Selective inhibition of HIV-1 reverse transcriptase-associated ribonuclease H activity by hydroxylated tropolones

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ABSTRACT

High-throughput screening of a National Cancer Institute library of pure natural products identified the hydroxylated tropolone derivatives β-thujaplicinol (2,7-dihydroxy-4-1(methylethyl)-2,4,6-cycloheptatrien-1-one) and manicol (1,2,3,4-tetrahydro-5-7-dihydroxy-9-methyl-2-(1-methylethenyl)-6H-benzocyclohepten-6-one) as potent and selective inhibitors of the ribonuclease H (RNase H) activity of human immunodeficiency virus-type 1 reverse transcriptase (HIV-1 RT). β-Thujaplicinol inhibited HIV-1 RNase H in vitro with an IC50 of 0.2 μM, while the IC50 for Escherichia coli and human RNases H was 50 μM and 5.7 μM, respectively. In contrast, the related tropolone analog β-thujaplicin (2-hydroxy-4-(methylethyl)-2,4,6-cycloheptatrien-1-one), which lacks the 7-OH group of the heptatriene ring, was inactive, while manicol, which possesses a 7-OH group, inhibited HIV-1 and E. coli RNases H with IC50 = 1.5 μM and 40 μM, respectively. Such a result highlights the importance of the 2,7-dihydroxy function of these tropolone analogs, possibly through a role in metal chelation at the RNase H active site. Inhibition of HIV-2 RT-associated RNase H indirectly indicates that these compounds do not occupy the nonnucleoside inhibitor-binding pocket in the vicinity of the DNA polymerase domain. Both β-thujaplicinol and manicol failed to inhibit DNA-dependent DNA polymerase activity of HIV-1 RT at a concentration of 50 μM, suggesting that they are specific for the C-terminal RNase H domain, while surface plasmon resonance studies indicated that the inhibition was not due to intercalation of the analog into the nucleic acid substrate. Finally, we have demonstrated synergy between β-thujaplicinol and calanolide A, a nonnucleoside inhibitor of HIV-1 RT, raising the possibility that both enzymatic activities of HIV-1 RT can be simultaneously targeted.

INTRODUCTION

Reverse transcriptase (RT)-associated ribonuclease H (RNase H) activity is responsible for both non-specified degrading the RNA strand of the RNA/DNA replication intermediate as well as specifically removing the minus (−) and plus (+) strand RNA primers (tRNA and the polypurine tract (PPT), respectively) from nascent DNA (1). The absolute requirement for RNase H activity for human immunodeficiency virus (HIV) replication (2,3) suggests that this might be an attractive target for the development of antiviral agents to complement DNA polymerase-based HIV-1 RT inhibitors currently in clinical use [reviewed in (4)]. In this respect, recent reports have documented several promising candidates effective at low micromolar concentrations, including hydrazones (5–7), tetragalloylglucopyranose (8), diketo acids (9) and N-hydroxyimides (10). Although it remains to be established that their mode of inhibition is through direct binding to the RNase H catalytic center, both diketo acids

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and N-hydroxylimides have been shown to inhibit an enzymatically active peptide derived from the RNase H domain of HIV-1 RT (9, 11). Thus, while antiviral activity of these select RNase H antagonists is yet to be demonstrated, sufficient evidence has accumulated to justify further screening for inhibitors of HIV-1 and HIV-2 RNase H. Moreover, although Klumpp et al. (10) have recently evaluated N-hydroxylimides for the inhibition of retroviral and bacterial RNases H, none of the more potent HIV-1 RNase H inhibitors reported to date has addressed selectivity with respect to the human counterpart. This issue is of particular importance, since recent data have indicated that disruption of the RNase H1 allele in mice confers a lethal embryonic defect (12). To take this issue into account, we recently described two simple, robust fluorescence-based methodologies which could be applied in parallel to retroviral, bacterial and human RNases H using the same assay platform, together with their use for high-throughput screening (13, 14). The availability of purified human RNase H1 has also allowed us to investigate the specificity of inhibitors uncovered from our current screening efforts.

Here, we describe two tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) derivatives with a 7-OH substitution, identified from high-throughput screening of National Cancer Institute libraries of natural products, which inhibit retroviral, bacterial and human RNases H. Inhibition of HIV-2 RNase H activity indirectly indicates that these compounds do not interact with the nonnucleoside-binding site located near the DNA polymerase catalytic center of this enzyme. The most potent of the hydroxylated tropolone analogs, β-thujaplicinol, derived from the heartwood of several cupressaceous plants (e.g. Thuja plicata, Thuja occidentalis and Chamaecyparis obtusa) (15–17), is active at sub-micromolar concentrations against the RNases H of HIV-1 and HIV-2 RT, while the DNA polymerase activity of either enzyme is unaffected at an inhibitor concentration of 50 μM. The same compound is ~30- to 250-fold less active against the human and Escherichia coli RNases H, respectively, demonstrating that selective inhibition of the retroviral enzyme can be achieved. Finally, we demonstrate here that β-thujaplicinol acts synergistically with calanolide A, a nonnucleoside inhibitor of HIV-1 RT (18, 19), opening the possibility of simultaneously targeting the DNA polymerase and RNase H functions of HIV-1 and HIV-2 RT.

A number of reports have demonstrated that tropolone derivatives elicit a variety of biological effects, including anti-tumor (20), insecticidal (21), antifungal (22, 23) and antimicrobial (24) activity, while their metal chelates have been shown to inhibit human influenza virus-induced apoptosis (25). Wakabayashi et al. (26) have investigated cytotoxic activity of tropolone derivatives against a variety of human oral tumor cell lines, and also indicated that they fail to protect MT-4 cells from HIV-1 infection.

MATERIALS AND METHODS

Tropolone derivatives

The structures of α-thujaplicin [2-hydroxy-3-(1-methylethyl)-2,4,6-cycloheptatrien-1-one], β-thujaplicin [2-hydroxy-4-(1-methylethyl)-2,4,6-cycloheptatrien-1-one] and γ-thujaplicin [2-hydroxy-5-(1-methylethyl)-2,4,6-cycloheptatrien-1-one], β-thujaplicinol [2,7-dihydroxy-4-(1-methylethyl)-2,4,6-cycloheptatrien-1-one], manicol [1,2,3,4-tetrahydro-2,7-dihydroxy-9-methyl-2-(1-methylethenyl)-6H-benzocyclohepten-6-one] and nootkatin [2-hydroxy-5-(3-methyl-2-butenyl)-4-(1-methylethyl)-2,4,6-cycloheptatrien-1-one] are provided in Figure 1. All compounds were members of a National Cancer Institute library of purified, natural compounds, and their identity and purity were confirmed by 1H-NMR. Each compound was prepared as a 20 mM stock solution in 100% dimethyl sulfoxide (DMSO) and diluted into the appropriate reaction buffer immediately prior to analysis.

Enzymes and enzyme assays

Recombinant, wild-type p66/p51 HIV-1 RT was expressed and purified as described previously (27). HIV-2 RT was prepared using the same E.coli expression system (27). E.coli RNase HI and recombinant human RNase H were prepared as described previously (28, 29). The strategy for high-throughput screening and confirmation of RNase H activity by capillary electrophoresis has recently been described by Parniak et al. (13) and Chan et al. (14), respectively. For HIV-1 RT, conditions for cleavage of the HIV-1 PPT RNA primer extended at its 3′ terminus by five deoxynucleotides have been described by Kvaratskhelia et al. (30). DNA-dependent DNA polymerase activity was analyzed on a 71 nt DNA to which a [32P]labeled 36 nt DNA primer was hybridized as described by Dash et al. (31). For qualitative analysis of PPT cleavage and DNA-dependent DNA polymerase activity, all tropolone derivatives were evaluated at a single concentration of 50 μM.

Surface plasmon resonance analysis

All experiments were performed with a Biacore 2000 optical biosensor at 25°C. CM5 Sensor chips with a carboxymethylated dextran matrix, EDC, NHS and P20 surfactant was obtained from Biacore AB, (Uppsala, Sweden), and streptavidin was from Pierce (Rockford, IL). Streptavidin was immobilized on the sensor chip surface to the level of ~4000–4500 RU in each flow cell. Biotinylated nucleic acid (12mer RNA–DNA hybrid in the form of a hairpin within which a thymidine residue of a connection tetraloop was biotinylated) was injected at a concentration of 50 nM in HBS buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.005% P20, pH 7.4) onto streptavidin-modified chip surface at a flow rate of 5 μl/min until the desired level (~1000–1500 RU) of immobilization was achieved. Blocking of unoccupied streptavidin with biotin and washing the chip with regeneration buffer (2 M NaCl) was performed as described previously (28). Experiments on inhibitor or marker binding to nucleic acid were carried out in Tris-buffered saline/DMSO running buffer (10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 0.005% P20, 5% DMSO, pH 8.0) using a flow rate of 50 μl/min. Typically, 50 μl of a marker or inhibitor (each separately or in mixtures of 2 or 3 simultaneously) were injected using the ‘kinject’ command defining a dissociation time of 2 min. After each round of injection, the system was intensively washed using running buffer. All experiments were performed in duplicate. Data transformation of the primary sensograms and overlay plots were prepared using the
BIASevaluation 3.0 software (Biacore AB). The response from the reference flow cell that does not contain nucleic acid was subtracted from that of the experimental flow cell.

**IC$_{50}$ and $K_i$ determinations**

Dose–response plots were prepared by measuring RNase H activity on a fluorescent RNA/DNA hybrid as described previously (13) while varying the inhibitor concentration by 2-fold dilutions from 100 µM to 0.19 nM. Curve fitting was performed using Sigma Plot. In order to determine the $K_i$ for β-thujaplicinol, the substrate concentration was varied from 200 to 1.56 nM while varying the inhibitor concentration from 1.56 to 0.024 nM at each substrate concentration. Hydrolysis was followed by fluorescence measurements at 40 s intervals over the 30 min reaction time using a Molecular Devices Spectramax Gemini EM fluorescence spectrometer. Slopes of the reaction curves were determined from the linear portion of the plots using SoftMAX Pro version 4.3 supplied with the instrument. Curve fitting and prediction of the mode of inhibition was performed using the Enzyme Kinetics module of Sigma Plot version 8 (SPSS, Chicago, IL).

**Synergy between RNase H and DNA polymerase inhibitors**

In order to determine whether the binding site for β-thujaplicinol overlapped that of nonnucleoside RT inhibitors, RNase H activity was measured in the presence of varying concentrations of tropolone inhibitor and varying concentrations of the nonnucleoside calanolide A (18) according to the method of Yonetani and Theorell (32) and Cook et al. (33). Data were processed using the Calcusyn software package (BioSoft, Cambridge, UK).

**RESULTS**

Tropolone derivatives specifically inhibit HIV-1 RNase H function

The structures of the tropolone derivatives used in the present study are provided in Figure 1. Since HIV-1 RT is a bifunctional enzyme, inhibition of RNase H activity might reflect direct binding to the RNase H domain or, alternatively, an allosteric effect through binding to the nonnucleoside-binding pocket close to the DNA polymerase domain, which has been shown previously to modulate RNase H function (34). Preliminary experiments were, therefore, designed to investigate the specificity of each analog for the RNase H activity of HIV-1 RT. In Figure 2A, RNase H activity was evaluated on a 5’[32P]labeled RNA–DNA chimera hybridized to its DNA complement, which mimics processing of the HIV-1 PPT primer from nascent DNA, following initiation of second or (+) strand synthesis. Using this substrate, we have previously demonstrated RNase H cleavage at the PPT RNA-U3 DNA junction (defined as −1), and additionally at positions −2 and −3 (30). For these initial experiments, a single analog concentration of 50 µM was employed, which resulted in almost complete inhibition of PPT processing by both
β-thujaplicinol (lane 4) and manicol (lane 7). In Figure 2B, the same derivatives were re-evaluated for their ability to inhibit DNA-dependent DNA polymerase activity on a duplex containing a 5'[^32]P]labeled 36 nt DNA primer hybridized to a 71 nt DNA template. At a single concentration of 50 μM, negligible inhibition of DNA polymerase activity was observed for all analogs. The combined data of Figure 2 thus indicate that inhibition of HIV-1 RNase H activity is not an consequence of analog binding to the DNA polymerase catalytic center and exerting an indirect effect, as has been reported for certain diketo acids and phenylhydrazones by Shaw-Reid et al. (9).

**Selectivity of inhibition**

Clearly, a desirable feature of an inhibitor of retroviral RT-associated RNase H activity would be selectivity with respect to its human counterpart. To address this issue, β-thujaplicinol and manicol were examined for their inhibitory effect on the RTs of HIV-1 and HIV-2 as well as human RNase H. Figure 3A indicates that β-thujaplicinol inhibited HIV-1 RT with an IC$_{50}$ of 0.21 ± 0.03 μM. For HIV-2 RT, the IC$_{50}$ for the same analog was ~3-fold higher (Figure 3C, 0.77 ± 0.08 μM). Such a result might reflect subtle differences in the architecture of RNase H domains of these two enzymes, which has been suggested from biochemical analysis of the purified RTs (35). More importantly, β-thujaplicinol inhibited human RNase H with an IC$_{50}$ of 5.7 ± 0.7 μM (Figure 3E), indicating ~30-fold selectivity for the HIV-1 enzyme. Although not shown here, an IC$_{50}$ value of ~50 μM was determined for *E. coli* RNase H, indicating that this enzyme was ~250-fold less sensitive to β-thujaplicinol inhibition. In Figure 3B, D and F, inhibition of retroviral and human RNases H by manicol was compared. While this analog was slightly less potent against HIV-1 RNase H (IC$_{50}$ = 0.60 ± 0.09 μM), 6-fold enhanced selectivity over the human enzyme was achieved (IC$_{50}$ = 3.5 ± 0.1 μM).

IC$_{50}$ values for tropolone and its derivatives are presented in Table 1. Interestingly, β-thujaplicin, which differs from β-thujaplicinol in that it lacks the hydroxyl function at position 7 of the heptatriene ring, was completely inactive against all enzymes tested, despite reports that it possesses metal chelating properties (36). Relocation of the hydroxyl function on the heptatriene ring produced a different effect, i.e. while γ-thujaplicin failed to inhibit retroviral RNases H, α-thujaplicin was weakly active, with an IC$_{50}$ value of 50 and 33 μM for the HIV-1 and HIV-2 enzymes, respectively.

**β-Thujaplicinol does not inhibit RNase H activity through intercalation**

Although DNA polymerase activity was unaffected, one possible mode of inhibition we had to consider was the intercalation of the planar tropolone derivatives into the RNA/DNA
hybrid, effectively making the substrate unavailable for enzyme binding. In order to eliminate this possibility, inhibitor binding to an immobilized RNA/DNA hybrid was investigated via surface plasmon resonance, which we have successfully used to investigate the binding properties of HIV-1 RT derivatives (37). As a control, binding of ethidium bromide to the immobilized hybrid was analyzed, the results of which are depicted in the sensorgram of Figure 4A. From this data, we calculated that 4–6 molecules of ethidium bromide intercalated into the hybrid with a $K_d$ of $1.5 \text{ mM}$. In contrast, data of Figure 4B, which presents the combined sensorgrams obtained when the RNA/DNA hybrid was incubated with $\alpha$-thujaplicin, $\beta$-thujaplicin and $\beta$-thujaplicinol, clearly indicate that they do not bind the substrate over a concentration range of 50 nM–10 mM.

**Mode of inhibition**

$\beta$-Thujaplicin and manicol were further investigated in order to gain insight into their mechanism of inhibition of HIV-1

**Figure 3.** Selectivity of RNase H inhibition. Dose–response curves for RNase I inhibition by $\beta$-thujaplicinol (A, C and E) and manicol (B, D and F) are presented. (A and B) HIV-1 RT; (C and D), HIV-2 RT; (E and F), human RNase H. $IC_{50}$ determinations are the results of triplicate assays.

**Table 1.** Inhibition of retroviral, bacterial and human RNases H by hydroxylated tropolone derivatives

| Compound          | $IC_{50}$ HIV-1 RT (µM) | $IC_{50}$ HIV-2 RT (µM) | $IC_{50}$ E.c. Rnase H (µM) | $IC_{50}$ Hu Rnase H (µM) |
|-------------------|-------------------------|--------------------------|----------------------------|---------------------------|
| Tropolone         | >100                    | >100                     | >100                       | 3.0                       |
| $\beta$-Thujaplicin | >100                   | >100                     | >100                       | >100                      |
| $\gamma$-Thujaplicin | >100                  | >100                     | >100                       | >100                      |
| Nootkatin         | >100                    | >100                     | >100                       | >100                      |
| $\alpha$-Thujaplicin | 50                    | 33                       | >100                       | 12                        |
| Manicol           | 60                      | 1.7                      | 40                         | 3.5                       |
| $\beta$-Thujaplicinol | 0.21                 | 0.77                     | 50                         | 5.7                       |

$IC_{50}$ values are the average of triplicate analyses.
RT/RNase H. From the Michaelis–Menten plot of Figure 5, a $K_i$ of 0.20 ± 0.07 µM was determined for $\beta$-thujaplicinol, and a separate study indicated a $K_i$ of 1.0 ± 0.4 µM for manicol (data not shown). The data presented in Figure 5 show a change in $V_{\max}$ without affecting the $K_m$, which is suggestive of mixed inhibition. Non-linear fitting of the data confirms that this is best fit to mixed-type inhibition compared with competitive or uncompetitive modes. With this type of inhibition, the antag- onist can bind either the free enzyme or the enzyme–substrate complex. This suggests that the tropolone derivatives could occupy (or create) a pocket near the RNase H active site, since access to that site would be impaired by the RNA/DNA duplex substrate.

$\beta$-Thujaplicinol and the nonnucleoside calanolide A occupy different sites on HIV-1 RT

The multifunctional nature of HIV-1 RT opens the possibility that antiviral agents could be separately targeted to the DNA polymerase and RNase H catalytic centers. In order to assess this, we investigated whether $\beta$-thujaplicinol would act in combination with calanolide A (18). This coumarin derivative, isolated from the tropical rainforest tree *Calophyllum lanigerum*, has been shown to inhibit HIV-1 RT activity by a mechanism similar to other nonnucleoside-based drugs (19). Figure 6 presents a Yonetani–Theorell plot of RNase H inhibition in the presence of both calanolide A and $\beta$-thujaplicinol. The data indicate that the inhibitors bind to two independent sites since the plot shows converging lines (32). In addition, HIV-2 is naturally resistant to NNRTIs; thus, inhibition of HIV-2 RNase H is additional, albeit indirect, evidence that $\beta$-thujaplicinol binds to an independent site. Independent binding sites for calanolide A and $\beta$-thujaplicinol were further supported by inhibitor-combination assays using the methods described by Chou and Talalay (38,39). The combination index (CI) derived for mixed inhibitors can be used to describe additivity (CI = 1), synergy (CI < 1) and antagonism (CI > 1). The inhibitor combination of $\beta$-thujaplicinol/calanolide A was found to be 0.50, i.e. the inhibition by a $\beta$-thujaplicinol/calanolide A combination is more potent than predicted by adding the effect of the individual inhibitors. This synergistic effect would not be observed with inhibitors competing for the same or closely positioned binding sites. In a control experiment, a CI of 1.8 was determined for a $\beta$-thujaplicinol/manicol combination, indicating antagonism. This would be predicted, since the two tropolone derivatives most likely compete for the same binding site.

**DISCUSSION**

In this communication, we demonstrate that the natural products $\beta$-thujaplicinol and manicol, derived from the heartwood of several cupressaceous trees, inhibit the RNase H activity of HIV-1 and HIV-2 RT at sub-micromolar concentrations without affecting DNA polymerase function of either enzyme. Moreover, the most potent inhibitor, $\beta$-thujaplicinol, is ~30-fold more active on HIV-1 RT/RNase H than on human RNase...
time, although manicol introduces a bulky substituent onto the heptatriene ring, it provides almost the same potency against HIV-1 RNase H as \( \beta \)-thujaplicinol. While addition of the 7-OH function enhances the specificity for HIV-1 and HIV-2 RT/RNase H, it has little to no effect on potency against the bacterial and human enzymes. Poor inhibition of \( E. coli \) RNase H and the consistent but modest inhibition of human RNase H by all tropolones tested suggests that if metal chelation is responsible for inhibition, the 7-OH function is critical for stabilizing an interaction, which is specific to the active-site geometry of the retroviral enzymes. Interestingly, a two-metal catalyzed catalytic mechanism has been proposed for HIV-1 RNase H, based on crystallographic data with the isolated Mn\(^{++}\)-doped domain showing two divalent cations at the active site (40). Although speculative, the potency we observe for \( \beta \)-thujaplicinol may reflect its ability to form a more stable complex with the divalent cation at both metal-binding sites. If an interaction with the divalent cation at the RNase H catalytic center is the basis for inhibition by \( \beta \)-thujaplicinol and manicol, it is interesting to note that these two compounds do not inhibit DNA polymerase function, despite the fact that the equivalent divalent cation is coordinated in this catalytic center by a triad of carboxyate residues (Asp110, Asp185 and Asp186). One possible explanation might be that the divalent cation is more tightly bound at the DNA polymerase catalytic center and thus more difficult to access. However, titration calorimetry studies suggest the contrary, namely that the divalent cation in the RNase H catalytic center is more tightly coordinated (41). Based on these observations, differences in the overall architecture of the metal-binding site might exclude these inhibitors from the polymerase catalytic center. Further studies of the interaction of \( \beta \)-thujaplicinol and manicol with HIV-1 and HIV-2 RT have the potential to clarify their mechanism of action. While inactive in cellular models of HIV-1 infection, these two compounds nonetheless will serve as useful probes of HIV-1 and HIV-2 RNase H enzymology. For example, recent studies have suggested that inhibition of HIV-1 RNase H activity might increase the rate at which chain terminators of DNA synthesis are excised, thus leading to enhanced nucleoside resistance (V. Pathak, personal communication). The availability of inhibitors selective for the RNase H activity of HIV-1 RT will allow this hypothesis to be directly evaluated \textit{in vitro}.

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