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Destabilization of the HIV-1 Reverse Transcriptase Dimer upon Interaction with *N*-Acyl Hydrazone Inhibitors

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ABSTRACT

N-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) inhibits both the DNA polymerase and ribonuclease H (RNase H) activities of the human immunodeficiency virus type 1 reverse transcriptase. In this study, we show that BBNH binding impacts on the stability of the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) heterodimer. The Gibbs free energy of dimer dissociation of HIV-1 RT is decreased in the presence of increasing concentrations of BBNH, resulting in a loss in stability of 3.8 kcal mol^{-1}. To evaluate whether this observed phenomenon was mediated by BBNH binding to one or more sites in RT, we synthesized a variety of BBNH analogs and identified (4-*t*-butylbenzoyl)-2-hydroxy-1-salicylyl hydrazone (BBSH) and (4,*N*,*N*-dimethylaminobenzoyl)-2-hydroxy-1-naphthyl hydrazone as specific inhibitors of RT DNA polymerase or RT RNase H activity, respectively. Interestingly, only BBSH provided significant destabilization of the HIV-1 RT dimer. The identification of these specific inhibitors, in combination with other biochemical data, suggests a model in which two molecules of BBNH bind per RT heterodimer. In this regard, only the binding of hydrazone molecules in the DNA polymerase domain activity elicits the observed destabilization of the HIV-1 RT heterodimer.

The multifunctional human immunodeficiency virus (HIV) type 1 reverse transcriptase (RT) catalyzes the conversion of single-stranded viral genomic RNA into double-stranded DNA. HIV-1 RT has RNA-dependent DNA polymerase activity (RDDP) to synthesize a (−)-strand DNA copy of the (+)-genomic RNA, RNase H activity to digest the RNA component of the resultant (+)RNA/(−)DNA duplex, and DNA-dependent DNA polymerase activity to synthesize (+)DNA using the (−)DNA as template. Numerous inhibitors of HIV-1 RT have been identified, 10 of which are in current clinical use as treatment for HIV infection (see Sluis-Cremer et al., 2000a and Parniak and Sluis-Cremer, 2000 for recent reviews). However, the majority of these inhibitors are directed at the DNA polymerase activity of RT, and they all function primarily at preintegrational stages of virus replication. Unfortunately, HIV-1 readily develops resistance to these inhibitors, and therefore, the identification of other compounds that exhibit novel mechanisms of action is essential.

The gene for HIV-1 RT encodes a 66-kDa polypeptide; however, the biologically relevant form of the enzyme is considered to be a heterodimer consisting of two subunits of 66 and 51 kDa. The latter subunit is derived from the former by proteolytic processing during HIV-1 assembly and maturation. Although the precise kinetics of HIV-1 RT processing and dimerization are not well characterized, it is clear that the expression of RNA-dependent DNA polymerase and RNase H activities requires a dimeric enzyme (Restle et al., 1990, 1992; Divita et al., 1995a,b). Therefore, inhibition of RT dimerization or the disruption of RT dimer stability could provide alternative targets for HIV-1 inhibition (Divita et al., 1994; Morris et al., 1999a,b; Sluis-Cremer et al., 2000b). In regard to the latter strategy of RT inhibition, we have recently shown that 1-{spiro[4-amino-2,2-dioxo-1,2-oxathiole-5,3′-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]]}-3-ethylthymine ($TSAOe^3T$) destabilizes the subunit interactions of both p66/p51 heterodimer and p66/p66 homodimer forms of HIV-1 RT (Sluis-Cremer et al., 2000b).

We previously identified *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH; Fig. 1, compound 1) as a multitarget inhibitor of HIV-1 RT, active

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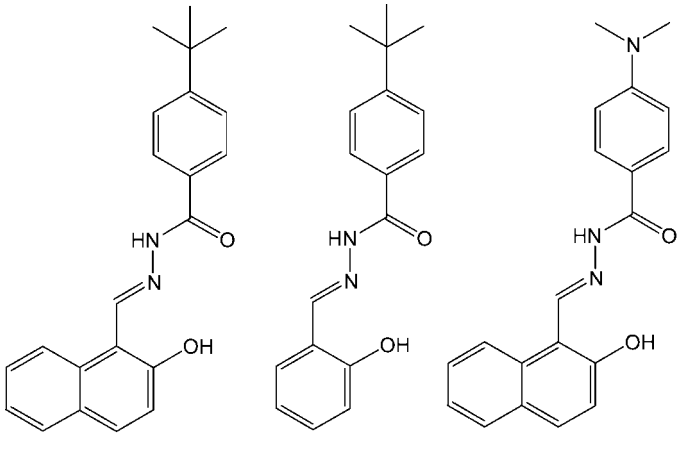
ABBREVIATIONS: HIV, human immunodeficiency virus; BBNH, *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone; BBSH, (4-*t*-butylbenzoyl)-2-hydroxy-1-salicylyl hydrazone; CD, circular dichroism; DABNH, (4,*N*,*N*-dimethylamino benzoyl)-2-hydroxy-1-naphthyl hydrazone; HPLC, high-performance liquid chromatography; RDDP, RNA-dependent DNA polymerase; RNase H, ribonuclease H; RT, reverse transcriptase; T/P, template-primer; $TSAOe^3T$, 1-{spiro[4-amino-2,2-dioxo-1,2-oxathiole-5,3′-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]]}-3-ethylthymine; NNRTI, nonnucleoside reverse transcriptase inhibitor.

against both the DNA polymerase and RNase H activities of the enzyme (Borkow et al., 1997). We have proposed that BBNH exerts its multitarget inhibition by binding to two spatially separate sites in RT, one in the DNA polymerase domain and the other in the RNase H domain (Borkow et al., 1997; Arion et al., 2002). Furthermore, we have shown that treatment of HIV-1 chronically infected H9 cells with an appropriately formulated iron chelate of BBNH [Fe(II-I)BBNH as SP1093V] resulted in a nascent virus that was significantly attenuated in infectivity, thus suggesting a postintegrational target for this inhibitor. However, viral p24 levels and HIV-1 protein processing in the nascent virions were unaffected by Fe(III)BBNH (Parniak et al., 1998). Although this compound targets RT, its enzymatic activities are not necessary for the postintegrational stages of HIV replication. Because RT is an obligatory dimer, we considered that BBNH might act at the level of RT dimer stability.

Our results indicate that BBNH considerably decreases the dimeric stability of p66/p51 HIV-1 RT. To determine whether BBNH binding to both the DNA polymerase and RNase H domains in RT was an important criterion for dimer destabilization, we synthesized a variety of BBNH analogs and identified (4-*t*-butylbenzoyl)-2-hydroxy-1-salicylyl hydrazone (BBSH), which binds specifically to the polymerase domain, and (4,*N*,*N*-dimethylaminobenzoyl)-2-hydroxy-1-naphthyl hydrazone (DABNH), which binds only to the RNase H domain. The structures of BBNH, BBSH, and DABNH are illustrated in Fig. 1. Only BBNH and BBSH provided significant destabilization of the HIV-1 RT dimer, suggesting that the interaction with a site in the RT DNA polymerase domain is important for the dimer-destabilizing activity of the compound.

Experimental Procedures

Materials. Recombinant HIV-1 RT p66/p51 heterodimer was expressed and purified essentially as described previously (Fletcher et al., 1996). [^{3}H]dNTPs, γ-[^{32}P]ATP, and the homopolymeric template/primers (T/P) poly(rA)-oligo(dG)$_{12-18}$ and poly(rC)-oligo(dG)$_{12-18}$ were products of Amersham Biosciences (Montreal, QC, Canada). Heteropolymeric T/P was prepared using an RNA transcript derived from pHIV-PBS and a synthetic 18-mer oligonucleotide primer (Arts et al., 1994). The RNase H substrate poly([^{3}H]rG)-poly(dC) was prepared as described previously (Starnes and Cheng, 1989). Nevirapine was obtained from Boehringer-Ingelheim (Canada) Ltd. (Laval, QC, Canada). BBNH and derivatives thereof were synthesized by standard methods (Edward et al., 1988). All other reagents were of the highest quality available and were used without further purification.



1 *2* *3*

Fig. 1. Chemical structures of BBNH (*1*), BBSH (*2*), and DABNH (*3*).

Assay of RT DNA Polymerase Activity. HIV-1 RT DNA polymerase activity was determined by a fixed time assay. Reaction mixtures (50 μl total volume) contained 50 mM Tris-HCl (pH 7.8, 37°C), 60 mM KCl, 10 mM $MgCl_2$, 5 μg/ml of either poly(rA)-oligo(dT)$_{12-18}$ or poly(rC)-oligo(dG)$_{12-18}$, and either 20 μM [^{3}H]TTP or 10 μM [^{3}H]dGTP. Reactions were initiated by the addition of 50 to 80 ng of RT (9–12 nM final concentration). Reaction mixtures were incubated at 37°C for 20 min and then quenched with 250 μl of ice-cold 10% trichloroacetic acid containing 20 mM sodium pyrophosphate. Quenched samples were left on ice for 20 min then filtered using 1.2-μm glass fiber Type C filter multiwell plates (Millipore Corp., Bedford, MA) and washed sequentially with 10% trichloroacetic acid containing 20 mM sodium pyrophosphate and with 100% ethanol. The extent of radionucleotide incorporation was determined by liquid scintillation spectrometry of the dried filters.

Assay of RT RNase H Activity. RT RNase H activity was assayed using either the homopolymeric poly([^{3}H]rG)-poly(dC) substrate or a 5′-^{32}P-labeled heteropolymeric RNA template (5′-GAUCUGAGCCUGGGAGCU-3′) annealed to a complementary DNA oligonucleotide (3′-CTAGACTCGGACCCTCGA-5′). Assays of RT RNase H activity using the homopolymeric substrate were carried out in reaction mixtures (50 μl total volume) that contained 50 mM Tris-HCl (pH 8.0, 37°C), 60 mM KCl, 10 mM $MgCl_2$, and 2 μg/ml poly([^{3}H]rG)-poly(dC). Reactions were initiated by the addition of RT (generally, 50 ng of p66/p51 RT, to provide a 9 nM final concentration), followed by 20 min incubation at 37°C. Reactions were quenched by placing the tubes on ice followed by the addition of 100 μl of cold 7% perchloric acid. After 30 min on ice, the reaction mixtures were centrifuged at 12000*g* for 15 min. The supernatants (100 μl) were carefully removed, and the radioactivity was determined by liquid scintillation analysis.

RT RNase H assays using the heteropolymeric RNA/DNA hybrid duplex were carried out in 10-μl aliquots, which included 50 mM Tris-HCl (pH 8.0, 37°C), 60 mM KCl, 10 mM $MgCl_2$, 7.5 pmol of DNA antisense oligonucleotide, and 2.5 pmol of ^{32}-P 5′end-labeled RNA. Reactions were initiated by the addition of RT (10 nM final concentration), incubated for 15 min (37°C), and then quenched by adding sequencing loading dye buffer (98% deionized formamide, 10 mM EDTA, 1 mg/ml bromphenol blue, and 1 mg/ml xylene cyanol). Reaction products were resolved on an 18% sequencing polyacrylamide gel containing 7M urea in Tris-Borate-EDTA buffer and visualized by autoradiography (Kodak X-OMAT film; Eastman Kodak, Rochester, NY).

PAGE Analysis of RT Processive DNA Synthesis in the Presence of Inhibitor. Assay conditions were identical to that described previously (Arion et al., 1996b). Briefly, HIV-1 RT was preincubated with ^{32}P-labeled heteropolymeric T/P at 37°C for 10 min. DNA synthesis was initiated by the simultaneous addition of dNTPs plus the polymerization trap heparin (0.2 mg/ml). Heparin competes with the binding of T/P for binding to free RT (Beard and Wilson, 1993). After appropriate incubation at 37°C in either the absence or presence of varying concentrations of RT inhibitors, reactions were quenched by addition of an equal volume of sequencing gel loading buffer. Samples were heated for 5 min at 100°C and then analyzed by denaturing PAGE using 16% polyacrylamide gels containing 7M urea. Products were visualized by autoradiography and quantified by densitometry.

Urea Denaturation of HIV-1 RT. HIV-1 RT (200 nM) was incubated with varying concentrations of urea (0–8M) in 50 mM Tris-HCl, pH 7.5, and 50 mM sodium sulfate for 1 h. Changes in protein structure and function were then assessed using a variety of probes. The intrinsic tryptophan fluorescence of the enzyme was monitored

using a fluorescence excitation wavelength of 295 nm and fluorescence emission wavelengths of 335 (in the absence of urea) and 352 nm (in the presence of 8M urea) using a T-format fluorescence spectrometer. Data were plotted as a ratio of the fluorescence emission values at 335 and 352 nm [i.e., F_{352}(unfolded)/F_{335}(folded)]. Size-exclusion high-performance liquid chromatography (HPLC) was carried out essentially as described previously (Sluis-Cremer et al., 2000). Circular dichroism experiments were carried out using a Jasco J-750 spectrometer (Tokyo, Japan), and data are reported as mean ellipticity values at 220 nm. For RT activity analyses, an aliquot of the denatured RT was briefly assayed (4 min) to evaluate the residual RT activity. Four-minute assays were used to ensure that no other parameters (such as T/P) affected the equilibrium between heterodimer and monomeric forms. Similar strategies have been successfully employed previously (Divita et al., 1995).

Urea-Mediated Dissociation of the HIV-1 RT Heterodimer. Urea denaturation of p66/p51 HIV-1 RT, in the presence or absence of various concentrations of *N*-acylhydrazones, was carried out essentially as described previously (Sluis-Cremer et al., 2000b). Denaturation isotherms, obtained by following the loss of RT RDDP and RNase H activities with increasing concentrations of denaturant, can be approximated by a two-state transition between dimer and monomer (Divita et al., 1994). Denaturation curves were evaluated according to a linear extrapolation method (Pace, 1986). Briefly, an equilibrium constant, K_d, was calculated at each point in the transition region of the denaturation isotherm according to the following expression (Bowie and Sauer, 1989; Timm and Neet, 1992): $K_d = P_t f_M^2/(1 - f_M)$, where P_t is the total protein concentration of RT and f_M is the fraction of monomeric protein. f_M was calculated from the urea dissociation isotherms using the algorithm: $f_M = (y_{100} - y)/y_{100}$), where y_{100} and y correspond to RT activity in the absence (i.e., 100% active RT) and presence of varying concentrations of urea, respectively. A linear dependence of the Gibbs free energy of monomer formation ($\Delta G = -RT \ln K_d$) on the denaturation concentration is assumed (Schellman, 1978): $\Delta G_D = \Delta G_D^{H_2O} - m$[urea], where $\Delta G_D^{H_2O}$ represents the difference in Gibbs free energy between the monomer and dimer transition in the absence of denaturant.

Conformational stability parameters were determined by iterative fitting of the denaturation curves to the above equations using the numerical analysis functions of σ-Plot 5.0 (SPSS Science, Chicago, IL) and a Levenberg-Marquardt least-squares algorithm.

Results

Urea Denaturation of the HIV-1 RT Heterodimer. Urea is a relatively strong denaturant, which at high concentrations favors the complete unfolding of polypeptide chains. To optimize the concentration of urea required to dissociate the RT subunits (but not promote significant unfolding of the polypeptide chains), we evaluated the changes in various structural and functional parameters of RT as a function of urea concentration (Fig. 2). In light of the structural complexity of the enzyme, a variety of probes were used to monitor changes occurring at different structural levels during the denaturation process. These included size-exclusion HPLC, tryptophan fluorescence (the 37 tryptophan residues in RT provide a "fingerprint" for tertiary structure), far-ultraviolet circular dichroism (CD; probe for protein secondary structure), and RT activity itself. The denaturation isotherms for each of these probes are illustrated in Fig. 2. The isotherms monitored by size-exclusion HPLC and RT activity are coincident, suggesting that loss of RT activity is a direct result of dissociation of the heterodimer and not a result of conformational change preceding dissociation. Analyses of the denaturation isotherms monitored by CD spectroscopy indicate that no significant changes in protein secondary are observed during the dimer-monomer transition. However, a small increase in the ratio of the tryptophan fluorescence emission at 335 and 352 nm is observed. This increase may result from the exposure of tryptophan residues that are buried in the RT dimer interface (five in the p66 subunit and two in the p51 subunit), as has been suggested previously (Divita et al., 1993). It was previously reported that the dissociation of RT subunits was accompanied by significant changes (increased fluorescence and large spectral shifts) in tryptophan fluorescence (Divita et al., 1993). However, in the present study, only small changes in fluorescence intensity and emission maximum wavelength were noted compared with that noted upon complete unfolding of the subunits (Fig. 2, inset). Our experiments suggest that the addition of a relatively low concentration of urea promotes dissociation of the subunits, and that this dissociation phenomenon can be monitored accurately by evaluating the loss of RT activity. Furthermore, it implies that no significant structural changes (or unfolding events) are associated with dissociation of the RT heterodimer subunits.

Urea Dissociation of HIV-1 RT Heterodimer in the Absence and Presence of BBNH. Because RT activity is dependent on the quaternary structure of the enzyme, we used this parameter to evaluate the effect of BBNH binding on the intrinsic stability of the HIV-1 RT dimer. Figure 3 illustrates dissociation isotherms of heterodimeric p66/p51 RT. In the absence of inhibitor, the transition from RT dimer

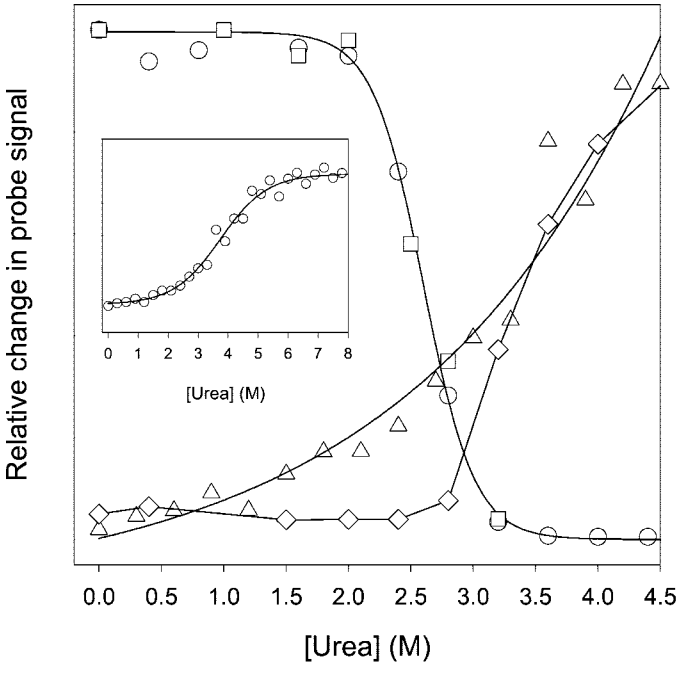


Fig. 2. Denaturation isotherms of HIV-1 RT monitored by various structural [Trp fluorescence (Δ), size-exclusion HPLC (□), CD (◇)] and functional probes [RT activity (○)]. RT (200 nM) was incubated in varying concentrations of urea (0–8M) for 1 h to allow for the establishment of a thermodynamic equilibrium. Trp fluorescence is reported as a ratio of the fluorescence emission value at 335 (folded protein) and 352 nm (8M urea denatured). Size-exclusion HPLC values are reported as percentage of heterodimeric RT as a function of urea concentration. CD values are reported as ellipticity values ($°\cdot cm^2\cdot dmol^{-1}$). RT activity is plotted as the residual (%) DNA polymerase activity as a function of urea concentration. Inset, urea denaturation transition of HIV-1 RT monitored by Trp fluorescence (F_{352}/F_{335}). This inset highlights that the increase in Trp fluorescence from 0 to 3M urea is small relative to the overall transition from folded RT heterodimer to unfolded polypeptide chains.

to monomer can be described by a sharp sigmoidal isotherm with a midpoint of denaturation ($[urea]_{1/2}$) at 2.30M urea. The Gibbs free energy of dimer dissociation in the absence of urea ($\Delta G_D^{H_2O}$) is estimated to be 10.1 kcal mol^{-1} for this transition, and the *m*-value (linear dependence of ΔG_D on urea concentration) is 3.6 kcal mol^{-1} M^{-1}. If HIV-1 RT is preincubated (20 min) with BBNH and then subjected to urea denaturation, the $[urea]_{1/2}$ value is shifted to lower urea concentrations. Values of 2.20, 1.85, and 1.76M were calculated for RT denaturation isotherms in the presence of 2, 10, and 25 μM of BBNH, respectively. Furthermore, the $\Delta G_D^{H_2O}$ and *m*-value are decreased to 6.3 kcal mol^{-1} and 2.3 kcal mol^{-1} M^{-1} in the presence of 25 μM BBNH, respectively. The change in *m*-value in the presence of BBNH may reflect differences in the solvent exposure of RT hydrophobic residues in the dimeric and monomeric states and/or alterations in the pathway by which the subunits dissociate due to ligand-induced changes in the subunit interactions (Sluis-Cremer et al., 2000).

Synthesis of BBNH Analogs and Characterization of Their RT-Inhibitory Properties. Previous biochemical data have suggested that BBNH binds to both the DNA polymerase and RNase H domains of HIV-1 RT (Borkow et al., 1997; Arion et al., 2002). To determine whether multisite binding is an important criterion for *N*-acylhydrazone–induced RT dimer destabilization, we used new BBNH analogs that were found to selectively inhibit either RT DNA polymerase or RNase H activity (Table 1), namely BBSH (Fig. 1, compound 2), which inhibits RT RDDP activity with a similar potency to BBNH but does not inhibit RT RNase H activity, and DABNH (Fig. 1, compound 3), which inhibits RT RNase H activity with a similar potency to BBNH (~4 μM) but is virtually inactive against RT RDDP activity ($IC_{50} \gg 20$ μM). Furthermore, we evaluated the ability of BBNH and BBSH to inhibit RT-containing mutations associated with resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs). NNRTI-resistant RT (including K103N, Y181C, Y188H, and E138K) remained sensitive to inhibition by BBNH and BBSH (data not shown), consistent with previously published data (Borkow et al., 1997).

DABNH did not inhibit the DNA polymerase activity of RT. However, DABNH was as effective as BBNH in inhibiting RT RNase H activity (Table 1). Neither compound altered the pattern of RNase H cleavage of the RNA strand in the RNA/DNA heteroduplex (Fig. 4).

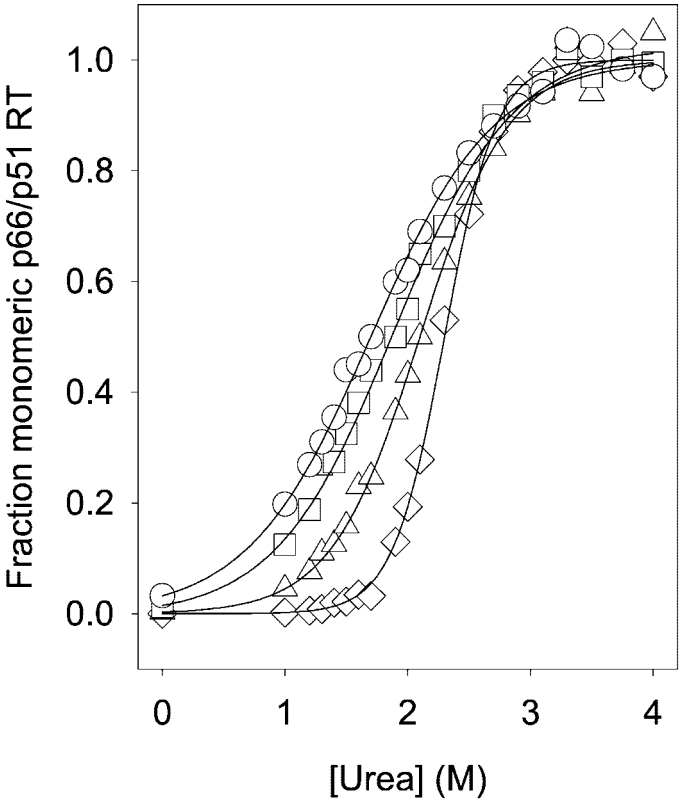


Fig. 3. Denaturation isotherms of HIV-1 RT in the absence (○) or presence of 2 (▲), 10 (□), or 25 μM (●) BBNH. Denaturation isotherms are averaged data calculated from both RT RDDP and RT RNase H activity. Standard errors ranged from 0.3 to 3% over the range of the measurements in the experiments. Fraction monomeric RT was calculated according to equation described under *Experimental Procedures*. Values for $\Delta G_D^{H_2O}$ were calculated to be 10.1 ± 0.1 kcal mol^{-1}, 8.3± kcal mol^{-1}, 7.05 ± 0.24 kcal mol^{-1}, and 6.3 ± 0.33 kcal mol^{-1} in the presence of 2, 10, and 25 μM BBNH, respectively.

Effect of BBNH Analogs on the Stability of RT Heterodimer Subunit Interactions. The curves for urea-induced dissociation of p66/p51 HIV-1 RT in the presence of varying concentrations of the *N*-acylhydrazones used in the present study showed that only BBNH and BBSH significantly destabilize the enzyme dimer in a concentration-dependent manner (Fig. 5; Table 2). The maximum change in $\Delta G_D^{H_2O}$ ($\Delta\Delta G$) for p66/p51 HIV-1 RT is 3.80 kcal mol^{-1} and 3.72 kcal mol^{-1} in the presence of BBNH and BBSH, respectively (Table 2). This observed decrease in $\Delta G_D^{H_2O}$ falls within the theoretical limit by which small-molecule ligand binding can contribute to the destabilization of macromolecular conformation (Freire, 1998). In contrast, the dissociation isotherms for the HIV-1 RT complex with DABNH are virtually identical to those obtained in the absence of inhibitor. It therefore seems that inhibitor binding to the DNA polymerase domain, but not to the RNase H domain, results in the observed destabilization of the RT dimer by *N*-acylhydrazones.

Effect of Inhibitor Concentration on RT DNA Polymerase Activity under Processive Conditions. NNRTIs, such as nevirapine, block RT-catalyzed DNA synthesis in regions of the RNA or DNA template that are rich in secondary nucleic acid structure (Arion et al., 1996b; Götte et al., 1999). This results in RT exhibiting a mode of DNA polymerization that is more distributive in the presence of certain NNRTIs under single processive-cycle conditions (Kopp et al., 1991; Olsen et al., 1994; Arion et al., 1996b). We have previously shown significant differences in the processivity of

TABLE 1

Inhibition of HIV-1 RT DNA polymerase and RNase H activities by various *N*-acylhydrazones

Values were determined using 1.7 nM RT, 5 μg/ml poly(rC)-oligo$(dG)_{12-18}$, and 20 μM dGTP.

Values were determined using 1.7 nM RT and 2 μg/ml poly([^{3}H]rG-poly(dC) as described by Starnes and Cheng (1989). IC_{50} values determined using heteropolymeric T/P yield similar values.

Inhibitor	IC_{50}	
	DNA Polymerase	RNase H
	μM	
BBNH	2.5 ± 0.6	3.5 ± 0.4
BBSH	4.7 ± 0.5	>50
DABNH	>40	4.0 ± 0.8

DNA synthesis carried out by HIV-1 RT in the presence of nevirapine and TSAOe^{3}T (Arion et al., 1996b). Because BBNH and BBSH destabilize RT subunit interactions, as does TSAOe^{3}T (Sluis-Cremer et al., 2000b), we examined the effect of *N*-acylhydrazones on RT-processive DNA polymerization to ascertain whether there may be any similarities between these inhibitors and TSAOe^{3}T.

We used a heteropolymeric RNA template comprising that sequence of HXB2D HIV-1 genomic RNA used for the synthesis of strong-stop (−)DNA (Arts et al., 1994). In the absence of inhibitor and under processive DNA polymerization conditions (i.e., in the presence of a polymerization trap), a number of polymerization products of different sizes are observed (Fig. 6A, lanes 1, 6, and 11). These may arise from transcriptional pausing and/or template-primer dissociation events that occur during RT-catalyzed primer extension (Huber et al., 1989; Abbotts et al., 1993). In the presence of *N*-acylhydrazones, the distribution of these polymerization products does not seem to be altered with respect to the uninhibited enzyme, although the amount of these products (band intensity) is reduced (Fig. 6A, lanes 2–5 and 7–10). There is no evidence of increased pausing and/or dissociation, and even at relatively high concentrations (24 μM) of inhibitor (either BBNH or BBSH), there is evidence of full-length DNA polymerization product (p + 173; Fig. 6, A and B). In contrast, nevirapine induces a more distributive mode of RT polymerization (Fig. 6A; lanes 12–15) in which there is an accumulation of short DNA products at position p + 2 through p + 7 (Fig. 6B), a region upstream of the primer binding site that is rich in secondary structure and results in a major pausing site during HIV-1 RT polymerization (Baudin et al., 1993; Isel et al., 1995). Furthermore, there is a complete absence of full-length DNA polymerization product (p + 173) at all concentrations of nevirapine tested.

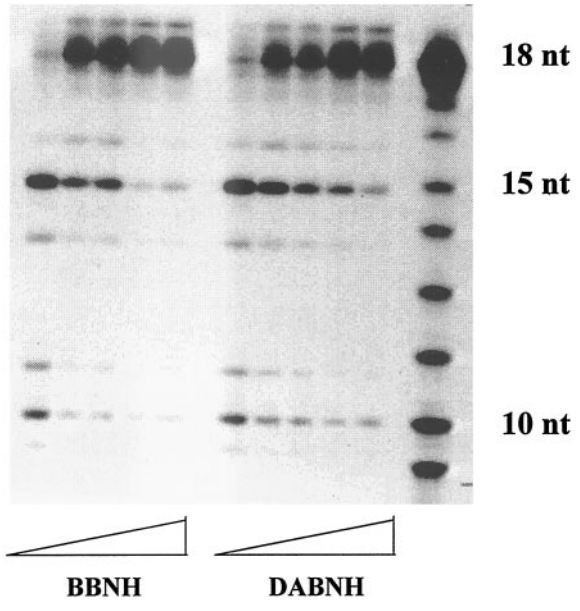


Fig. 4. Inhibition of RT RNase H activity by BBNH (lanes 1–5) and DABNH (lanes 6–10). A 5′-^{32}P-labeled 18 nucleotide heteropolymeric RNA template (18-mer) was annealed to complementary 18 nucleotide DNA oligonucleotide. Reactions were carried out as described under *Experimental Procedures* using 2.5 pmol of RNA template and 10 nM HIV-1 RT. IC_{50} values of ~4 μM were calculated for both compounds.

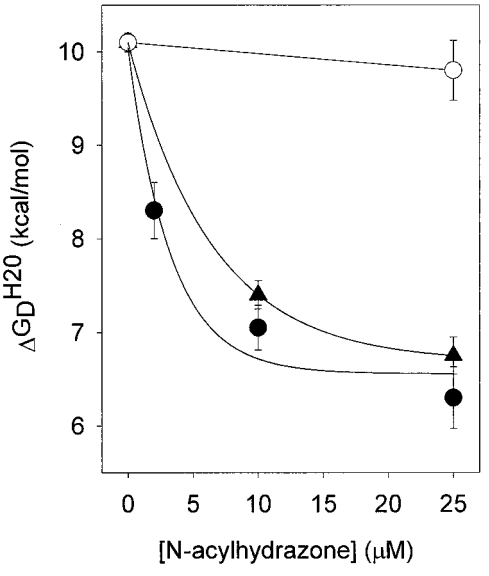


Fig. 5. Dependence of HIV-1 RT $\Delta G_D^{H_2O}$ on concentration of BBNH (○), BBSH (Δ), or DABNH (●). The lines for the *N*-acylhydrazone concentration dependence are calculated as single-exponential decay curves ($y = y_0 + ae^{-bx}$). The asymptotic value of $\Delta G_D^{H_2O}$ is 6.3 kcal mol^{-1} for BBNH [$\Delta(\Delta G_D^{H_2O})$ = 3.55 kcal mol^{-1}], 6.68 kcal mol^{-1} for BBSH [$\Delta(\Delta G_D^{H_2O})$ = 3.42 kcal mol^{-1}], and 9.8 kcal mol^{-1} for DABNH [$\Delta(\Delta G_D^{H_2O})$ = 0.3 kcal mol^{-1}].

Discussion

To date, the majority of all compounds described that inhibit HIV-1 RT target the DNA polymerase activity of the enzyme (Parniak and Sluis-Cremer, 2000). BBNH is unique in that it inhibits both the DNA polymerase and RNase H activities of the enzyme (Borkow et al., 1997). We previously suggested that this multifunctional inhibition resulted from the binding of two molecules of BBNH per molecule of HIV-1 RT at two spatially separate sites. We proposed that one molecule bound close to the nonnucleoside RT inhibitor binding pocket in the polymerase domain of the enzyme, whereas the second molecule interacted with the spatially distinct RNase H active site (Borkow et al., 1997). Our recent molecular modeling and mutagenesis studies further support this hypothesis (Arion et al., 2002). BBNH binding to the RNase H domain of HIV-1 RT seems to require an aromatic stacking interaction between Tyr-501 and the naphthyl ring of BBNH. Mutation of Tyr-501 to Arg severely attenuates the ability of BBNH to inhibit the RNase H activity of HIV-1 RT. In contrast, the DNA polymerase activity of these mutant enzymes remained as sensitive to inhibition of BBNH as the wild-type enzyme.

We now show that BBNH binding to HIV-1 RT impacts on the protein-protein interactions of the heterodimeric enzyme by decreasing the Gibbs free energy of dimer destabilization by 3.8 kcal mol^{-1}. To evaluate whether this observed effect was mediated by BBNH binding to one or both sites in RT, we synthesized more than 100 analogs of BBNH and evaluated their ability to inhibit RT RNase H and DNA polymerase activities. From these analogs, we identified two compounds that specifically inhibited either the RT DNA polymerase (BBSH) or RNase H (DABNH) activity of RT. As indicated above, we recently demonstrated that the binding of hydrazone molecules in the RT RNase H domain involves an important π-π stacking interaction between the phenol ring of

Tyr-501 and the second ring of the naphthyl moiety of BBNH. The observation that BBSH, a BBNH analog with a reduced ring system (Fig. 1), is unable to inhibit RT RNase H is consistent with this model. At present, the structural features of DABNH that give rise to its unique inhibitory properties are not understood; however, crystallographic studies are in progress to evaluate its binding interaction with RT. Nonetheless, the identification of these BBNH analogs has unequivocally demonstrated that *N*-acylhydrazone compounds can be developed that target either activity, and furthermore, it has enabled us to identify that their binding to the RT DNA polymerase domain exerts the observed destabilization of heterodimeric HIV-1 RT. It should be noted that BBNH and BBNH do not dissociate the RT heterodimer subunits; instead, their binding seems to induce conformational changes that impact on the protein-protein interactions of the enzyme and consequently affect the DNA polymerase active site. We previously reported that TSAOe^{3}T, a highly modified nucleoside based compound, inhibits RT DNA polymerase activity via an analogous mechanism (Sluis-Cremer et al., 2000b).

We previously showed that the binding of other NNRTIs, such as nevirapine and UC781, to RT do not destabilize RT subunit interactions (Sluis-Cremer et al., 2000b). Interestingly, Tachedjian et al. (2001) recently reported that several NNRTIs (including nevirapine and UC781) actually enhance

TABLE 2

Thermodynamic parameters calculated from HIV-1 RT dimer dissociation isotherms in the presence of different *N*-acylhydrazones

Data were calculated as described under *Experimental Procedures* and are an average of at least two separate experiments, each carried out in duplicate. $C_{1/2}$ is the concentration of urea at the midpoint of the denaturation isotherm. k_D was calculated from the relationship $\Delta G_D^{H}{}_{20} = -RT \ln k_D$.

Inhibitor	$C_{1/2}$	$\Delta G_D^{H}{}_{20}$	$\Delta(\Delta G_D^{H}{}_{20})$	k_D
	M	*kcal/mol*$^{-1}$		*M*
Control	2.30	10.1	0[c]	7.57×10^{-8}
5 μM BBNH	2.20	8.3	1.8	1.40×10^{-6}
10 μM BBNH	1.85	7.1	3.0	9.80×10^{-6}
25 μM BBNH	1.76	6.3	3.8	3.61×10^{-5}
10 μM BBSH	1.90	7.4	2.7	6.00×10^{-6}
25 μM BBSH	1.80	6.7	3.4	1.88×10^{-6}
25 μM DABNH	2.25	9.8	0.3	12.30×10^{-8}

[a] Value relative to control.

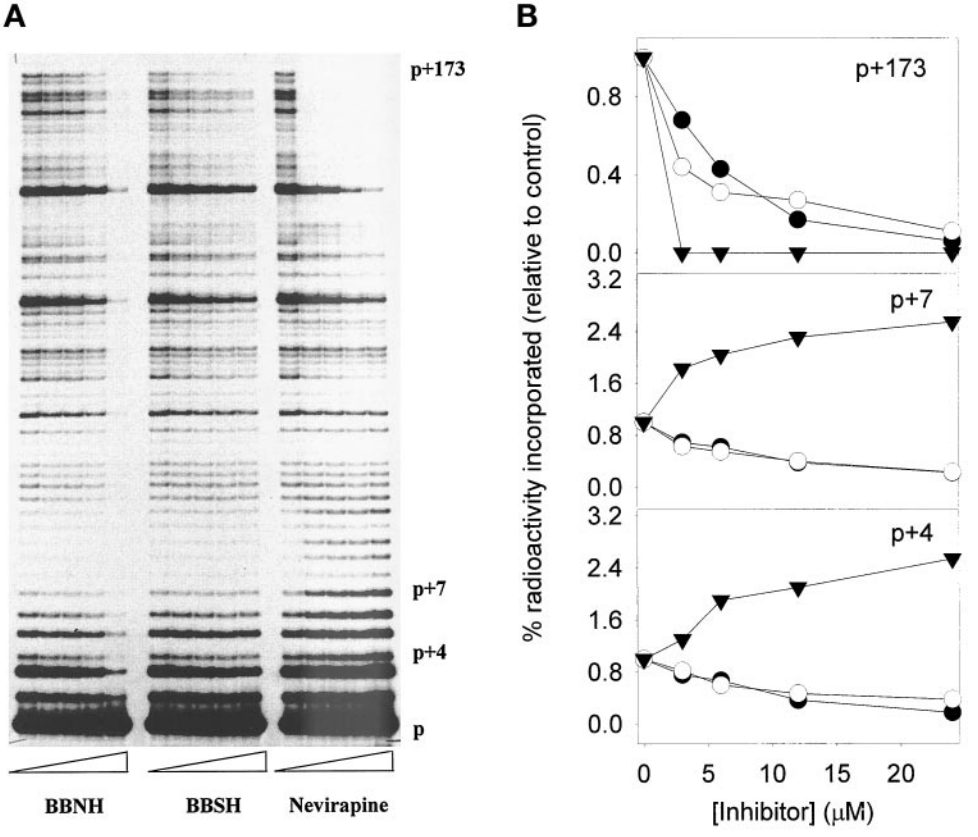


Fig. 6. Effect of inhibitor concentration on RT RDDP product distribution under single processive cycle conditions. A, autoradiogram of polymerization product profiles in the presence of BBNH (lanes 1–5), BBSH (lanes 6–10), and nevirapine (lanes 11–15). The concentration of inhibitor in each series is 0, 3, 6, 12, and 24 μM, respectively. The IC_{50} for BBNH, BBSH, and nevirapine on heteropolymeric T/P are 3, 2.5, and 1 μM, respectively. Experimental conditions are as described under *Experimental Procedures*. $p + 173$ represents the final DNA polymerization product. Arrows at sites $p + 4$ and $p + 7$ indicate regions at which increased dissociation is observed in the presence of nevirapine. B, graphical representation of the variation of selected polymerization products with increase in BBNH (●), BBSH (○), or nevirapine (▼). Percentage of RT polymerization product was determined by densitometric scanning of appropriate gel bands.

the dimerization of HIV-1 RT. The mechanism by which this observed enhancement arises remains unclear. One hypothesis suggested that NNRTI binding stabilizes the RT heterodimer and thus shifts the dimerization binding equilibrium to heterodimer formation (Tachedjian et al., 2001). However, this hypothesis is not supported by the wealth of crystal structure data currently available, and evaluation of the buried surface area in the dimer interface of HIV-1 RT in the absence of inhibitors with that of the enzyme complexed with various NNRTIs does not correlate with the observed enhancement described by Tachedjian et al. (2001) (Menéndez-Arias et al., 2001). This is consistent with our observations that NNRTI binding to purified dimeric HIV-1 RT does not impact significantly on the dimeric structure of the enzyme (Sluis-Cremer et al., 2000b).

TSAOe^{3}T also destabilizes RT subunit interactions, and our modeling studies suggested that TSAOe^{3}T could bind to a site that is overlapping with, but distinct from, the NNRTI binding site (Sluis-Cremer et al., 2000b). X-ray diffraction studies show that BBNH is a flat planar molecule (Lanthier et al., 1997), and preliminary modeling studies suggest that it cannot be readily accommodated in the NNRTI binding pocket. We propose that *N*-acylhydrazones may bind to RT at a site close to, but distinct from, the NNRTI binding pocket in a manner analogous to TSAOe^{3}T, and that this difference in binding relative to other NNRTIs may impact on RT subunit interaction stability. This different mode of binding is consistent with the differences in BBNH and TSAOe^{3}T inhibition of RT RNA-dependent DNA synthesis under processive conditions compared with other NNRTIs, such as nevirapine. Neither TSAOe^{3}T (Arion et al., 1996b) nor BBNH or BBSH promote dissociation and/or stalling of RT on a heteropolymeric RDDP template. The distribution of DNA synthesis products, including full-length product, do not differ significantly from that noted with uninhibited enzyme (except for the reduction in product levels). In contrast, nevirapine prevents the similar conditions (Arion et al., 1996a). On the other hand, nevirapine prevents the formation of full-length DNA product (p + 173), and there is a corresponding accumulation of smaller polymerization products in regions of the RNA template [that is, formation of full-length DNA product (p + 173) with a corresponding accumulation of smaller polymerization products in regions of the RNA template that are rich in secondary structure]. NNRTI binding alters the conformation of the "thumb domain" and displaces the orientation of the "primer grip" region in RT (Kohlstaedt et al., 1992; Ding et al., 1995a,b; Ren et al., 1995a,b). These regions are essential for the correct positioning of the primer terminus in the active site and also for RT DNA polymerization processivity and translocation (Jacques et al., 1994; Ghosh et al., 1996; Powell et al., 1997; Wohrl et al., 1997). Thus, the NNRTI-induced changes in RT structure can provide a plausible explanation for the increased enzyme dissociation and polymerization stalling during viral DNA synthesis in the presence of nevirapine under processive conditions. The exact molecular mechanism whereby *N*-acylhydrazones (and/or TSAOe3T) destabilize RT structure and how this destabilization process affects RT DNA polymerization remain unclear. It is of interest to note that neither TSAOe^{3}T nor BBSH inhibit RT RNase H activity despite the inhibitor-induced weakening of the RT subunit interactions. This suggests that although the RT dimer form may be obligatory for the activity of RNase H (Restle et al., 1990, 1992), small changes in the stability of the subunit interactions of RT do not impact on this activity, even though they provide considerable alterations of the DNA synthetic capacity of the enzyme.

In summary, we have now identified two structurally diverse classes of small organic molecules, TSAOe^{3}T (Sluis-Cremer et al., 2000b) and certain *N*-acylhydrazones, that act as "molecular crowbars" to destabilize the interactions of the RT dimer subunits. These molecules illustrate that small organic compounds can indeed impact on protein-protein interactions and may provide useful leads in the search for additional such compounds for use as antiviral agents.

References

Abbotts J, Bebenek K, Kunkel TA, and Wilson SH (1993) Mechanism of HIV-1 reverse transcriptase. Termination of processive synthesis on a natural DNA template is influenced by the sequence of the template-primer stem. *J Biol Chem* **268:**10312–10323.

Arion D, Borkow G, Gu Z, Wainberg MA, and Parniak MA (1996a) The K65R mutation confers increased DNA polymerase processivity to HIV-1 reverse transcriptase. *J Biol Chem* **271:**19860–19864.

Arion D, Fletcher RS, Borkow G, Camarasa M-J, Balzarini J, Dmitrienko GI, and Parniak MA (1996b) Differences in the inhibition of human immunodeficiency virus type 1 reverse transcriptase DNA polymerase activity by analogs of nevirapine and [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (TSAO) *Mol Pharmacol* **50:**1057–1064.

Arion D, Sluis-Cremer N, Min K-L, Abram ME, Fletcher RS, and Parniak MA (2002) Mutational analysis of Tyr-501 of HIV-1 reverse transcriptase. Effects on ribonuclease H activity and inhibition of this activity by *N*-acylhydrazones. *J Biol Chem* **277:**1370–1374.

Arts EJ, Li X, Gu L, Kleiman L, Parniak MA, and Wainberg MA (1994) Comparison of deoxyoligonucleotide and tRNA^{Lys-3} as primers in an endogenous human immunodeficiency virus-1 in vitro reverse transcription/template switching reaction. *J Biol Chem* **269:**14672–14680.

Baudin F, Marquet R, Isel C, Darlix J-L, Ehresmann B, and Ehresmann C (1993) Functional sites in the 5′ region of human immunodeficiency virus type 1 RNA form defined structural domains. *J Mol Biol* **229:**382–397.

Beard WA and Wilson SH (1993) Kinetic analysis of template-primer interactions with recombinant forms of HIV-1 reverse transcriptase. *Biochemistry* **32:**9745–9753.

Borkow G, Fletcher RS, Barnard J, Arion D, Motakis D, Dmitrienko GI, and Parniak MA (1997) Inhibition of the ribonuclease H and DNA polymerase activities of HIV-1 reverse transcriptase by *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone. *Biochemistry* **36:**3179–3185.

Bowie JU and Sauer RT (1989) Equilibrium dissociation and unfolding of the arc repressor dimer. *Biochemistry* **28:**7139–7143.

Ding J, Das K, Moereels H, Koymans L, Andries K, Janssen PAJ, Hughes SH, and Arnold E (1995b) Structure of HIV-1 RT/TIBO R86183 complex reveals similarity in the binding of diverse nonnucleoside inhibitors. *Nat Struct Biol* **2:**407–415.

Ding J, Das K, Tantillo C, Zhang W, Clark Jr AD, Jessen S, Lu X, Hsiou Y, Jacobo-Molina A, Andries K, et al. (1995a) Structure of the HIV-1 reverse transcriptase in a complex with the non-nucleoside inhibitor α-APA R95845 at 2.8Å resolution. *Structure* **3:**365–379.

Divita G, Restle T, and Goody RS (1993) Characterization of the dimerization process of HIV-1 reverse transcriptase heterodimer using intrinsic protein fluorescence. *FEBS Lett* **324:**153–158.

Divita G, Restle T, Goody RS, Chermann J-C, and Baillon JG (1994) Inhibition of human immunodeficiency virus type 1 reverse transcriptase dimerization using synthetic peptides derived from the connection domain. *J Biol Chem* **269:**13080–13083.

Divita G, Rittinger K, Geourjon C, Deléage G, and Goody RS (1995a) Dimerization kinetics of HIV-1 and HIV-2 reverse transcriptase: a two step process. *J Mol Biol* **245:**508–521.

Divita G, Rittinger K, Restle T, Immendörfer U, and Goody RS (1995b) Conformational stability of dimeric HIV-1 and HIV-2 reverse transcriptases. *Biochemistry* **34:**16337–16346.

Edward JT, Gauthier M, Chubb FL, and Ponka P (1988) Synthesis of new acylhydrazones as iron-chelating compounds. *J Chem Eng Data* **33:**538–540.

Fletcher RS, Holleschak G, Nagy E, Arion D, Borkow G, Gu Z, Wainberg MA, and Parniak MA (1996) Single step purification of HIV-1 recombinant wild type and mutant reverse transcriptase. *Protein Expression Purif* **7:**27–32.

Freire E (1998) Statistical thermodynamic linkage between conformational and binding equilibria. *Adv Protein Chem* **51:**255–279.

Ghosh M, Jacques PS, Rodgers DW, Ottman M, Darlix JL, and Le Grice SF (1996) Alterations to the primer grip of p66 HIV-1 reverse transcriptase and their consequences for template-primer utilization. *Biochemistry* **35:**8553–8562.

Götte M, Arion D, Cellai L, Parniak M, and Wainberg M (1999) Effects of nucleoside and non-nucleoside reverse transcriptase inhibitors on initiation of HIV-1 plus-strand DNA synthesis. *The Fourth European Conference on Experimental AIDS Research (ECEAR '99)*; Tampere, Finland; 1999 June 18–21. pp 13–18, Bologna, Italy, Monduzzi Editore.

Huber EH, McCoy JM, Seerah JS, and Richardson CC (1989) Human immunodefi-

ciency virus 1 reverse transcriptase. Template binding, processivity, strand displacement synthesis and template switching. *J Biol Chem* **264:**4669–4678.
Isel C, Ehresmann C, Keith G, Ehresmann B, and Marquet R (1995) Initiation of reverse transcription of HIV-1: secondary structure of the HIV-1 RNA/$tRNA_3^{Lys}$ (template/primer). *J Mol Biol* **247:**236–250.
Jacques PS, Wohrl BM, Darlix JL, and Le Grice SF (1994) Mutating the "primer grip" of p66 HIV-1 reverse transcriptase implicates tryptophan-229 in template-primer utilization. *J Biol Chem* **269:**26472–26478.
Kohlstaedt LA, Wang J, Friedman JM, Rice PA, and Steitz TA (1992) Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science (Wash DC)* **256:**1783–1790.
Kopp EB, Miglietta JJ, Shrutkowski AG, Shih CK, Grob PM, and Skoog MT (1991) Steady state kinetics and inhibition of HIV-1 reverse transcriptase by a non-nucleoside dipyridodiazepinone, BI-RG-587, using a heteropolymeric template. *Nucleic Acids Res* **19:**3035–3039.
Lanthier CM, Parniak MA, and Dmitrienko GI (1997) Inhibition of carboxypeptidase A by *N*-(4-t-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone. *Bioorg Med Chem Lett* **7:**1557–1562.
Menéndez-Arias L, Abraha A, Quinones-Mateu ME, Mas A, Camarasa MJ, and Arts EJ (2001) Functional characterization of chimeric reverse transcriptases with polypeptide subunits of highly divergent group M and O HIV-1 strains. *J Biol Chem* **276:**27470–27479.
Morris MC, Berducou C, Mery J, Heitz F, and Divita G (1999b) The thumb domain is essential for activation of HIV-1 reverse transcriptase. *Biochemistry* **38:**15097–15103.
Morris MC, Robert-Hebmann V, Chaloin L, Mery J, Heitz F, Devaux C, Goody RS, and Divita G (1999a) A new potent HIV-1 reverse transcriptase inhibitor. *J Biol Chem* **274:**24941–24946.
Olsen DB, Carroll SS, Culberson JC, Shafer JA, and Kuo LC (1994) Effect of template secondary structure on the inhibition of HIV-1 reverse transcriptase by a pyridinone non-nucleoside inhibitor. *Nucleic Acids Res* **22:**1437–1443.
Pace CN (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol* **131:**266–280.
Parniak MA and Sluis-Cremer N (2000) Inhibitors of HIV-1 reverse transcriptase. *Adv Pharmacol* **49:**67–109.
Parniak MA, Borkow G, Sluis-Cremer N, Klinsky E, and Alakov V (1998) Inhibition of multiple stages of HIV-1 replication in vitro by SP 1000-Fe(III)BBNH formulations. *Antiviral Ther* **3 (Suppl 5):**8.
Powell MD, Ghosh M, Jacques PS, Howard KJ, Le Grice SF, and Levin JG (1997) Alanine-scanning mutations in the "primer grip" of p66 HIV-1 reverse transcriptase result in selective loss of RNA priming activity. *J Biol Chem* **272:**13262–13269.
Ren J, Esnouf R, Garman E, Somers D, Ross C, Kirby I, Keeling J, Darby G, Jones Y, Stuart D, et al (1995a) High-resolution structures of HIV-1 RT from RT-inhibitor complexes. *Nat Struct Biol* **2:**293–302.
Ren J, Esnouf R, Hopkins A, Ross C, Jones Y, Stammers D, and Stuart D (1995b) The structure of HIV-1 reverse transcriptase complexed with 9-chloro-TIBO: lessons for inhibitor design. *Structure* **3:**915–926.
Restle T, Müller B, and Goody RS (1990) Dimerization of human immunodeficiency virus type 1 reverse transcriptase. *J Biol Chem* **265:**8986–8988.
Restle T, Müller B, and Goody RS (1992) RNase-H activity of HIV-1 reverse transcriptase is confined exclusively to the dimeric forms. *FEBS Lett* **300:**97–100.
Schellman JA (1978) Solvent denaturation. *Biopolymers* **17:**1305–1322.
Sluis-Cremer N, Arion D, and Parniak MA (2000a) Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). *Cell Mol Life Sci* **57:**1408–1422.
Sluis-Cremer N, Dmitrienko GI, Balzarini J, Camarasa M-J, and Parniak MA (2000b) Human immunodeficiency virus type 1 reverse transcriptase dimer destabilization by 1-{spiro[4″-amino-2″,2″-dioxo-1″,2″-oxathiole-5″,3′-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]]}-3-ethylthymine. *Biochemistry* **39:**1427–1433.
Starnes MC and Cheng Y (1989) Human immunodeficiency virus reverse transcriptase-associated RNase H activity. *J Biol Chem* **264:**7073–7077.
Tachedjian G, Orlova M, Sarafianos SG, Arnold E, and Goff SP (2001) Nonnucleoside reverse transcriptase inhibitors are chemical enhancers of dimerization of the HIV type 1 reverse transcriptase. *Proc Natl Acad Sci USA* **98:**7188–7193.
Timm DE and Neet KE (1992) Equilibrium denaturation studies of mouse beta-nerve growth factor. *Protein Sci* **1:**236–244.
Wohrl BM, Krebs R, Thrall SH, Le Grice SFJ, Scheidig AJ, and Goody RS (1997) Kinetic analysis of four HIV-1 reverse transcriptase enzymes mutated in the primer region of p66. Implications for DNA synthesis and dimerization. *J Biol Chem* **272:**17581–17587.

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