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†

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ABSTRACT: The nonnucleoside inhibitor binding pocket is a well-defined region in the p66 palm domain of the human immunodeficiency virus type-1 reverse transcriptase (HIV-1 RT). This binding pocket opens toward the interface of the p66/p51 heterodimer and we have investigated whether ligand binding at or near this site induces structural changes that have an impact on the dimeric structure of HIV-1 RT. 1-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)-3-ethylthymine (TSAOe^{3}T) was found to destabilize the subunit interactions of both the p66/p51 heterodimer and p66/p66 homodimer enzymes. The Gibbs free energy of dimer dissociation ($\Delta G_D^{H_2O}$) is decreased with increasing concentrations of TSAOe^{3}T, resulting in a loss in dimer stability of 4.0 and 3.2 kcal/mol for the p66/p51 and p66/p66 HIV-1 RT enzymes, respectively. This loss of energy is not sufficient to induce the dissociation of the subunits in the absence of denaturant. This destabilizing effect seems to be unique for TSAOe^{3}T, since neither the tight-binding inhibitor UC781 nor nevirapine showed any effects on the stability of HIV-1 RT dimers. TSAOe^{3}T was unable to destabilize the subunit interactions of the E138K mutant enzyme, which exhibits significant resistance to TSAOe^{3}T inhibition. Molecular modeling of TSAOm^{3}T into the nonnucleoside inhibitor binding pocket of wild-type RT suggests that it makes significant interactions with the p51 subunit of the enzyme, a feature that has not been observed with other types of nonnucleoside inhibitors. The observed destabilization of the dimeric HIV-1 RT may result from structural/conformational perturbations at the reverse transcriptase subunit interface.

Reverse transcriptase (RT; EC 2.7.7.49)[1] of the human immunodeficiency virus type 1 (HIV-1) is a multifunctional enzyme that exhibits RNA-dependent DNA polymerase (RDDP), DNA-dependent DNA polymerase (DDDP), and ribonuclease H (RNase H) activities. HIV-1 RT is an asymmetric heterodimer composed of two subunits of 66 and 51 kDa (*1*). The p66 subunit shows an overall architectural similarity to the Klenow fragment of *Escherichia coli* DNA polymerase I. The p51 subunit is derived from the p66 polypeptide by proteolytic cleavage of the C-terminal (RNase H) domain. Whereas the p66 subunit adopts an "open", catalytically competent conformation that can accommodate the RNA/DNA template (*2*), the p51 subunit is "closed" and is assumed to play a largely structural role in the RT heterodimer (*1*−*3*). Neither the p66 nor the p51 subunit can carry out RDDP activity as a monomer; dimerization of the HIV-1 RT subunits is therefore essential for the fully multifunctional enzyme (*4*−*6*).

Since RT is crucial for HIV-1 replication, the enzyme has proven an attractive target for chemotherapy. Several classes of RT inhibitors have been identified. One class includes dideoxynucleoside (ddN) analogues such as 3′-azido-3′-deoxythymidine (AZT) (*7*). The ddN inhibitors require cellular phosphorylation for activity, are competitive with respect to dNTP substrates, and inhibit viral replication primarily by acting as terminators of nascent viral DNA synthesis. Another class of compounds, the nonnucleoside

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[1] Abbreviations: DDDP, DNA-dependent DNA polymerase; DMSO, dimethyl sulfoxide; HIV-1, human immunodeficiency virus type 1; NNI, nonnucleoside inhibitor; NNIBP, nonnucleoside inhibitor binding pocket; RDDP, RNA-dependent DNA polymerase; RNase H, ribonuclease H; RT, reverse transcriptase; SEC−HPLC, size-exclusion chromatography−high-performance liquid chromatography; TBDMS, *tert*-butyldimethylsilyl; T/P, template/primer; TSAOe^{3}T, 1-{spiro[4″-amino-2″,2″-dioxo-1″,2″-oxathiole-5″,3′-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]]}-3-ethylthymine; UC781, *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furanocarbothiamide.

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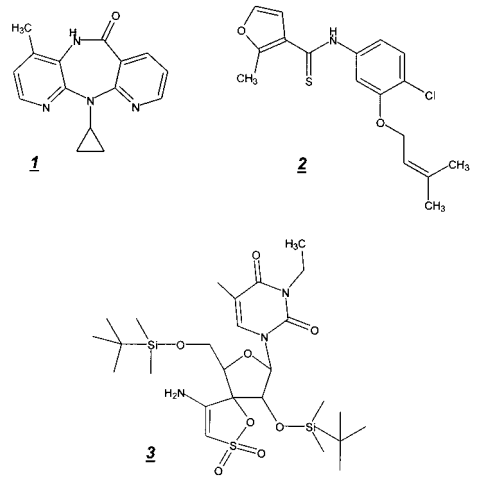

FIGURE 1: Structures of nevirapine (**1**), UC781 (**2**), and TSAOe^{3}T (**3**).

inhibitors (NNI) such as nevirapine (*8*; Figure 1A) and the (thio)carboxanilides (*9*−*12*) such as the tight-binding inhibitor UC781 (Figure 1B), are hydrophobic compounds with diverse structural features. The NNI do not require cellular modification for antiviral activity and show mixed-type or uncompetitive inhibition with respect to dNTP substrates. NNI generally bind to a defined site on RT, termed the nonnucleoside inhibitor binding pocket (NNIBP), that is close to but distinct from the polymerase active site.

Certain inhibitors do not readily fall into one of these two major classes; such compounds include the TSAO derivatives (*13*, *14*), such as 1-{spiro[4″-amino-2″,2″-dioxo-1″,2″-oxathiole-5″,3′-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]]}-3-ethylthymine (TSAOe^{3}T) (Figure 1C). TSAO inhibitors are highly modified nucleoside-based compounds that show some inhibition characteristics of NNI (*15*). HIV-1 resistance to TSAO derivatives results from a unique mutation (E138K) not normally noted in resistance to NNI or dideoxynucleoside analogue drugs (*16*). Interestingly, the relevant mutation in resistance to TSAO occurs in the p51 subunit of RT (*17*, *18*). We have previously shown significant differences in the processivity of DNA synthesis carried out by HIV-1 RT in the presence of nevirapine and TSAOe^{3}T (*19*), which led us to suggest that TSAOe^{3}T may not bind to the well-defined NNI binding pocket of HIV-1 RT, but rather may bind to a region of RT close to and partially overlapping with this site.

An interesting aspect of the NNIBP is that it opens toward the dimer interface of the p66/p51 RT heterodimer (*1*). Comparison of the crystal structures of unliganded RT (*20*) with that of the enzyme complexed with various NNI (*1*, *21*−*23*) indicates that inhibitor binding induces the repositioning of certain protein structural elements. In this report, we describe studies designed to test whether ligand-induced structural changes have an impact on the dimeric structure of HIV-1 RT. Our results indicate TSAOe^{3}T markedly decreases the stability both of the p66/p51 RT heterodimer and the p66/p66 RT homodimer. This dimer destabilization may be unique for TSAO, since other NNI such as nevirapine or UC781 do not alter RT dimer stability, and further reinforces the unique inhibitory properties of the TSAO analogues compared to other anti-HIV agents.

MATERIALS AND METHODS

Recombinant HIV-1 RT p66/p51 heterodimers and p66/p66 homodimers were expressed and purified essentially as described (*24*). The thiocarboxanilide nonnucleoside UC781 was a generous gift from Dr. W. G. Brouwer (Uniroyal Chemical Research Laboratories, Guelph, Ontario, Canada). The methyl and ethyl derivatives of TSAO, TSAOm^{3}T and TSAOe^{3}T, were prepared as previously described (*14*). Nevirapine was obtained from Boehringer-Ingelheim. The homopolymeric template/primers (T/P) poly(rA)·oligo(dT)$_{12-18}$ and poly(rC)·oligo(dG)$_{12-18}$ were products of Pharmacia (Montreal, QC, Canada). [^{3}H]dGTP and [^{3}H]TTP were purchased from Amersham. All other reagents were of the highest quality available and were used without further purification.

Assay of RT RDDP Activity. HIV-1 RT RNA-dependent DNA polymerase (RDDP) 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 $MgCl_2$, up to 4 M urea, 1 mM dithiothreitol, 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 2.5 μM [^{3}H]dGTP. Reactions were initiated by the addition of 80−100 ng of RT, which had been preincubated (20 min) with the desired concentration of urea (0−4 M). Reaction mixtures were incubated at 37 °C for 20 min and then quenched with 250 μL of ice-cold 10% trichloroacetic acid (TCA) containing 20 mM sodium pyrophosphate. Quenched samples were left on ice for 20 min, then filtered on Whatman 934-AH glass fiber filters, and washed sequentially with 10% TCA containing 20 mM sodium pyrophosphate and with ethanol, and the extent of radionucleotide incorporation was determined by liquid scintillation spectrometry.

A similar approach was used to assess the effect of various NNI on the stability of HIV-1 RT. Enzyme was preincubated (20 min) with variable concentrations of NNI dissolved in dimethyl sulfoxide (DMSO), prior to the addition of urea as described above. The NNI was also present in the denaturation and reaction mixtures so that the final concentration of NNI in the assay was identical to that used for preincubation with enzyme. The maximum concentration of DMSO in the reaction assays was 3%, and control experiments verified that the RT RDDP activity was not affected by this concentration of DMSO. All experiments were carried out at least two times, in duplicate.

Size-Exclusion High-Performance Liquid Chromatography. The hydrodynamic volume of HIV-1 RT in the absence and in the presence of varying concentrations of urea and TSAOe^{3}T was measured at room temperature by analytical SEC−HPLC on a 7.8 × 300 mm column of Phenomenex Biosep SEC-S3000 and a mobile phase of 50 mM Tris-HCl (pH 7.8, 37 °C) containing 60 mM KCl and 10 mM $MgCl_2$ and a flow rate of 1 mL/min. The sample injection volume was 20 μL and elution profiles were recorded simultaneously at 220 and 280 nm. The column was calibrated using molecular mass markers for gel filtration chromatography (Sigma) ranging in size from 12 000 to 200 000 Da.

Data Analysis of Denaturation Isotherms. HIV-1 RT RDDP activity is dependent on the dimeric structure of the enzyme (*4*). Thus the denaturation isotherms, obtained by following the loss of RT RDDP activity with increasing

concentrations of urea, can be approximated by a two-state transition between dimer and monomer (*5*). Denaturation curves were evaluated according to a linear extrapolation method (*25*). Briefly, an equilibrium constant, K_D, was calculated at each point in the transition region of the denaturation isotherm according to the following expression (*26*, *27*):

$$K_D = P_t f_M^2/(1 - f_M)$$

where P_t is the total concentration of RT and f_M is the fraction of monomeric protein. A linear dependence of the Gibbs free energy of monomer formation ($\Delta G = -RT \ln K_D$) on the denaturation concentration is assumed (*28*):

$$\Delta G_D = \Delta G_D^{H_2O} - m[\text{denaturant}]$$

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 SigmaPlot 5.0 (Jandel Scientific) and a Levenberg−Marquardt least-squares algorithm.

Molecular Modeling of the Binding of TSAO to HIV-1 RT. Coordinates for the structure of TSAOm^{3}T were obtained by single-crystal X-ray diffraction. These coordinates are provided as Supporting Information. The crystal structure shows that the TSAO furanose ring is in a 2′-*endo* conformation that is stabilized by intramolecular hydrogen bonding between the C-5′ oxygen atom and the amino group of the sultone ring. The amino-group nitrogen is trigonal planar, indicating substantial delocalization of the nitrogen lone pair electrons into the unsaturated sultone system. This suggests that the TSAO amino group is not very basic and is probably unprotonated at physiological pH.

A model for the interaction of TSAOm^{3}T with HIV-1 RT was constructed from the X-ray crystallographic coordinates for the free enzyme reported by Rodgers et al. (*20*; Brookhaven Protein Data Bank entry 1hmv). Docking and energy minimization experiments were carried out with the Anneal function of Sybyl 6.2 (Tripos Inc., St. Louis, MO). Charges were calculated by the Gasteiger−Huckel method, and iterative minimization was carried out with the Tripos force field until the energy difference between iterations was less than 0.01 kcal/mol/Å.

RESULTS

Inhibition of HIV-1 RT RDDP Activity by NNI. NNI are hydrophobic compounds with diverse structural features (see Figure 1) that function by interacting with a hydrophobic pocket on the p66 subunit of RT that is near to, but distinct from, the catalytic site (*1*, *21*−*23*). In general, the inhibition profiles due to NNI binding are noncompetitive and/or uncompetitive with respect to the normal RT substrates (*9*, *12*, *29*, *30*). In vitro IC_{50} values range from <10 nM for tight-binding inhibitors such as UC781 to 1 μM for rapid equilibrium-type inhibitors such as nevirapine (Table 1). Although TSAO inhibitors are nucleoside-based structures, they are highly modified and exhibit inhibition kinetics characteristic of NNI (*15*). Both TSAOm^{3}T and TSAOe^{3}T exhibit similar inhibitory potency (*15*).

Table 1: Inhibition of HIV-1 RT p66/p51 Heterodimer and p66/p66 Homodimer by Various NNIs[a]

RT form	IC_{50} (μM)		
	UC781	nevirapine	TSAOe^{3}T
wild-type p66/p51	0.01 ± 0.001	0.8 ± 0.01	1.1 ± 0.05
wild-type p66/p66	0.01 ± 0.001	1.5 ± 0.02	0.7 ± 0.01
E138K p66/p51	0.01 ± 0.002	0.8 ± 0.15	44.7 ± 4.5

[a] Assays were carried out with poly(rC)·oligo(dG)$_{12-18}$ and [^{3}H]dGTP as described under Materials and Methods. Values are the averages from two separate experiments, each carried out in duplicate.

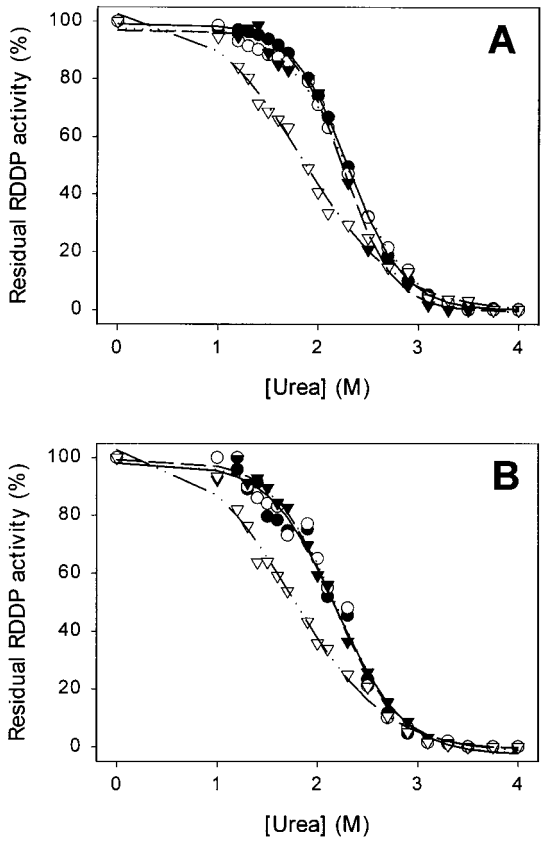

FIGURE 2: Denaturation isotherms of HIV-1 p66/p51 (A) and p66/p66 (B) RT in the absence and presence of various NNIs: HIV-1 RT (○); HIV-1 RT + 10 nM UC781 (●); HIV-1 RT + 2 μM nevirapine (▼); and HIV-1 RT + 2 μM TSAOe^{3}T (▽). Experiments were carried out as described under Materials and Methods. Standard errors ranged from 0.3% to 3% over the range of measurements in the experiments.

The E138K mutation provides about 40-fold resistance to TSAOe^{3}T but does lead to resistance to either UC781 or nevirapine (Table 1), consistent with previous data (*16*−*18*).

Urea Denaturation of HIV-1 RT. The RDDP activity of HIV-1 RT requires the dimeric structure of the enzyme (*4*). This activity therefore provides an excellent probe for studying changes in RT structure that might have an impact on the subunit interactions of HIV-1 RT. Figure 2 illustrates denaturation isotherms of the p66/p51 heterodimer (Figure 2A) and the p66/p66 homodimer (Figure 2B) of HIV-1 RT. These isotherms show sharp sigmoidal transitions that are approximated by a two-state model in which two populations, dimers and monomers, exist at equilibrium. Similar assumptions have been made for the acetonitrile-mediated dissocia-

Table 2: Thermodynamic Parameters Calculated from Urea Dissociation Isotherms[a]

inhibitor	$[urea]_{1/2}$[b] (M)	m (kcal mol^{-1} M^{-1})	$\Delta G_D^{H_2O}$ (kcal/mol)	$\Delta(\Delta G_D^{H_2O})$ (kcal/mol)	K_D[c] (M)
		p66/p51 Heterodimer			
none	2.30	3.63	9.7		1.5×10^{-7}
1 μM nevirapine	2.31	3.65	9.7	0[d]	1.5×10^{-7}
10 μM nevirapine	2.35	3.70	10.0	−0.3	9.0×10^{-8}
10 nM UC781	2.26	3.76	9.8	−0.1	1.25×10^{-7}
0.5 μM TSAOe^{3}T	2.10	2.93	7.5	2.2	5.2×10^{-6}
2 μM TSAOe^{3}T	1.80	2.36	6.1	3.6	5.0×10^{-5}
10 μM TSAOe^{3}T	1.70	2.35	5.7	4.0	9.7×10^{-5}
		p66/p51 E138K Mutant RT			
none	2.22	3.60	9.35	0.35[d]	2.6×10^{-7}
20 μM TSAOe^{3}T	2.24	3.68	9.4	0.3	2.3×10^{-7}
		p66/p66 Homodimer			
none	2.15	3.06	7.9		2.7×10^{-6}
1 μM nevirapine	2.14	3.08	7.9	0[e]	2.7×10^{-6}
10 nM UC781	2.20	3.06	7.9	0	2.7×10^{-6}
0.5 μM TSAOe^{3}T	1.80	2.23	5.3	2.6	1.8×10^{-4}
2 μM TSAOe^{3}T	1.75	1.99	4.8	3.1	4.1×10^{-4}
10 μM TSAOe^{3}T	1.75	1.94	4.7	3.2	4.9×10^{-4}

[a] Data were calculated as described under Materials and Methods and are an average of at least two separate experiments, each carried out in duplicate. [b] Concentration of urea at the midpoint of the denaturation isotherm. [c] Calculated from the relationship $\Delta G_D^{H_2O} = -RT \ln K_D$. [d] Value relative to wild-type p66/p51 heterodimer. [e] Value relative to wild-type p66/p66 homodimer.

tion of HIV-1 and HIV-2 RT (*5*). Our calculated values for the free energy of dissociation in the absence of urea ($\Delta G_D^{H_2O}$) are 9.7 kcal/mol for the p66/p51 heterodimer and 7.9 kcal/mol for the p66/p66 homodimer (Table 2), values that correspond well with previous data (10 kcal/mol for p66/p51 HIV-1 RT and 8.1 kcal/mol p66/p66 HIV-1 RT) (*5*). The calculated $\Delta G_D^{H_2O}$ values are independent of the T/P used in the assay, with identical parameters obtained with either poly(rA)·oligo(dT)$_{12-18}$ or poly(rC)·oligo(dG)$_{12-18}$ (data not shown). The m values (linear dependence of ΔG_D on urea concentration) for these transitions are about 3.6 kcal mol^{-1} M^{-1} (urea), indicating a highly cooperative two-state dimer/monomer dissociation process (*25*). The values for $[urea]_{1/2}$, $\Delta G_D^{H_2O}$, and m values were generally lower for the p66/p66 RT homodimer compared to the p66/p51 heterodimer (Table 2). This indicates that the subunit interactions of the homodimer are weaker than those of the heterodimer, consistent with earlier observations (*5*).

TSAOe^{3}T destabilizes the dimeric structure of HIV-1 RT in a concentration dependent manner (Figure 3). The maximum change in $\Delta G_D^{H_2O}$ ($\Delta\Delta G$) in the presence of TSAOe^{3}T is 4.0 kcal/mol for the p66/p51 RT heterodimer and 3.2 kcal/mol for the p66/p66 RT homodimer. In contrast, the dissociation isotherms for the HIV-1 RT complex with either nevirapine or UC781 are virtually identical to those obtained in the absence of inhibitor (Table 2).

The E138K mutation gives 40-fold resistance to TSAOe^{3}T; the compound was unable to destabilize the subunit interactions of this mutant RT (Table 2).

SEC−HPLC of HIV-1 RT. SEC−HPLC is useful for studying dimer dissociation due to its ability to resolve changes in the hydrodynamic volumes of macromolecules and, therefore, to detect the presence of monomeric species, provided they are kinetically stable within the time scale of the chromatographic run. Figure 4 shows elution profiles of HIV-1 p66/p51 RT in the presence of both urea and TSAOe^{3}T. We were unable to demonstrate physical dissociation of RT subunits upon preincubation of HIV-1 RT with a saturating concentration of TSAOe^{3}T (Figure 4B), in contrast to previously reported results (*31*).

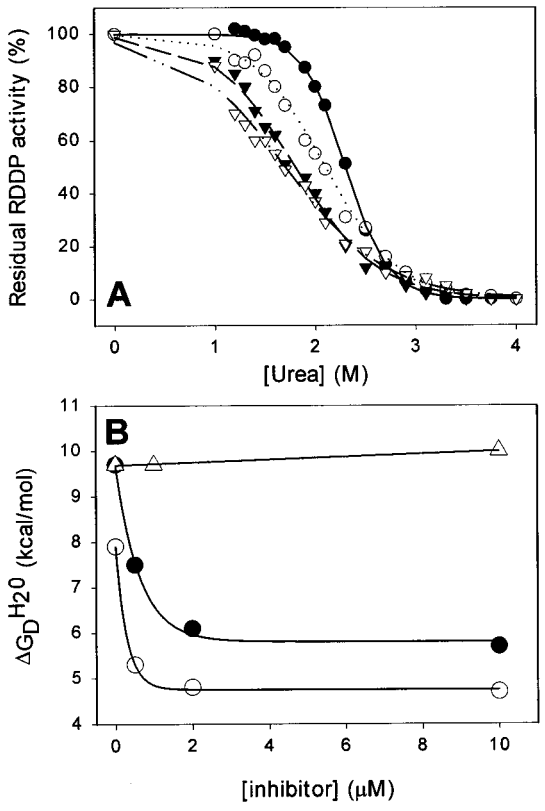

FIGURE 3: (A) Denaturation isotherms of p66/p51 RT in the absence (●) or the presence of 0.5 μM (○), 2 μM (▼), or 10 μM (▽) TSAOe^{3}T. (B) Dependence of $\Delta G_D^{H_2O}$ on NNI concentration. p66/p51 RT heterodimer + nevirapine (△); p66/p51 RT heterodimer + TSAOe^{3}T (●); p66/p66 RT homodimer + TSAOe^{3}T (○). The lines for the TSAOe^{3}T concentration dependence are calculated single-exponential decay curves ($y = y_0 + ae^{-bx}$). The asymptotic value of $\Delta G_D^{H_2O}$ in the presence of TSAOe^{3}T is 4.0 kcal/mol for the p66/p51 RT heterodimer and 3.2 kcal/mol for the p66/p66 RT homodimer. Experiments were carried out as described under Materials and Methods. Standard errors ranged from 0.3% to 3% over the range of measurements in the experiments.

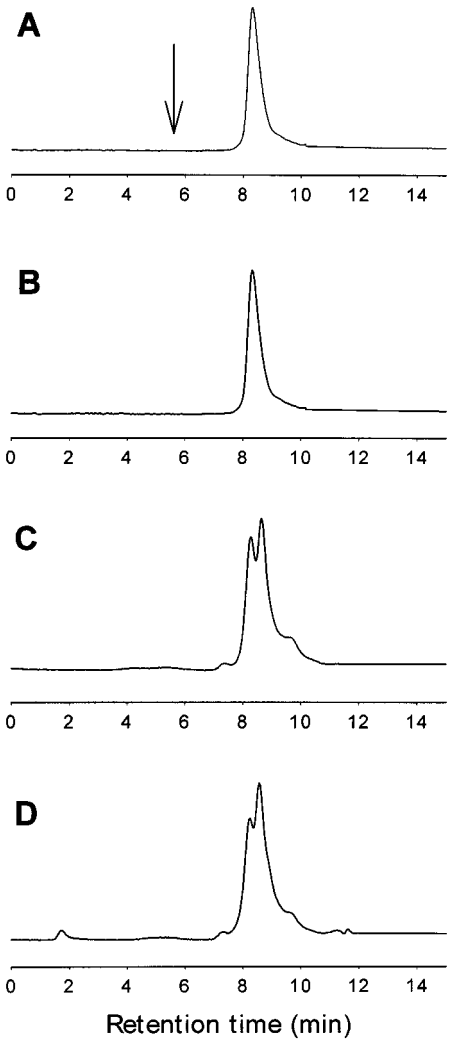

FIGURE 4: SEC−HPLC elution profiles of HIV-1 p66/p51 RT. (A) p66/p51 RT heterodimer (200 μg) (retention time = 8.32 min). The arrow indicates V_0, the void volume, as determined from the elution of blue dextran. (B) p66/p51 RT heterodimer (200 μg) preincubated with a 5-fold molar excess of TSAOe^{3}T (conditions as described in ref *31*). The retention time of RT is 8.32 min. (C) p66/p51 RT heterodimer (200 μg) preincubated with 2.5 M urea ($\sim$[urea]$_{1/2}$ value) and then subjected to SEC−HPLC in a buffer with the same concentration of urea. The retention times of the two peaks are 8.3 and 8.64 min, yielding estimated molecular masses of 117 and 60 kDa, respectively. (D) p66/p51 RT heterodimer (200 μg) preincubated with a 5-fold molar excess of TSAOe^{3}T + 2.5 M urea and then subjected to SEC−HPLC in a buffer with the same concentration of urea. Retention times of the two major peaks are identical to those in panel C.

In the presence of 2.5 M urea (a concentration equivalent to [urea]$_{1/2}$ calculated from the dissociation isotherms), two species are detected in the absence of TSAOe^{3}T (Figure 4C). The first, with a retention time of 8.32 min, corresponds to the p66/p51 RT heterodimer (117 kDa). The second, with a retention time of 8.64 min, corresponds to an apparent molecular mass of 60 kDa and is due to the p66 and p51 monomers (these monomeric species cannot be resolved under the chromatographic conditions used). With the same concentration of urea but in the presence of saturating concentrations of TSAOe^{3}T (Figure 4D), the extent of the monomer fraction is substantially increased, implying that RT subunit interactions are weakened in the presence of this inhibitor, consistent with the urea-induced dissociation isotherms (Figure 3).

Molecular Modeling of the Binding of TSAO to HIV-1 RT. Docking of the C-2′-*endo* conformation of TSAOm^{3}T at the interface of the p66 and p51 RT subunits near the amino acid residues of the NNIBP indicated a very good shape complementarity between TSAO and this region of RT (Figure 5). The model indicates that TSAO compounds bind to RT rather differently from other NNIs (*1*, *21*−*23*), primarily by making extensive interactions with the p51 subunit. The amino group forms a hydrogen bond with the carboxylate of E138 of the p51 subunit, consistent with the observed resistance of HIV-1 to TSAO analogues due to mutations of this RT residue (*17*, *18*). The model shows very substantial van der Waals contacts between p51 subunit residues (E28, I31, K32, P133, S134, I135, and T139) and the methyl groups of the *tert*-butyldimethylsilyl (TBDMS) group at C-5′ of the inhibitor. Significant contacts with p66 subunit residues include close contact between a sultone oxygen and the ϵ-amino of K103 and favorable contacts between K172, P176, V179, and Y181 with the C-4′ TBDMS group of TSAO.

DISCUSSION

NNIs are a diverse group of hydrophobic compounds that bind to a well-defined region in the p66 palm subdomain of HIV-1 RT. This binding pocket is predominantly hydrophobic in nature with substantial aromatic character (Y181, Y188, F227, W229, and Y232), but also contains several hydrophilic residues (K101, K103, S105, D192, and E224 of the p66 subunit and E138 of the β7−β8 loop of the p51 subunit). NNI binding induces certain subtle shifts in the secondary structural elements β4, β7, and β8; these affect the positioning of the catalytic YMDD motif and the thumb subdomains of the enzyme (*1*, *21*−*23*). However, no gross structural alterations are evident in the RT dimer, and the overall fold in the various RT complexes remains similar. Our data showing that NNI such as nevirapine and UC781 do not induce any significant changes in the dimeric stability of either the p66/p51 or p66/p66 HIV-1 RT enzymes are consistent with the minor structural perturbations upon binding of these NNI.

TSAO compounds, however, have highly modified nucleoside structures that are significantly bulkier than nevirapine or UC781 (Figure 1). Although nucleoside-based, TSAO analogues do not bind competitively with dNTP substrates but rather show gross inhibition kinetics characteristic of NNI. Our previous data (*19*) suggested that TSAOe^{3}T might interact interact with RT differently from other NNI. The present studies show the binding of TSAOe^{3}T to dimeric HIV-1 RT results in a marked destabilization of the dimeric structure of the enzyme, unlike the other NNI tested, and provides further evidence that TSAO may bind to RT in a unique manner.

We previously suggested that TSAO might bind to a site close to, but not precisely within, the well-characterized NNI binding pocket of RT (*19*). Our molecular modeling suggests that TSAO makes multiple contacts with residues in the p51 subunit (Figure 5), unlike other NNI (*1*, *21*−*23*). On the basis of this model, the TSAO-induced changes in RT dimer stability likely arise from significant conformational perturbations that affect the p66/p51 interface. It is possible that

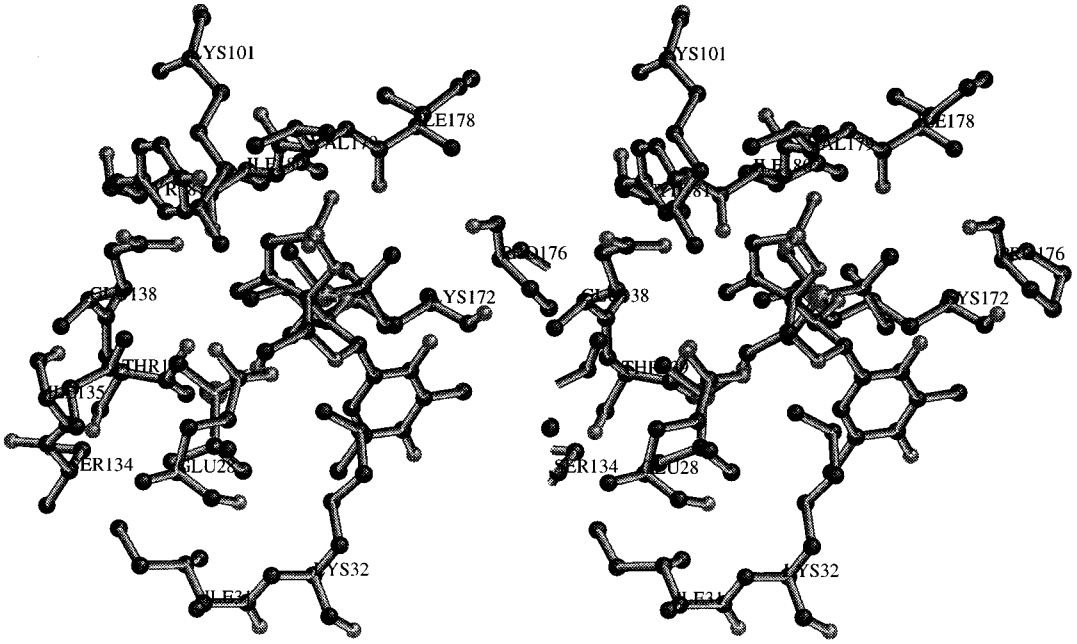

FIGURE 5: Stereoview of the model for the interaction of TSAOm^{3}T with residues in the proximity of the NNIBP of HIV-1 RT.

the multiple interactions of TSAO with residues in both RT subunits leads to a diminution of favorable subunit−subunit contacts, thereby decreasing the strength of the intersubunit contacts. Residue E138 of the p51 subunit, which is mutated in HIV-1 resistance to TSAO (*17*, *18*), is one of seven residues that represent 30% of the interfacial area of the heterodimer (*32*). These residues lie within regions of the protein that are constrained upon dimerization. The E138K mutation, which results in resistance to TSAO resistance (*16*−*18*) results in only a small change in RT subunit interaction strength relative to wild-type enzyme (Table 2). Interaction of TSAO with one or more of these dimer interface residues might then destabilize the RT dimer by disrupting a crucial interface interaction. The observed TSAO-dependent change in the midpoint of the denaturation curves ($[urea]_{1/2}$) and changes in the *m* value (linear dependence of ΔG_D on urea concentration) (Table 2) are consistent with this possibility. Changes in $[urea]_{1/2}$ can be attributed to differences in the specific interactions at the subunit interface when TSAOe^{3}T is bound. The *m* value is a more complex parameter, and changes in this value may be related to differences in the solvent exposure of RT hydrophobic residues in the dimeric and monomeric states (*28*, *33*, *34*), as well as alterations in the pathway by which the subunits dissociate due to ligand-induced changes in the subunit interactions (*35*). Since HIV-1 RT is an obligate dimer, it is intriguing to speculate that the mechanism of TSAO-mediated inhibition is due to disruption of the intersubunit contacts. In this regard, the E138K mutation results in 40-fold resistance to TSAOe^{3}T; the compound is unable to disrupt the intersubunit contacts of this mutant.

It is well documented that while small molecule ligands can stabilize or destabilize the conformation of a macromolecule, there are intrinsic limits (dictated by molecular weights and binding affinities of the ligand, as well as practical or experimental constraints) to which a ligand can alter this conformation. In general, a single ligand molecule binding to a large macromolecule can result in a maximum contribution to ΔG of about 3−4 kcal/mol (*36*), similar to that noted for our experimentally derived $\Delta G_D^{H_2O}$ values in the presence of TSAOe^{3}T. Since the strength of the RT p66/p51 subunit association is in the range of 10 kcal/mol (*5*; the present work), the decreased subunit interaction induced upon binding of TSAOe^{3}T is unlikely to lead to dissociation of the subunits, as has been previously suggested (*31*). Indeed, we were unable to observe such dissociation in the present studies.

HIV-1 RT dimer stability and/or dimerization during virus assembly may be interesting targets for antiviral intervention. Investigators have previously suggested that a 19-residue peptide homologous to RT subunit interface sequences might be a useful inhibitor of these processes (*6*). Unfortunately, peptide therapeutics can have significant pharmacokinetic problems, such as lack of oral bioavailability. Our present studies suggest that small organic molecules, which generally have better pharmacokinetic properties, may also be useful as RT dimerization inhibitors. TSAOe^{3}T is a useful lead in the search for additional such compounds. It is interesting that TSAO is equally effective at destabilizing the heterodimer and homodimer forms of RT. The kinetics of HIV-1 RT heterodimer formation during virus assembly and maturation are not clearly defined. It is possible that this may proceed through a p66/p66 RT homodimer intermediate. The ability to affect both homo- and heterodimer forms of RT may be an important factor in the potency of small molecule dimerization inhibitors against HIV-1.

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SUPPORTING INFORMATION AVAILABLE

Three figures and seven tables showing X-ray crystallographic data for TSAOm^{3}T. This material is available free of charge via the Internet at http://pubs.acs.org.

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