Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1461–1466

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

5-ALKYL-2-[(METHYLTHIOMETHYL)THIO]-6-(BENZYL)-PYRIMIDIN-4-(1H)-ONES AS POTENT NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS OF S-DABO SERIES

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Received 20 February 1998; accepted 1 May 1998

Abstract: Novel dihydroalkoxybenzyloxopyrimidine (S-DABO) derivatives targeting the non-nucleoside inhibitor (NNI) binding site of human immunodeficiency virus (HIV) reverse transcriptase (RT) have been synthesized using a novel computer model for the NNI binding pocket and tested for their RT inhibitory activity in cell-free assays using purified recombinant HIV RT as well as for their anti-HIV activity in HTLVIIIB-infected peripheral blood mononuclear cells. Our computational approach allowed the identification of several ligand derivatization sites for the generation of more potent S-DABO derivatives. Our lead S-DABO derivative, 5-isopropyl-2-[(methylthiomethyl)thio]-6-(benzyl)-pyrimidin-4-(1H)-one (compound **3**), elicited potent anti-HIV activity with an IC_{50} value of less than 1nM for inhibition of HIV replication without any evidence of cytotoxicity and an unprecedented selectivity index of >100,000.

Design of potent inhibitors of HIV reverse transcriptase (RT) has been a focal point in translational AIDS research efforts.[1] Among the promising inhibitors are the nonnucleoside inhibitors (NNIs), which include tetrahydroimidazobenzodiazepinethione(TIBO) compounds,[2] 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio) thymine (HEPT) derivatives,[3] bis(heteroaryl)piperazine (BHAP) analogs,[4] 2'-5'-bis-*O*-(*tert*-butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1", 2"-oxathiole-2", 2"-dioxide) pyrimidine (TSAO),[5] and phenethylthiazolylthiourea (PETT) derivatives.[6] More recently, dihydroalkoxybenzyloxopyrimidines (DABO) were found to inhibit HIV-1 replication.[7] NNIs have been found to bind to a specific allosteric site of HIV-1 RT near the polymerase site and interfere with reverse transcription by altering either the conformation or mobility of RT, thereby leading to a noncompetitive inhibition of the enzyme.[8,9]

The NNI binding site of HIV-1 RT is among the most extensively studied drug binding pockets.[8,9] The high resolution crystal structures of HIV-1 RT from NNI-RT complexes have shown distinct properties of the NNI binding pocket within the three-dimensional structure of HIV-1 RT, which can be utilized for structure-based rational drug design.[8-10] However, each reported structure revealed a unique binding pattern indicating that rational drug design efforts should not rely on one particular crystal structure. We have constructed a model for the three-dimensional structure of the RT-DNA complex based on the available backbone structure of RT-DNA complex and full structure of RT complexed with several NNI compounds. Structural information from several complexes were combined to provide a suitable working model since no experimental data regarding the crystal structure of RT-DNA-NNI complexes has been reported. We have used the NNI binding site coordinates of 9 individual RT-NNI structures to generate a composite molecular surface revealing a larger than

0960-894X/98/$19.00
PII: S0960-894X(98)00250-9

presumed NNI binding pocket.[11] In the present study we have utilized this pocket, together with docking and a structure-based semi-empirical score function, as a guide for the synthesis and analysis of structure- activity relationships for new derivatives of dihydroalkoxybenzyloxopyrimidines (S-DABO) as potent NNI of HIV-1 RT (**Figure 1**).

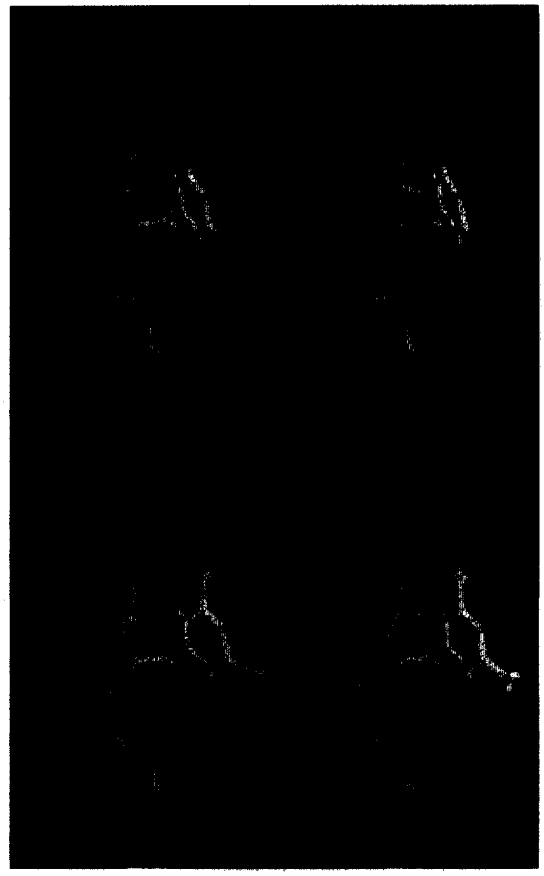
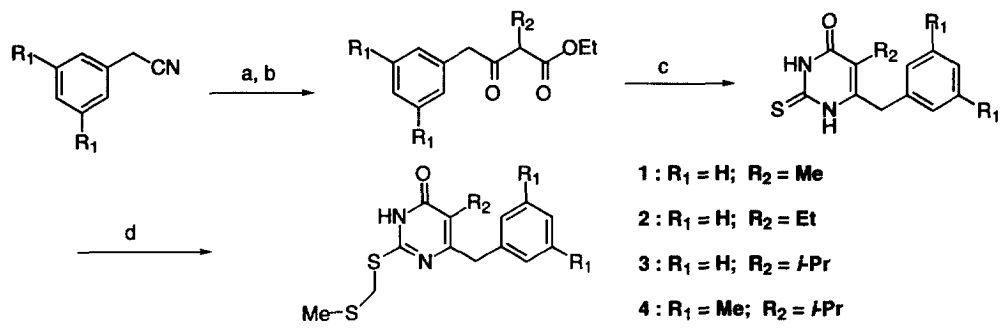


Figure 1. The composite binding pocket of the NNI active site of HIV-1 RT. Grid lines represent the collective van der Waals surface of 9 different inhibitor crystal structures superimposed in the active site and highlight the available space for binding (inhibitor structures include HEPT, MKC, TNK, APA, Nevirapine, N-ethyl Nevirapine derivative, 8-Cl TIBO, and two 9-Cl TIBO compounds, with PDB access codes rti, rt1, rt2, hni, vrt, rth, hnv, rev and tvr, respectively). The surface is color-coded for hydrogen bonding (red), hydrophobic (gray) and hydrophilic (blue) groups of the superimposed inhibitors. The hydrogen atoms were not included. The side-by-side stereoview of (**A**) compound **3**, (**B**) compound **4** which have been docked into the active-site of RT/9-Cl-TIBO complex (PDB access code: rev) and transformed into the composite binding pocket based on the matrix used in the pocket construction. The uracil ring of MKC-442 in the crystal structure is in the same position as the ring in the DABO models (**3** and **4**). The N1-substituted group on MKC-442 is located in the same region of the binding site as the S2-substituted group of our DABO models. The calculated K_i values for compounds **3** and **4** were 0.5 μM and 0.06 μM, respectively, based on our established procedure involving a modified LUDI function.[11]

5-Alkyl-2-[(methylthiomethyl)thio]-6-(benzyl)-pyrimidin-4-(1H)-one derivatives **1-4** were prepared as illustrated in **Scheme 1**. Ethyl-2-alkyl-4-(phenyl)-3-oxobutyrate was obtained from the commercially available phenyl acetonitrile by a previously described method.[7] The β-ketoester was condensed with thiourea in the presence of sodium ethoxide to furnish the corresponding thiouracil. The reaction of thiouracil with methylthiomethyl chloride in *N,N*-dimethylformamide (DMF) in the presence of potassium carbonate afforded compounds **1-4** in moderate yields.[12]

Scheme 1



Reagents and conditions: (a) R_2-CHBrCOOEt/Zn/THF, (b) HCl (aq), (c) $(H_2N)_2CS$/Na/EtOH, (d) DMF, K_2CO_3, Chloromethyl methyl sulfide, 15 h.

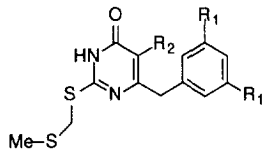
Design and Activity of S-DABO Compounds

Our analysis of HEPT and MKC-442 (HEPT derivative), both which are active NNI compounds, revealed that the N1 substituents of HEPT derivatives actually occupy the same region as the thio (S2) substituents of S-DABO compounds. This observation suggested that (1) NNI design efforts should avoid simultaneous substitutions on both N1 and S2 because they would compete with each other for the same space in the binding pocket (**Figure 1A**), consistent with the recently reported lack of significant activity of some N1 and S2 double-substituted compounds,[7] and (2) conclusions from quantitative structure-activity relashionship (QSAR) studies of potent HEPT derivatives could also be applied to new designs of S-DABO derivatives. We therefore designed and modeled novel S-DABO derivatives in the NNI binding site of a RT crystal structure by use of a molecular docking procedure in the Affinity module with the Insight II program.[13] We then compared the final positions of the docked molecules with the composite binding pocket to check the fit within the pocket (**Figure 1A**). These comparisons showed that S-DABO compounds snugly fit into our composite binding pocket and revealed multiple sites which can be used for future incorporation of larger functional groups. The docked S-DABO molecule (compound **3**) showed significant space surrounding the 6-benzyl ring. We proposed that efficient use of this space by strategically designed functional groups would lead to high affinity binding and ultimately result in better inhibitors of RT. To this end, we synthesized compound **4**, which differs from compound **3** by the addition of two methyl groups to the 6-benzyl ring thereby providing more hydrophobic contact with the pocket (**Figure 1B**).

Compounds **1-4** were tested for RT inhibitory activity in cell-free assays using purified recombinant HIV RT the Quan-T-RT assay system (Amersham, Arlington Heights, IL), which utilizes the scintillation proximity assay principle[14] as well as by in vitro assays of anti-HIV activity in HTLVIIIB-infected peripheral blood mononuclear cells[15] (**Table 1**). Larger compounds which better fill the composite binding pocket showed better IC_{50} values in cell-free RT inhibition assays. This is reflected by the enhancement of the inhibitory

activity with the addition of progressively larger groups such as methyl (**1**), ethyl (**2**), and isopropyl (**3**) at the C-5 position of the thymine ring (see **Table 1**). The same trend was also observed in HIV replication assays using peripheral blood mononuclear cells. Compound **4** which differs from compound **3** by the addition of two methyl groups to the 6-benzyl ring providing a better hydrophobic contact with the NNI binding pocket was slightly more potent than compound **3** in inhibiting recombinant HIV RT. However, compound **4** failed to inhibit HIV replication in HTLVIIIB-infected cells as effectively as compound **3** despite its lower IC_{50} values for recombinant RT in cell-free assays, probably due to differences in cellular uptake and intracellular metabolism of these two compounds. As shown in **Table 1**, the IC_{50}[p24] values were significantly better than the IC_{50}[rRT] values in cell-free RT inhibition assays. These differences are likely due to the fact that the treatment of cells with the compounds may result in drug accumulation within the cell, resulting in much higher intracellular concentrations.

Table 1. Inhibitory effects of S-DABO derivatives (compounds **1-4**) on the enzymatic activity of purified recombinant HIV RT, p24 production in HIV-infected peripheral blood mononuclear cells, and viability of peripheral blood mononuclear cells. A microculture tetrazolium assay (MTA), using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2*H*-tetrazolium hydroxide, was performed to quantitate cytotoxicity. All data are in μM and represent concentrations required to inhibit by 50% the activity of recombinant HIV RT (IC_{50} [rRT]), HIV replication, as measured by assays of p24 production (IC_{50} [p24]), or cellular proliferation, as measured by MTA (IC_{50} [MTA]).[15]



Compound Number	R_1	R_2	IC_{50}[rRT] (μM)	IC_{50} [p24] (μM)	IC_{50}[MTA] (μM)
1	H	Me	18.8	4.5	>100
2	H	Et	9.7	0.8	>100
3	H	*i*-Pr	6.1	<0.001	>100
4	Me	*i*-Pr	4.8	0.01	>100
MKC-442			N.D	0.004	>100
AZT			>100	0.004	100

A structural analysis showed that the Tyr183 residue of the HIV RT is located in the catalytic region which has a conserved YMDD motif characteristic of reverse transcriptases. Therefore, the displacement of this tyrosine residue can interfere with catalysis and render the HIV-1 RT protein inactive. It has been suggested that bulky substituents at the 5th position of the thymine ring could indirectly accomplish this goal by displacing Tyr181 which is near Tyr183.[9] Our composite binding pocket shows that there is sufficient space for at least a 3-carbon group. The addition of a methyl, ethyl or isopropyl group at the 5th position of the thymine ring could lead to a higher affinity for the relatively hydrophobic environment. The hydrophobic contact is likely to increase as hydrophobic groups at the 5th position get bulkier. As it binds to the site, the ethyl or isopropyl group can cause the nearby Tyr181 residue to rotate away from the inhibitor. This change in conformation in

turn should affect the positions of the neighboring Tyr183 and Tyr188 which may contribute to the inactivation of HIV-1 RT.

Our lead S-DABO derivative, 5-isopropyl-2-[(methylthiomethyl)thio]-6-(benzyl)-pyrimidin-4-(1*H*)-one (compound **3**), elicited potent anti-HIV activity with an IC_{50} value less than 1nM for inhibition of HIV replication, as measured by p24 production in HIV-infected human peripheral blood mononuclear cells and showed no detectable cytotoxicity with IC_{50}[MTA] values of >100μM for cellular proliferation (**Table 1**). In contrast to all previously published DABO derivatives which inhibited HIV replication with selectivity indices <1,000 and were less active than AZT and MKC-442,[7] compound **3** was >4-fold more active than AZT as well as MKC-442 and abrogated HIV replication in peripheral blood mononuclear cells at nanomolar concentrations with an unprecedented selectivity index (= IC_{50}[MTA]/IC_{50}[p24]) of >100,000. Encouraged by these promising results, we will continue to use the composite binding pocket geometry for the structure-based design of novel NNIs of RT as potentially more active anti-HIV agents.

Acknowledgments

This work was supported in part by Grant No. 1996-18 from Alexander Parker Pharmaceuticals, Inc.

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12. Selected data for Compounds **1**: Yield 62%; mp 148-149 °C ; ^{1}H NMR ($CDCl_3$) δ 2.10 (s, 3H), 2.14 (s, 3H), 3.91 (s, 2H), 4.29 (s, 2H), 7.29-7.26 (m, 5H), 12.20 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 10.7 (CH_3), 15.5 (SCH_3), 36.6 (CH_2Ph), 41.0 (SCH_2), 116.7 (C-5), 137.6-126.4 (Ph), 155.2 (C-6), 162.0 (C-4), 165.1 (C-2); MS (CI) 293.1 (M+1).

2: Yield 65%; mp 124-126 °C; ^{1}H NMR ($CDCl_3$) δ 1.08 (t, 3H), 2.12 (s, 3H), 2.58 (q, 2H), 3.91 (s, 2H), 4.26 (s, 2H), 7.28-7.26 (m, 5H), 12.30 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.1 (CH_3), 15.4 (SCH_3), 18.7 (CH_2), 36.4 (CH_2Ph), 40.3 (SCH_2), 122.4 (C-5), 138.0- 126.3 (Ph), 155.4 (C-6), 161.5 (C-4), 165.2 (C-2); MS (CI) 307.1 (M+1).

3: Yield 57%; mp 116-117 °C; ^{1}H NMR ($CDCl_3$) δ 1.22 (d, 6H), 2.07 (s, 3H), 3.03 (q, 1H), 3.88 (s, 2H), 4.21 (s, 2H), 7.24-7.13 (m, 5H), 12.43 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 15.4 (SCH_3), 19.6 (CH_3), 28.0 (CH), 36.3 (CH_2Ph), 40.9 (SCH_2), 125.3 (C-5), 138.3- 126.3 (Ph), 155.5 (C-6), 161.1 (C-4), 164.5 (C-2); MS (CI) 321.1 (M+1).

4: Yield 67%; mp 116-120 °C; ^{1}H NMR ($CDCl_3$) δ 1.28 (d, 6H), 2.15 (s, 3H), 2.27 (s, 6H), 3.10 (q, 1H), 3.88 (s, 2H), 4.31 (s, 2H), 6.84 (s, 3H), 12.42 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 15.3 (SCH_3), 19.6 (CH_3), 21.2 (CH_3), 28.0 (CH), 36.3 (CH_2Ph), 40.8 (SCH_2), 125.2 (C-5), 138.0-126.5 (Ph), 155.4 (C-6), 161.3 (C-4), 164.7 (C-2); MS (CI) 349.2 (M+1).

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