TSAO Compounds: The Comprehensive Story of a Unique Family of HIV-1 Specific Inhibitors of Reverse Transcriptase

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Abstract: Emergence of drug-resistant viral strains is one of the major milestones and the main cause for the failure of antiretroviral therapy. Combination of different anti-HIV agents has become the standard clinical practice to keep the viral load at low or even undetectable levels and to prevent emergence of virus-drug resistance. Among the human immunodeficiency virus (HIV) reverse transcriptase (RT) inhibitors, the so called nonnucleoside RT inhibitors (NNRTIs) have gained a definitive place in the treatment of HIV infections in combination with nucleoside analogue RT inhibitors (NRTIs) and HIV protease inhibitors (PIs). The virus can be markedly suppressed for a relatively long period of time when exposed to multiple drug combination therapy (highly active antiretroviral therapy, HAART). TSAO derivatives are a peculiar group of highly functionalized nucleosides that belong to the so-called nonnucleoside RT inhibitors (NNRTIs). They exert their unique selectivity for HIV-1 through a specific interaction with the p51 subunit of HIV-1 RT. They are the first small molecules that seem to interfere with the dimerization process of the enzyme. This review covers the work carried out with this unique class of specific inhibitors of HIV-1 reverse transcriptase, including structure activity relationship studies (SAR), its mechanism of action, resistance studies, model of interaction with the enzyme, etc.

Keywords: Reverse transcriptase, Non-nucleoside RT inhibitors, TSAO compounds, resistance, dimerization.

INTRODUCTION

There is no doubt that the eradication or efficient control of the AIDS epidemic is still one of the most important challenges at the start of the 21st century. Although there is currently no cure for AIDS, a variety of drugs are available for treatment of the Human immunodeficiency virus type (HIV) infection. To date, six nucleoside reverse transcriptase (RT) inhibitors (NRTIs), one nucleotide reverse transcriptase inhibitor (NtRTI), three nonnucleoside RT inhibitors (NNRTIs), four protease inhibitors and recently a fusion inhibitor are approved for treatment of AIDS [1-9]. A number of other RT inhibitors, protease inhibitors but also drugs targetting other sites of the virus replication cycle, are currently subject of clinical trials [10,11].

Reverse Transcriptase (RT) is a key enzyme in the life cycle of HIV which is responsible for the conversion of genomic viral RNA into double-stranded proviral DNA, a crucial step in the replication cycle of HIV [12, 13]. HIV RT is an asymmetric heterodimer consisting of two subunits, p66 and p51 [14]. Crystal structures of unliganded RT [15], RT-DNA complexes [16], and RT-inhibitor complexes [17] have consistently shown that, despite the amino acid identity in both subunits, the polymerase subdomains (called fingers, palm, thumb, and connection) are arranged differently in each one, with p66 forming a large active-site cleft and p51 forming an inactive closed structure that provides structural support to the polymerase domain of p66 [18-20]. The catalytic site (located at this subunit) where polymerization occurs contains a triad of aspartic acid residues at positions 110, 185 and 186 [21]. At least two Mg^{2+} ions may assist with polymerization by binding to the carboxyl groups of these aspartic acids [22,23]. The RT heterodimer represents the biologically active form of the enzyme, while the monomeric subunits have only low catalytic activity [24]. Indeed the p66 and p51 monomers are catalytically virtually inert and it is only through dimer formation that enzymatic activity is restored [24]. For the process of native dimerization a two-step model has been proposed in which the dimers are in equilibrium with the folded inactive monomers [25]. The first step is a concentration-dependent association of p66 and p51 that yields an inactive intermediate that slowly isomerizes to the "mature" heterodimeric form of the enzyme [26].

Several classes of RT inhibitors have been developed:

1. The nucleoside RT inhibitors (NRTIs)

They, upon metabolic activation (phosphorylation by cellular kinases to their 5'-triphosphate forms), bind at the

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polymerase active site (the substrate-binding site) of RT [1, 27], acting then either as competitive substrates/inhibitors with respect to the natural substrates (dNTPs) and/or as DNA chain terminators [27]. Six nucleoside analogues have been officially approved for clinical use as anti-HIV drugs "Fig. (**1**)" zidovudine (AZT), zalcitabine (ddC), didanosine (ddI), stavudine (d4T), lamivudine (3TC), and abacavir (ABC) [4, 8]. These drugs show important clinical benefits, but also have important drawbacks including development of resistance and severe dose-limiting toxic side effects, mostly due to interaction with cellular DNA polymerases and/or interference with nucleotide pools [1].

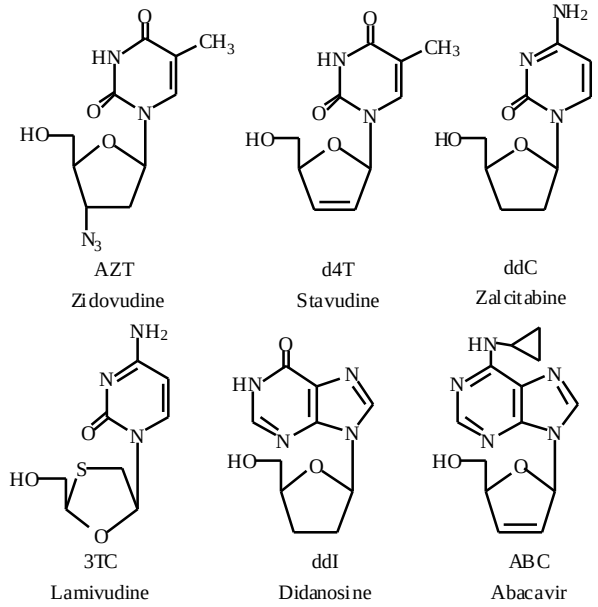


Fig. (1). Structures of approved nucleoside RT inhibitors (NRTIs).

2. The nucleotide RT inhibitors (NtRTIs) [acyclic nucleoside phosphonates (ANPs)]

Adefovir (PMEA) and tenofovir (PMPA) "Fig. (**2**)", are members of this class of potent and selective inhibitors of HIV RT. These compounds are converted to their active (diphosphorylated) metabolites by cellular enzymes. These metabolites are potent inhibitors of HIV RT which act upon incorporation into the growing viral DNA chain as DNA chain terminators [1, 27]. Thus their mechanism of antiretroviral action is virtually similar to that of NRTIs such as AZT and ABC. They are resistant to catabolic degradation such as dephosphorylation, due to the presence in their stucture of the phosphonate group (isopolar with respect to the phosphate). Oral prodrug forms of adefovir as adefovir dipivoxil [Bis(POM)PMEA] and tenofovir as tenofovir disoproxyl fumarate [Bis(POC)PMPA] "Fig. (**2**)", have been used in clinical trials [28]. Tenofovir disoproxyl fumarate has recently been approved for the treatment of HIV infections and adefovir dipivoxil for HBV infections.

3. The nonnucleoside RT inhibitors (NNRTIs)

To this important class of RT inhibitors belong nevirapine, delavirdine and efavirenz "Fig. (**3**)". They are approved for the clinical treatment of HIV-1 infection in combination with NRTIs and/or protease inhibitors [6-9]. NNRTIs are structurally diverse (more than 30 different classes have been described) but they all are targeted exclusively at the HIV-1 RT at an allosteric, non-substrate binding site ("hydrophobic pocket") that is close (on average, ≈ 15Å) but distinct from the substrate binding site (NRTI-binding site). This "pocket" does not exist in ligand-free RT, and has not been reported in RTs other than HIV-1 RT [16a, 29-31]. NNRTIs do not need to be metabolically activated and are noncompetitive with respect to both substrate and the template/primer [1, 27].

Crystal structures of RT/NNRTI complexes revealed that, upon binding of an NNRTI with the HIV-1 RT, the template/primer undergoes a repositioning in the protein, leading to a displacement of the binding groove by approximately 2 Å away from the active binding site. This, in turn, results in a markedly decreased enzyme activity in the presence of the NNRTI. Thus, NNRTIs inhibit the HIV-1 RT enzyme due to distortion of the polymerase-active site, a conclusion that has been supported by both structural [32] and kinetic [33] studies. NNRTIs are highly potent and specific inhibitors of HIV-1 replication [5, 7, 8]. They do not inhibit the transcriptases of other lentiviruses including HIV-2, Simian immunodeficiency virus (SIV) and Feline immunodeficiency viruse (FIV) or other (retro)viruses.

TSAO DERIVATIVES: A UNIQUE CLASS OF NNRTIS

Within the NNRTIs TSAO compounds represent a unique family of antiretroviral compounds first described in the early 90's by our research groups at the CSIC (Madrid, Spain) and the Rega Institute (Leuven, Belgium) [34-36]. TSAO are highly functionalised nucleosides, being this the first feature of their "uniqueness". However, mechanistically, TSAO compounds behave as all the other NNRTIs, inhibiting HIV-1 replication, but not the replication of HIV-2 or of other (retro)viruses [34a, 35a]. They are targeted at HIV-1 reverse transcriptase at a nonsubstrate binding site. They do not inhibit other DNA polymerases such as the cellular DNA polymerases α, β, γ and HSV-1 (herpes simplex virus type 1) DNA polymerase [37, 38]. TSAO compounds are relatively nontoxic to human cells; however, like the other NNRTIs; they select for highly resistant HIV-1 strains [39, 40].

The prototype compound of this family is the [1-[2',5'-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]thymine]-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2"-dioxide) named as TSAO-T "Fig. (**3**)" [34b]. TSAO-T is endowed with potent anti-HIV-1 activity, that is not dependent on the cell line (EC_{50}: 0.017-0.058 μM) [34a]. TSAO-T inhibits noncompetitively RT when poly(C) • oligo(dG) is used as the template/primer and dGTP as the natural substrate [37a, 38]. No marked inhibitory effect is noted with other artificial templates. In contrast, the other NNRTIs usually inhibit HIV-1 RT in the presence of various template/primers, although they usually prefer poly(C) • oligo(dG) as the template/primer for optimal inhibition.

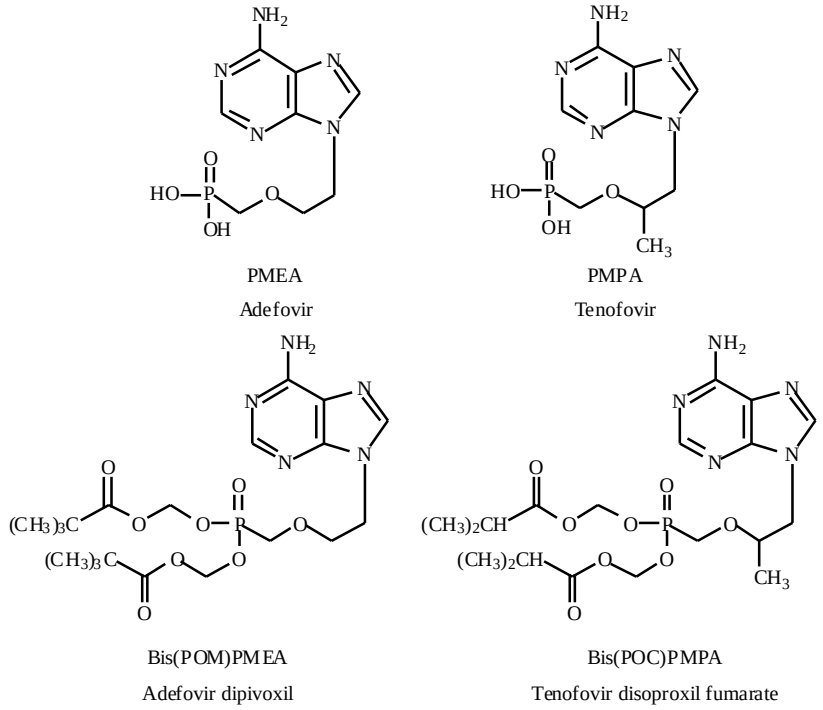


Fig. (2). Structures of acyclic nucleoside phosphonates (ANPs).

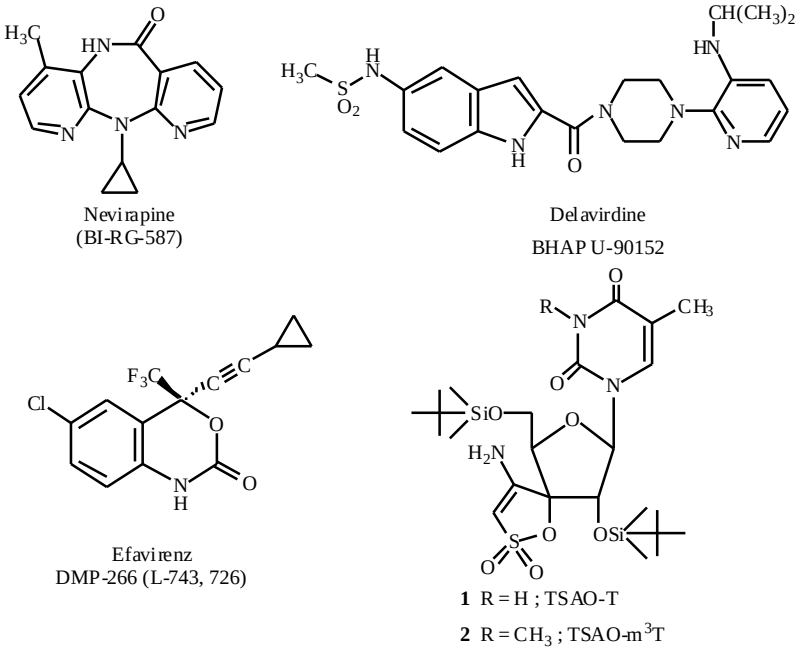


Fig. (3). Structures of nonnucleoside RT inhibitors (NNRTIs).

SAR Studies in the TSAO Compounds

Since we first synthesized TSAO-T in 1992 [34], several modifications have been carried out on this family of compounds that have helped to reveal the structural requirements of TSAO derivatives for their optimal interaction with the HIV-1 RT.

In contrast to other NNRTIs this family of compounds must fulfill a strict nucleosidic nature, that is, an intact ribose moiety with very stringent structural requirements. Thus, all TSAO derivatives need the concomitant presence in their molecules of a 3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide) moiety and *tert*-butyldimethylsilyl (TBDMS) groups at both 2' and 5' positions of the sugar moiety, in

nucleosides of *ribo* configuration, as a prerequisite for antiviral activity [34, 41]. The corresponding *xylo* isomers were devoid of any antiviral activity [42]. Change of the 3'-spiro moiety to the 2' position of the ribose, resulted in annihiliation of the antiviral activity. Removal of the lipophilic (TBDMS) groups, either at 2', at 5' or at both positions, gave inactive TSAO derivatives at subtoxic concentrations (EC_{50}: 30-1000 μM) [34b]. The corresponding 2'- or 5'-deoxy analogues were also inactive. Replacement of the 2'-TBDMS by other groups mimicking either the lipophilic or the steric properties of TBDMS led to TSAO compounds with 2- to 20-fold reduced anti-HIV-1 activity [43a], while a similar replacement at the 5'-position rendered antivirally inactive TSAO compounds, thus, pointing to a more critical role of the 5'-substituent for activity. The only group that restored some activity (although drastically reduced) is the *tert*-hexyldimethylsilyl group (EC_{50}: 0.8-2.0 μM) [43b].

In contrast, the base part of TSAO molecules is less stringent in its structural requirements, but seems to play a modulatory role of the activity/cytoxicity of the TSAO compounds. The thymine of the prototype TSAO-T can be considerably changed without compromising the anti-HIV-1 activity. It has been replaced by a number of other pyrimidines (i.e. uracil, 5-ethyluracil, cytosine, 5-methylcytosine, 5-substituted pyrimidines, etc), purines (adenine, hypoxanthine, xanthine, etc) or several 4- and /or 5-substituted-1,2,3-triazoles [34-36a, 37b, 37c, 44-46]. These base-modified TSAO derivatives showed potent anti-HIV-1 activities. In general, the TSAO-purine derivatives were 3- to 5- fold less potent than the most active TSAO-pyrimidines or TSAO-1,2,3-triazoles. Interestingly, alkyl or alkenyl substitutions at the N-3 position of TSAO-pyrimidines or at the N-1 position of TSAO-purines attenuated cytotoxicity (10- to 20- fold), without affecting the anti-HIV-1 activity [34-36a, 37b, 37c, 44, 45], resulting in compounds with higher selectivity indices ($SI = CC_{50}/EC_{50}$). As an example TSAO-m^3T "Fig. (**3**)", the N-3-methyl analogue of TSAO-T, showed a 20-fold higher SI than that of TSAO-T ($SI_{TSAO\text{-}m3T}$: 4088 *vs* $SI_{TSAO\text{-}T}$: 227). It should be pointed out that further modifications carried out at position N-3 of the thymine base in the prototype compound TSAO-T, led to novel analogues bearing at their N-3 position different amino acids (i.e. Ala, Val, Asp and Glu) with unprotected carboxylic functions at that position "Fig. (**4**)" [47]. Although, none of these new analogues showed an improved activity/toxicity profile (EC_{50}: 0.05-10 μM; CC_{50}: >250 μM) with respect to that of TSAO-T or TSAO-m^3T, they gained an interesting activity against HIV-2 (EC_{50}: 25-140 μM). These new TSAO lead compounds should be further pursued to develop TSAO molecules with greater potency and broader spectrum of anti-HIV activity.

Several members among the TSAO-substituted triazoles showed potent anti-HIV-1 activity comparable to that of the TSAO prototype (TSAO-T). Thus, the 5-substituted amido-, methylamido-, and dimethylamido-1,2-3-triazole derivatives "Fig. (**4**)" showed potent anti-HIV-1 activity (EC_{50}: 0.056-0.52 μM). In particular, the 5-dimethylamido-1,2-3-triazole TSAO derivative (**9**) emerged as the most active triazole TSAO compound [46]. This was considered as a novel lead to develop further TSAO derivatives with potent anti-HIV-1 activity, and new series of analogues possessing different hydrocarbon shapes capping the amide at C-5 were prepared [48]. Several of these new compounds showed not only potent anti-HIV-1 activities but also a decreased or no cytotoxicity which resulted in more selective compounds than that of the parent TSAO-triazole prototype (**9**), as in the case of **10** and **11**. (SI_{10}: 1470; SI_{11}: 1190 *vs* SI_9: 333).

Further studies aimed at gaining deeper insights into the role that the thymine base of TSAO-T may play in the interaction of TSAO compounds with HIV-1 RT, were conducted. In particular, these studies were directed to assess the role that aromaticity and different fragments mimicking parts or the whole thymine base of TSAO-T may play in the above mentioned interaction with the RT enzyme. Thus, in 1998 we reported on the synthesis and anti-HIV-1 activity of a series of 3-spiro sugar derivatives. These compounds were substituted at the anomeric position with non aromatic rings

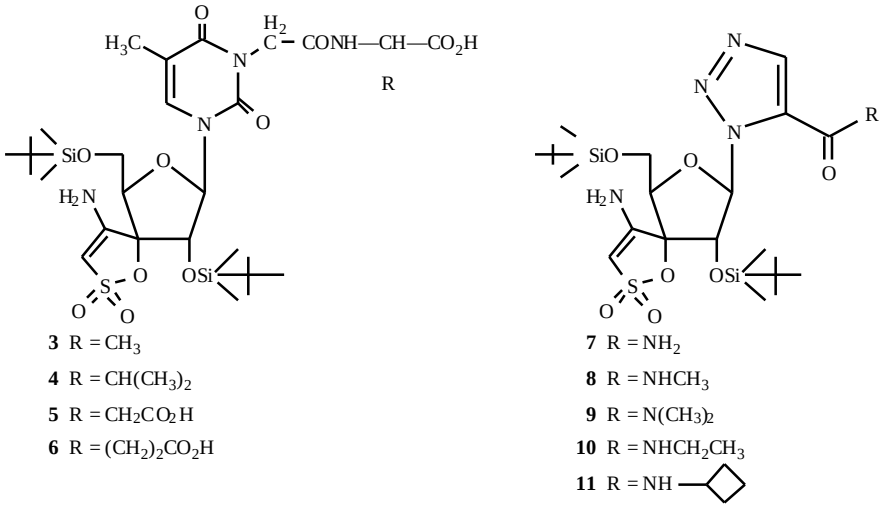
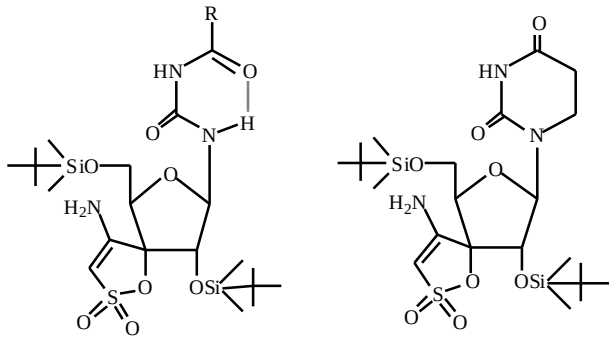


Fig. (4). Structures of base modified TSAO-T derivatives (**3-6**) and TSAO-triazole derivatives (**7-11**).

or with amine, amide, urea or thiourea moieties, derived from a systematic disassemblage of the molecular architecture of the thymine ring that mimic parts or the whole thymine base of TSAO-T [49]. Also, a dihydrouracil-TSAO analogue and *O*-glycosyl 3-spiro sugar derivatives substituted at the anomeric position with aromatic and nonaromatic lipophilic moieties (i.e. methyloxy or benzyloxy groups) were prepared. Compounds substituted at the anomeric position with an azido, amino or methoxy group, respectively, were devoid of marked antiviral activity (EC_{50}:10-200 μM). However, the substituted urea sugar derivatives led to an increase in antiviral potency (EC_{50}: 0.35-4 μM). Among them are those urea derivatives that mimic most closely the intact TSAO-T molecule (**12-14**) "Fig. (**5**)" retained the highest antiviral activity. Also, the dihydro-uracil TSAO derivative (**15**) retained pronounced anti-HIV-1 activity. None of the compounds showed any anti-HIV-2 activity. These abasic TSAO derivatives represent the first examples of sugar derivatives that interact in a specific manner with HIV-1 RT [49]. Urea derivatives, which can mimic to a large extent both the shape and the electrostatic potential of the thymine ring, can effectively replace this nucleic acid base when incorporated into a TSAO molecular framework with only moderate loss of activity. The abasic TSAO analogue (**14**) is just one order of magnitude less active than the prototype TSAO-T, and our calculations suggest that this may be due to the energy penalty involved in breaking the intramolecular hydrogen bond in order to adopt a suitable conformation for binding into the enzyme.



12 R = CH_3

13 R = Ph

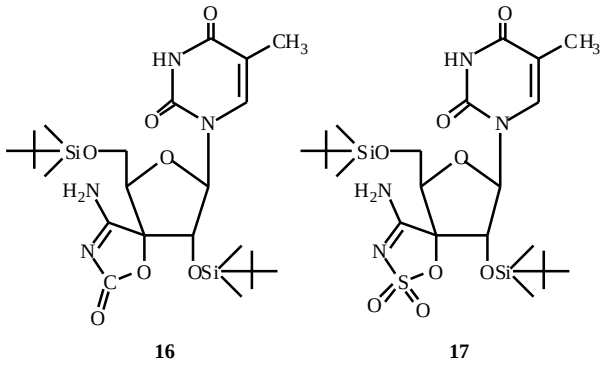
14 R = CH_2=C(CH_3)

15

Fig. (5). Examples of abasic TSAO derivatives (**12-14**) and 5,6-dihydrouracil-TSAO (**15**).

Our experimental data on TSAO derivatives strongly suggest a specific interaction of the amino group of the 3'-spiro moiety of TSAO molecules with the carboxylic group of a glutamic acid residue at position 138 of the p51 subunit of HIV-1 RT [18, 39, 40, 50,51]. This residue is located at the top of the finger domain of the p51 subunit of HIV-1 RT, that is part of the binding pocket of the HIV-1-specific RT inhibitors at the p66 subunit [16a, 30, 31].

The 3'-spiro moiety of TSAO derivatives was replaced by other 3'-spiro rings like 4-amino-2-oxazolone (**16**) or 4-amino-1,2,3-oxathiazole-2,2-dioxide (**17**) "Fig. (**6**)" that maintain a NH_2 group at the same position as the 4"-NH_2 group in the prototype compound TSAO-T, to allow the specific interaction of these new TSAO analogues with the carboxylic group of glutamic acid 138 of HIV-1 RT [52]. However, such replacements resulted in a decrease in the anti-HIV-1 activity by two orders of magnitude ($EC_{50,\ 16}$: 5.3-13 μM and $EC_{50,\ 17}$: 5.3 μM *vs* $EC_{50,TSAO-T}$: 0.04-0.06 μM), in spite of the fact that these novel TSAO analogues fullfilled the structural requirements of the TSAO family for activity. From comparative NMR studies, no significant conformational differences were detected in solution between TSAO-m^3T and these new spiro analogues that may account for the differences observed in their inhibitory activity against HIV-1 RT. Comparative studies based on theoretical calculations of the hydrophobicity, the solvation free energies and molecular electrostatic potentials (MEP) of the three spiro rings (aminooxathioledioxide, amino-oxazolone and amino-1,2,3-oxathiazoledioxide) showed that the calculated hydrophobicity (log P) values, dipole moments and the electrostatic contributions to the solvation free energies of the three spiro ring systems were also similar [53]. However, the differences found in the calculated MEPs of the spiro systems between TSAO-m^3T and its analogues suggest that the different electrostatic surroundings of the 4"-amino group of the spiro moiety in the analogues may be responsible for a detrimental electrostatic interaction of the spiro rings with the Glu-B138 of RT [53].



16 **17**

Fig. (6). Structures of 3'-spiro modified-TSAO analogues.

Several sugar-modified 3'-spiro-TSAO derivatives including allofuranosyl-TSAO analogues [54], compounds bearing L-sugars [55] or with inverted configuration at the C-4' stereocenter [56] were also prepared. However, none of these sugar modified TSAO compounds showed marked antiviral efficacy (EC_{50} >10 μM).

Metabolism and Pharmacokinetics of TSAO Derivatives

The cellular uptake, metabolism and pharmacokinetic properties of the N-3-methyl-substituted TSAO-T derivative TSAO-m^3T were investigated [57]. The compound is very stable in physiological solutions (i.e. PBS pH 7.2) at room temperature and no intracellular metabolites (besides the parent compound) were detected in at least three different human cell lines upon prolonged exposure of the cells to the compound. Uptake of TSAO-m^3T by human CEM cells is drug concentration-dependent and increased proportionally

with increasing initial extracellular TSAO- m^3T concentrations up to 20 μg/mL. Within 6 hr of incubation, the cells were almost completely saturated with the test compound. Further incubation up to 72 hr did not markedly increase the intracellular concentration of the compound. The pronounced lipophilicity of TSAO-m^3T (partition coefficient in octanol/water: >>20) is most likely responsible for the rapid uptake of the compound into human T-lymphocytes. No intracellular metabolic conversion of TSAO-m^3T was observed in CEM, MT-4 or MOLT-4 cells. In contrast to the lack of metabolism of TSAO-m^3T in cell cultures, the compound seems to be metabolized *in vivo*. Upon intravenous bolus administration of the compound to mice at 0.75 mg/kg, TSAO-m^3T was rapidly cleared from the plasma in a mono-exponential manner (half-life: 22 min; distribution volume: 9.5 L/kg; total body clearance: 17.8 L/hr/kg) [57].

TSAO-m^3T mainly accumulated in the lungs, followed by the heart, kidney and liver. The reason for the preferential accumulation of the compound in the lungs is still unclear. In view of the neurological disorders associated with AIDS, it is important to note that the brain contains low but significant levels of TSAO-m^3T. Although TSAO-m^3T levels in the brain are low, they may suffice to achieve a therapeutic effect since TSAO-m^3T was found to be antivirally effective at a 50% inhibitory concentration as low as 30 ng/ mL [34a, 58]. Thus, our data indicate that TSAO-m^3T is able to cross the blood-brain barrier, and thus may be able to inhibit virus replication in the brain compartment.

Significant amounts of different metabolites of TSAO-m^3T were detected in most tissues, the liver, kidney and spleen being the organs that showed the most extensive metabolism. The principal metabolites identified were TSAO-m^3T derivatives in which the *t*-butyldimethylsilyl moiety at O-2' and/or O-5' had been split-off. The free base N^3-methylthymine was not detected [58].

Resistance Development of HIV-1 Against TSAO Compounds

One of the major problems of the NNRTIs is the rapid emergence of virus drug resistance [59]. HIV-1 resistance to NNRTIs is primarily asociated with mutations in the HIV-1 RT gene usually located between codons 98 and 108, between codons 179 and 190 and between codons 225 and 236 [60]. All these mutations are lining the lipophilic NNRTI-specific binding pocket in the p66 subunit of the RT.

In cell culture, TSAO derivatives rapidly select for TSAO-resistant HIV-1 strains. All TSAO-resistant HIV-1 strains sequenced (more than 20) contain one amino acid change at position 138 of their RT [39]. In all cases glutamic acid was invariably replaced by lysine. This mutation consistently occurs regardless of the TSAO congener used for selection [40a]. No other mutations have ever been found in the sequenced domain of the RT gene of the TSAO-resistant HIV-1 strains obtained in cell culture. The Glu138Lys RT mutant HIV-1 strains are highly resistant to TSAO inhibition, but they retain marked to full sensitivity to most other NNRTIs, as well as to nucleoside RT inhibitors (NRTIs) and to the acyclic nucleoside phosphonates [39, 40b, 61, 62]. This provides direct evidence that 138-Glu must play a crucial role in the recognition of TSAO derivatives by the enzyme.

By site-directed mutagenesis we demonstrated that this glutamic acid residue at position 138 of the p51 subunit, but not of the p66 subunit of HIV-1 RT, determines the sensitivity and resistance of the enzyme to the inhibitory effects of the TSAO derivatives [51]. So far, no other HIV-1-specific inhibitors are reported to preferentially select for HIV-1 strains that are mutated at that position.

Although TSAO derivatives do not seem to select for mutant HIV-1 strains containing amino acid changes at Tyr181Cys, Tyr188Cys and Val106Ala in their RT, they considerably lose activity against such mutant HIV-1 strains, while retaining substantial activity agains Leu100Ile and Lys103Asn mutants [63]. These data strongly suggest the presence of additional interaction points between the TSAO molecules and the p66 RT subunit (that may serve either as direct interaction sites for the TSAO compounds or at least help in maintaining the desirable conformation of the TSAO binding site in the RT heterodimer). TSAO compounds are the only molecules within the group of NNRTIs for which aminoacids at both HIV-1 RT subunits (p66 and p51) are needed for optimal interaction with the enzyme.

Site-directed Mutagenesis Studies of HIV-1 RT at Amino Acid Position 138

Although the Glu138 amino acid is highly conserved in HIV-1 strains, recently, the presence of the Glu138Ala mutation in several samples of drug-treated but TSAO-inexperienced patients has been reported [64]. This Glu138Ala RT mutated virus also showed a decreased sensitivity to TSAO derivatives.

In a study we sought to address the question why the Glu138Lys RT mutation invariably appeared under TSAO selective pressure [65]. Seven RT mutants (i.e., Ala, Asp, Gln, Gly, Lys, Phe, and Tyr) at position 138 were constructed by site-directed mutagenesis. The mutant Glu138Asp, Glu138Lys, Glu138Gln, Glu138Ala, and Glu138Gly RTs retained marked catalytic activity. In contrast, the Glu138Phe and Glu138Tyr RT mutants showed poor RNA-dependent DNA polymerase activity (30 and 4% of wild-type, respectively). TSAO derivatives lost their inhibitory activity against all mutant enzymes, except against the closely related Glu138Asp RT mutant that remained as sensitive to TSAOs as did wild-type RT. Other NNRTIs, including delavirdine, emivirine, and the thiocarboxanilide UC-781, and the NRTI ddGTP retained pronounced inhibitory activity against all mutant enzymes (Table **1**). When the amino acid mutations at position 138 of RT were introduced in recombinant virus clones, the sensitivity/ resistance spectrum obtained toward the TSAOs and other NNRTIs was similar to those observed for the isolated recombinant mutant enzymes (Table **2**). The Glu138Lys RT mutant virus had the most marked resistance to TSAOs, followed by the Glu138Gln, Glu138Phe, Glu138Gly, Glu138Tyr, and Glu138Ala virus mutants. The Glu138Asp

Table 1. Sensitivity of HlV-l Wild-Type G1u138 and Mutant Recombinant RTs to the inhibitory Effects of NNRTIs and ddGTP.

	IC_{50} (μM)[a]							
Compound	**Glu138 (Wild Type)**	**Glu138Asp**	**Glu138Ala**	**Glu138Lys**	**Glu138Gln**	**Glu138Gly**	**Glu138Tyr**	**Glu138Phe**
TSAO-T	2.5 ± 0.6	1.5 ± 0.2	77 ± 8	>850	>85	>850	>850	>85
TSAO-m^3T	0.9 ± 0.2	0.7 ± 0.1	81 ± 4	>83	>830	>83	>83	>830
TSAO-triaz.	23 ± 7	12 ± 4	>90	>90	>90	>90	>90	>900
Delavirdine	0.39 ± 0.05	0.5 ± 0.2	2.1 ± 0.1	1.8 ± 0.3	1.0 ± 0.8	1.4 ± 1.2	0.34 ± 0.02	0.3 ± 0.00
Emivirine	0.1 ± 0.05	0.1 ± 0.1	0.47 ± 0.03	3 ± 1	0.5 ± 0.1	0.5 ± 0.2	0.39 ± 0.02	0.57 ± 0.02
UC-781	0.03 ± 0.00	0.05 ± 0.02	0.2 ± 0.1	0.2 ± 0.1	0.15 ± 0.08	0.31 ± 0.02	0.06 ± 0.00	0.28 ± 0.02
ddGTP	0.07 ± 0.01	0.05 ± 0.03	0.09 ± 0.04	0.02 ± 0.00	0.02 ± 0.00	0.10 ± 0.02	0.14 + 0.01	0.10 ± 0.01

[a] IC_{50}, or 50% inhibitory concentration required to inhibit the enzyme activity by 50%, using poly(rC) • o1igo(dG) as the template-primer and [2.8-^{3}H]dGTP as the radiolabeled substrate. The IC_{50} was determined using *E. coli* extracts as the source of the wild-type and mutant RTs. For the G1u138Phe mutant RT purified enzyme was used because *E. coli* extract did not show sufficient activity. The data are means of two to three independent experiments (means ± SD).

Table 2. Sensitivity of HlV-l Wi1d-Type G1u138 and Mutant Recombinant Strains to NNRTIs and ddG in CEM Cell Cultures.

	EC_{50} (μM) for recombinant mutant HIV-1 strains[a]							
Compound	**Glu138 (Wild Type)**	**Glu138Asp**	**Glu138Ala**	**Glu138Lys**	**Glu138Gln**	**Glu138Gly**	**Glu138Tyr**	**Glu138Phe**
TSAO-T	0.024±0.015	0.013±0.004	0.52±0.19	>3	2.1±1.4	0.76±0.48	0.53±0.38	2.5±1.6
TSAO-m^3T	0.035±0.019	0.021±0.018	0.41±0.09	>17	12±7	1.2±0.7	0.34±0.24	11±7
Delavirdine	0.008±0.007	0.009±0.006	0.07±0.04	0.05±0.02	0.04±0.03	0.02±0.01	0.007±0.003	0.004±0.001
Emivirine	0.0021±0.0016	0.0018±0.0009	0.023±0.008	0.042±0.023	0.011±0.004	0.006±0.0034	0.0043±0.0019	0.0044±0.0019
UC-781	0.0009±0.0003	0.0013±0.0006	0.009±0.006	0.010±0.0033	0.007±0.007	0.005±0.002	0.0013±0.0001	0.002±0.001
Efavirenz	0.003±0.002	0.0015±0.0003	0.005±0.001	0.0041±0.0001	0.007±0.002	0.003±0.001	0.0014±0.0001	0.0013±0.0003
ddG	3±2	4±3	11±8	4±1	5±2	6±4	3±2	1.6±0.3

[a] EC_{50}, or 50% Effective concentration, or compound concentration required to inhibit HIV-1-induced cytopathicity in CEM cell cultures by 50%. The data are means of at least four to six two independent experiments (means ± SD).

RT mutant virus kept full sensitivity to the TSAO derivatives. Mixtures of Glu138Lys RT mutant virus with the other virus clones mutated at the 138 position resulted in all cases, except for the Glu138Asp and Glu138Gly RT mutant viruses, in an outgrowth of the Glu138Lys RT mutant virus. Among the Glu138 RT mutants investigated, the Glu138Lys RT proved most resistant to TSAO derivatives. It also was among the most catalytically efficient enzymes, compared with the majority of the mutants made, and resulted in highly replication-competent virus. Moreover, the G→A transition mutation that is necessary to create the mutated Glu138Lys codon is known to occur preferentially and more easily than other base changes. Those three findings may explain why the Glu138Lys RT mutant virus strains but not virus strains containing other amino acids at position 138 invariably emerge in cell cultures under TSAO drug pressure.

Effect of dNTP Pool Imbalances on Resistance Development Against TSAO Derivatives

The RNA genome of the lentivirus human immunodeficiency virus type 1 (HIV-1) is significantly richer in adenine nucleotides than the statistically equal distribution of the four different nucleotides that is expected [66, 67]. This compositional bias may be due to the guanine-to-adenine (G→A) nucleotide hypermutability of the HIV genome, which has been explained by dCTP/dTTP pool imbalances during reverse transcription [68]. The adenine nucleotide bias together with the poor fidelity of HIV-1 reverse transcriptase markedly enhances the genetic variation of HIV and may be responsible for the rapid emergence of drug-resistant HIV-1 strains. In 1998 Balzarini and co-workers [69] hypothesized that the low fidelity of HIV-1 RT on the one hand, and the adenine nucleotide hypermutability bias on the other hand, could be exploited to manipulate and

redirect the mutational pattern of resistance of HIV-1 to antiviral drugs by influencing the dNTP pools of the target cells. To provide experimental evidence for this novel concept, TSAO derivatives (i.e. TSAO-m^3T and TSAO-5-dimethylamino-1,2,3-triazole) were used. It was shown that it is possible to shift the appearance of the TSAO-characteristic Glu138Lys (AAG codon) RT mutation to a Glu138Gly (GGG) RT mutation under TSAO-m^3T or TSAO-triazole drug pressure when the ratio of cellular endogenous dCTP/ dTTP pools is increased in the presence of exogenous dCyd [69]. However, the G→A transition mutation that occurs in the Glu138 codon (GAG) when converted to the Glu138Lys codon (AAG) is known as a favorable hypermutation that may preferentially and more easily occur than any other base changes [particularly the opposite A→G transition mutation [68c] that takes place when the Glu138 codon (GAG) is converted to the Glu138Gly codon (GGG) under elevated dCTP/dTTP levels [69]. This consideration raises the possibility that increasing the dCTP/dTTP ratio may be of long-term therapeutic benefit, which represents an interesting issue for further exploration. It would not be unlikely that this phenomenon may also contribute to the predominant appearance of the Glu138Lys RT mutation but not the Glu138Gly RT mutation in cell culture.

From the above study it was concluded that it is possible to counteract the mutational bias and particularly the adenine-over-guanine nucleotide preference of HIV-1 by changing the endogenous dNTP pool levels/ratios in HIV-1-infected cells by using antimetabolic drugs. Forcing the virus to change its inherent nucleotide bias may lead to better control of viral drug resistance development.

Combination of TSAO Compounds and NNRTIs and NRTIs in HIV-1 Infected Cell Cultures

From a therapeutical point of view, the development of resistance is one of the major milestones in the treatment of AIDS patients. It is now widely accepted that the best strategy to fight against resistance is the use of combination therapy. It has been proposed that a rational approach toward drug combination may be based on the choice of drugs that do not select for mutations resulting in cross-resistance of the virus for the combined drugs [70].

TSAO derivatives have been used in several double and triple drug combination studies with both NNRTIs [e.g. thiocarboxanilides (UC), BHAP U-88204 and U-90152, TIBO R82913, Nevirapine (BI-RG-587) and emivirine (MKC-442), etc] and NRTIs lamivudine (3TC) [63, 71, 72]. When 3TC, TSAO-m^3T and the thiocarboxanilide UC10 were used individually, they rapidly led to the emergence of drug-resistant HIV-1 mutants (Glu138Lys for TSAO-m^3T, Met184Val for 3TC, and Lys103Thr/Asn for UC10) in cell culture [63, 71, 72]. When 3TC was combined with either TSAO-m^3T or UC10 or UC42, emergence of drug-resistant virus was markedly delayed or even fully suppresed. The concomitant presence of the Glu138Lys and Met184Val mutations was noted in the RT of these mutant viruses that emerged under combination therapy of 3TC with either TSAO-m^3T or UC10, but the UC10 resistance mutation Lys103Thr/Asn was no longer detected, which may mean that it must have been suppressed upon combining UC10 with 3TC [63]. It was found that combination of TSAO-m^3T, UC10 or UC42 with 3TC resulted in marked potentiation of the anti-HIV-1 activity and suppress virus breakthrough in cell culture at compound concentrations that were ≥ 1-2 orders of magnitude lower than when the drugs were used individually [63, 71, 72]. Triple-drug combinations containing 3TC plus TSAOm^3T and BHAP and MKC-442 further potentiate the suppressive effect of the double combinations on HIV replication, and indeed resulted in complete suppression of virus breakthrough in HIV-1-infected CEM cell cultures at drug concentrations that readily allowed virus replication when the single drugs were administered as "monotherapy". Similar results were observed when BHAP U-88204 was added to the combination of UC42 with TSAO-m^3T [71].

TSAO Compounds in "Knocking-out" Studies

It has been proposed that an approach to prevent emergence of resistant virus (and apparent clearance of the virus from the culture) would be the use of "knocking-out" concentrations of the HIV-1-specific inhibitors (NNRTIs) [8a, 73]. When added to the HIV-1-infected cell cultures from the start of the infection at sufficiently high concentrations, the compounds may completely suppress virus replication and thus prevent the virus from becoming resistant. At a concentration of 2.5 μg/ml, TSAO-m^3T suppress virus breakthrough ("knock out" the virus) for 10 to 15 days, although this represents a shorther time period than that observed with other NNRTIs. The "knocking-out" principle also holds for HIV-1 RT mutant strains. Thus in cell cultures infected with the TSAO-resistant Glu138Lys mutant, virus replication could be completely suppressed by micromolar concentrations of NNRTIs (i.e., TIBO, BHAP, nevirapine) [74]. Interestingly, the concentrations required to knock out the virus can be significantly reduced when well-defined combinations of NNRTIs or combinations of NNRTIs with AZT or 3TC are used. For example when TSAO-m^3T (0.4 μg/ml) and 3TC (0.05-0.1 μg/ml) were combined, they were able to prevent virus breakthrough for more than 52 days. However, when used individually at the same concentrations they could not prevent virus breakthrough for more than a few days. Similar effects have been reported with two NNRTIs (i.e., TSAO-m^3T and thiocarboxanilide UC42). When combined, virus breakthrough could be suppressed for more than 77 days at drug concentrations (0.1 μg/ml for UC42 and 1 μg/ml for TSAO-m^3T) at which the individual compounds delayed virus breakthrough for only 20 and 25 days, respectively [71]. Virus breakthrough could be suppressed for even longer, and at lower drug concentrations, if a third NNRTI (BHAP) was added to the combination of UC42 with TSAO-m^3T, which points to the feasibility of triple drug combinations in preventing virus breakthrough and resistance development [71].

TSAO-T as Part of Potential Multifunctional Inhibitors of HIV-1 RT

Heterodimers [TSAO-T]-[NRTI]

It was suggested that an approach to combination therapy would be the use of dimers resulting from the linking of a

NNRTI and a NRTI through an appropriate spacer, in an attempt to combine the inhibitory capacity of these two different classes of molecules [30, 31]. Due to the NNRTI, the dimers might be highly specific to HIV-1 RT and due to the presence of the NRTI, the dimers might lower the speed of emergence of virus-drug resistance. It has been demonstrated that the binding of the NNRTIs to the HIV-1 RT results in an even tighter binding of the natural substrates (dNTPs) to the enzyme [33]. This cooperative interaction between the substrate (dNTP)-binding site and the non-substrate (NNRTI)-binding site may be interpreted as indicative of a synergistic action between the NNRTIs and ddNTPs. The interaction between the two binding sites may provide a means to increase the effectiveness of different drugs when combined, or of a single drug when combining the functionalities of a non-nucleoside RT Inhibitor (NNRTI) and a 2',3'-dideoxynucleoside or an acyclic nucleoside phosphonate RT inhibitor (NRTI) [33].

Crystallographic studies revealed that the NRTI binding site and the NNRTI binding pocket are close enough (≈ 10-19Å) [30] to be reached by one single molecule that combines in its structure the functionalities of both kinds of inhibitors. With this aim, and in an attempt to combine the HIV-inhibitory capacity of NRTI analogues and NNRTIs we reported, in 1995, for the first time the synthesis and anti-HIV-activity of a series of heterodimers of the general formula [NRTI]-$(CH_2)_n$-[NNRTI] [75]. The heterodimers had in their structure a NRTI such as AZT combined with a NNRTI such as TSAO-T or HEPT, linked through an appropriate spacer between the N-3 of the thymine base of both compounds. As spacer, we used aliphatic chains of different lenghts in order to obtain a heterodimer possessing an optimum distance between both active principles (NRTI and NNRTI) "Fig. (**7**)". The [TSAO-T]-$(CH_2)_n$-[AZT] heterodimers proved markedly inhibitory to HIV-1. However, they were less potent inhibitors than the parent compounds from which they were derived [75]. Also, if AZT was replaced by thymidine in the heterodimer molecules, potent anti-HIV-1 activity was observed. However, the corresponding [HEPT]-$(CH_2)_n$-[AZT] dimers were inactive. The best compound in the series of prepared compounds was the [TSAO-T]-$(CH_2)_3$-[AZT] heterodimer (EC_{50}: 0.38±0.07 μM) "Fig. (**7**)". None of the dimers were endowed with anti-HIV-2 activity.

To obtain better insights in the feasibility of this heterodimer approach and to increase the inhibitory efficacy of the test compounds against HIV-1 RT, novel analogues of the [AZT]-$(CH_2)_3$-[TSAO-T] prototype were prepared "Fig. (**7**)". In these novel series, other NRTIs such as d4T, and an expanded range of linkers with different conformational freedom and nature were synthesized [76]. Other attachment sites for these linkers on the base part of the NRTI analogue were explored, by anchoring the linker at the C-5 position of the thymine of the NRTI and at the N-3 position of the thymine base of TSAO-T. Moreover, in order to circumvent the dependence of the NRTI moiety of the heterodimer on activation by cellular nucleoside kinases, novel heterodimers in which the NRTI contained a masked monophosphate group (phosphoramidate) at the 5'-position were also prepared. Among the novel heterodimers, several derivatives showed potent anti-HIV-1 activity, which proved comparable, or even superior, to that of the AZT heterodimer prototype. The nature of the NRTI was important for the anti-HIV-1 activity. In particular, the d4T heterodimer derivative containing a propyl linker between the N-3 positions of the thymine bases of TSAO-T and d4T (**18**) was 5- to 10- fold more inhibitory to HIV-1 (EC_{50}: 0.018±0.03 μM) than the corresponding AZT heterodimer prototype and even 2-fold more potent than the unsubstituted TSAO-T parent compound. In general the nature of the spacer and the position of the linker on the NRTI could be changed while keeping the antiviral activity. The phosphoramidate series of compounds had no improved anti-HIV-1 activity over the corresponding non-phosphorylated analogues. Intriguingly,

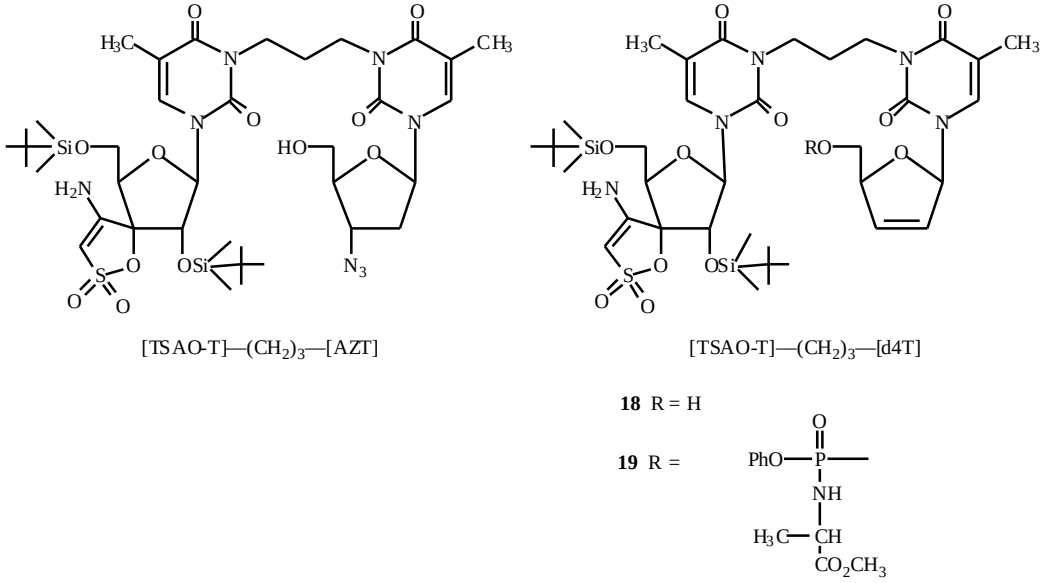


Fig. (7). Examples of [TSAO-T]—$(CH_2)_n$—[NRTIs] heterodimers.

the phosphoramidate heterodimer (**19**) (containing d4T as the nucleoside) had marginal, but significant, anti-HIV-2 activity (EC_{50}: 15 μM), whereas the corresponding AZT derivative had not.

TSAO Bidentate Inhibitors

A series of TSAO derivatives that contained groups with bivalent ion-chelating properties were also prepared [77]. The design of such molecules was based on the reported measurable interaction between the inhibitors binding pocket (NNRTIs) and the Mg^{2+} binding site [33]. It was proposed that attaching an NNRTI to molecules that should bind specifically to the nearby metal ion-binding site might create bidentate inhibitors with a stronger affinity, even for mutant enzyme forms. The TSAO derivatives prepared combine in their structure the functionality of a NNRTI (TSAO-T) and a metal chelating moiety (a β-diketone group and enol-ether groups) linked by an appropriate spacer (polymethylene linkers of different lengths) "Fig. (**8**)" at the N-3 position of the thymine [78, 79]. Some of the new compounds preserved their anti-HIV-1 activity (EC_{50}: 0.06-0.97 μM) being comparable to that of the parent compound TSAO-T, but display a markedly increased antiviral selectivity (SI_{21}: 2170; SI_{20}: 1250 *vs* SI_{TSAO-T}: 233), owing to lower cytotoxicity (CC_{50}: ≥ 130->250 μM) [77].

Hybrids [TSAO-T]-[Foscarnet]

In vitro combination experiments of TSAO-m^3T with Foscarnet (PFA, a potent inhibitor of the TSAO-characteristic HIV-1 RT/138 Lys mutant) [27] "Fig. (**8**)" resulted in a very pronounced suppression of virus replication and related virus breakthrough in the CEM cell cultures. However, foscarnet shows poor penetration into cells due to its polyanionic nature at physiological pH. These findings led us to prepare "hybrids" that combine TSAO derivatives and foscarnet in a single molecule through a labile covalent ester bond (**22**) "Fig. (**8**)" [80]. The major consideration in the design of these hybrids was to explore whether the highly lipophilic TSAO molecule may serve as driving-force of PFA into the cells thus resulting in an improvement of cell membrane permeability of the latter. The [TSAO-T]-[PFA] conjugate may also escape extracellular hydrolysis and once inside the cell would liberate the parent compounds. Therefore, we prepared [TSAO-T]-[PFA] conjugates containing the parent drugs linked via ester bonds to permit hydrolytic cleavage at physiological pH, or enzyme-mediated catalysis. Several [TSAO-T]-[PFA] conjugates proved markedly inhibitory to HIV-1 (EC_{50}: 0.47-0.82 μM) and also showed moderate activity against TSAO-resistant (EC_{50}: 20-50 μM) and PFA-resistant (EC_{50}: 0.16 μM) HIV-1 strains. Compounds were stable in PBS and in human serum. The conjugate (**22**) regenerated the parent compounds in CEM cell extracts.

Molecular Model of Interaction of TSAO-T with HIV-1 RT

Despite the similar binding geometry observed for several chemically diverse NNRTIs in their complexes with HIV-1 RT, considerable variation exists in the non-nucleoside binding pocket of the enzyme [17, 81]. Co-crystallization attempts of HIV-1 RT with TSAO derivatives have been unsuccessful so far. As already mentioned, the HIV-1 resistance to TSAO compounds is determined by the

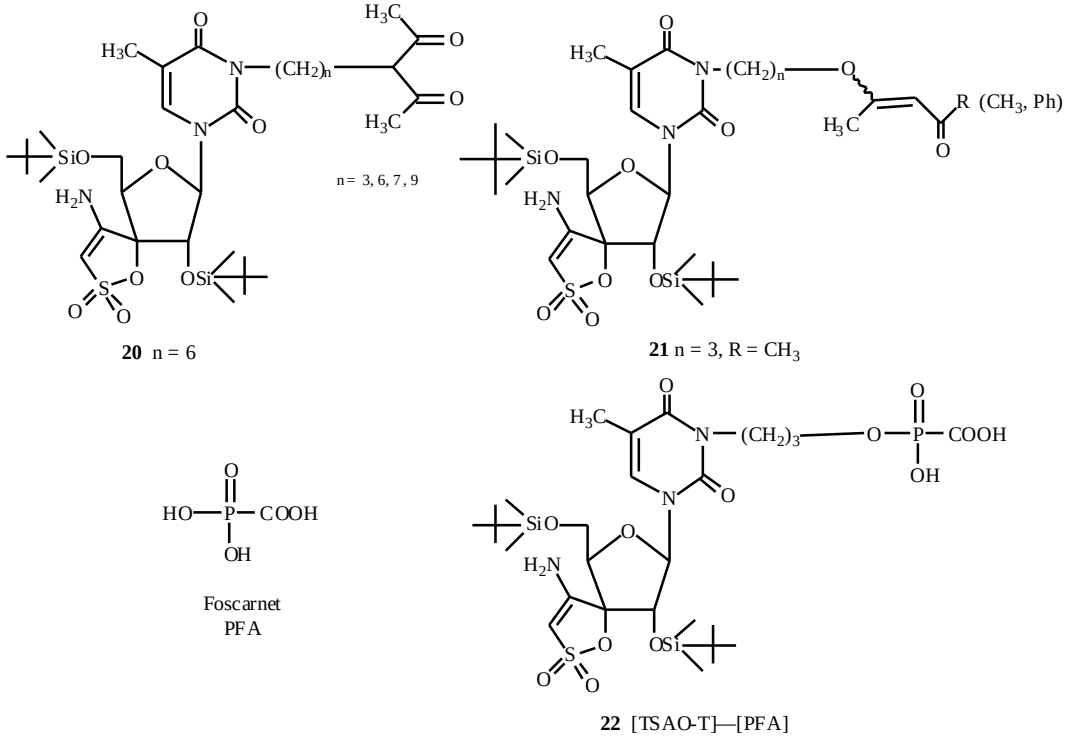
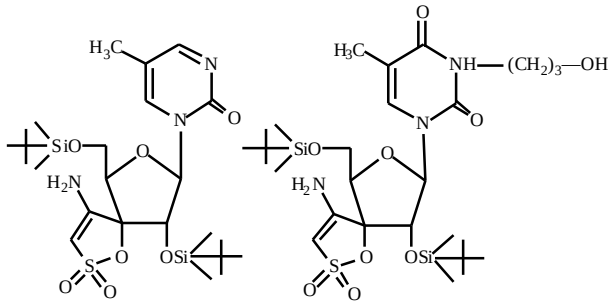


Fig. (8). Examples of TSAO-bidentated inhibitors and [TSAO-T]—[PFA] conjugates.

Glu138Lys mutation (in the β7-β8 loop of the p51 subunit) [40a, 51]. Recently, it has been shown that TSAO-T and its N3-ethyl derivative (TSAO-e^{3}T) destabilize the p66/p51 HIV-1 RT heterodimer and the p66/p66 homodimer in a concentration-dependent manner leading to a loss in their ability to bind DNA [20, 82]. However, TSAO-e^{3}T was unable to destabilize the subunit interactions of the Glu138Lys mutant enzyme. These results strongly suggest a binding mode for TSAO that may substantially deviate from that of other non-nucleoside RT inhibitors.

A preliminary molecular model of the HIV-1 RT-TSAO complex placed TSAO-T near the interface between the p66 and p51subunits with most of the molecule inside the standard NNRTI binding pocket in p66 [41a, 48, 49]. This model located the essential 5'-TBDMS substituent of TSAO-T into the cavity formed by Tyr181, Tyr188 and Trp229 and placed the thymine ring close to Tyr318, with substituents on the N3 position pointing toward a channel connecting the binding pocket with the solvent. In this orientation, the spiro amino group was able to form a good hydrogen bond with the carboxyl group of Glu138 in the p51 subunit, and this important interaction was thought to lie at the origin of the Glu138Lys mutation detected under the selective pressure of TSAO derivatives. Despite the relative success of this model in accounting for many experimental findings, some data argued against it. For the purpose of validation, we designed modifications on both the inhibitor and the enzyme. On the one hand, it was inferred from the model that removal of the O4 oxygen of the pyrimidine ring in TSAO-T would lead to an increase in binding affinity, since the negative electrostatic potential generated in this region of the inhibitor was detrimental for binding as this oxygen was close to the main chain carbonyl group of His-A235. However the TSAO compound (**23**) "Fig. (**9**)", resulting from the removal of the oxygen atom from position 4 of the thymine ring in the prototype compound, resulted in a partial loss of activity rather than in the expected increment ($EC_{50,\ 23}$: 0.23 μM vs $EC_{50,\mathbf{TSAO\text{-}T}}$: 0.05 μM) [83]. On the other hand, the model suggested that mutation of Arg172 to Ala would decrease the affinity for TSAO compounds but would not affect the binding of other NNRTIs, because the negative electrostatic potential generated by the solvent-exposed sulfone group of TSAO could give rise to a favorable interaction energy with the positively charged side chain of Arg172 (or Lys172 in other HIV-1 strains). However, again, the experimental results did not corroborate the molecular model. Instead, replacement of the arginine at position 172 by an alanine in RT led to an unanticipated improvement in the inhibitory activity of TSAO analogues [83]. Undoubtedly, our early model of interaction was strongly biased by attempts to make use of the NNRTI binding pocket, as seen in many complexes solved by X-ray crystallography. This led us to explore alternative binding modes and to study the energetics of dimerization and the rationale for the effects of TSAO-T on RT dimer stabilization [83]. The docking strategy consisted of extending the putative binding site further away from the standard NNRTI binding pocket and using all the reported X-ray crystal structures of the enzyme, both complexed with other inhibitors and in the apo form, as input for an automated docking program. This led us to propose a novel binding mode for TSAO-m^{3}T that is quite distinct from that of "classical" NNRTIs [83, 84]. In the updated model, TSAO-m^{3}T straddles between subunits binding at the p66/p51 interface but it does not make use of the NNRTI binding pocket.



23 **24**

Fig. (9). Base modified TSAO compounds.

According to the updated molecular model, TSAO-m^{3}T binds at the interface between the two subunits "Fig. (**10**)" in such a way that the strong dipole moment of the spiro group (μ = 8.4 debye) [53] is properly aligned in the field created by the positive electrostatic region emanating from Lys101 and Lys103 in the p66 subunit and the negatively charged Glu138 in the p51 subunit. This Glu138 is at hydrogen bond distance of the 4"-amino of the spiro group of TSAO-m^{3}T. An additional polar interaction (hydrogen bond) is formed between the O4 of the thymine ring and the hydroxyl group of Thr139 in the p51 subunit (which explains why removal of this O4 is detrimental to activity). The remaining stabilizing interactions are hydrophobic and exploit two cavities present at the enzyme interface that provide binding pockets for the 2'- and 5'-TBDMS substituents of TSAO derivatives. In the p51 subunit, the side chains of Ile31, Val35, and the hydrocarbon side chains of Lys32 and Ser134 provide a large hydrophobic surface that accomodates the indispensable 5'-TBDMS substituent. In the p66 subunit, the hydrophobic pocket is made up mainly by Pro176 and Val179, both of which interact with the 2'-TBDMS group.

The new molecular model is in agreement with the biochemical evidence and with known structure-activity and mutagenesis data for this class of inhibitors including a R172A mutant that had not been described previously. The feasibility of the proposed binding mode was assessed by analyzing the results of nanosecond MD simulations for the complexes of TSAO-m^{3}T with reduced models of the wild-type and Arg172Ala mutant RT enzymes in the presence of water molecules. The cost of desolvating the side chain of this arginine residue was shown to be unfavorable for complex formation, and this is suggested to be the main reason accounting for the unanticipated improved binding of TSAO-m^{3}T to the Arg172Ala mutant enzyme.

According to the docking model, additional interactions at or near the p51/p66 interface could result in compounds with improved binding affinity over the prototype TSAO-T. In the light of the present model, the N3 substituents of TSAO derivatives would be found running parallel to the subunit interface and mostly exposed to the solvent. A structure-based modification on the lead compound TSAO-T

consisting of introducing a -$(CH_2)_3$-OH substituent in the thymine N3 atom (compound **24**) "Fig. (**9**)" resulted in a 2–6 fold better inhibitor, consistent with the prediction that an additional hydrogen bond could be formed with the carbonyl group of Pro140. This putative extra hydrogen bond with the protein backbone may be partially offset by the inherent flexibility of the methylene linker and by competition with surrounding water molecules.

How do TSAO Derivatives exert Their Inhibitory Action?

With regard to the mechanism by which TSAO derivatives exert their inhibitory action, it has been suggested that the presence of the drug at the interface between the two subunits may perturb the dynamics of subunit association and/or prevent the conformational changes necessary for loading the p66 subunit on to the template-primer. In fact, it has been demonstrated that the heterodimeric enzyme shows a reduced DNA binding ability in the presence of 5-fold molar excess of TSAO-T and also that TSAO-e^3T can induce the dissociation of RT into inactive monomers under certain experimental conditions. By studying the residues that are involved in dimer formation, we have identified the β7-β8 loop in the p51 subunit as an important component of the dimerization interface [83]. Interestingly, Glu138, the residue that is found mutated to Lys in TSAO-resistant viruses, is located in this loop, and our results with the Glu138Lys mutant enzyme are consistent with the experimental results that this substitution drastically reduces the affinity of TSAO derivatives and at the same time does not lead to gross changes in dimerization binding energy. Nevertheless, dissociation of the subunits does not appear to take place in the absence of denaturant, and the concentrations of TSAO-m^3T that are necessary to produce this effect are higher than those needed to display inhibitory activity [20, 82].

For RT inhibition by classical NNRTIs it has been suggested that the most dramatic change in the protein relative to the apoenzyme is the concerted movement of strands β6, β9, and β10, which contain the functional aspartic acid residues (Asp110, Asp185, and Asp186) that comprise part of the polymerase active site [32]. By comparing our simulations of wild-type HIV-1 RT in the presence and in the absence of bound TSAO-m^3T, a similar disorganization of the catalytic aspartates is apparent "Fig. (**10**)" although it is achieved through a different mechanism [83]. The β7-β8 loop remains essentially unchanged but the "pulling" interaction of TSAO-m^3T with Lys101 and Lys103 gives rise to changes in both the β5 and β6 strands. On the other hand, the "pushing" interaction with Val179 and Pro176 determines a displacement of the β9 and β10 strands and the α-helix immediately preceding β9. Although these results should be viewed with caution, given the limitations of the reduced models used, it is tempting to speculate that the presence of TSAO at the subunit interface may preclude

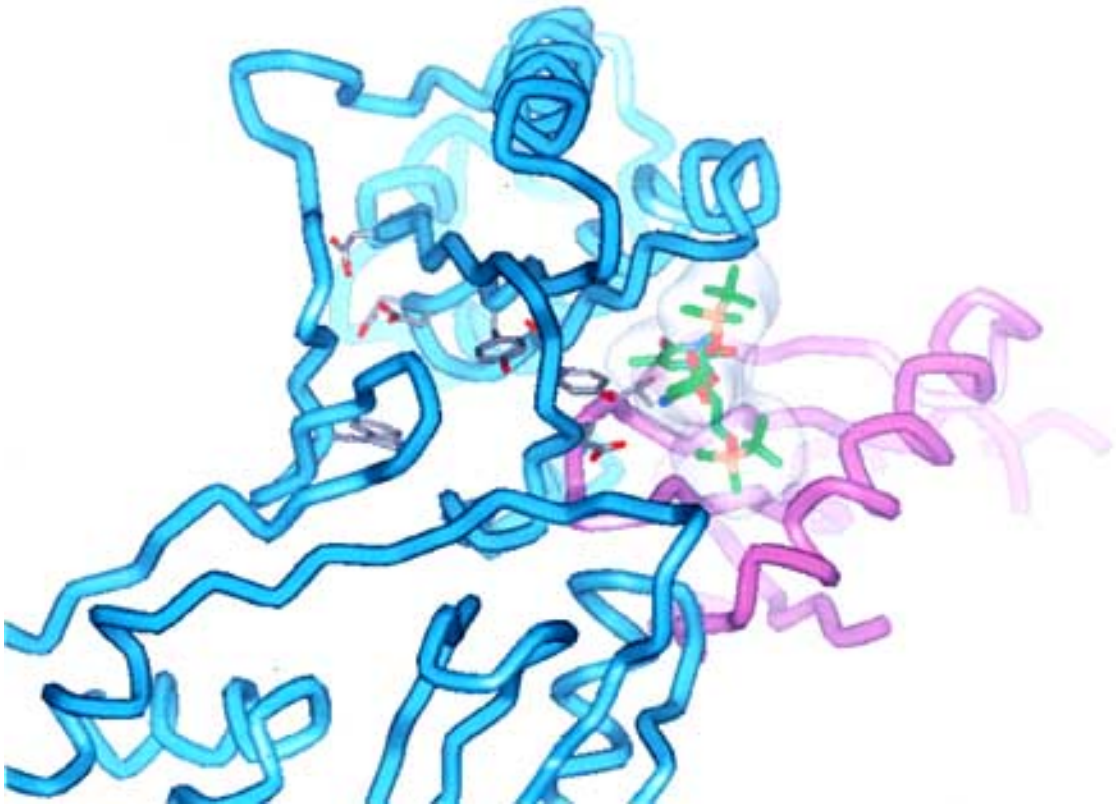


Fig. (10). Putative binding site of TSAO-m^3T (shown as sticks with C atoms coloured in green, and enveloped in a semi-transparent van der waals surface) at the interface between the p66 (Cα trace in cyan) and p51 (Cα trace in pink) subunits. The side chains of Glu138, Tyr181, Tyr188, Trp229 and the three catalytic aspartate residues are also displayed as sticks with C atoms coloured in grey.

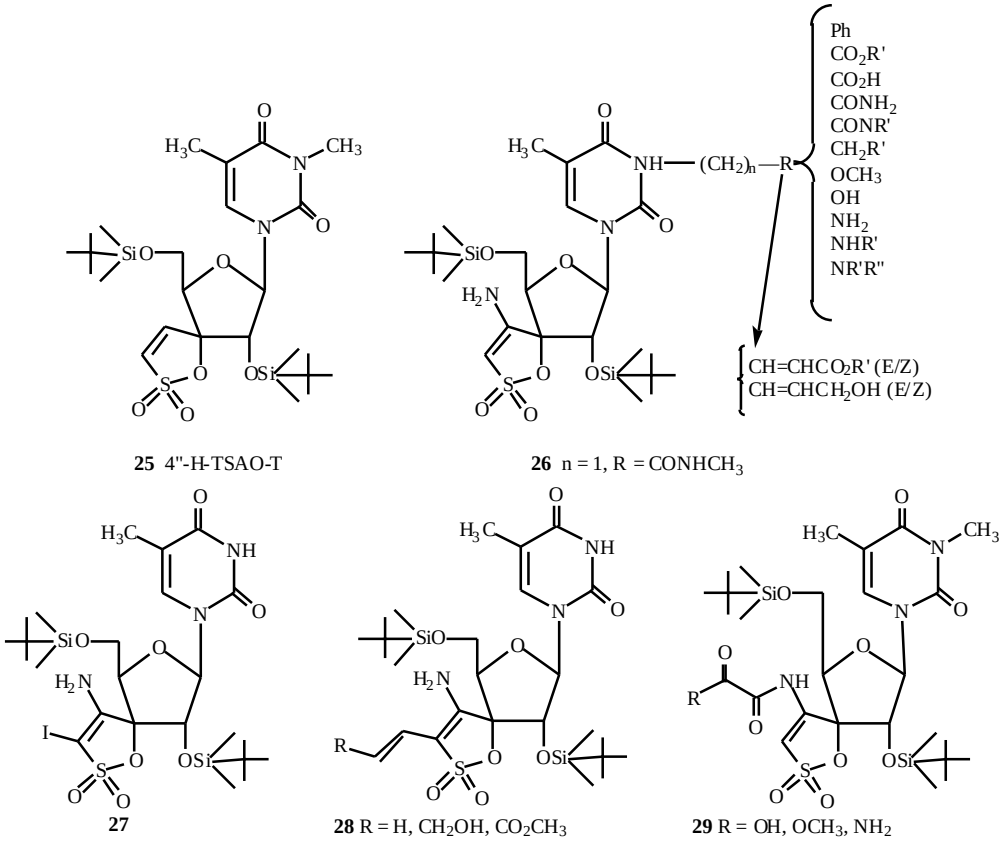
adoption of the catalytically active conformation by the enzyme. In this dynamic context, the presence of additional residues not directly in contact with the TSAO molecule could be important in the inhibition mechanism as cross-resistance to TSAO derivatives has been shown in virus mutants selected by other NNRTIs, such as those involving RT amino acid positions 106, 181 and 188, which are within the "classical" NNRTI binding site [85].

The current model points to a well-defined part of the dimerization interface as a novel target for inhibitor design [83]. To our knowledge, TSAO-T is the first example of a small non-peptidic molecule that can interfere with the dimerization process and can thus be used as a lead compound for the design of an entirely new family of RT inhibitors that would act independently of both the active site and the NNRTI binding pocket.

Exploring Dimerization of RT as a Mode of Inhibition of the Enzyme. Towards "Second Generation" TSAO Derivatives

TSAO-deaminated (4"-H-TSAO-T)

Based on the hypothesis, supported by our experimental data and molecular modelling studies, that a specific interaction exists between the Glu138 of the p51 subunit and the 4"-amino group of the 3'-spiro moiety of the TSAO molecules, we considered of interest to remove this amino group and evaluate the effect of this modification on the activity and resistance profile of the corresponding deaminated TSAO derivative. Therefore, we synthesized the first TSAO molecule that lacked the amino group at the 3'-spiro moiety (**25**) "Fig. (**11**)" [86]. This compound kept HIV-1 specificity (NNRTIs characteristic), it proved inhibitory to HIV-1 replication in cell cultures (EC_{50} = 0.15-0.53 μM) and was also inhibitory against HIV-1 RT (IC_{50} = 3.3 μM). Moreover, it was devoid of any activity either against HIV-2 RT or HIV-2 replication. Compound **25** was able to select for virus strains that proved to escape the drug pressure. The virus strain that emerged under escalating drug pressure of **25** was used for sensitivity/resistance testing against a variety of NNRTIs (including TSAO derivatives) and NRTIs. This TSAO molecule showed an aberrant resistance spectrum. Interestingly, this drug resistant strain kept full sensitivity to all NRTI and NNRTIs, including TSAO compounds that contain the NH_2 group at the spiro moiety. Resistance was observed only against the deaminated TSAO derivative **25** in the presence of which it was selected. Even more intriguing is the observation that the drug also lost inhibitory potential against virus mutants that contain certain NNRTIs specific mutations in the reverse transcriptase. The latter observations point to an interaction with the NNRTI pocket of the RT. However, when sequencing the RT gene of the drug resistant virus strain, any of the well known NNRTIs specific mutations in the RT gene including 138-Lys (characteristic for TSAO resistance) were not detected.



25 4"-H-TSAO-T

26 n = 1, R = $CONHCH_3$

27

28 R = H, CH_2OH, CO_2CH_3

29 R = OH, OCH_3, NH_2

Fig. (11).

It is still unclear whether a mutation has appeared downstream of the region where the NNRTI mutations are located (i. e. RNAseH domain). So, the deaminated TSAO derivative represents a new type of molecule with HIV-1 specificity, but most likely with another mechanism of action than that of the classical NNRTIs.

TSAO Compounds Modified at the N-3 Position

In the light of the updated molecular model of interaction of TSAO derivatives with HIV-1 RT, the N-3 substituents of the pyrimidine ring would run parallel to the subunit (p66/p51) interface [83]. Structure-base modifications were introduced in the prototype compound (TSAO-T) in order to get compounds with additional interactions with aminoacids at/or near the p51-p66 interface [87]. Several N-3-substituted TSAO derivatives were prepared. These novel TSAO analogues contained different functional groups that might give additional interaction with one or more aminoacids of the dimer interface and thus might disrupt crucial interface interactions "Fig. (**11**)". Thus, compounds having at the N-3 position functional groups of different nature (alkyl, aryl, acid, ester, amido, ether, halogen, alcohol, amino, etc.), linked to this position through flexible polymethylene linkers of different lenght (n = 1-4), were prepared [87]. Compounds with restricted conformational flexibility in the linker were also prepared by introduction of *cis* or *trans* double bonds. All the compounds synthesized showed pronounced activity against HIV-1 replication (EC_{50} = 0.02-0.76 μM) while being inactive against HIV-2 [82]. The N-3 methylcarboxamide TSAO compound (**26**) proved 2-6 fold more active than the prototype TSAO-T and did not show toxicity, thus being the most selective TSAO derivative prepared so far (Selectivity index CC_{50}/EC_{50}: 10000).

3"-Substituted TSAO Derivatives

Following with the rational of preparing TSAO compounds that may give additional interactions with aminoacids at/or near the p51-p66 interface, next we focused our attention on modifications of the 3'-spiro moiety of TSAO derivatives. According to the molecular model of interaction of TSAO derivatives with HIV-1 RT, this moiety represents the closest part of the molecule to the interface between the p51 and p66 subunit domains, in fact it is buried in the subunit interface [83]. Based on this hypothesis, we introduced different functional groups at position 3" of the spiro ring of the prototype compound (TSAO-T) and its N-3 methyl derivative (TSAO-m^3T) that may give additional interactions with aminoacids adjacent to the Glu138 of the p51 subunit [88]. Thus, several 3"-substituted TSAO derivatives carrying differently substituted halogen, alkenyl, alkynyl, allyl, aromatic and heteroaromatic groups were prepared "Fig. (**11**)". In these novel analogues, the 4"-amino group of the sultone spiro moiety was maintained to allow the crucial interaction of TSAO derivatives with the Glu138 of the p51 subunit of the HIV-1 RT. The compounds synthesized were evaluated for their inhibitoty effect on HIV-1 and HIV-2 replication in cell culture. The introduction of a bromine and particularly an iodine at the 3"-position (**27**) conferred the highest anti-HIV-1 activity (EC_{50}: 0.05 ± 0.03 μM) being comparable to that of the prototype compound TSAO-T. In contrast, the presence at this position of (un)substituted vinyl, alkynyl, phenyl or thienyl groups markedly diminished the anti-HIV-1 activity ($EC_{50,27}$: 0.98-4.0 μM). Surprisingly, several of the 3"-alkenyl substituted TSAO derivatives (**28**, "Fig. (**11**)") gained also anti-HIV-2 activity at subtoxic concentrations (EC_{50}: 1.2-7.3 μM), an observation that is very unusual for NNRTIs including TSAO derivatives. The structure-activity relationship to have both anti-HIV-1 and anti-HIV-2 activity in the TSAO molecules is currently unclear. When the compounds were evaluated on their inhibitory activity against recombinant HIV-2 RT, they were found to be inactive at 500 μM. In addition, the most active anti-HIV-2 compounds were also inactive (> 500 μM) against a mutant HIV-1 RT that contains the TSAO-characteristic Glu138Lys resistance mutation. Taken together, the data point to a different mechanism of action of some of the 3"-substituted TSAO derivatives. The NNRTI (SJ-3366) has been reported to inhibit both HIV-1 and HIV-2 replication, and to inhibit HIV-1 RT but not HIV-2 RT [89]. This compound was found to interfere with entry of HIV as a second mechanism of action. Investigations on the mechanism of action of the TSAO derivatives against HIV-2 is currently ongoing but initial data may suggest that they seem to interfere with cellular transcription rather than virus entry.

4"-Substituted TSAO Compounds

Finally, we focused on "second generation" TSAO molecules directed against TSAO-resistant strains. We prepared TSAO compounds bearing at the 4" position of the spiro moiety different carbonyl functionalities (including keto, carboxylic acid, ester, amide and urea groups) that may interfere, by hydrogen bond formation, with the amino group of Lys138 in TSAO-resistant HIV-1 strains [90]. The N-acyl derivatives bearing oxalyl substituents at 4" position (**29**, "Fig. (**11**)") showed potent activity against HIV-1 replication (EC_{50} = 0.03-0.06 μM) while being inactive against HIV-2. Moreover, the TSAO compound bearing a free carboxylic acid residue (**29**, R = OH) shows moderate activity (IC_{50}: 20-50 μM) against mutant Lys138 HIV-1 RT (TSAO-characteristic resistance mutation). From the biological results it seems that the presence of two neighbouring carbonyl groups at the 4" position of the spiro moiety is important for activity against both HIV-1 wild type and HIV-1 TSAO-resistant viruses. Recently it has been found that the β carbonyl group (not directly attached to the 4"-amino group), seems to be important for the activity of these compounds since either monocarbonyl TSAO derivatives or non-neighbouring dicarbonyl TSAO compounds (i.e. ureido derivatives etc) were devoid of antiviral activity ($EC_{50,}$: 0.6 μM *vs* >50 μM).

CONCLUSIONS

In this review we have tried to gather and summarize the work developed in our laboratories, during the last twelve years, that led to the discovery and study of a unique class of specific inhibitors of HIV-1 reverse transcriptase, the TSAO family of compounds.

Since the discovery of TSAO-T, more than 500 derivatives have been prepared and evaluated against HIV-1

and HIV-2 replication in cell culture and HIV-1 RT. Due to the high degree of functionalization, the presence of stereogenic centres and the presence of both acid and base sensitive groups, in these nucleosidic molecules, their synthesis has been a real challenge. It has not always been straightforward, and it has required the development of mild and stereoselective synthetic procedures, especially in those TSAO-derivatives modified in the spiro moiety (an aminospirooxathioledioxide, previously unknown and first described by us [91]).

Since our initial reports in 1992 on the first TSAO compounds and their specific inhibition of HIV-1 RT, the structure of these "aberrant" nucleosides has been the subject of many questions. Why do these nucleosides behave as NNRTIs?; Why is the presence of the *tert*-butyldimethylsilyl moiety mostly at 5'-position so crucial for activity?; Why, if they behave as NNRTIs, do they not share the typical "butterfly" three dimensional structure characteristic for many NNRTIs?; Why do they always and consistently select for the Glu138Lys mutation, that is not described with any other NNRTI?.

During these twelve years we have tried to address and solve some of these questions, and some of the answers are contained in this review. In this process, we have found new features that may allow the clasification of TSAO compounds as a unique class of HIV-1 RT inhibitors. Besides their highly functionalized nucleoside structure, TSAO compounds are among the very few HIV-1 RT inhibitors for which aminoacids at both subunits of RT are needed for optimal interaction. Moreover, they are the first small molecules that seem to interfere with the dimerization process of the enzyme, which suggests a new and different mechanism of inhibition of HIV-1 RT with regards to the other known NNRTIs. Finally, because dimerization of HIV-RT is a prerequisite for enzymatic activity, interference with the dimerization process could constitute an alternative strategy for RT inhibition. Based on our biological results and on the molecular model of interaction, we believe that with the TSAO molecule, we have now an important tool in hand to rationally design potent dimerization inhibitors.

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