Structure−Activity Relationships of [2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)thymine Derivatives as Inhibitors of HIV-1 Reverse Transcriptase Dimerization

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The polymerase activity of HIV-1 reverse transcriptase (RT) is entirely dependent on the heterodimeric structure of the enzyme. Accordingly, RT dimerization represents a target for the development of a new therapeutic class of HIV inhibitors. We previously demonstrated that the *N*-3-ethyl derivative of 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)-thymine (TSAO-T) destabilizes the inter-subunit interactions of HIV-1 RT [Sluis-Cremer, N.; Dmietrinko, G. I.; Balzarini, J.; Camarasa, M.-J.; Parniak, M. A. *Biochemistry* **2000**, *39*, 1427−1433]. In the current study, we evaluated the ability of 64 TSAO-T derivatives to inhibit RT dimerization using a novel screening assay. Five derivatives were identified with improved activity compared to TSAO-T. Four of these harbored hydrophilic or aromatic substituents at the N3 position. Furthermore, a good correlation between the ability of the TSAO-T derivatives to inhibit RT dimerization and the enzyme's polymerase activity was also observed. This study provides an important framework for the rational design of more potent inhibitors of RT dimerization.

Introduction

To date, 20 anti-HIV agents have been approved by the United States Food and Drug Administration for the management of HIV-1 infected individuals. These antiviral agents can be divided into only four different therapeutic classes: nine are inhibitors of HIV-1 protease, eight are nucleoside or nucleotide reverse transcriptase inhibitors, three are non-nucleoside reverse transcriptase inhibitors (NNRTI[a]), and one is a viral fusion inhibitor. However, due to the high replication rate of HIV-1 as well as the genetic plasticity inherent to the virus, emergence of viral resistance to these antiretroviral agents is inevitable. Furthermore, because many of the compounds from the same therapeutic class exhibit similar chemical structures and mechanisms of action, the emergence of viral resistance to one drug frequently results in the cross-resistance to other drugs.[1,2] Therefore, the identification of additional viral targets and the development of new classes of antiviral compounds are essential in the fight against HIV/AIDS.

HIV-1 reverse transcriptase (RT) is a multifunctional enzyme responsible for the conversion of the viral single-stranded RNA genome into double-stranded DNA. To facilitate this process, RT exhibits two enzymatically distinct activities: a DNA polymerase activity that synthesizes DNA using either RNA or DNA templates (termed RNA-dependent (RDDP) or DNA-dependent DNA polymerase (DDDP) activity, respectively) and a ribonuclease H (RNase H) activity that degrades the RNA strand of RNA/DNA hybrids.[3] HIV-1 RT is an asymmetric heterodimer composed of a 560 amino acid, 66 kDa subunit (p66) and a 440 amino acid, 51 kDa (p51) subunit.[4,5] The p51 polypeptide is derived from the p66 by proteolytic cleavage of its C-terminal RNase H domain.[6] The p66/p51 HIV-1 RT heterodimer contains one DNA polymerization active site and one RNase H active site, which both reside in the p66 subunit at spatially distinct regions. Several studies have demonstrated that HIV-1 RT is an obligate dimer. Monomeric forms of both subunits are devoid of DNA polymerase activity.[7,8] Accordingly, dimerization of HIV-1 RT represents a novel target for the identification of a new therapeutic class of antiviral agents, and several classes of small molecules and peptides have been identified as RT dimerization inhibitors.[9−15]

[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)thymine (TSAO-T) derivatives represent a unique class of compounds that are potent and highly specific HIV-1 RT inhibitors. Although TSAO derivatives are highly functionalized nucleosides, they behave as allosteric inhibitors of HIV-1 RT. They were originally thought to target the same nonsubstrate binding site as all the other NNRTIs.[16] However, TSAO compounds are one of a few NNRTIs that require amino acids in both HIV-1 RT subunits (p66 and p51) for optimal interaction with the enzyme. Molecular modeling studies have shown that the TSAO molecules bind at the p66/p51 subunit interface at a position close to but distinct from the NNRTIs binding pocket.[13,17] According to these models, the 4″-amino group of the spiro sultone moiety of TSAO derivatives is within hydrogen bonding distance of the glutamic acid residue at position 138 in the p51 subunit of HIV-1 RT, and this important interaction is thought to be responsible for the E138K mutation detected under the

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[a] Abbreviations: RT, reverse transcriptase; TSAO-T, [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)thymine; NNRTI, non-nucleoside reverse transcriptase inhibitor; RDDP, RNA-dependent DNA polymerase; RNase H, ribonuclease H; HTS, high-throughput screening.

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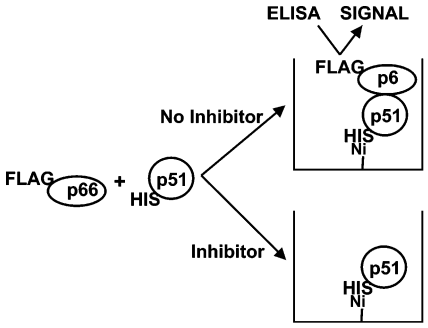


Figure 1. Schematic for the in vitro HTS assay developed to detect FLAG−p66/HIS−p51 RT dimer formation. Purified FLAG−p66 and HIS−p51 are added in equimolar concentrations to the wells of a 96-well His-Select nickel coated plate in the absence or presence of drug. The RT subunits interact with one another to form FLAG−p66/HIS−p51 heterodimers, which immobilize to the plate via the nickel−HIS interaction. Unbound FLAG−p66 RT is removed from the wells by extensive washing. FLAG−p66/HIS−p51 dimer formation is quantitated by ELISA using primary antibodies specific for the FLAG peptide on the N-terminus of p66 RT.

selective pressure of TSAO derivatives.[18] In this regard, we demonstrated that E138 in the p51 subunit, but not in the p66 subunit, of HIV-1 RT determines the sensitivity and resistance of the enzyme to this class of compounds.[19] Furthermore, we previously demonstrated that the N3-ethyl derivative of TSAO-T could destabilize the inter-subunit interactions in p66/p51 HIV-1 RT by altering the dimer/monomer thermodynamic equilibrium of the enzyme.[13] Therefore, TSAO compounds may be considered as the first small non-peptide molecules that interfere with the enzyme's dimerization process. In the current study, we have conducted extensive structure−activity relationship studies on TSAO-T derivatives to identify the salient structural features that are important for their ability to inhibit HIV-1 RT dimerization.

Results and Discussion

Development of an in Vitro High-Throughput Screening (HTS) Assay for HIV-1 RT Dimerization. Several biochemical assays have been developed that specifically detect the inter-subunit interactions in p66/p51 RT. Some of these assays are based on the physical separation of monomers and dimers as determined by analytical ultracentrifugation[20] and gel filtration.[13,21] Others include the intrinsic tryptophan fluorescence of the enzyme,[21,22] chemical cross-linking of subunits,[23] yeast-two hybrid assays,[24] and RT activity itself.[7,13,14,21] Although these methods detect dimerization, they either lack specificity or are not easy to perform. Moreover, most of them are not amenable for HTS for the identification of RT dimerization inhibitors. In light of this, we developed an in vitro HTS assay that is suitable for assessing a large number of compounds for their ability to inhibit HIV-1 RT dimerization. This assay is schematically depicted in Figure 1. The p66 and p51 subunits of RT are separately expressed and purified as either N-terminal FLAG (FLAG−p66) or hexa-histidine (HIS−p51) fusion proteins. These are then added in equimolar concentrations to the wells of a 96-well His-Select high-sensitivity nickel coated plate (Sigma-Aldrich) in 50 mM Tris-HCl (pH 7.2, 25° C) containing 50 mM Na_2SO_4. The RT subunits interact with one another to form FLAG−p66/HIS−p51 heterodimers, which are immobilized to the plate via the nickel−histidine interaction. Unbound FLAG−p66 RT is removed from the wells by extensive washing. FLAG−p66/HIS−p51 dimer formation is quantitated by ELISA using primary antibodies specific for the FLAG peptide on the N-terminus of p66 RT (see Materials and Methods). Control reactions to evaluate nonspecific FLAG−p66 interactions were carried out in which equimolar concentrations of FLAG−p66 and HIS−GST were added to the well and processed as described above. This assay is sensitive and robust and demonstrates a signal-to-noise (S/N) ratio of 25.6 and a Z' factor of 0.70. The S/N ratio defines the assay mainly on the basis of assay window, that is, the difference between the assay background and maximal signal (see Materials and Methods for the equation used to calculate S/N ratio). The Z' factor has been widely accepted as a very useful way of assessing the statistical performance of an assay and is a relative indication of the separation of the signal and background populations.[25] It is a dimensionless parameter that ranges from 1 (infinite separation) to <0. Signal and background populations start to overlap when $Z' = 0$. In general, the acceptance criteria for a quality assay is one in which the $Z' > 0.4$ and S/N ratio = 10.[25,26] In this regard, our in vitro HIV-1 RT dimerization assay meets and exceeds these criteria.

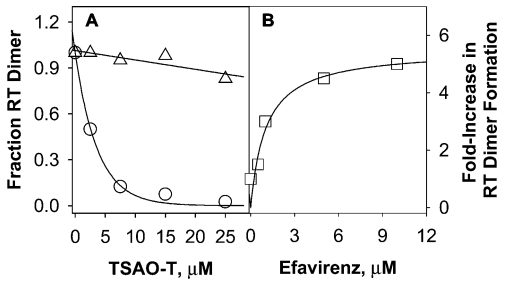
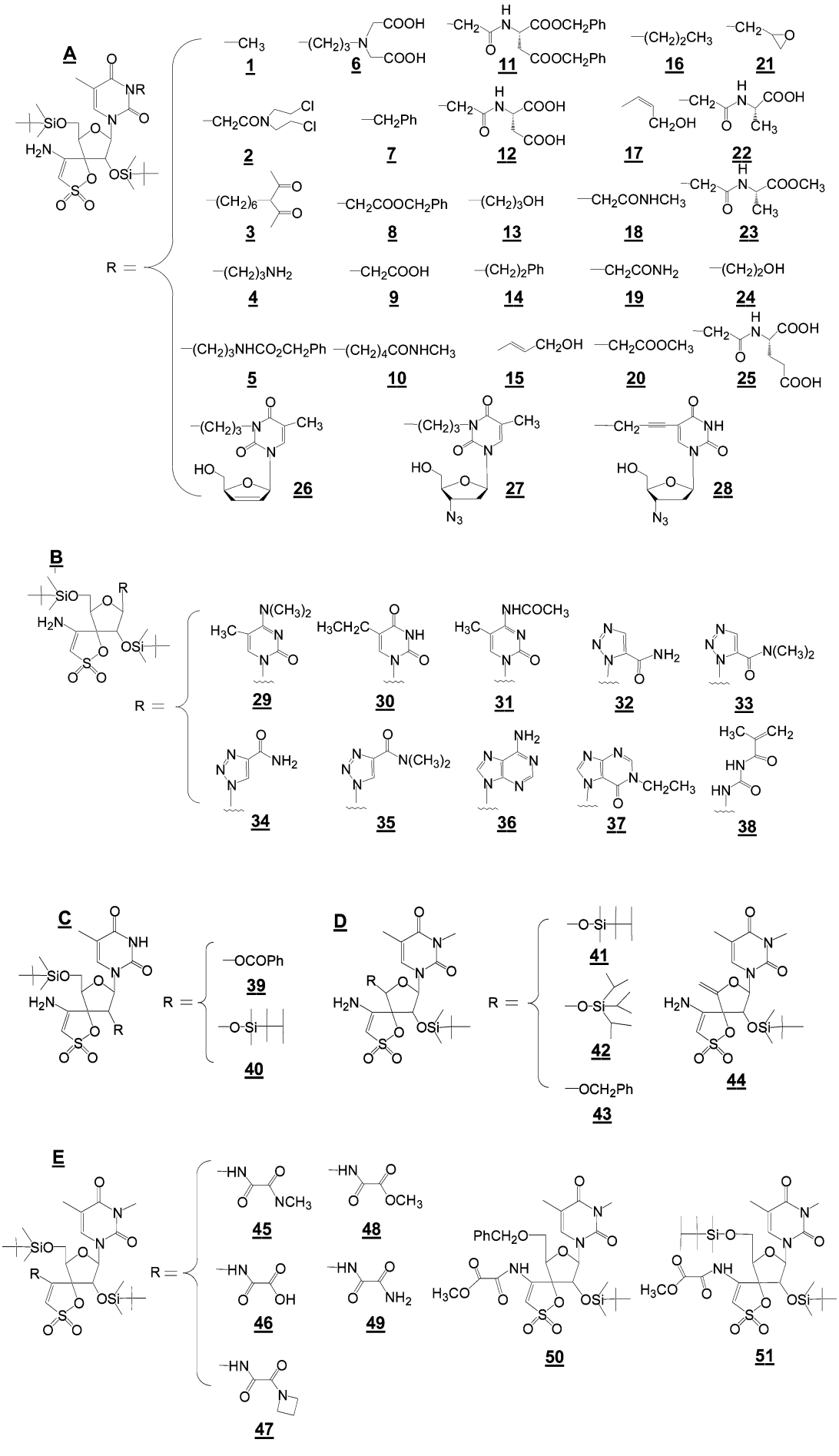


Figure 2. Effects of TSAO-T and efavirenz on FLAG−p66/HIS−p51 RT dimer formation. Assays were carried out as described in Materials and Methods, using increasing concentrations of TSAO-T (panel A) or efavirenz (panel B). The IC_{50} for TSAO-T was calculated to be 6.7 ± 1.3 μM. In panel A, experiments were carried out using wt p51 RT (○) and E138K p51 RT (△). In panel B, only wt p51 RT was used (□).

Inhibition of HIV-1 RT Dimerization by TSAO-T Derivatives. The HTS assay described above can readily be used to evaluate the ability of compounds to inhibit or enhance FLAG−p66/HIS−p51 RT heterodimer formation. In this regard, we previously demonstrated that the N3-ethyl derivative of TSAO-T (TSAO-e^{3}T) destabilizes both the p66/p51 and p66/p66 dimeric forms of HIV-1 RT.[13] In the current study, we first evaluated the ability of the parent compound (TSAO-T) to inhibit in vitro FLAG−p66/HIS−p51 RT heterodimer formation. Assays were conducted in which increasing concentrations of TSAO-T were added to the reaction mixture and the fraction of FLAG−p66/HIS−p51 RT dimer formation was calculated for each drug concentration (Figure 2A). An IC_{50} value of 6.6 ± 1.3 μM for TSAO-T was determined from these experiments. The specificity of the TSAO-T interaction with RT was established by using HIS−p51 RT that contained E138K, a mutation that confers a high level of resistance to TSAO compounds.[18] In these experiments, TSAO-T was unable to inhibit FLAG−p66/HIS−p51 RT dimer formation (Figure 2A). Furthermore, experiments that were carried out with the NNRTI efavirenz demonstrated that this ligand enhanced RT dimer formation, a result that is consistent with past studies.[27,28] Structural and mechanistic models for the ability of TSAO-T to inhibit RT dimerization versus the ability of efavirenz to enhance RT dimerization have been described elsewhere.[15,49,50] To gain a better understanding of the structural features of TSAO-T that allow it to bind to HIV-1 RT and inhibit heterodimer formation, 64 structurally different TSAO-T derivatives were screened. These derivatives can be broadly grouped into seven categories (Figure 3): (i) N-3-modified derivatives (Figure 3A), (ii) base-modified deriva-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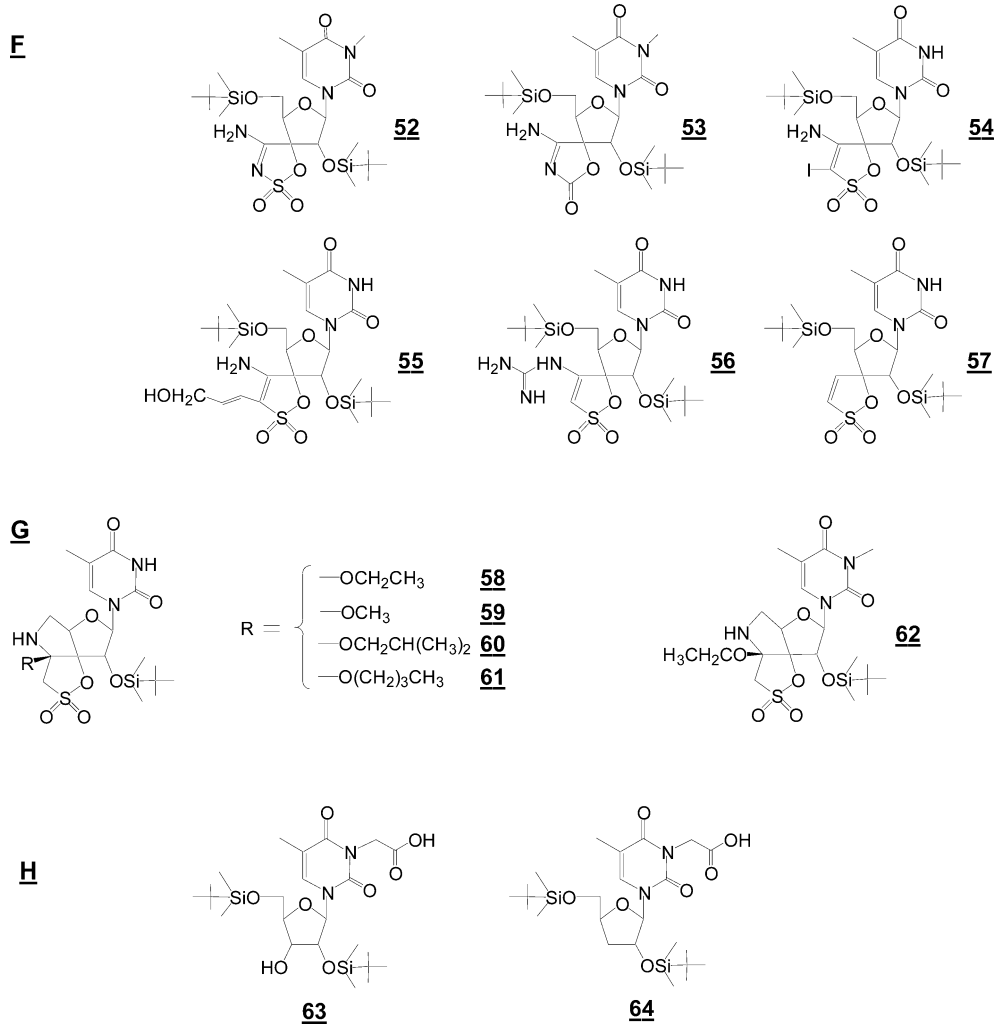

Figure 3. Chemical structures of TSAO-T derivatives: (A) N-3-modified derivatives; (B) base-modified derivatives; (C) 2′-modified derivatives; (D) 5′-modified derivatives; (E, F) spiro-modified derivatives; (G) tricyclic TSAO nucleosides; (H) N-3-carboxymethyl nucleosides.

tives (Figure 3B), (iii and iv) 2′- and 5′-modified derivatives (Figure 3C,D), (v) spiro-modified derivatives (Figure 3E,F), and (vi) tricyclic TSAO nucleosides (Figure 3G). In this study, we also included N-3 carboxymethyl nucleosides (Figure 3H), which represent the second example of nucleoside analogues that specifically inhibit HIV-1 RT, although they interact differently with HIV-1 RT than the TSAO derivatives.[29,30] The nucleoside derivatives were assessed for their ability to inhibit RT dimerization in assays carried out in 96-well plates. Each plate included six control reactions (FLAG−p66, HIS−p51, and 3% DMSO), six background reactions (FLAG−p66, HIS−GST, and 3% DMSO), and duplicate control experiments that included 4, 8, and 16 μM TSAO-T, respectively. All nucleoside derivatives were screened at three concentrations: 4, 8, and 16 μM. However, eight additional compounds (compounds **18**−**25**) were screened at a later date using only a single drug concentration of 8 μM. The results of the screens are shown in Figure 4. Of the 64 compounds tested, only 7 compounds, numbers **13**, **14**, **15**, **16**, **17**, **54**, and **64**, were found to be more active than TSAO-T. Five of these harbored modifications at the N-3 position of the thymine base (compounds **13**, **14**, **15**, **16**, and **17**), one had a modified spiro group (compound **54**), and one was an N-3-carboxymethyl nucleoside (compound **64**). Several other derivatives (i.e., compounds **1**, **4**, **6**, **9**, **10**, **11**, **12**, **18**, **19**, **20**, **21**, **23**, **24**, and **63)** were found to exhibit anti-RT dimerization activity comparable with TSAO-T. All of these derivatives harbored modifications at the N-3 position of the thymine base of TSAO-T, except for the carboxymethyl nucleoside compound **63** (Figure 3). Compared with the prototype molecule TSAO-T, other TSAO compounds modified at the base, at 2′- or 5′-positions, or at the spiroaminosultone ring all demonstrated a decreased capacity to inhibit FLAG−p66/HIS−p51 RT dimer formation. The tricyclic derivatives were also less active than TSAO-T in the RT dimerization assay.

To date, a crystal structure of HIV-1 RT complexed with a TSAO derivative has not been solved. However, a binding interaction between an N-3-methyl derivative of TSAO-T (TSAO-m^{3}T) and HIV-1 RT has been proposed based on modeling studies.[13,17] In these models, TSAO-m^{3}T binds at the interface between the two subunits in such a way that the strong dipole moment of the spiro group of TSAO-m^{3}T is aligned in the field created by the positive electrostatic region emanating from K101 and K103 in the p66 subunit and the negatively charged E138 in the p51 subunit. The spiro amino group is in hydrogen bonding distance with E138 in the p51 subunit of HIV-1 RT, and the O4 of the thymine ring interacts with the hydroxyl group of T139 in the p51 subunit. The remaining stabilizing interactions are hydrophobic and exploit two cavities present at the enzyme interface that provide binding pockets for the 2′- and 5′-*tert*-butyldimethylsilyl substituents of TSAO derivatives. The numerous contacts that have been identified for the 2′- and 5′-*tert*-butyldimethylsilyl substituents may account for the very stringent requirement for this group at this position[17] and may provide a partial explanation for why

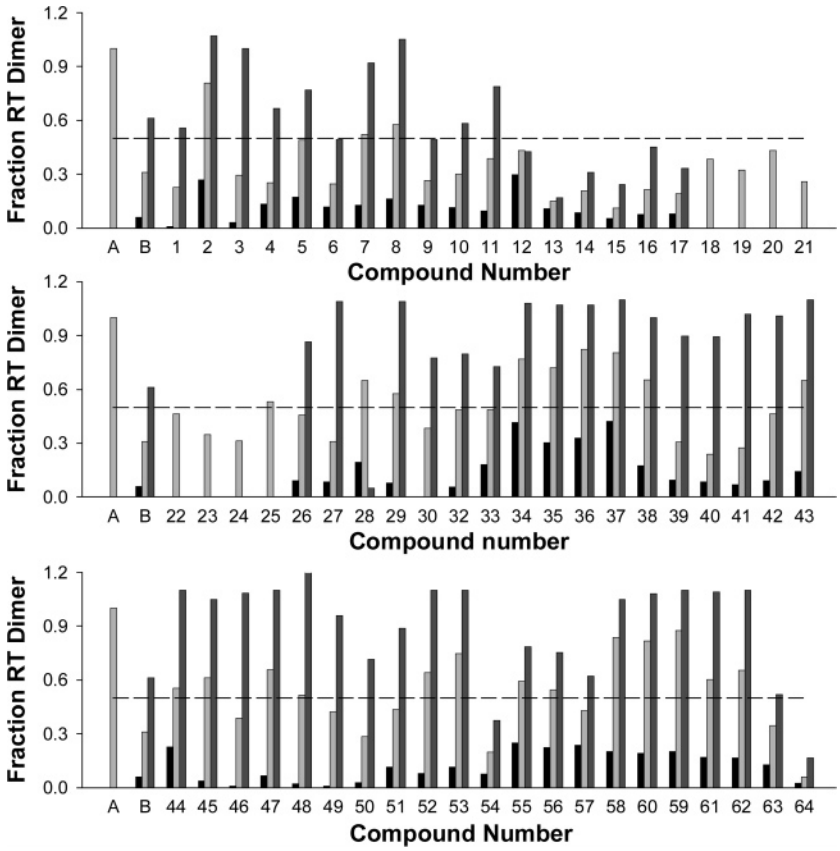

Figure 4. Anti-RT dimerization activities of 64 TSAO-T analogues at 4 (dark gray bars), 8 (light gray bars), and 16 μM (black bars), respectively. Data are compared to the FLAG−p66/HIS−p51 dimerization signal determined in the absence of drug (A) and to the FLAG−p66/HIS−p51 dimerization signal in the presence of 4, 8, and 16 μM TSAO-T (B). The dashed horizontal line indicates 50% inhibition.

structural variation at this position (compounds **39**−**43**) leads to a loss of activity. The most "buried" part of the TSAO molecule in the RT subunit interface is the spiroaminosultone moiety. Interestingly, derivative **54**, which is a 3″-iodo-substituted analogue, is a more potent inhibitor of RT dimerization than TSAO-T (see Figure 4). Therefore, the spiroaminosultone moiety represents a unique site in the molecule that can be modified to design new more potent TSAO derivatives. Our data also demonstrate that modifications at the N-3 position on the thymine base are well tolerated. In our model, the N-3 substituent runs parallel to the subunit interface and is mostly exposed to the solvent.[17] Interestingly, compounds bearing an N-3 hydroxyl group (e.g., **13**, **15**, and **17**) were 2−4-fold more potent than TSAO-T. Recent studies have demonstrated that hydrophilic modifications at the N-3 position of thymine may afford additional interactions with Pro140 and Lys49 from the p51 subunit,[31] thus providing a rational explanation for the increased activity observed with these compounds. Interestingly, aromatic or alkyl substituents at the N-3 position (compounds **14** and **16**) also have a more pronounced effect as RT dimerization inhibitors than TSAO-T. Finally, it is worth mentioning that the N-3 carboxymethyl derivatives **63** and, specially, the 3′-deoxy derivative **64** rank among the most potent RT dimerization inhibitors. These derivatives, as mentioned previously, represent the second example of nucleoside analogues that specifically inhibit HIV-1 RT.

Inhibition of HIV-1 RT Dimerization by TSAO Derivatives: Comparison with Inhibition of RDDP and Antiviral Activity. In this study, we have demonstrated that TSAO-T derivatives can inhibit the protein−protein interactions of HIV-1 RT. However, TSAO-T analogues also inhibit the DNA polymerase activity of RT in vitro and demonstrate appreciable antiviral activity. To evaluate whether the structure−activity relationship of this class of compounds are similar for each of these activities, we selected 23 TSAO-T analogues and characterized their ability to inhibit HIV-1 RT dimerization, RDDP activity, and antiviral activity in detail. These 23 compounds included the seven compounds (**13**−**17**, **54**, and **64**) identified in the HTS screen that exhibited better activity than TSAO-T. A further eight compounds (compounds **1**, **4**, **6**, **9**, **10**, **19**, **21**, and **63**) were selected that demonstrated activity comparable to TSAO-T and eight compounds (compounds **12, 22**, **30**, **32**, **33**, **50**, **55**, and **56**) that were less active than TSAO-T. IC_{50} or EC_{50} values were determined for each of these compounds for each of the activities (Table 1). An excellent correlation ($r^2 = 0.93$) between the IC_{50} values determined for RT dimerization and RT RDDP activity was observed (Figure 5). This result suggests that the structure−activity relationship for both of these activities is similar. This data provides additional support for our hypothesis that these compounds inhibit HIV-1 RT by disrupting the inter-subunit interactions of the enzyme. All the TSAO-T derivatives also inhibited viral replication with EC_{50} values ranging from 0.03 to 8.3 μM (Table 1). Interestingly, compounds **13** and **15**, which are both more potent inhibitors of RT dimerization compared with TSAO-T, also exhibited better antiviral activities than the parent compound (Table 1). However, the overall correlation between the EC_{50} values determined for antiviral activity and IC_{50} values determined for RT dimerization were not as striking as those observed in Figure 5 ($r^2 = 0.4$; data not shown). This could possibly be attributed to additional pharmacokinetic parameters, such as cellular uptake and metabolic stability, which complicate the structure−activity relationship analyses. Nevertheless, our data clearly demonstrate

Table 1. Inhibition Data for TSAO-T Derivatives

	IC_{50} or EC_{50} (μM)			CC_{50} (μM)
compound	dimerization[b]	RDDP[c]	MT-4[d]	MT-4[e]
TSAO-T	6.7 ± 1.3	1.0 ± 0.2	0.06 ± 0.03	14 ± 2
13	1.6 ± 0.8	0.7 ± 0.1	0.03 ± 0.003	4 ± 0.3
14	3.0 ± 1.2	0.8 ± 0.1	0.84 ± 0.14	2 ± 0.4
15	1.9 ± 0.9	0.5 ± 0.1	0.04 ± 0.004	4 ± 0.1
16	4.3 ± 1.3	0.5 ± 0.1	0.26 ± 0.08	75 ± 19
17	3.1 ± 0.9	1.6 ± 0.2	0.32 ± 0.02	36 ± 6
54	3.5 ± 1.0	8.0 ± 0.3	0.12 ± 0.01	4 ± 0.2
64	1.1 ± 1.3	5.7 ± 0.3	6.39 ± 1.15	17 ± 3
1	5.7 ± 1.4	1.3 ± 0.2	0.06 ± 0.01	240 ± 91
4	7.2 ± 2.1	4.4 ± 0.9	0.52 ± 0.31	14 ± 8
6	5.2 ± 0.9	10.6 ± 1.4	8.32 ± 6.23	123 ± 18
9	5.5 ± 1.7	3.2 ± 0.4	6.22 ± 4.69	>155
10	6.8 ± 1.8	3.2 ± 0.4	0.05 ± 0.01	>250
19	7.0 ± 3.1	1.7 ± 0.8	0.07 ± 0.04	18 ± 1
50	7.2 ± 2.2	5.4 ± 0.9	4.5 ± 0.7	4 ± 0.5
21	5.0 ± 2.0	2.4 ± 0.5	0.11 ± 0.11	56 ± 8
63	6.6 ± 2.7	6.6 ± 1.0	5.35 ± 0.69	19 ± 1
12	11.9 ± 3.8	9.1 ± 1.3	6.29 ± 3.23	>250
22	12.0 ± 3.9	3.1 ± 1.1	5.70 ± 0.16	>250
30	8.5 ± 3.9	3.4 ± 1.3	0.06 ± 0.06	20 ± 8
32	10.1 ± 4.2	8.4 ± 1.9	0.52 ± 0.07	15 ± 1
33	11.7 ± 2.9	6.4 ± 1.8	0.04 ± 0.03	12 ± 5
55	16.0 ± 4.0	4.8 ± 1.3	6.63 ± 2.93	17 ± 1
56	14.0 ± 3.3	3.8 ± 0.7	0.96 ± 0.10	87 ± 10

[a] Data are averages from at least three independent experiments ± standard deviation. [b] IC_{50} for inhibition of FLAG−p66/HIS−p51 RT dimerization. [c] IC_{50} for inhibition of HIV-1 RT RDDP activity. [d] EC_{50} for inhibition of viral replication in MT-4 cells. [e] CC_{50} for inhibition of MT-4 cell viability.

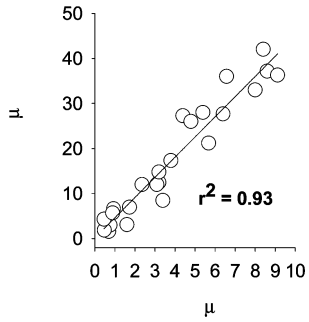

Figure 5. Correlation between IC_{50} values determined for the ability of TSAO-T derivatives to inhibit FLAG−p66/HIS−p51 RT dimerization and RDDP activity. Assays were carried out as described in Materials and Methods. Values are provided in Table 1.

that TSAO-T derivatives can be developed that exhibit improved activity against both RT dimerization and HIV-1 viral replication.

Conclusions

In this study, we have carried out the first structure−activity relationship study for a novel class of compounds that inhibit HIV-1 RT dimerization. Our results clearly demonstrate that some N-3-substituted TSAO-T derivatives act better than TSAO-T at disrupting the inter-subunit interactions of HIV-1 RT, are more potent inhibitors of the enzyme's DNA polymerase activity, and also exert potent antiviral activity. These results provide an important framework from which new TSAO-T derivatives can be designed that will act as more potent inhibitors of HIV-1 RT dimerization.

Materials and Methods

Inhibitors. All TSAO compounds were synthesized as described previously.[29−47] The syntheses of compounds **2**, **15**, **16**, **17**, and **21** will be published in a separate paper. Efavirenz was obtained from the NIH AIDS Research and Reference Reagent Program.

Cloning, Expression, and Purification of HIS−p51. The gene for the p51 subunit of HIV-1 RT was amplified from xxLAI proviral DNA[48] and cloned into pBAD-HisB (Invitrogen). The following forward and reverse primers were used: 5′-CTCTCTCTCGAGACCCATTAGTCCTATTGAAACT-3′ and 5′-CTTCAAGCTTTTAGAACGTTTCTGCTCCTAC-3′. These primers generate flanking restriction sites (underlined sequence) for the enzymes *Xho*I and *Hin*dIII. The resulting PCR fragment (1.3 kb) was double digested with *Xho*I and *Hin*dIII and ligated into the pBAD-HisB vector to generate pBAD-HIS−p51. Clones were verified by DNA sequencing. pBAD-HIS−p51 was transformed into *Escherichia coli* TOP10 cells, and the cells were grown overnight at 37 °C in 100 mL of Luria Broth (LB) medium containing 100 μg/mL of ampicillin. This was used to inoculate 1 L of Power Prime Broth (Athena Environmental Sciences, Baltimore, MD) containing 100 μg/mL of ampicillin, which was incubated at 37 °C with vigorous shaking until the culture reached an OD_{600} of 0.5 before protein expression was induced by the addition of 0.1% L-arabinose. Cells were grown overnight at 30 °C, harvested by centrifugation (10 000 rpm), suspended in lysis buffer (20 mL of 50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 1 mM β-mercaptoethanol), and lysed via two passages through a French press (Thermo Electron Corp., Ashville, NC). The resultant lysate was clarified by centrifugation and mixed with 1 mL of Talon metal affinity resin (Biosciences Clontech., Palo Alto, CA). Following 1 h incubation at 4 °C, the affinity resin was washed 3 times with lysis buffer to remove unbound proteins. HIS−p51 was eluted with 1× elution buffer (50 mM sodium phosphate buffer, 250 mM imidazol, 1 mM β-mercaptoethanol, pH 6.0). Purified HIS−p51 was dialyzed overnight against dialysis buffer (50 mM Tris-HCl, 25 mM NaCl, 1 mM β-mercaptoethanol, and 10% glycerol, pH 7.0) at 4 °C, concentrated using 30 kDa Vivaspin concentrators (Vivascience, Hannover, Germany), and then stored in 50% glycerol at −80 °C for subsequent use. Protein concentration was determined by Bradford assay (Sigma-Aldrich, Saint Louis, MO) using bovine serum albumin as a standard.

Cloning, Expression, and Purification of FLAG−p66. The gene for the p66 subunit of HIV-1 RT was amplified from xxLAI proviral DNA and cloned into pT7-FLAG (Sigma-Aldrich, Saint Louis, MO). The following forward and reverse primers were used: 5′-TTCAAGCTTCCCATTAGTCCTATTG AAACTG-3′ and 5′-TAACGAGCTCTTATAGTACTTTCCTGATTCC-3′. *Hin*dIII and *Sac*I (both underlined) restriction sites were incorporated in the forward and reverse primers, respectively. The resulting PCR fragment (1.7 kb) was double digested with *Hin*dIII and *Sac*I and ligated into pT7-FLAG to generate pT7-FLAG−p66. Clones were verified by DNA sequencing. pT7-FLAG−p66 was transformed into *E. coli* BL21 star (DE3) cells (Invitrogen), which were grown overnight at 37 °C in 100 mL of LB medium containing 100 μg/mL of ampicillin. This was used to innoculate 1 L of Power Prime Broth containing 100 μg/mL ampicillin; cells were grown to mid-log phase ($OD_{600} \approx 0.5$) before protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were grown overnight at 4 °C, harvested by centrifugation, and suspended in 20 mL of 1× TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 1 mM phenylmethylsulfonylfluoride (PMSF) and two tablets of protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN). Cells were lysed and clarified as described for HIS−p51. The supernatant was incubated with 5 mL of anti-FLAG M2 affinity gel (Sigma-Aldrich Corp., Saint Louis, MO) for 1 h on a platform shaker at +4 °C. The resin was harvested by centrifugation and then washed three times with TBS buffer to remove unbound proteins. FLAG−p66 was eluted by incubating the resin with FLAG peptide (Sigma-Aldrich, Saint Louis, MO) for 30 min. Purified FLAG−p66 was dialyzed overnight against dialysis buffer as described above and then concentrated and stored in 50% glycerol at −80 °C. Protein concentration was determined as described above.

Cloning, Expression, and Purification of HIS−GST. The gene for the *Schistosoma japonicum* glutathione S-transferase (GST) was amplified from pGEX-2T (GE Healthcare) and cloned into pBAD-HisB (Invitrogen). The following forward and reverse primers were

used: 5′-TACTCGAGATCCCCTATACTAGGTTATTG-3′ and 5′-TACTCGAGATCCCCTATACTAGGTTATTG-3′. The resulting PCR fragment was double digested with *Xho*I and *Hin*dIII and ligated into the pBAD-HisB vector to generate pBAD-HIS−GST. Clones were verified by DNA sequencing. pBAD-HIS−GST was transformed into *E. coli* TOP10 cells, and protein expression and purification were carried out as described for HIS−p51.

In Vitro HIV-1 RT Dimerization Assay. HIS−p51 (0.4 μM) was mixed with FLAG−p66 (0.4 μM) in a total volume of 200 μL of binding buffer (50 mM Tris-HCl, pH 7.0, 50 mM Na_2SO_4, 3% DMSO), in the presence and absence of drug, and incubated in His-Select high-capacity nickel coated plate (Sigma-Aldrich, Saint Louis, MO) on a platform shaker at room temperature for 16 h. Reactions that included efavirenz were incubated for 4 h. Control reactions included HIS−GST (0.4 μM) combined with FLAG−p66 (0.4 μM). Unbound protein was removed by washing four times with 200 μL of TBST buffer (TBS, 0.1% Tween-20). To detect bound FLAG−p66, 200 μL of mouse monoclonal anti-FLAG antibody (Sigma-Aldrich, Saint Louis, MO), diluted 1:2000 in TBST, was added to each well and incubated for 1 h. Thereafter the plate was washed 5 times with wash buffer, and then 200 μL of the peroxidase-conjugated goat anti-mouse antibody (Sigma-Aldrich, Saint Louis, MO), diluted 1:10000 in TBST, was added and incubated for 1 h. The plate was then washed 5 times with wash buffer, and then 200 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) was added to each well for 10 min. Reactions were stopped with 100 μL of stop reagent (Sigma-Aldrich, Saint Louis, MO). Absorbance was read at 450 nm (OD_{450}). All incubations were performed at room temperature.

Statistical Analyses of Dimerization Assay. Statistical analyses of the signal-to-noise ratios of the assay were carried out by calculating the mean (M) and standard deviations (SD) for the signal and background reactions and then determining the signal-to-noise ratio (S/N) using the following equation:[26] $S/N = (M_{signal} 2M_{background})/[(SD_{signal})^2 + (SD_{background})^2]^{1/2}$.

The Z' value, which is a relative indication of the separation of the signal and background populations, was calculated from data from three 96-well plates, which were filled with both positive (signal) and negative (background) controls, using the following equation:[25] $Z' = 1 - 3(SD_{signal} + SD_{background})/|M_{signal} - M_{background}|$.

RNA-Dependent DNA Polymerase (RDDP) Assays. Fixed time assays were used for HIV-1 RT-associated RDDP activity. Briefly, reactions were carried out in 50 mM Tris-HCl, pH 7.8 (37 °C), 50 mM KCl, 10 mM $MgCl_2$, and 5 μg/mL poly(rA)−oligo$(dT)_{12-18}$ and 20 μM [^{3}H]TTP and variable concentrations of ligand dissolved in DMSO (3% final concentration). Reactions were initiated by the addition of 50 ng of wild-type RT, incubated for 20 min at 37 °C, and then quenched with 250 μL of ice-cold 10% trichloroacetic acid (TCA) containing 20 mM sodium pyrophosphate. Quenched samples were filtered, and the extent of radionucleotide incorporation was determined by liquid scintillation spectrometry.

Antiviral Assays. MT-4 cells (3×10^5 cells per milliliter) were infected with HIV-1 at ~100 $CCID_{50}$ (50% cell culture infective dose) per milliliter of cell suspension. Then, 100 μL of the infected cell suspension was transferred to microtiter plate wells and mixed with 100 μL of the appropriate dilutions of test compounds. HIV-induced cytopathicity was determined by trypan blue dye exclusion in the HIV-infected cell cultures after 5 days. The 50% effective concentration (EC_{50}) of the test compounds was defined as the compound concentration required to reduce cell viability by 50%. The 50% cytostatic or cytotoxic concentration (CC_{50}) was defined as the compound concentration required to reduce the number of viable MT-4 cells in mock-infected cell cultures by 50%.

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Supporting Information Available: Elemental analytical data for compounds **2**, **15**, **16**, **17**, and **21.** This material is available free of charge via the Internet at http://pub.acs.org.

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