Inhibition of HIV-1 Ribonuclease H by a Novel Diketo Acid, 4-[5-(Benzoylamino)thien-2-yl]-2,4-dioxobutanoic Acid*

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Human immunodeficiency virus-type 1 (HIV-1) reverse transcriptase (RT) coordinates DNA polymerization and ribonuclease H (RNase H) activities using two discrete active sites embedded within a single heterodimeric polypeptide. We have identified a novel thiophene diketo acid, 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid, that selectively inhibits polymerase-independent RNase H cleavage (IC50 = 3.2 μM) but has no effect on DNA polymerization (IC50 > 50 μM). The activity profile of the diketo acid is shown to be distinct from previously described compounds, including the polymerase inhibitor foscarnet and the putative RNase H inhibitor 4-chlorophenylhydrazone. Both foscarnet and the hydrazone inhibit RNase H cleavage and DNA polymerization activities of RT, yet neither inhibits the RNase H activity of RT containing a mutation in the polymerase active site (D185N) or an isolated HIV-1 RNase H domain. In some cases, these inhibitors have been shown to affect both the polymerase and RNase H activities of RT (8, 9), which superficially suggests that these compounds may bind at both active sites. However, several studies have demonstrated that there is significant interdomain communication between the polymerase and RNase H domains of RT (e.g. Ref. 12), and potential allosteric effects on RNase H function are usually not ruled out.

In this report we have employed a series of RT polymerase and RNase H mutants to characterize the effects of several known inhibitors on each enzymatic function and to explore the behavior of a novel diketo acid inhibitor of HIV-1 RNase H: 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid (Compound I, Fig. 1). We demonstrate here that inhibitors that bind to the polymerase domain, such as foscarnet, can inhibit the RNase H activity in the absence of polymerization. The phenylhydrazone (Compound II) previously reported to be an RNase H inhibitor (10, 11) is shown to work by this mechanism. In contrast, 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid selectively binds to and inhibits the RNase H domain of HIV-1 RT. The metal-dependent behavior of this novel RNase H inhibitor is analogous to the mechanism of action proposed for HIV-1 integrase inhibitors of the same structural class (13, 14). These studies demonstrate that inhibitors of RNase H function can be either direct or allosteric modulators of activity and suggest that prototype structures that have proven successful in developing integrase inhibitors with potent antiretroviral activity can be exploited to develop selective inhibitors of the RNase H activity of HIV-1 RT.

EXPERIMENTAL PROCEDURES

Reagents—All buffer components were obtained from either Sigma-Aldrich or Ambion, Inc. unless specified. 4-[5-(Benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid (Compound I) was synthesized according to published methods (15). 4-Chlorophenylhydrazone of mesoxalic acid (Compound II) was synthesized according to well established methods (16). 3-[[4,7-Dichloro-1,3-benzoazol-2-yl]methyl]amine-5-ethyl-6-

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Inhibition of HIV-1 RNase H by DKA

Cleavage reactions were conducted as described under “Experimental Procedures.” Values represent the mean and S.D. of at least three independent experiments.

| Compound | Wild type, IC50 in MgCl2 | D185N mutant, IC50 in MgCl2 | Isolated RNase H (p15-EC), IC50 in MnCl2 |
|----------|--------------------------|-----------------------------|----------------------------------------|
| Compound I | 3.2 ± 1.7               | 8.8 ± 1.8                   | 4.7 ± 3.2                               |
| Compound II | 6.1 ± 1.0               | >100                        | >100                                    |
| Compound III | >100                    | >100                        | >100                                    |
| Foscarnet | 1.8 ± 0.7               | >100                        | >100                                    |
| AZT-TP    | >100                    | >100                        | >100                                    |

RESULTS AND DISCUSSION

Inhibition of Polymerase-independent RNase H Cleavage—There are striking structural and functional homologies between HIV-1 integrase and the RNase H domain of HIV-1 RT. Potent and specific compounds containing a diketo acid motif have been shown to inhibit HIV-1 integrase in vitro and in cell culture (13). As the purported mechanism of these inhibitors invokes sequestration of the active site divalent metals in integrase, it has been hypothesized that a similar mechanism could be exploited for other phosphotransferase enzymes. Screening a library of diketo acids we identified 4-[[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid (Compound I) as a novel inhibitor of the RNase H activity of HIV-1 RT.

In RNase H cleavage assays, the activity of Compound I against full-length wild type HIV-1 RT (IC50 = 3.2 μM, Table I) was as potent as the most active RNase H inhibitors reported previously (8, 23). Inhibition of RNase H by Compound I was independent of the order of addition to the assay; reactions initiated by either enzyme or substrate behaved identically. To further characterize this activity, Compound I was also evaluated in RNase H cleavage assays using an RT polymerase active site mutant (D185N) and a chimeric isolated HIV-1 RNase H domain, p15-EC. As expected for a bona fide RNase H inhibitor, the activity of the diketo acid against p15-EC (IC50 = 4.7 μM) was comparable with wild type HIV-1 RT (Table I, Fig. 2) and was only slightly weaker when assayed against the D185N mutant (IC50 = 8.8 μM). Compound I did not inhibit E. coli RNase HI activity under similar conditions when tested at concentrations up to 50 μM. Neither the nucleoside RT inhibitor (NRTI) AZT-TP nor the non-nucleoside RT inhibitor (NNRTI; Compound III) inhibited the RNase H activity of the wild type, D185N, or p15-EC enzymes at concentrations up to 100 μM (Table I).

The HIV-1 RT inhibitor foscarnet and a previously described methylpyridin-2-(1H)-one (Compound III) inhibited the RNase H activity of the wild type, D185N, and equilibrated at 30 °C with a stirring speed of 490 rpm, a total of 25 aliquots (5 μl each) were injected into the cell. The heat released with each injection was measured, and heats of reaction were determined by integration of peak areas. Base-line correction, peak integration, and binding parameters (stoichiometry, Kd and ΔH°) were performed using the ORIGIN analysis software. Gibbs free energy (ΔG° = −RT ln(Kd)) and entropy of binding (ΔS° = ΔH° − ΔC°) were calculated from measured experimental values. Samples were run in triplicate and results were averaged to obtain binding parameters.

**FIG. 1.** Chemical structures of the five compounds used in this study.
RNase H inhibitor, 4-chlorophenylhydrazone (Compound II), were also effective inhibitors of the RNase H activity of HIV-1 RT. However, in contrast to the diketo acid, both foscarnet and the phenylhydrazone inhibited RNase H cleavage only when the assays were conducted with full-length wild type RT (IC₅₀ > 100 μM) and not with either the D185N mutant or p15-EC (IC₅₀ > 100 μM). The lack of activity observed for foscarnet and Compound II with p15-EC cannot be attributed to the unique manganese requirement for this enzyme. Foscarnet, Compound I, and Compound II were comparably active in RNase H assays using full-length RT when assayed in either 6 mM MgCl₂ (IC₅₀ = 2–6 μM) or in 2 mM MnCl₂ (IC₅₀ = 50–100 mM). Although the effect of foscarnet on RNase H function was initially surprising because this compound is well known as a pyrophosphate mimetic inhibitor of RT polymerase, the lack of activity observed with the D185N mutant is consistent with binding at the polymerase active site.

These results strongly suggest that foscarnet and Compound II are polymerase inhibitors which indirectly affect RNase H activity. Their affect on RNase H activity may be due to an indirect change in either RT conformation or nucleic acid positioning. An allosteric effect on the RNase H function is not unprecedented; several NNRTIs have been shown to enhance RNase H activity. In particular, Compound III (data not shown) and nevirapine (12) increase RNase H cleavage rates and alter the position and number of cleavage products observed in vitro.

Characterization of Allosteric Inhibitors with HIV-1 RT Polymerase—To substantiate that the allosteric effect of foscarnet and Compound II on RNase H function is as a result of binding to the polymerase domain of RT, the compounds were evaluated in HIV-1 RT polymerization assays using wild type HIV-1 RT and an active site RNase H mutant (D443N). Wild type HIV-1 RT polymerase activity was inhibited by foscarnet and Compound II as well as AZT-TP and the NNRTI Compound III, but not by Compound I (Table II). The potencies listed are consistent with previously reported values for foscarnet, AZT-TP, and Compound III (17, 24). In contrast to the effect of D185N mutation on RNase H inhibition, polymerase inhibition
by foscarnet and Compound II was not affected by the RNase H active site mutation, D443N.

Under these conditions, Compound II was a moderately potent polymerase inhibitor (IC_{50} = 2.3 μM). The negligible change in potency observed with the RNase H active site D443N mutant (IC_{50} = 2.1 μM) is consistent with the observation that both foscarnet and Compound II are affected by the D185N mutation and likely bind at or near the polymerase active site. Although in previously published work Compound II did not inhibit DNA polymerization (10), the reaction conditions were not optimal for evaluation; DNA synthesis is not rate-limiting when enzyme is equivalent or in excess of substrate, thus explaining the apparent discordance from results presented here.

Unlike foscarnet and the hydrazone, the diketo acid did not inhibit RNA-dependent DNA polymerization of wild type or D443N RT (IC_{50} > 50 μM). Although all three compounds had activity in RNase H cleavage assays using wild type RT, these results suggest that only Compound I is a selective inhibitor of HIV-1 RNase H catalysis.

**Metal-dependent Interactions Observed by Isothermal Titration Calorimetry**—Isothermal titration calorimetry experiments were conducted with the isolated RNase H domain hybrid protein (p15-EC) to characterize the interaction between the diketo acid (Compound I) and the RNase H domain of HIV-1 RT. Although no binding was observed either in the absence of metal or in the presence of Mg^{2+} (Fig. 3B), a robust signal corresponding to the heat released was detected in the presence of MnCl_{2} (Fig. 3, A and B). The divalent cation specificity observed for Compound I binding to p15-EC is consistent with the strict manganese requirement for p15-EC catalytic activity and supports the hypothesis that Compound I might interact with metal ions in the RNase H active site. As expected, no change in the heat of reaction was detected when either foscarnet or Compound II were titrated into buffer containing p15-EC (data not shown).

For Compound I, the fitted parameters (Fig. 3B, insert) derived from the ITC studies suggest that there is one binding site (n = 0.9 ± 0.2) on the RNase H domain for the diketo acid with an exothermic reaction upon association as shown by the negative molar entropy and enthalpy changes. In addition, the dissociation constant calculated from the ITC analysis (8.9 ± 3.5 μM) is consistent with the IC_{50} measured in enzymatic activity assays (3.2 μM, Table I); the slight difference between the values may be due to a shift in solvent proton concentration (pH 7.0 vs. pH 7.8, respectively). As the ITC studies were performed in the absence of nucleic acid substrate, the observation that binding is metal-dependent and that the calculated K_{D} is comparable with the observed IC_{50} suggests nucleic acid is not required for either binding of the inhibitor or the active site metals.

In summary, we have demonstrated that compounds from the diketo acid structural class can inhibit HIV-1 RNase H catalysis in vitro. Compound I is distinct from previously described RNase H inhibitors, and it selectively inhibits RNase H cleavage without affecting RNA-dependent DNA polymerization. Isothermal titration calorimetry studies confirm a direct interaction with the isolated RNase H domain and demonstrate a requirement for divalent cation. The suggestion that the thiophene diketo acid may interact with metals in the RNase H active site is consistent with the metal sequestration mechanism by which similar compounds affect HIV-1 integrase activity. Compound I also inhibits HIV-1 integrase in vitro (1.9 μM); however, the activity relationship for RNase H inhibition is clearly distinct from integrase, as previously described integrase inhibitors with the diketo acid pharmacophore have no effect on RNase H activity (Refs. 13 and 14 and data not shown). Although Compound I does not inhibit HIV-1 replication in cell culture, it provides an important proof of concept for direct inhibitors of the RNase H activity of HIV-1 RT. This work also outlines methods to distinguish indirect affects on RNase H function by compounds that bind to the polymerase domain and to identify selective RNase H inhibitors that may ultimately lead to the development of novel chemotherapeutics for HIV-1 infection.

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