Journal of Medicinal Chemistry Letter

J. Med. Chem. **2009,** *52,* 5781–5784 **5781**
DOI: 10.1021/jm900597q

RNase H Active Site Inhibitors of Human Immunodeficiency Virus Type 1 Reverse Transcriptase: Design, Biochemical Activity, and Structural Information†

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Received May 7, 2009

Abstract: Pyrimidinol carboxylic acids were designed as inhibitors of HIV-1 RNase H function. These molecules can coordinate to two divalent metal ions in the RNase H active site. Inhibition of enzymatic activity was measured in a biochemical assay, but no antiviral effect was observed. Binding was demonstrated via a solid state structure of the isolated p15-Ec domain of HIV-1 RT showing inhibitor and two Mn(II) ions bound to the RNase H active site.

The inhibition of enzyme-mediated processes associated with the life cycle of the human immunodeficiency virus type 1 (HIV-1[a]) has led to major advancements in the treatment of patients suffering from AIDS. Nonetheless, the hallmark of this human pathogen is the ease with which it is capable of adapting to selection pressures via genetic mutation. Hence, the search for new members of established drug classes as well as the discovery of agents to inhibit HIV via novel mechanisms of action continue to be important research goals worldwide.[1]

One of the three virally encoded enzymes is HIV-1 reverse transcriptase (HIV-1 RT). This enzyme is responsible for the reverse transcription of single-stranded viral RNA into double-stranded DNA, utilizing RNA- and DNA-dependent polymerization activities. Additionally, the enzyme catalyzes the hydrolysis of RNA phosphodiester bonds in the RNA/DNA hybrids produced during RNA-dependent DNA synthesis.[2] These two functions are linked to two distinct and spatially well-resolved active sites within the same protein. Exploitation of binding at or near the polymerase active site has proven fruitful for the development of nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors (NNRTIs), respectively. On the other hand, inhibition of the RNase H function of HIV-1 RT has proven to be more difficult.[3] Although inhibitors of biochemical activity have been described as early as 1990, no approved drugs or clinical development candidates have emerged.[4]

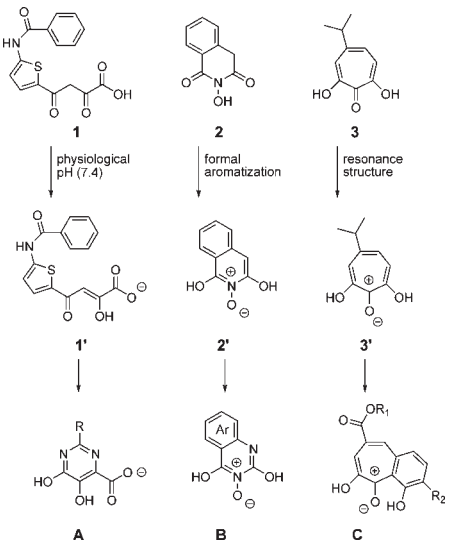

Figure 1. Electronic analysis of inhibitor classes in the context of dual metal chelation.

More recently, the effect of NNRTIs on the efficiency and specificity of RNase H-mediated cleavage events (likely through allosteric effects) has become a novel avenue for research.[5] Recent crystallographic studies of the RNase H protein from *Bacillus halodurans* have elucidated the metal requirements and ligand sphere events in catalysis of endonucleolytic phosphodiester hydrolysis.[6] This information, together with results obtained with mixtures of divalent metals in assays of HIV-1 RT RNase H function, supports a bimetallic mode of action involving Mg(II).[7] The enzymatic active site contains four carboxylate residues, creating an environment capable of stabilizing two Mg ions in close proximity. Chelators of these two ions in the enzyme active site are therefore regarded as potential inhibitors of the function of HIV-1 RNase H.

Three different scaffold types have been reported as RNase H active site metal chelators and are depicted in Figure 1.[8] α,γ-Diketo acids have often served as starting points for the design and optimization of inhibitors against enzymes reliant on a two-metal mechanism of action for endonucleolytic phosphodiester hydrolysis such as influenza endonuclease, HIV-1 integrase, and FLAP endonuclease.[9] The structure of a moderately potent α,γ-diketo acid (**1**), the result of a study targeting the RNase H function of HIV-1 RT, is depicted in Figure 1.[10] *N*-Hydroxyimides have been reported as potent inhibitors of HIV-1 RNase H function, acting via active site metal chelation. These compounds were first described as inhibitors of influenza endonuclease, but their high potency in biochemical assays of HIV-1 RNase H inhibition make these structures valuable leads against this target.[11] Selected hydroxytropolones and especially the natural product β-thujaplicinol have been reported as potent inhibitors of HIV-1 RNase H.[12] This class has been explored for inhibition of multiple HIV-1 enzymes including HIV-1 integrase and HIV-1 RT polymerase, albeit with low to moderate selectivities and potencies.[13] Earlier, hydroxytropolones were shown to have inhibitory properties toward inositol monophosphatase. An inhibitory mechanism invoking dual metal chelation was described.[14]

An overarching connection among the three inhibitor classes emerges upon analysis of different resonance and

†The atomic coordinates and structure factors have been deposited in the Protein Data Bank for p15-Ec in complex with compound **9**; PDB code 3HYF.

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[a] Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; NNRTI, non-nucleoside reverse transcriptase inhibitor; SAR, structure−activity relationship.

Published on Web 09/10/2009

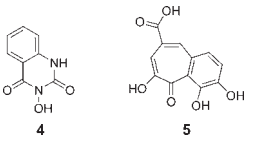


Figure 2. Modified scaffolds derived from literature leads.

tautomeric forms of the core scaffolds, as depicted in Figure 1. Limited SAR on the *N*-hydroxyimide class has revealed the absolute requirement for one enolizable proton within the six-membered imide ring structure.[7] Consistent with this, an *iso*-quinoline *N*-oxide is proposed as the biologically relevant tautomer (**2′**). A similar distribution of electron density around the triad of coordinating oxygen atoms is found in the 6-π aromatic resonance structure of the hydroxytropolone (**3′**). From both these species, a single deprotonation yields a doubly negatively charged triad of oxygen atoms. The α,γ-diketo acid system can be analyzed in a similar way: at physiological pH, the carboxylic acid is deprotonated, as in structure **1′**. The acidity of the 1,3-dicarbonyl system is anticipated to be sufficient to generate small quantities of dianions under these conditions. Supporting evidence for the biological relevance of the dianion is found in studies of influenza endonuclease inhibition by α,γ-diketoacids, where reduction of the pH of the assay to below the second pK_a of the diketoacid sharply decreased the inhibitory activity.[15] In all of these cases, therefore, a doubly anionic species may be necessary for effective coordination of a putative inhibitor to both Mg ions in the structural environment of the enzyme active sites.

While this analysis allows for the creation of a unifying metal-chelating pharmacophore model for HIV RNase H, scaffolds **1**−**3** all present intrinsic problems as starting points for inhibitor design, mainly with respect to their stability under aqueous conditions.[16] For example, compound **2** decomposes to uncharacterized more polar species in aqueous solution at pH 7.4 at room temperature over hours (unpublished results). The instability problem was solved via the introduction of a nitrogen atom to yield the bicyclic aromatic *N*-hydroxy quinazolinedione **4** (Figure 2).[17] In the hydroxytropolone series, the chelating scaffold was modified via attachment of an aromatic ring, yielding compound **5**, with a geometrically altered oxygen triad (Figure 2). This modification however resulted in a significant drop in potency (Table 1). The enzyme-mediated synthesis of this revised scaffold did not allow for a convenient exploration of SAR.[18] Tautomeric structures of the stabilized scaffolds **A**, **B**, and **C**, emphasizing the pharmacophore model for dual metal chelation, are depicted in Figure 1, and potencies of key examples are reported in Table 1.

Pyrimidinol carboxylic acid derivatives were recently reported as stable substitutes for α,γ-diketo acids and α,γ-diketo amides.[19] Hence, this scaffold was selected to study the inhibition of HIV-1 RNase H, for it had already been shown that customization of dual metal chelation via modification of the acidity of the central hydroxyl group was feasible.[20] The synthesis of compounds in this series utilized the published four-step route outlined in Scheme 1.

Anticipating that the carboxylate and both phenolic hydroxyl groups would be important for dual metal chelation, investigations focused on variations of the 2-substituent. A simple first set of analogues is depicted in Figure 3, with corresponding biochemical data in Table 1. All compounds presented here were evaluated in a biochemical RNase H assay as described in the literature.[21]

Table 1. Biochemical RNase H Inhibitory Potency of the Compounds[a]

compd	HIV-1 RNase H IC_{50} [μM]	hu RNase H IC_{50} [μM]
1	6.5/13.2 (3.2)	nd
2	0.12/0.15 (0.6−1)	nd
3	0.60/0.72 (0.21)	nd
4	5.9/6.1	nd
5	22.4/23.9	nd
6	11.4/14.5	> 100/ > 100
7	2.1/2.9	> 100/ > 100
8	9.6/10.7	> 100/ > 100
9	1.2/1.7	> 100/ > 100
10	0.9/1.4	78/70
11	0.17/0.18	48/49
12	70.0/74.0	nd

[a] Inhibitory activities were determined via a fluorescence-based RNase H assay (ref 21) using either HIV-1 RT or human RNase H enzyme. Numbers in parentheses for compounds **1**−**3** are IC_{50}s reported in the original publications and are cited for comparison. All compounds had >95% purity with exception of **1** (92.4%). nd = not determined.

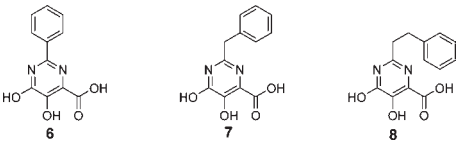
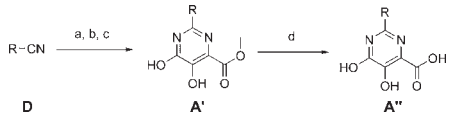


Figure 3. Simple pyrimidinol carboxylic acids with C2 variants.

Scheme 1. General Access to Pyrimidinol Carboxylic Acids[a]



[a] (a) NH_2OH (2×), MeOH, 50 °C, 14 h; (b) DMAD (1.05×) $CHCl_3$, 60 °C; (c) *m*-xylenes, 130 °C 4 h; (d) NaOH, MeOH, H_2O, then HCl aq.

These compounds proved informative for establishing initial SAR within this series. A 2-phenyl substituted analogue (**6**) displayed moderate inhibitory activity in the RNase H cleavage assay with an IC_{50} value of 12.9 μM (average of two experiments). The introduction of a methylene linker provided the most active compound (**7**) in this initial set (IC_{50} 2.5 μM), contrasting with the SAR for inhibition of HCV polymerase, which showed a clear preference for a phenyl or thiophene group at C2 optimally substituted at the ortho position.[20] This observation is suggestive of a secondary binding interaction of the side chain that could be exploited for affinity and selectivity. Hence, further exploration focused on benzyl variants of **7**. Examples of key inhibitors are depicted in Figure 4. Disubstituted analogues **9** and **10** proved a little more potent when compared to **7**. The most active inhibitor within this series was compound **11** (IC_{50} 0.18 μM), which is more than 10-fold more potent than the initial lead **7**. Consistent with the dual metal chelating pharmacophore model, only marginal inhibitory activity was observed for compound **12**, the methyl ester of compound **7**. All pyrimidinol carboxylic acids exhibited minimal to no inhibitory activity against the human RNase H enzyme (Table 1) consistent with good selectivity for the viral enzyme.

Time of addition experiments were studied with compound **9** to support the mechanistic metal dependence of the inhibitor. The assay was performed at a high enzyme to substrate ratio to capture a large burst amplitude upon initiation of the reaction (Figure 5A). Preincubation of the enzyme with inhibitor and Mg before substrate addition significantly quenched the initial

burst of catalysis (Figure 5C). When the Mg ions were omitted in this preincubation period (Figure 5B), enzymatic activity more closely resembled the inhibitor free conditions, rendering strong support for a metal dependent mechanism of action for this inhibitor class with the physiologically relevant Mg. The results are in agreement with data from similar experiments using β-thujaplicinol.[22]

Compound **9** was successfully crystallized in the active site of the truncated HIV-1 RNase H domain (p15-Ec) from a solution containing Mn(II) ions as a surrogate for Mg(II). In agreement with early structural work, two Mn(II) ions are observed.[23] The inhibitor is coordinated to both metal ions, and the carboxylate is coordinated to the metal ion that is mechanistically associated with the leaving group of the hydrolysis step (i.e., the 3′ hydroxyl group of the sugar), corresponding to metal B in the *B. halodurans* structure.[24] In that work, the authors propose that the primary catalytic role of metal B is to lower the activation barrier to the transition state by destabilizing the enzyme−substrate ground state. Metal A, by contrast, serves to activate a molecule of water for nucleophilic attack. In the present structure, this metal ion is coordinated by the two phenolic oxygen atoms of compound **9**. The coordination geometry of metal A is octahedral, with the remaining ligands derived from active site carboxylates and two apical water molecules. This geometry is the most common in solid-state structures of Mg(II) coordinated by oxygen ligands and is presumably energetically preferred.[25] The ligands for metal B are less symmetrically disposed, with geometry akin to that seen in the *B. halodurans* structure, where the perturbation is proposed as the basis for the destabilization of the enzyme−substrate ground state. In addition to the chelation of the inhibitor to the metal ions, a binding interaction is apparent between the C2 aromatic substituent and the imidazole of histidine 539, consistent with the improved potency observed with the methylene linker. The distance from the center of the phenyl ring to the edge of the imidazole is 3.8 Å and is best characterized as an edge-on π interaction. The side chain orientation of histidine 539 depicted in Figure 6 is consistent with the short distance between the Nε of the histidine and the outer phenolic oxygen (2.7 Å) of the ligand as well as the water network in the crystal structure.

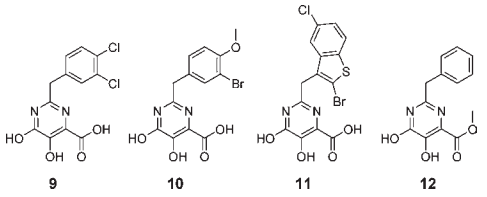


Figure 4. Structures of pyrimidinol carboxylic acid derivatives.

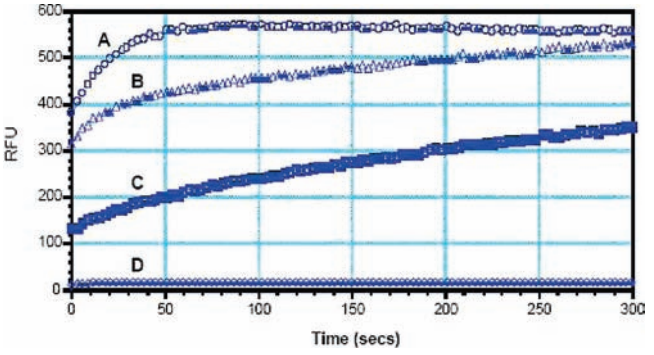


Figure 5. Time of addition experiment. HIV-1 RT was preincubated in DMSO with Mg (A), or **9** without Mg (B), or **9** with Mg (C). Enzymatic reactions were initiated by combining with substrate solutions. All experiments used otherwise identical conditions (60 nM enzyme, 100 nM substrate, 5 mM $MgCl_2$, 25 μM of **9**, 2 min preincubation). There is a 10 s delay between addition of the substrate and recording of the first time point. No hydrolysis was observed without enzyme (D).

It should be emphasized that the structure was obtained with Mn(II) ions in the active site and that an analogous binding mode for inhibition of the enzyme can only be inferred under the conditions of the biochemical assay, where only Mg is present. The structure discussed here is consistent with data from another metal chelating scaffold with respect to the nature and stoichiometry of metals and inhibitors in isolated HIV-1 RNase H domains.[26]

None of the pyrimidinol carboxylic acids discussed herein displayed activity in a multicycle MT-4 HIV infectivity assay at concentrations up to 100 μM. One or more reasons might account for this lack of antiviral activity: insufficient intrinsic potency, low cell permeability, and high protein binding, for example, may all play a role. Modulation of physicochemical properties, particularly those affecting binding to the bovine serum albumin in the medium of the assay, was key to achieving high cellular potency for a series of structurally

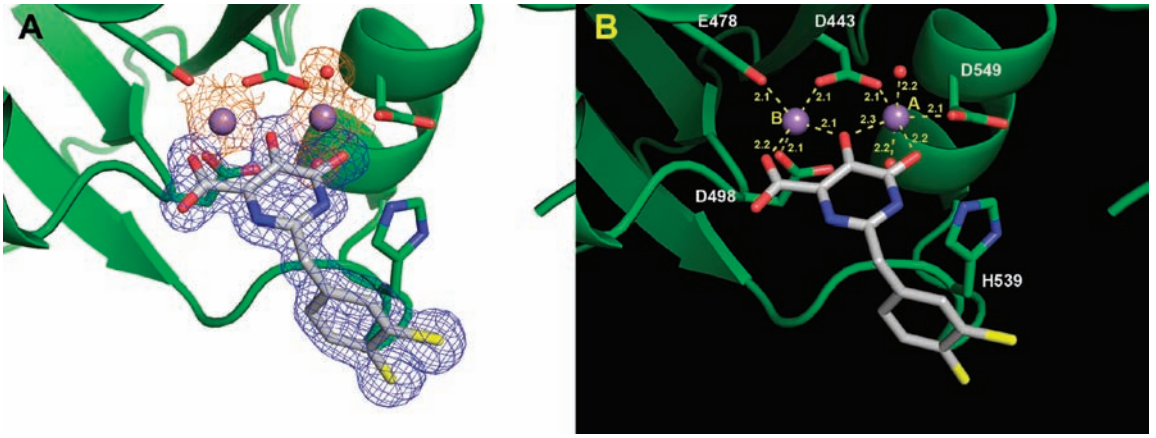


Figure 6. Crystal structure of inhibitor **9** bound in the active site of RNase H at 1.7 Å resolution. (A) Composite omit map contoured at 1.5σ showing **9** (blue) and both Mn atoms with the two tightly coordinated water molecules to metal A (orange). (B) Depiction of the complex highlighting the metal coordination. Interatomic distances are indicated in Å. Key amino acids are labeled (HIV-1 RT numbering).

related pyrimidinol-based HIV-1 integrase inhibitors.[27] Additionally, access to the RNase H active site in the virion might be limited. Finally and more speculatively, the overall stability of a complex wherein the coordination geometry of metal B is similar to that responsible for the elevation of the ground-state energy during catalytic phosphodiester hydrolysis might be intrinsically too low for useful levels of inhibition to be observed.

Acknowledgment. We thank Mary McGrath, Wei Huang, and Swami Swaminathan for insightful discussions, as well as David Cowfer, Mona Cai, Amy Kwok, James Nugteren, and Brian Stephens for analytical support.

Supporting Information Available: Experimental procedures, analytical and spectral characterization data, crystallographic information. This material is available free of charge via the Internet at http://pubs.acs.org.

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