-ml glass test tubes, were added 1 μg of the internal standard (R82150) in 100 μl of methanol, 0.5 ml of 2 N H_2SO_4, and 4 ml of heptane-isoamyl alcohol (95:5, vol/vol). The samples were mixed in a rotary mixer at 10 rpm for 10 min. After centrifugation (1,000 × *g* for 10 min), the organic layers were discarded after aspiration and the aqueous layers were alkalinized with 150 μl of concentrated ammonia. The samples were then extracted twice with 2 ml of the heptane-isoamyl alcohol mixture. After centrifugation, the organic layers were aspirated and combined in 5-ml glass test tubes and 100 μl of 0.1 N H_2SO_4 and 50 μl of methanol were added. The samples were vortexed for a few seconds, rotated, and centrifuged. Aliquots of 100 μl of the aqueous layer were then transferred to 0.2-ml conical polypropylene microvials, and 35-μl aliquots were injected by a Perkin-Elmer ISS-100 autosampler in a Perkin-Elmer series 410 liquid chromatograph equipped with a Perkin-Elmer LC 235 DAD UV detector operating at 315 nm. The separations were achieved on a reversed-phase column (15 cm by 2.1 mm), packed with 5-μm-diameter particle octyldecyl silane Hypersil (Shandon) by the balanced-density procedure by means of an air-driven fluid pump (Haskel). The samples were eluted at ambient temperature with 0.1 M ammonium acetate–methanol–acetonitrile (36:32:32) at a constant flow rate of 0.9 ml/min. Area integrations, calculations, and plotting of the chromatograms were carried out by a Nelson series 3000 chromatography data system. Retention times for R82150 and R82913 were 1.5 and 2.8 min, respectively. Extraction recoveries exceeded 90% for both TIBO compounds.

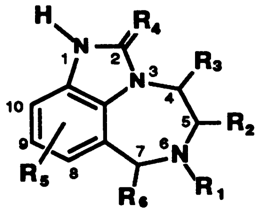
RT assays. In the exogenous RT assay (12), wherein a homopolymeric template was used, the reaction mixture (50 μl) contained 50 mM Tris-HCl (pH 8.4), 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM dithiothreitol, and 0.05% (wt/vol) Triton X-100. The template poly(C) and the primer oligo$(dG)_{12-18}$ were used at a concentration of 40 and 6 μg/ml, respectively. The DNA-directed DNA polymerase activity of RT was measured with poly(dC) as the template and oligo$(dG)_{12-18}$ as the primer and used at the same concentrations. Templates and primers (Pharmacia, Uppsala, Sweden) were annealed at room temperature (10 min) prior to the RT assays. Recombinant HIV-1 RT p66/p51 (*Saccharomyces cerevisiae*) was a kind gift of P. J. Barr (Chiron Corporation) (8) and was used at a concentration of 72 ng/ml. In the endogenous RT assay (13), wherein the viral RNA functioned as the template, the reaction mixture (50 μl) consisted of 50 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 100 mM KCl, 4 mM dithiothreitol, 30 μg of bovine serum albumin per ml, 0.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], and 0.01% (wt/vol) Triton X-100. Of the four deoxynucleoside triphosphates, three were used at a saturating concentration of 100 μM, while the tritium-labeled dGTP (Amersham, Brussels, Belgium) was used at a concentration of 2.5 μM. Specific activity was 11 Ci/mmol (1 Ci = 37 GBq). A similar concentration of dGTP was used in the exogenous reaction.

RESULTS

Anti-HIV activities of TIBO derivatives. The anti-HIV-1 activities of newly synthesized TIBO derivatives (Table 1; Fig. 1) were determined with MT-4 cells which were infected with the III_B(LAI) strain at a multiplicity of infection that completely destroyed the cells by day 5 postinfection. Of these new congeners, three TIBO derivatives were identified with EC_{50}s between 1 and 5 nM. R86183, R87027, and R86775 contain an 8-chloro or 8-bromo substituent in the phenyl moiety and possess a dimethyl or diethylallyl substituent at the N-6 position of the diazepin ring. The potencies are about 10 times higher than that of the unsubstituted prototype TIBO derivative R82150 (EC_{50}, 44 nM). Also the 8-methyl-substituted congener R84674 had a higher activity (EC_{50}, 14 nM). The three most potent compounds (R86183, R87027, and R86775) had selectivity indices ranging from 2,353 up to 30,000 (Table 1).

From a series of 9-chloro-substituted TIBO derivatives, R86162, which contains a diethylallyl substituent at the N-6 position, emerged as the most potent inhibitor (EC_{50}, 15 nM). This diethylallyl substitution within the 9-chlorine series of sulfur-containing TIBO derivatives only slightly enhanced the

TABLE 1. SAR for inhibition of HIV-1 cytopathicity and cytotoxicity in MT4 cells by TIBO derivatives



Compound	R_1	R_2	R_3	R_4	R_5	R_6	EC_{50}[a] (nM)	CC_{50}[b] (μM)	SI[c]	Relative potency
R14458	—CH_2—CH=CH_2	(±)-CH_3	H	O	H	H	59,000	620	10	1
R78305	—CH_2—CH=CH_2	(+)-(S)-CH_3	H	O	H	H	67,000	680	10	1
R78819	—$CH_2C(CH_3)$=CH_2	(+)-(S)-CH_3	H	O	H	H	14,000	110	8	4
R80902	—CH_2—CH=$C(CH_3)_2$	(+)-(S)-CH_3	H	O	H	H	4,200	470	110	14
R81886	—CH_2—$C(CH_3)$=CH_2	(±)-CH_3	H	O	9-Cl	H	4,700	17	4	13
R86080	—CH_2—CH=$C(CH_2CH_3)_2$	(±)-CH_3	H	O	9-CH_3	H	310	20	65	190
R85386	—CH_2—CH=$C(CH_2CH_3)_2$	(−)-(S)-CH_3	H	O	9-Cl	H	25	22	880	2,400
R82150	—CH_2—CH=$C(CH_3)_2$	(+)-(S)-CH_3	H	S	H	H	44	550	12,000	1,300
R84963	—CH_2—CH=$C(CH_3)_2$	(±)-CH_3	H	S	H	—CH_3(*trans*)	39	81	2,100	1,500
R84914	—CH_2—CH=$C(CH_3)_2$	(±)-CH_3	H	S	H	—CH_3(*cis*)	790	74	94	75
R80806	—CH_2—CH_2—CH_3	(±)-CH_3	H	S	H	H	240	560	2,300	240
R82913	—CH_2—CH=$C(CH_3)_2$	(+)-(S)-CH_3	H	S	9-Cl	H	33	34	1,000	1,800
R86085	—CH_2—CH_2-cyclopropyl	(−)-(S)-CH_3	H	S	9-Cl	H	330	36	110	180
R86777	—CH_2—CH=$C(CH_3)_2$	(−)-(S)-CH_3	H	S	9-Cl	H	92	19	210	640
R85787	—CH_2—cyclobutyl	(−)-(S)-CH_3	H	S	9-Cl	H	43	28	650	1,400
R86162	—CH_2—CH=$C(CH_2CH_3)_2$	(−)-(S)-CH_3	H	S	9-Cl	H	15	12	800	4,000
R86154	—CH_2—$C(CH_3)$=CH_2	H	(+)-(S)-CH_3	S	9-Cl	H	450	24	53	130
R86150	—CH_2—CH_2-cyclopropyl	H	(−)-(S)-CH_3	S	9-Cl	H	2,200	>810	>370	27
R85255	—CH_2—CH=$C(CH_3)_2$	(+)-(S)-CH_3	H	S	9-Cl, 10-Cl	H	25	45	1,800	2,400
R86183	—CH_2—CH=$C(CH_3)_2$	(+)-(S)-CH_3	H	S	8-Cl	H	4.6	140	30,000	13,000
R87027	—CH_2—CH=$C(CH_2CH_3)_2$	(+)-(S)-CH_3	H	S	8-Cl	H	5.1	12	2,400	12,000
R86775	—CH_2—CH=$C(CH_3)_2$	(+)-(S)-CH_3	H	S	8-Br	H	3.0	53	18,000	20,000
R84674	—CH_2—CH=$C(CH_3)_2$	(+)-(S)-CH_3	H	S	8-CH_3	H	14	80	5,700	4,200

[a] Determined by the MTT procedure 5 days postinfection. The data were based on 2 to 50 determinations. On the average, the upper and lower limits of the 95% confidence intervals ranged between 0.24 and 4.22 times the median EC_{50}.
[b] After a 5-day incubation in the presence of the compound.
[c] SI, selectivity index (ratio of CC_{50} to EC_{50}).

anti-HIV-1 activity compared with the reference compound R82913. Among the oxygen-containing TIBO derivatives, R85386 (EC_{50}, 25 nM) proved to be the most potent. The addition of a second chlorine at position 10 of the phenyl moiety yielded a product (R85255) that was about as active the mono-substituted analog (EC_{50}, 25 nM).

The introduction of a methyl substituent (R_6) at position C-7 led to a new potent congener, R84963 (EC_{50}, 39 nM). The relative stereochemistry of the C-5 and C-7 methyl substituents is important for anti-HIV activity, since the *cis* analog (R84914) is about 20 times less active than the *trans* analog (R84963). However, the two compounds exhibit similar cytotoxicities (50% cytotoxic concentrations, [CC_{50}], 74 and 81 μM, respectively).

Plastic adherence effects of R82913. It was found accidentally that the EC_{50} of R82913 varied significantly as a function of the starting concentration prior to dilution in 96-well microtiter trays. HPLC analysis of drug samples diluted in 96-well trays revealed that R82913 exhibited a strong plastic adherence effect (Fig. 2). If a single tip is used, the compound which is adhered to the plastic leaks back to the wells at higher dilutions. However, if tips are changed after each dilution step, the opposite occurs, i.e., the compound is actually lost through adherence to the disposed plastic tips. This phenomenon occurs only with highly potent compounds, which have to be diluted over a wide range of concentrations, and with highly hydrophobic substances (e.g., R82913). R82150, which has 70-fold higher solubility in water than R82913, exhibits markedly less plastic adherence (data not shown). The difference in hydrophobicity between the two TIBO compounds was also apparent in their log P (partition coefficient) values, which, determined at 25°C in *N*-octanol-phosphate-buffered saline, pH 7.4, were 3.51 and 4.34, respectively. To overcome this problem, a dilution protocol in which tips were changed every three dilution steps was worked out. Figure 2 shows that, even for highly hydrophobic compounds such as R82913, dilutions up to 100,000-fold that accurately reflect the theoretical concentrations can be prepared. The activities of all compounds in this study, including R86183, were determined by this new dilution protocol.

Correlation of anti-HIV-1 activity, anti-RT activity, and cytotoxicity. All 22 TIBO derivatives in Table 1 were examined for their capacity to inhibit a poly(C) · oligo$(dG)_{12–18}$-directed recombinant RT reaction. A comparison of their EC_{50} for HIV-1 RT activity and their EC_{50} for HIV-1 replication in cell culture revealed that modifications that resulted in increased antiviral potency at the cellular level also led to increased RT inhibition (Fig. 1). Three new TIBO derivatives with 8-chloro or 8-bromo substitutions (R86183, R87027, and R86775) were the most potent inhibitors of HIV-1 RT (with EC_{50} in the 30 to 60 nM range) and of HIV-1 in cell culture. Only the anti-RT activity of R86150, as measured under these assay conditions,

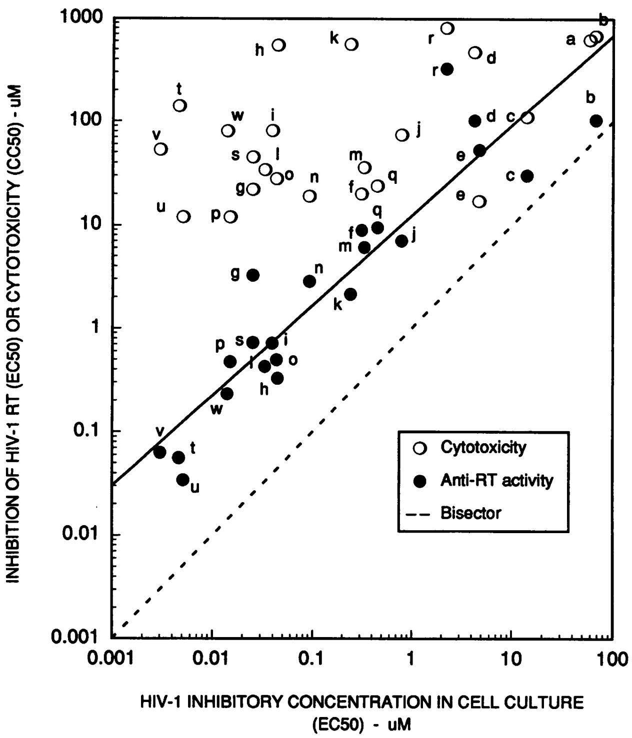


FIG. 1. Correlation between inhibitory effects of a series of TIBO derivatives on HIV-1 replication in MT-4 cells and either HIV-1 recombinant poly(C) · oligo(dG)$_{12-18}$-directed RT activity or cytotoxicity in MT-4 cells. Dashed line, line of equal potency (bisector). The data were based on 2 to 50 determinations. On the average, the upper and lower limits of the 95% confidence intervals ranged between 0.24 and 4.2 times the median EC$_{50}$. a, R14458; b, R78305; c, R78819; d, R80902; e, R81886; f, R86080; g, R85386; h, R82150; i, R84963; j, R84914; k, R80806; l, R82913; m, R86085; n, R86777; o, R85787; p, R86162; q, R86154; r, R86150; s, R85255; t, R86183; u, R87027; v, R86775; w, R84674.

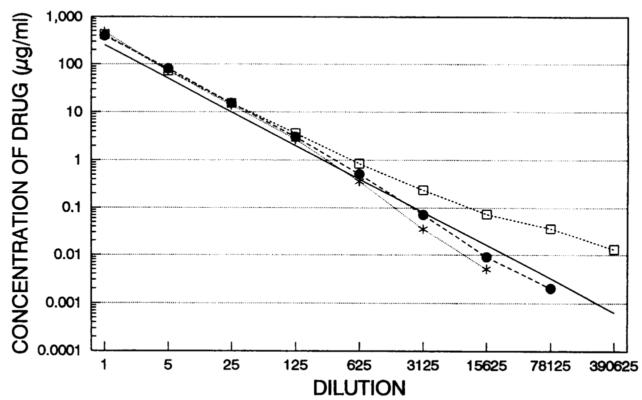


FIG. 2. Effects of different dilution protocols on concentrations of R82913, as measured by HPLC analysis. □, no tip change after each dilution; *, tips changed after each dilution; ●, tips changed every 3 dilutions. Solid line, theoretical concentration.

was higher than would be expected on the basis of its EC$_{50}$ in cell culture (Fig. 1). The EC$_{50}$ for inhibition of HIV-1 in cell culture were on the average 10- to 15-fold lower than the EC$_{50}$ for inhibition of HIV-1 RT activity. In contrast with the correlation between the EC$_{50}$ for HIV-1 replication in cell culture and HIV-1 RT activity (r, 0.91 [power ($y = ax^b$) regression]), the EC$_{50}$ for HIV-1 replication in cell culture did not correlate with the cytotoxic effects (CC$_{50}$) of the compounds (Fig. 1). The new TIBO prototype R86183 was also examined for its inhibitory effect on the RT of HIV-1 under different experimental conditions, and its anti-RT activity profile was compared with that of TIBO derivative R82150. When the exogenous template-primer poly(C) · oligo(dG)$_{12-18}$ was used, R86183 achieved 50% inhibition of the incorporation of radiolabeled dGMP at a concentration of 57 nM. TIBO R82150 exerted this effect at a fivefold higher concentration (EC$_{50}$, 254 nM). Similar results were obtained for the endogenous system, in which the viral RNA functioned as the template for new DNA synthesis catalyzed by the RT from the detergent-disrupted virions with EC$_{50}$ for R86183 and R82150 of 50 and 139 nM, respectively. The effect on the DNA-dependent DNA polymerase activity of the enzyme was assessed by using poly(dC) · oligo(dG)$_{12-18}$ as the template-primer. The TIBO derivatives R82150 and R86183 inhibited this reaction at a concentration of 12.3 and 0.496 µM, respectively.

Antiviral activity profile of TIBO R86183. TIBO R86183 was chosen as the prototype molecule of this new series of 8-substituted TIBO compounds, and its antiviral activity was compared with those of DDI and AZT (Table 2). The activity and dose-response curve of R86183 in PBL (EC$_{50}$, 4.6 nM) were comparable to those of AZT (EC$_{50}$, 2.1 nM), while the EC$_{50}$ of DDI was markedly higher (212 nM) (Table 2). TIBO R86183 achieved full protection at a concentration of about 50 to 100 nM (16 to 32 ng/ml). These concentrations were also fully protective in MT-4 cells (data not shown). Also, in the non-human T-cell leukemia virus type I transformed CD4$^+$ T-cell line CEM, for which anti-HIV-1 activity was monitored by fluorescence-activated cell sorter (FACS) analysis of viral antigen expression, a similar EC$_{50}$ (4.3 nM) was observed for R86183. In MT-4 cells, the Haitian strain RF and the Zairian strain NDK exhibited similar sensitivities to R86183. Two clinical HIV-1 isolates, 2749M and 2750M, had EC$_{50}$ of 7.8 and 0.3 nM, respectively. The HE strain, containing a valine-to-aspartic acid mutation at amino acid position 179 (38), proved about sixfold less sensitive compared with the III$_B$(LAI) strain. An HIV-1 strain (13MB1) isolated with the TIBO derivative R82913 and containing a leucine-to-isoleucine mutation at amino acid position 100 of the RT gene was about 400-fold less sensitive to R86183 (EC$_{50}$, 1,700 µM) compared with the parent strain III$_B$(LAI). On the other hand, an HIV-1 strain carrying a tyrosine-to-cysteine mutation at amino acid position 181 (strain 13CN1) had an EC$_{50}$ of 130 nM. A similar activity was seen for an HIV-1 strain (39MH1) isolated with the α-APA derivative R89439 which contained a Val-106→Ala RT mutation. No or marginal activity was found with R86183 against the HIV-2 strains ROD and EHO and the SIV strains MAC$_{251}$ and mndGB1. Interestingly, R86183 was found to protect MOLT-4 cells against the cytopathic effect of the SIV agm3 strain at EC$_{50}$ and EC$_{90}$ of 1.8 and 10 µM, respectively (Table 2).

Combination studies. The anti-HIV-1 activity of combinations of R86183 with prototype compounds of other classes of HIV-1 inhibitors was investigated with MT-4 cells by the (3-4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (26) MTT procedure. Optimal synergy was seen for the combinations of R86183 with AZT and DDI. When R86183 was combined with the HIV protease inhibitor Ro31-8959, merely additive effects became apparent. The volumes of synergy and antagonism were calculated, at 95% confidence intervals, and

TABLE 2. Antiretroviral spectra of R86183, DDI, and AZT[a]

Virus	Cells	R86183			DDI			AZT		
		EC_{50}[b] (nM)	CC_{50} (μM)	SI[c]	EC_{50} (nM)	CC_{50} (μM)	SI	EC_{50} (nM)	CC_{50} (μM)	SI
HIV-1										
III_B(LAI)	MT-4[d]	4.6	140	30,000	4,800	1,000	210	0.5	3.5	7,000
III_B(LAI)	PBL[e]	4.6	>50	>11,000	210	>250	>1,200	2.1	52	25,000
III_B(LAI)	CEM[f]	4.3	18	4,200	1,400	760	540	1.1	38	35,000
RF[g]	MT-4	12	140	12,000	4,000	1,000	250	5.2	3.5	670
NDK[h]	MT-4	2.5	140	56,000	14,000	1,000	71	1.7	3.5	2,100
2749M[i]	MT-4	7.8	140	18,000	1,900	1,000	526	7.5	3.5	467
2750M[i,j]	MT-4	0.3	140	470,000	3,000	1,000	330	7.5	3.5	467
HE[k]	MT-4	28	140	5,000	9,400	1,000	110	0.9	3.5	3,900
13MB1[l]	MT-4	1,700	140	82	1,800	1,000	560	0.6	3.5	5,800
13CN1[m]	MT-4	130	140	1,100	6,800	1,000	150	1.1	3.5	3,200
39MH1[n]	MT-4	137	140	1,000	2,000	1,000	500	3.7	3.5	940
HIV-2										
ROD	MT-4	>20,000	140	<7	18,000	1,000	56	2.6	3.5	1,300
EHO	MT-4	>10,000	140	<14	14,000	1,000	71	2.2	3.5	1,600
SIV										
MAC_{251}	MT-4	26,000	140	5	5,300	1,000	190	1.8	3.5	1,900
mndGB[o]	MOLT-4	>50,000	>50	>1	12,000	>250	>21	1.1	>4	>3,600
agm3[p]	MOLT-4	1,800	>50	>30	11,000	>250	>23	1.1	>4	>3,600

[a] The data are based on 2 to 50 determinations. On the average, the upper and lower limits of the 95% confidence intervals ranged between 0.24 and 4.22 times the median 50% values.
[b] The data are median values for at least two experiments.
[c] SI, selectivity index (ratio of CC_{50} to EC_{50}).
[d] The concentrations were determined 5 days postinfection by the MTT procedure.
[e] The concentrations were determined by measuring HIV-1 p24 core protein production 7 days postinfection. Cytotoxicity was determined by the MTT procedure.
[f] HIV-1 antigen expression was determined by FACS analysis.
[g] Haitian strain.
[h] Zairian strain.
[i] HIV-1 clinical isolate from Europe with a short passage history in PBL.
[j] Contains a threonine-to-isoleucine mutation at amino acid position 165 of HIV-1 RT.
[k] Belgian strain, containing a valine-to-aspartic acid mutation at a amino acid position 179 of HIV-1 RT (25).
[l] Resistant to TIBO R82913; obtained after serial passage of III_B(LAI) in MT-4 cells; contains a leucine-to-isoleucine mutation at amino acid position 100 of the HIV-1 RT.
[m] Resistant to TIBO R82913; obtained after serial passage of NDK in CEM cells: contains a tyrosine-to-cysteine mutation at amino acid position 181 of the HIV-1 RT.
[n] Resistant to α-APA R89439; obtained after serial passage of HE in MT-4 cells: contains a valine-to-alanine mutation at amino acid position 106 of the HIV-1 RT.
[o] Derived from mandrills. The concentrations were determined by the MTT procedure.
[p] Derived from African green monkeys. The concentrations were determined by the trypan blue dye exclusion method.

are expressed in percent square micrograms per milliliter (Fig. 3). Combinations of ribavirin with DDI and AZT were included as reference combinations and were clearly synergistic and antagonistic, respectively. The combination of R86183 with the RT inhibitor phosphonoformic acid was slightly antagonistic (Fig. 3).

DISCUSSION

The TIBO derivatives were the first examples of potent and selective HIV-1 inhibitors shown to specifically interact with the RT of HIV-1 (25). Guided by structure-activity relationship (SAR) analysis, we have now developed a new series of TIBO derivatives which exhibit a potency higher than those of the prototype compounds of the first series. They all contain a substituent (chlorine, bromine, or methyl) at the 8 position of the phenyl moiety. These compounds have a 10,000- to 20,000-fold increase in potency relative to the original lead compound R14458. TIBO R86183, R87027, and R86775 have emerged as the most potent and selective compounds with selectivity indices up to 30,000. In addition, the SAR analysis revealed some new features of the TIBO pharmacophore, i.e., the stereochemical requirement of the C-7 methyl substituent of TIBO derivative R84963. A stereospecific requirement was previously demonstrated for the methyl group at position C-5 (19).

During this SAR study we became aware of the high tendency of the TIBO derivative R82913 to adhere to plastic surfaces. This phenomenon was not observed for other potent, but more hydrophilic, compounds such as AZT and R82150 and may be attributed to the high hydrophobicity of compounds such as R82913. It leads to deviation from the theoretical concentrations when hydrophobic compounds are diluted over wide concentration ranges by using either the same tip or different tips for each dilution step. Guided by HPLC analysis of the actual concentrations, we established a dilution protocol that is suitable for hydrophilic as well as hydrophobic compounds. This protocol will be particularly useful when high-capacity in vitro evaluations are required and working conditions do not allow the use of glass containers or needles.

Our previous studies have indicated that TIBO derivatives exert their inhibitory effects on HIV-1 replication in cell culture through a novel, highly specific interaction with HIV-1 RT (12, 25). In these studies we found a correlation between the anti-HIV-1 activity of the TIBO derivatives in cell culture and their capacity to inhibit poly(A) · oligo(dT)- or a poly(C) · oligo(dG)-directed RT activity. Yet, these experiments were performed on a small number of compounds, and

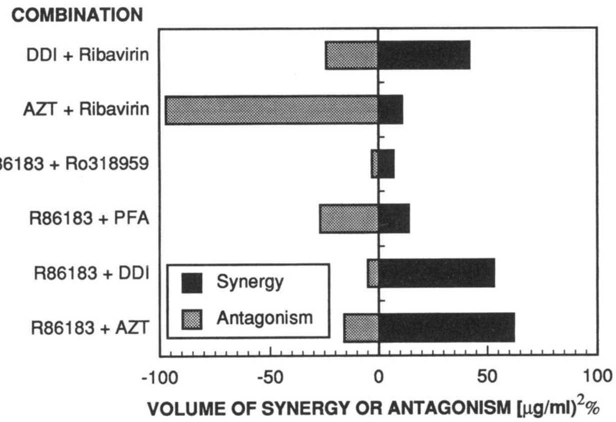


FIG. 3. Volumes of synergy and antagonism of drug combinations determined by the MacSynergy II program using 95% confidence limits. Anti-HIV-1 III_B activity in MT-4 cells was determined by the MTT procedure. PFA, phosphonoformic acid.

the correlation that was found deviated for the most active TIBO congener, R82913. This observation can now be explained by the plastic adherence effect of R82913 mentioned above. We have now investigated the capacity of a larger number of TIBO derivatives to inhibit the poly(C) · oligo(dG)-directed RT reaction. This template-primer was previously shown to allow the most effective RT inhibition by TIBO derivatives (12, 25). Furthermore, the finding of TIBO derivatives that deviate from this linearity may be indicative of compounds with good anti-HIV activity yet other template-primer preferences. Overall, a close correlation was found: i.e., structural modifications that led to a more effective inhibition of HIV-1 replication in cell culture also led to a more efficient inhibition of HIV-1 RT. This sharply contrasted with the absence of any correlation with cytotoxicity. Only RT inhibition by R86150 was much lower than expected on the basis of its anti-HIV activity in cell culture. Whether this is linked to the choice of the template-primer and/or novel substitutions in this molecule needs to be further investigated. R86183 was found to be a potent inhibitor of HIV-1 RT with an EC_{50} below 100 nM in both the endogenous system and the exogenous system. When the RT inhibition by R86183 was compared with that of R82150, the new TIBO derivative R86183 appeared to be less discriminative between RNA- and DNA-dependent DNA polymerase activities. Whether this would be typical for 8-substituted compounds and points to slightly different interaction with the putative TIBO site on HIV-1 RT needs to be further investigated.

When R86183 was combined with 2′,3′-dideoxynucleoside analogs such as AZT and DDI, the combinations proved to be synergistic in regard to antiviral effects in vitro. Synergistic interactions have been demonstrated for combinations of AZT with other TIBO-like compounds such as the HEPT derivative 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil (2), dipyridodiazepinone BI-RG-587 (31), pyridinone derivatives (16), and bis(heteroaryl)piperazines (33). An additive effect was observed for the combination of R86183 with the HIV-1 protease inhibitor Ro31-8959. Combination of R86183 with the PP_i analog phosphonoformic acid was slightly antagonistic. Whether this is related to the observation of Goldman et al. (16) that phosphonoformic acid is capable of displacing TIBO-like compounds such as L-697,639 from RT complexes is a subject for further study. In addition, this may suggest that, although the TIBO-like compounds are a distinct pharmacological class of HIV-1 RT inhibitors, their interaction with RT somehow also affects the process that leads to inhibition by 2′,3′-dideoxynucleoside and PP_i analogs.

R86183 was found to be active against an HIV-1 strain containing the Tyr-181→Cys mutation in the RT gene. This mutation is rapidly selected for in vitro and/or in vivo by several nonnucleoside RT inhibitors (NNRT inhibitors) including pyridinones (23), the dipyridodiazepinone inhibitor nevirapine (BI-RG-587) (32), the α-APA derivative R89439 (24), and some TIBO derivatives (21, 24). In fact, this has created the notion that the various NNRT inhibitors all behave similarly in that the mutants selected by these compounds display cross-resistance to other compounds of this class. However, recent observations show that some of these mutants can display large differences in sensitivity for different compounds. This is, for instance, seen with the Leu-100→Ile RT mutant which is highly resistant to TIBO derivatives but very sensitive to α-APA derivatives (24). Several NNRT inhibitors also display differences in the mutants they select for, as shown for the bis(heteroaryl)piperazine compounds (Pro-236→Leu) (33) and [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) derivatives (Glu-138→Lys) (5, 7). In these cases, mutant viruses were not cross-resistant to the other NNRT inhibitors. The former mutation even increased the sensitivity to other NNRT inhibitors. Whereas the TIBO derivative R86183 does select for drug-resistant HIV-1 variants, these do not contain the Tyr-181→Cys mutation (data not shown). Whereas the full characterization of R86183-resistant HIV-1 strains is in progress, it is interesting that the shift of the chlorine atom from the 9 position (R82913) to the 8 position (R86183) results in a difference in the resistance pattern. The current generation of NNRT inhibitors rapidly select for HIV-1 drug-resistant variants. The mutations in these strains are confined to a hydrophobic region near the catalytic center which constitutes the NNRT drug-binding pocket. The differential sensitivity profile of HIV-1 variants with mutations in the pocket region and the differential resistance profile suggest that NNRT inhibitors, depending on their chemical structure, display quantitative and/or qualitative differences in their interaction with the amino acids that constitute this pocket. It is therefore not inconceivable that combinations of different NNRT inhibitors with complementary properties can be worked out to facilitate interaction with the different variants of this pocket. Whether this will result in a mere selection of rarer variants and further delay of the occurrence of resistance or ultimately prevent its occurrence completely because of constrains put on the enzyme functions remains to be seen. On the other hand, the knowledge gained from SAR and resistance studies combined with the advances made in the elucidation of the three-dimensional structure may give new insights in the development of newer generations of NNRT inhibitors with an improved antiviral spectrum. Whether combination strategies with inhibitors interacting with other RT regions (e.g., dideoxynucleoside analogs) are successful in this regard is now under investigation.

The earlier prototype TIBO R82913 has been investigated in vivo in a phase I clinical trial aimed primarily at obtaining information regarding pharmocokinetics and side effects in patients with AIDS (28). In that study, the $CD4^+$ cell count and, in particular, the p24 antigenemia showed a favorable trend in patients in whom higher trough levels were attained. Since R86183 is between 5- and 10-fold more potent as an inhibitor of HIV-1 replication in vitro and because it is has, after oral administration, a better pharmacokinetic profile than

R82913 (data not shown), it is a potential new candidate for studies of efficacy in patients with HIV infection and disease.

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