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HIV-1 REVERSE TRANSCRIPTASE INHIBITION BY A DIPYRIDODIAZEPINONE DERIVATIVE: BI-RG-587

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Abstract—The dipyridodiazepinone derivative 6,11-dihydro-11-cyclopropyl-4-methyldipyrido[2,3-*b*:2′,3′-*e*]-[1,4]diazepin-6-one (BI-RG-587) selectively inhibits human immunodeficiency virus type 1 (HIV-1) replication by suppressing HIV-1 reverse transcriptase activity. Both RNA- and DNA-dependent polymerase associated activities of this enzyme were found to be inhibited by BI-RG-587 in a pattern dependent on the template used. The lowest IC_{50} values were obtained using poly(rC)-oligo$(dG)_{12-18}$ and poly(dA)-oligo$(dT)_{12-18}$ as template-primer. For the RNA-dependent activity poly(rC)-oligo$(dG)_{12-18}$ and dGTP appeared to enhance the inhibition of the RNA-dependent enzyme activity by BI-RG-587, with the effect of poly(rC)-oligo$(dG)_{12-18}$ dominating that of dGTP. Poly(rA)-oligo$(dT)_{10}$ seemed to decrease the inhibition whereas poly(rU)-oligo$(dA)_{12-18}$ or poly(rG)-oligo-$(dC)_{12-18}$ had no effect. dATP, dTTP and dCTP, three nucleotide triphosphates, also had no impact on the inhibition. Differences were observed for the template-dependent action of BI-RG-587 against the DNA-dependent enzyme activity. Both substrates were required to allow the inhibition by BI-RG-587 in the poly(dC)-oligo$(dG)_{12-18}$ and dGTP reaction, whereas only the template and enzyme interaction seemed to be necessary for the poly(dA)-oligo$(dT)_{12-18}$ and dTTP reaction. The different behaviors of DNA- and RNA-dependent DNA polymerase activities could indicate either the presence of different active sites for distinct activities or the presence of a unique active site with different configurations depending upon the template used. Also, BI-RG-587 showed a mutually exclusive inhibition when combined with two other classes of HIV-1 RT inhibitors represented by phosphonoformic acid and 3′-azido-3′-dideoxythymidine triphosphate.

Human immunodeficiency virus type 1 (HIV-1†) [1, 2] is believed to be responsible for acquired immunodeficiency syndrome (AIDS). Much effort is being made to find new chemotherapeutic agents that might result in more potent as well as less toxic drugs than 3′-azido-3′-dideoxythymidine (AZT) [3] for the treatment of AIDS.

A number of compounds with high specificity against HIV-1 virus have been identified recently. A non-nucleoside dipyridodiazepinone derivative, 6,11-dihydro-11-cyclopropyl-4-methyldipyrido[2,3-*b*:2′,3′-*e*]-[1, 4]diazepin-6-one (BI-RG-587), inhibits HIV-1 replication selectively with extremely low cytotoxic effects [4, 5]. A novel class of tetrahydroimidazo [4,5,1-*jk*] [1,4] - benzodiazepin -2 (1*H*) -one and -thione (TIBO) derivatives with the leading compound R82150 [6], and the 6-substituted acyclouridine derivative 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio)thymine (HEPT) [17] and its analogues have similar activity.

The viral target for these compounds appears to be the reverse transcriptase (RT) enzyme. This is a virus encoded protein essential for viral replication having three associated activities [8]: RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity, and RNAse H activity. This set of compounds was shown to have no effect on the viral replication cycle until DNA synthesis [9, 10], where the activity appears to be on HIV-1 RT catalyzed action. Moreover, they appear ineffective against HIV-2 RT, cellular DNA polymerase α, β, γ and other viral RT [4, 11, 12]. In the present study we elucidated the effect of BI-RG-587 on HIV-1 RT RNA- and DNA-dependent DNA polymerase associated activity using different template-primers. We further explored the BI-RG-587 mechanism of action by performing kinetic studies. In addition, the kinetic interaction of BI-RG-587 with other HIV-1 RT inhibitors such as phosphonoformic acid (PFA) and 3′-azido-3′-dideoxythymidine triphosphate (AZTTP) was also examined against HIV-1 RT activity.

METHODS

Poly(rA)-oligo$(dT)_{10}$, poly(rC)-oligo$(dG)_{12-18}$, poly(dC)-oligo$(dG)_{12-18}$, and poly(dA)-oligo-$(dT)_{12-18}$ were purchased from Pharmacia. Poly(rG), poly(rU), oligo$(dC)_{12-18}$, and oligo$(dA)_{12-18}$ were purchased from Pharmacia and annealed; the ratio template-primer was 4 to 1. dTTP, [^{3}H]dTTP (62 Ci/mmol), [^{3}H]dCTP (26 Ci/mmol), and [^{3}H]dATP (16 Ci/mmol) were acquired from ICN, and [^{3}H]-dGTP (11.75 Ci/mmol) was purchased from

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† Abbreviations: HIV-1, human immunodeficiency virus type 1; BI-RG-587, 6,11-dihydro-11-cyclopropyl-4-methyldipyrido[2,3-*b*:2′,3′-*e*]-[1,4]diazepin-6-one; IC_{50}, concentration inhibiting 50% of control; AZT, 3′-azido-3′-dideoxythymidine; AZTTP, 3′-azido-3′-dideoxythymidine triphosphate; PFA, phosphonoformic acid; AIDS, acquired immunodeficiency syndrome; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; RT, reverse transcriptase; HIV-2, human immunodeficiency virus type 2; $K_{I\,int}$, $K_{I\,intercept}$, and $K_{I\,slp}$, $K_{I\,slope}$.

Table 1. Template-primer dependence of HIV-1 RT inhibitors

Template-primer	Units used	Relative rate	BI-RG-587	R82150	PFA	HEPT
			IC_{50} (μM)			
RNA-dependent DNA polymerase associated activity						
Poly(rC)-oligo(dG)$_{12-18}$	4×10^{-3}	30	0.082 ± 0.01	0.078 ± 0.007	3.9 ± 0.8	23 ± 6
Poly(rA)-oligo(dT)$_{10}$	1.6×10^{-3}	100	0.650 ± 0.02	0.530 ± 0.035	0.2 ± 0.01	49 ± 2
Poly(rU)-oligo(dA)$_{12-18}$	0.16	0.4	0.185 ± 0.02	0.180 ± 0.01	170 ± 20	56 ± 10
Poly(rG)-oligo(dC)$_{12-18}$	0.32	0.1	0.125 ± 0.03	0.130 ± 0.02	260 ± 50[a]	33 ± 10[a]
DNA-dependent DNA polymerase associated activity						
Poly(dC)-oligo(dG)$_{12-18}$	4×10^{-3}	68	0.240 ± 0.005	0.265 ± 0.01	1.6 ± 0.08	53 ± 10
Poly(dA)-oligo(dT)$_{12-18}$	0.32	0.5	0.090 ± 0.005	0.090 ± 0.007	5.0 ± 0.6	37 ± 5

A unit was defined as the amount of enzyme necessary to incorporate 1 nmol of [^{3}H]dTMP into the poly(rA)-oligo(dT)$_{10}$ template in 1.0 min at 37° [14]. All the reaction rates were normalized to the reaction rate obtained using poly(rA)-oligo(dT)$_{10}$. The calculations of IC_{50} (concentration inhibiting 50% of control) and standard deviations were done from at least three determinations, with the exception of two indicated cases (a) in which only two determinations were performed and the average values ± range are presented. The reaction mixture used is described in Methods with the exception of dTTP (final concentration 30 μM), dATP (final concentration 100 μM) and poly(rU)-oligo(dA)$_{12-18}$ (final concentration 0.085 OD_{260} units/mL).

DuPont. dATP, dCTP, dGTP and PFA were acquired from Sigma. BI-RG-587 was provided by Boehringer Ingelheim, and HEPT and AZTTP by Dr. R. Schinazi of Emory University.

HIV-1 reverse transcriptase was expressed from a recombinant plasmid in bacteria as 66 and 51 kDa protein dimer according to D'Aquila and Summers [13]. The standard reaction mixture for the RNA- and DNA-dependent enzyme assays contained in a 50 μL volume: 50 mM Tris–HCl, pH 7.8, 50 mM KCl, 6 mM $MgCl_2$, 0.1 mg/mL heat-inactivated bovine serum albumin, 1 mM dithiothreitol, 0.5 OD_{260} units/mL template-primer, 10 μM [^{3}H]-dNTP (1 Ci/mmol) and 1.6×10^{-3}–0.32 units of HIV-1 RT (specific activity 80 units/mg). A unit was defined as the amount of enzyme necessary to incorporate 1 nmol of [^{3}H]dTMP into the poly(rA)-olido(dT)$_{10}$ template in 1.0 min at 37° [14]. The samples were incubated for 30 min at 37°, and all reaction were carried out in the linear range. Aliquots of 40 μL were spotted on glass fiber filters (Whatman GF/A) and processed for determination of trichloroacetic acid-insoluble radioactivity as described [14].

The analysis of kinetic study was made according to Lineweaver–Burk plots; v was expressed as pmol/min, and [S] as OD_{260} units/mL (template-primer) or as μM (dNTP). $K_{i\text{int}}$ and $K_{i\text{slp}}$ were calculated by replotting the intercept or the slope versus the inhibitor concentration. The dual inhibitor studies were performed according to Segel [15].

RESULTS

Since it was reported that HIV-1 RT inhibitors such as PFA, R82150 and phosphorothioate oligonucleotide show different degrees of enzyme inhibition related to the template-primer used [11, 16, 17], the inhibition of HIV-1 RT RNA-dependent DNA polymerase associated activity by BI-RG-587 using four different templates was examined (Table 1). The RT reaction velocity was highly dependent upon which template-primer was used. In comparison with poly(rA)-oligo(dT)$_{10}$, the reaction rate was about 30% using poly(rC)-oligo(dG)$_{12-18}$ and less than 5% using either poly(rG)-oligo-(dC)$_{12-18}$, or poly(rU)-oligo(dA)$_{12-18}$. Higher concentrations of poly(rU)-oligo(dA)$_{12-18}$ (<0.1 OD_{260} units/mL) could even inhibit the reaction. To achieve a significant incorporation of radiolabeled deoxyribonucleotide, different amounts of enzyme were used with different templates (Table 1). However, in all instances the amount of substrate incorporated was less than 10% of the amount available in the reaction mixture and well within the linear range.

BI-RG-587 revealed a template-dependent degree of RT inhibition, achieving the lowest IC_{50} value using poly(rC)-oligo(dG)$_{12-18}$ as template-primer. An 8-fold higher IC_{50} value was obtained using poly(rA)-oligo(dT)$_{10}$, while a 1- or 2-fold higher IC_{50} was observed with the other two template-primers (Table 1).

A very similar picture was shown by R82150 with the strongest inhibition achieved with poly(rC)-oligo(dG)$_{12-18}$ as template-primer and with IC_{50} values very close to the BI-RG-587 IC_{50} values. A different and more pronounced pattern of template dependence was observed using PFA as an inhibitor. In this case the lowest IC_{50} value was attained using poly(rA)-oligo(dT)$_{10}$ as template-primer; an approximately 19-fold increase of IC_{50} was observed with poly(rC)-oligo(dG)$_{12-18}$. Much higher IC_{50} values were found when either poly(rU)-oligo(dA)$_{12-18}$ or poly(rG)-oligo(dC)$_{12-18}$ were used as template-primer. HEPT did not show a high template-dependent inhibition pattern with only a 2-fold difference between the lowest and the highest IC_{50} value.

The template-primer-dependent inhibition of these compounds against the HIV-1 RT DNA-dependent DNA polymerase associated activity was also observed using poly(dC)-oligo(dG)$_{12-18}$ or poly(dA)-oligo(dT)$_{12-18}$ as template (Table 1). The reaction rate with poly(dA)-oligo(dT)$_{12-18}$ was less

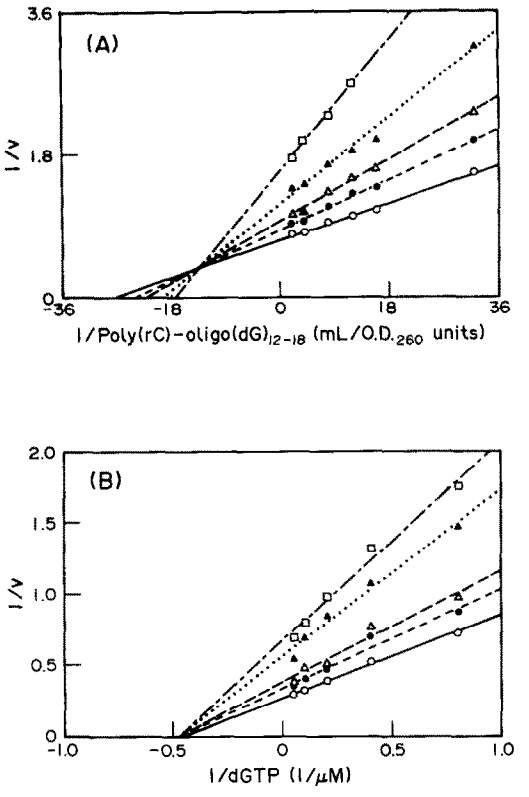

Fig. 1. Lineweaver–Burk plots of the inhibition of the HIV-1 RT RNA-dependent DNA polymerase associated activity by BI-RG-587 using the poly(rC)-oligo(dG)$_{12-18}$ and dGTP reaction. Panel A: poly(rC)-oligo(dG)$_{12-18}$ was used as the variable substrate and dGTP (10 μM) as the fixed substrate in the presence of increasing concentrations of BI-RG-587. The K_{Islp} was calculated to be 45 nM and the K_{Iint} 95 nM. Panel B: poly(rC)-oligo-dG)$_{12-18}$ was used as the fixed substrate (0.5 OD_{260} units/mL) and dGTP as the variable substrate in the presence of increasing concentrations of inhibitor. K_{Islp} and K_{Iint} values were 73 nM. BI-RG-587 concentrations in both plots were: 0 (○), 12.5 nM (●), 25 nM (△), 50 nM (▲), 100 nM (□).

than 5% of the velocity obtained using poly(dC)-oligo-(dG)$_{12-18}$. The lowest IC_{50} value was achieved using Poly(dA)-oligo(dT)$_{12-18}$. In contrast, PFA had an opposite pattern showing that the enzyme was more sensitive to this drug using poly(dC)-oligo(dG)$_{12-18}$ than poly(dA)-oligo(dT)$_{12-18}$ as template-primer. No significant template-dependent inhibition was observed with HEPT.

HIV-1 RT catalyzes the incorporation of dNTPs into the template-primer by forming an enzyme–template-primer–dNTP complex [18, 19]. Utilizing dGTP as the fixed saturating substrate and poly(rC)-oligo(dG)$_{12-18}$ (K_m = 0.034 OD_{260} units/mL) as the variable substrate, a mixed-type inhibition was observed according to a Lineweaver–Burk plot (Fig. 1A), with a K_{Iint} value very close to the IC_{50} value. A non-competitive inhibition pattern (Fig. 1B) was observed using dGTP (K_m = 2.1 μM) as the variable substrate, which was also observed by others [4]. A similar inhibition behavior was observed using either poly(rA)-oligo(dT)$_{10}$ (K_m = 0.025 OD_{260} units/mL) or dTTP (K_m = 10 μM) as the variable substrate in the poly(rA)-oligo(dT)$_{10}$ and dTTP reaction (Table 2). Non-competitive inhibitions by BI-RG-587 were obtained in the reaction with poly(rU)-oligo-(dA)$_{12-18}$ and poly(rG)-oligo(dC)$_{12-18}$ as template-primers, when either template or dNTP was a variable substrate (Table 2). Poly(rU)-oligo(dA)$_{12-18}$ and poly(rG)-oligo(dC)$_{12-18}$ had K_m values of 0.33 and 0.13 OD_{260} units/mL, respectively, while the K_m values calculated for dATP and dCTP were 70 and 3.3 μM, respectively.

Different patterns of inhibition of HIV-1 RT by BI-RG-587 were found by using various DNA template-primers. With poly(dC)-oligo(dG)$_{12-18}$ (K_m = 0.03 OD_{260} units/mL) or dGTP (K_m = 2.5 μM) as variable substrates in the poly(dC)-oligo(dG)$_{12-18}$ and dGTP reaction (Fig. 2, A and B), uncompetitive inhibition patterns were observed. When poly(dA)-oligo(dT)$_{12-18}$ (K_m = 0.08 OD_{260} units/mL) or dTTP (K_m = 20 μM) was used in the poly(dA)-oligo-(dT)$_{12-18}$ and dTTP reaction as variable substrates, the patterns of inhibition to the variable substrate were uncompetitive and non-competitive, respectively (Table 2).

BI-RG-587, PFA and AZTTP represent three different classes of RT inhibitors. Since a synergistic inhibition of HIV-1 replication by AZT and BI-RG-587 has been reported [5], it was of interest to examine the interaction of these compounds on HIV-1 RT. Dual inhibitor studies were conducted in the presence of different concentrations of BI-RG-587 and the simultaneous presence of increasing concentrations of PFA or AZTTP (Fig. 3), using poly(rC)-oligo(dG)$_{12-18}$ and poly(rA)-oligo(dT)$_{10}$ as template-primer, respectively. In both cases the slope of the lines remained unchanged. This suggested, kinetically speaking, the mutually exclusive nature of inhibition of HIV-1 RT by BI-RG-587 and PFA or AZTTP.

DISCUSSION

The potency of inhibition of HIV-1 RT by BI-RG-587 was shown to be dependent on the template used. The template-dependent inhibition by HIV-1 RT by other non-nucleosides has already been reported [11, 16, 17]. This template-dependent inhibition did not appear to be dependent on the different reaction velocity shown by each template, or on the incorporation of purine versus pyrimidine into the elongated DNA. The template-dependent inhibition could be due to subtle differences in the conformation of the enzyme when it interacts with different templates. The templates could induce conformational changes that may either facilitate or hinder the degree of the inhibition by the non-nucleoside RT inhibitors. Certain aspects of our studies were also examined by others [4, 16]; our results are in basic agreement except for the reported IC_{50} values for R82150 [6, 11] that were higher than ours when poly(rA)-oligo(dT)$_{12-18}$ (5.9 μM) or poly(rC)-oligo-(dG)$_{12-18}$ (0.34 μM) was used as template, and for the reported inability of R82150 to inhibit the HIV-1 RT DNA-dependent DNA polymerase activity

Table 2. Inhibition kinetics of HIV-1 RT by BI-RG-587

Fixed substrate	Variable substrate	K_m	Inhibition pattern	K_{islp} (nM)	K_{iint} (nM)
RNA-dependent DNA polymerase associated activity					
dGTP	Poly(rC)-oligo(dG)$_{12-18}$	0.034	Mixed	45	95
dTTP	Poly(rA)-oligo(dT)$_{10}$	0.025	Mixed	150	800
dATP	Poly(rU)-oligo(dA)$_{12-18}$	0.33	Non-comp.	165	165
dCTP	Poly(rG)-oligo(dC)$_{12-18}$	0.13	Non-comp.	160	160
Poly(rC)-oligo(dG)$_{12-18}$	dGTP	2.1	Non-comp.	73	73
Poly(rA)-oligo(dT)$_{10}$	dTTP	10.0	Non-comp.	750	750
Poly(rU)-oligo(dA)$_{12-18}$	dATP	70.0	Non-comp.	200	200
Poly(rG)-oligo(dC)$_{12-18}$	dCTP	3.3	Non-comp.	90	90
DNA-dependent DNA polymerase associated activity					
dGTP	Poly(dC)-oligo(dG)$_{12-18}$	0.03	Uncomp.		210
dTTP	Poly(dA)-oligo(dT)$_{12-18}$	0.08	Uncomp.		85
Poly(dC)-oligo(dG)$_{12-18}$	dGTP	2.5	Uncomp.		205
Poly(dA)-oligo(dT)$_{12-18}$	dTTP	20.0	Non-comp.	105	105

K_{islp} and K_{iint} were determined by replotting the slope and intercept values obtained in Lineweaver–Burk plots versus the inhibitor concentration. The K_m values of the variable substrates, calculated by Lineweaver–Burk plots, were expressed as OD_{260} units/mL (template-primers) and μM (dNTPs). The standard reaction mixture presented in Methods (template-primer 0.5 OD_{260} units/mL and dNTP 10 μM) was used with the exception of dTTP (final concentration 30 μM), dATP (final concentration 200 μM) and poly(rU)-oligo(dA)$_{12-18}$ (final concentration 0.085 OD_{260} units/mL) when used as fixed substrates.

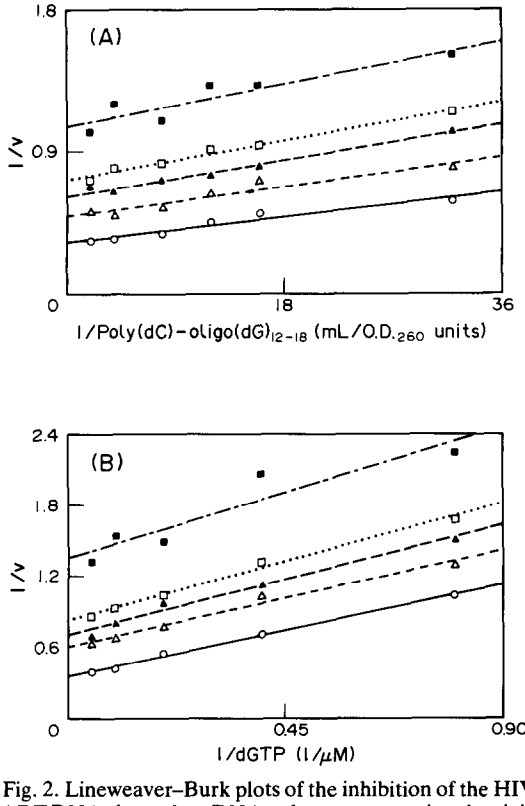

Fig. 2. Lineweaver–Burk plots of the inhibition of the HIV-1 RT DNA-dependent DNA polymerase associated activity by BI-RG-587 using the poly(dC)-oligo(dG)$_{12-18}$ and dGTP reaction. Panel A: poly(dC)-oligo(dG)$_{12-18}$ was used as the variable substrate and dGTP (10 μM) as the fixed substrate in the presence of different concentrations of BI-RG-587. K_{iint} was calculated to be 210 nM. Panel B: poly(dC)-oligo(dC)$_{12-18}$ was used as fixed substrate (0.5 OD_{260} units/mL) and dGTP as the variable substrate in the presence of increasing concentrations of inhibitors. The K_{iint} value was 205 nM. BI-RG-587 concentrations in both plots were: 0 (○), 50 nM (△), 100 nM (▲), 200 nM (□) and 400 nM (■).

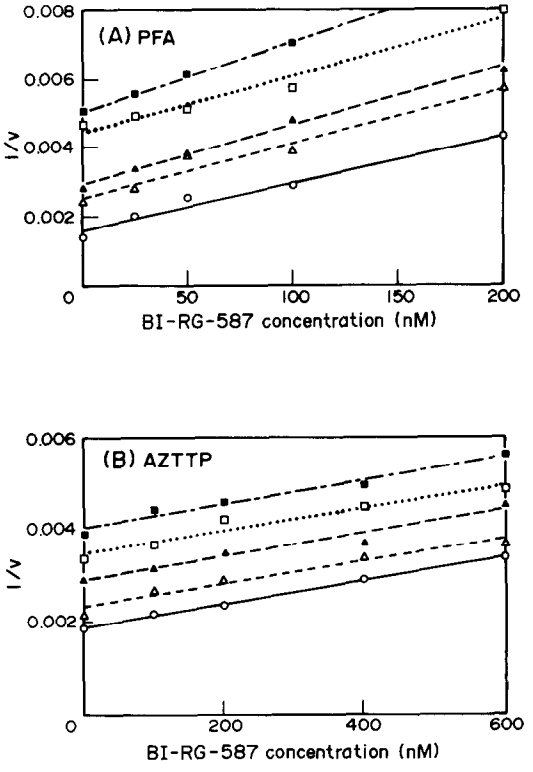

Fig. 3. Inhibition of HIV-1 RT RNA-dependent DNA polymerase by a combination of BI-RG-587 and other HIV-1 RT inhibitors. Panel A: combination of BI-RG-587 and PFA. Poly(rC)-oligo(dG)$_{12-18}$ and dGTP were used in a standard reaction. The concentrations of PFA were: 0 (○), 2.5 μM (△), 5 μM (▲), 10 μM (□), and 15 μM (■). Panel B: combination of BI-RG-587 and AZTTP. Poly(rA)-oligo(dT)$_{10}$ and dTTP were used in a standard reaction. The concentrations of AZTTP were: 0 (○), 2 nM (△), 4 nM (▲), 6 nM (□) and 8 nM (■).

[11]. These discrepancies could be due to different enzyme sources or assay conditions.

It is known that AZTTP is a chain-terminator agent which is incorporated into the DNA [20], competes with dTTP and is uncompetitive versus template-primer [21, 22]. Similarly, HEPT analogues appear to compete with dTTP, but are non-competitive when either poly(rA)-oligo(dT) or dGTP (using poly(rC)-oligo(dG) fixed substrate) is used as the variable substrate [12]. PFA has a non-competitive pattern of inhibition when varying dTTP, and an uncompetitive pattern versus the template-primer (poly(rA)-oligo$(dT)_{12-18}$) [16]. BI-RG-587 appeared to be different from the inhibitors mentioned above; in fact, different degrees and types of inhibition were observed when several template-primers for the RNA- and DNA-dependent reactions were used. The K_{islp} values obtained for the RNA-dependent DNA polymerase reaction, with fixed concentrations of dNTP, appeared to be close to 150 nM. The exception of the reaction with dGTP and poly(rC)-oligo$(dG)_{12-18}$ (K_{islp} = 45 nM) suggested that the presence of dGTP may potentiate the interaction between BI-RG-587 and enzyme. Since it is believed that the binding of dNTP to the enzyme is taking place after the binding of the template to the enzyme [18], dGTP may possess a particular ability to interact with the enzyme before the template and to affect the drug inhibition. It is interesting to note that there was an enhanced binding of BI-RG-587 analog to the HIV-1 RT after photoinactivation in the presence of dGTP [23].

A mixed-type inhibition was observed when poly(rC)-oligo$(dG)_{12-18}$ and poly(rA)-oligo$(dT)_{10}$ were used as variable substrates. Since the K_{islp} value indicates the interaction between drug and enzyme, whereas the K_{iint} value indicates the interaction between drug, enzyme and template, this result indicated that the interaction of these templates with the enzyme could affect the potency of drug inhibition. Poly(rC)-oligo$(dG)_{12-18}$ enhanced the inhibition of BI-RG-587 whereas poly(rA)-oligo$(dT)_{10}$ decreased this inhibition. Poly(rU)-oligo$(dA)_{12-18}$ and poly(rG)-oligo$(dC)_{12-18}$ showed no impact on this inhibition. When both poly(rC)-oligo$(dG)_{12-18}$ and dGTP were present in the reaction, poly(rC)-oligo$(dG)_{12-18}$ appeared to dominate the effect of the dGTP. This was suggested by the fact that, considering the possibility that dGTP may be able to interact with the enzyme before the template, the K_{iint} value was bigger than the K_{islp} value and it was close to the IC_{50} value.

When the template-primers were used as fixed substrate in the reaction, non-competitive patterns were observed. Furthermore, the K_{iint} and K_{islp} values were consistent with the K_{iint} calculated using the templates as variable substrate and with the IC_{50} values.

Marked differences were observed in the pattern of inhibition for the DNA- and RNA-dependent DNA polymerase associated activities. When poly(dC)-oligo$(dG)_{12-18}$ and dGTP were used in the reaction, both template and dGTP were required to interact with the enzyme to allow the inhibition by BI-RG-587. When poly(dA)-oligo$(dT)_{12-18}$ and dTTP were used, only poly(dA)-oligo$(dT)_{12-18}$ was required for the inhibition of this enzyme activity. A different behavior in the processivity of the HIV-1 RT in two DNA polymerase associated activities has already been reported [19, 24]. Together with the observations made here, it could indicate either the presence of different active sites for distinct enzyme activities, or the presence of a unique active site with different configurations depending upon the template used. Structure determination of the enzyme could allow one to distinguish between these two possibilities.

The inhibition studies with BI-RG-587 showed mutually exclusive kinetic interactions with PFA, AZTTP and R82150 (data not shown), which were similar to the reported interaction between PFA and AZTTP [25]. This may indicate that the binding of one of these inhibitors to the enzyme may form an inactive complex. Then the second inhibitor either cannot bind the enzyme, or the binding does not affect the inhibition. Analysis of these interactions with an isobologram method showed a simple additive inhibition (data not shown). This suggests that the synergistic effect reported in the inhibition of HIV-1 by the combination of AZT and BI-RG-587 [5] may be due either to an unknown interaction that enhances the metabolic pathway of these drugs into the cells, or to the fact that the biological observation and the *in vitro* enzyme assay are not correlated linearly. However, a synergistic enhancement of the inhibition of the viral target by these compounds cannot explain the biological observation.

In summary, BI-RG-587 displayed a template-dependent inhibition of both the RNA- and DNA-dependent DNA polymerase activities associated to HIV-1 RT. A similar pattern was also observed with R82150. In the kinetic study the inhibition of these activities by BI-RG-587 was affected by different template-primers and by dGTP. Marked differences were observed in the inhibition of the two DNA polymerase activities implying either the presence of different active sites for distinct activities or the presence of a unique active site with a different configuration depending upon the template-primer. BI-RG-587 showed a mutually exclusive inhibition with PFA, AZTTP and R82150, suggesting that the binding of one drug could create an inactive complex such that the second drug either could not bind the enzyme or this binding does not affect the inhibition. These results could suggest that in the presence of the natural (endogenous) template at a minimum effective dosage of BI-RG-587 the inhibition of both RNA- and DNA-dependent associated activities would occur. The preferential inhibition event of the DNA chain elongation for the RNA-dependent reaction could be the incorporation of dGMP, whereas for the DNA-dependent reaction the preferential site could be the incorporation of dTMP. However, at a higher concentration of the drug all the incorporations could be inhibited.

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