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Active site and allosteric inhibitors of the ribonuclease H activity of HIV reverse transcriptase

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Abstract

Despite the wealth of information available for the reverse transcriptase (RT)-associated ribonuclease H (RNaseH) domain of lentiviruses, gammaretroviruses and long terminal repeat containing retrotransposons, exploiting this information in the form of an RNaseH inhibitor with high specificity and low cellular toxicity has been disappointing. However, it is now becoming increasingly evident that the two-subunit HIV-1 RT is a highly versatile enzyme, undergoing major structural alterations in order to interact with, position and ultimately hydrolyze the RNA component of an RNA/DNA hybrid. Thus, in addition to targeting the RNaseH active site, identifying small molecules that bind elsewhere and disrupt catalysis allosterically by impairing conformational flexibility is gaining increased attention. This review summarizes current progress towards development of both active site and allosteric RNaseH inhibitors.

The **ribonuclease H** (RNaseH) domain of retroviral **reverse transcriptase** (RT), in addition to nonspecifically hydrolyzing the RNA strand of the RNA/DNA replication intermediate, catalyzes highly specific hydrolytic events that are critical to synthesis of integration-competent double-stranded proviral DNA from the RNA genome of the infecting particle [1]. Prominent among these is precise removal of the RNA primers that initiate (-) and (+) strand DNA synthesis (a host-coded tRNA and the polypurine tract, respectively), since these events ultimately define 5' and 3' long terminal repeat sequences essential for efficient integration of viral DNA. With respect to (+) strand synthesis, generating the polypurine tract 3' terminus also mandates a mechanism whereby this sequence is accurately recognized when embedded within the replication intermediate. The observation over two decades ago that mutating active site residues of the RNaseH domain of HIV-1 RT eliminates activity [2] and results in loss of virus infectivity [3] demonstrates the necessity for this function and that the retrovirus-associated activity cannot be complemented by a host enzyme. Together, these observations define the C-terminal RT-associated RNaseH

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domain as an additional and important target in the development of future combination antiretroviral regimens.

For the **nucleoside-** and **non-nucleoside-derived DNA polymerase inhibitors** (NRTIs and NNRTIs, respectively), there is a wealth of data to guide structure-based drug design since the complex of HIV-1 RT containing the NNRTI nevirapine was solved in 1992 by Kohlstaedt and Steitz [4]. In contrast, high-resolution structures of HIV-1 RT containing an inhibitor bound to the RNaseH active site have only recently become available following the initial report in 2009 by Himmel *et al.* [5]. Although the ease with which the current generation of RNaseH active site inhibitors can be displaced from their binding site in the presence of the nucleic acid substrate represents a major obstacle, the recently-reported structure of HIV-1 RT containing an RNA/DNA hybrid and an NNRTI (Figure 1A) [6] provides a plausible model where the hybrid has ready access to the RNaseH active site (Figure 1B). This model indicates that significant structural alterations within and between the p66 and p51 subunits of the parental p66/p51 heterodimer (Figure 1) are a prerequisite to correctly accommodating the duplex, thus, it may be possible to identify non-active site inhibitors that occupy a site within, or close to, the RNaseH domain and restrict conformational flexibility. Indeed, although a high-resolution co-crystal structure is unavailable, recent data suggest that vinyllogous ureas and thienopyrimidinones might fulfill this requirement. The goal of this article is to extend previous reviews by providing an updated account of progress towards developing HIV-1 RNaseH inhibitors that interact outside the RNaseH active site. The reader is also encouraged to read recent reviews by Tramontano and Di Santo [7], and Ilina *et al.* [8].

Metal-chelating active site inhibitors

N-hydroxyimides

N-hydroxyimides were initially reported to inhibit the endonuclease-dependent influenza RNA-dependent RNA polymerase. 2-hydroxy-4*H*-isoquinoline-1,3-dione (compound **1**, Figure 2) was shown to compete with the RNA substrate in a metal-dependent mode ($IC_{50} = 15 \mu M$) suggesting a direct interaction between its oxygen atoms and the active-site metal ions [9]. This observation provided a pharmacophore model comprising three oxygen atoms able to mimic the presumed interactions of the substrate, nucleophile and leaving group oxygens in the transition state with the metal ions present in the active site of influenza virus endonuclease [9]. Since HIV-1 RNaseH activity also involves a two metal ion mechanism, *N*-hydroxyimides were subsequently tested for their effect on HIV-1 RNaseH and the prototype, compound **1**, was inhibitory, with an IC_{50} value of 0.6–1 μM [10]. Loss of activity when the hydroxyl group was replaced with a methoxy or an amino function demonstrates a requirement for the oxygen ligands. Compound **1** was inactive against *Escherichia coli* RNase HI, and inhibited RNA-dependent DNA polymerase activity of HIV-1 RT only at significantly higher concentrations. Preliminary structure–activity relationship (SAR) data suggested that substitutions on the phenyl moiety increased both potency and selectivity [10] while crystallographic studies indicate that the flexible ‘His-loop’ near the RNaseH C-terminus was stabilized in the presence of **1** [11].

Compound **1** was subsequently used to synthesize derivatives with a variety of substitutions in position 7 of the isoquinoline ring in an effort to obtain dual HIV-1 RNaseH and integrase (IN) inhibitors [12]. Although the most potent RNaseH inhibitors were equally active on IN, all compounds of this series exhibited high cytotoxicity in cell culture. A second series, obtained by synthesizing analogs substituted at position 4 by alkyl and arylalkyl chains and generating more amphiphilic molecules [13], showed little improvement and, while some compounds inhibited HIV-1 IN in the low micromolar range, none inhibited viral replication due to their cytotoxicity [13]. Physicochemical studies on *N*-hydroxyimides showed results consistent with a 1:1 stoichiometry of the Mg^{2+} complexes and that metal binding was strictly dependent on the enolization ability of the compounds [12]. Further ^3H - and ^{13}C -NMR, IR and ESR studies on **1** and analogs bearing electron-withdrawing or electron-donating moieties in position 4 of the isoquinoline ring confirmed this model and their facile enolization [14]. More recently, substituted 3-hydroxyquinolin-2(1*H*)-ones were reported to selectively inhibit HIV-1 RNaseH [15]. *N*-(4-fluorobenzyl)-3-hydroxy-2-oxo-1,2-dihydroquinoline-4-carboxamide (compound **2**, Figure 2) inhibited HIV-1 RNaseH with an IC_{50} value of 19 μM , but was cytotoxic (CC_{50} = 29 μM). UV spectrophotometry, ^1H NMR and ^{13}C NMR spectrometry, together with studies on reduction of the intrinsic fluorescence of the isolated RNaseH domain, confirmed the ability of **2** to chelate the Mg^{2+} ions in the RNaseH catalytic site [15]. Further SAR studies showed that halogen substitution at position 6 of **2** did not significantly alter RNaseH inhibition, while the introduction of a halogen in position 8 significantly reduced its inhibitory potency [15].

Diketo acids

The two-metal-ion active site binding pharmacophore model elaborated for *N*-hydroxyimides also explained the mode of action by which 2,4-diketo acids (DKAs) interacted with the influenza endonuclease active site [9]. DKAs were previously reported to inhibit influenza virus endonuclease [16] and HIV-1 IN [17] and since these two enzymes, together with RNaseH, are members of the polynucleotidyl phosphotransferase enzyme family, they were also tested on HIV-1 RNaseH by Merck laboratories [18]. 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid (BTDBA, compound **3**, Figure 2) was shown to: inhibit HIV-1 RNaseH with an IC_{50} value of 3.2 μM , independent of the order of addition; inhibit a hybrid protein comprising the *E. coli* RNaseH scaffold and the HIV-1 RNaseH domain (p15-EC) with an IC_{50} value of 4.7 μM ; lack inactivity on the *E. coli* RNaseH. Isothermal titration calorimetry showed a BTDBA dissociation constant of 8.9 μM , supporting the hypothesis that BTDBA might interact with metal ions in the RNaseH active site independently of the presence or absence of the nucleic acid substrate [18]. BTDBA has been subsequently modeled into the HIV-1 RNaseH domain assuming the basic interaction of the DKA group with the metal ions of the active site [11]. According to this model, its benzoylamide moiety may extend towards Trp266, Leu422 and Trp426 of the p51 subunit. Notably, BTDBA has been shown to inhibit HIV-1 RT-catalyzed strand transfer activity synergistically with either an NRTI, an NNRTI and phosphonoformic acid (PFA, Foscarnet). Consistent with the requirement of both DNA polymerase and RNaseH activities for DNA strand transfer, these results suggest that future cell-penetrant regimens combining RT-associated DNA polymerase and RNaseH inhibitors may have therapeutic benefit [19].

Starting from a parallel screen for HIV-1 IN inhibitors [20,21] the DKA derivative 6-[1-(4-fluorophenyl)methyl-1*H*-pyrrol-2-yl]-2,4-dioxo-5-hexenoic acid ethyl ester (RDS 1643, compound **4**, Figure 2) was shown to inhibit the HIV-1 RNaseH activity with an IC₅₀ value of 13 μM [22]. RDS 1643 did not inhibit RDDP, AMV and *E. coli* RNaseH activities, while it inhibited HIV-1 IN at higher concentrations (IC₅₀ = 90 μM). Further biochemical characterization indicated that RDS 1643 activity was substrate-independent, and spectrophotometric analysis indicated it interacted with the active site divalent metal ions [22]. Notably, RDS 1643 inhibited replication of both wt and NNRTI-resistant viruses with EC₅₀ values of 7–14 μM and a selective index of 5.

Hydroxytropolones

2-hydroxy-2,4,6-cycloheptatrien-1-one (tropolone) derivatives with a 7-OH substitution, such as 2,7-dihydroxy-4-(methylethyl)-2,4,6-cycloheptatrien-1-one (β-thujaplicinol, compound **5**, Figure 3) and 1,2,3,4-tetrahydro-5–7-dihydroxy-9-methyl-2-(1-methylethenyl)-6*H*-benzocyclohepten-6-one, or manicol, (compound **6**, Figure 3) were first identified as potent and selective HIV-1 RNaseH inhibitors via high-throughput screening of a National Cancer Institute library of pure natural products [23]. β-thujaplicinol derives from the heartwood of several Cupressaceae plants (e.g., *Thuja plicata*, *Thuja occidentalis* and *Chamaecyparis obtusa*), inhibiting HIV-1, HIV-2, *E. coli* and human RNaseH activities with IC₅₀ values of 0.2, 0.77, 50 and 5.7 μM, respectively. Manicol more potently inhibited HIV-2 and human RNases H (IC₅₀ = 1.7 and 3.5 μM, respectively) than the HIV-1 and *E. coli* enzymes (IC₅₀ = 60 and 40 μM, respectively). Importantly, the related tropolone analog 2-hydroxy-4-(methylethyl)-2,4,6-cycloheptatrien-1-one (β-thujaplicin) that lacks the 7-OH group of the heptatriene ring was inactive, suggesting that the 2,7-dihydroxy tropolone function is important for metal chelation at the RNaseH active site [23]. β-thujaplicinol and manicol were also shown to inhibit the HIV-1 IN-catalyzed strand transfer reaction in the micromolar range (IC₅₀ = 21 and 71 μM, respectively) by chelating one or two Mg²⁺ in the active site [24]. Pre-steady state biochemical studies further demonstrated that β-thujaplicinol is unable to inhibit RNaseH cleavage if added after the RT-substrate complex is formed, suggesting that the RNA/DNA substrate may block access to the RNaseH active site, and confirmed that it does not inhibit HIV-1 RDDP activity [25]. It was also proposed that, in addition to coordinating one of the active site divalent cations, the central tropolone ring of β-thujaplicinol could form π-stacking interactions with the side chain of Tyr501, suggesting that its binding site could lie between the RNaseH active site and Tyr501 of the RNaseH primer grip [25]. Subsequent studies provided biochemical evidence that β-thujaplicinol is a slow-binding RNaseH inhibitor and suggested that the presence of a bound RNA:DNA substrate is necessary to achieve its maximal binding to RT [5]. A 2.80 Å resolution co-crystal of HIV-1 RT containing β-thujaplicinol confirmed that it indeed binds at the RNaseH active site: all three oxygen atoms of the β-thujaplicinol tropolone ring coordinate the two divalent cations and the inhibitor forms ionic or hydrogen bond interactions with the side chains of residues Glu478, Asp498, His539 and Asp549 [5]. Overall, it has been proposed that β-thujaplicinol forms a stable inhibitory complex with both RT and RNA:DNA substrate that directly prevents the attacking water and the scissile phosphate from being properly positioned for catalysis.

Hydroxytropolone derivatives have been synthesized by different research teams. A series of monosubstituted, disubstituted, 3,7-dihydroxytropolones, previously shown to inhibit inositol monophosphatase by binding the two Mg^{2+} ions of the catalytic site [26], was shown to inhibit both HIV-1 RNaseH and RDDP functions, as well as both HIV-1 IN 3'-processing and strand transfer activities, in the low-micromolar range [27]. The most potent derivative, the 4-monosubstituted 3,7-dihydroxytropolone, SP47 (compound **7**, Figure 3), inhibited HIV-1 RT and IN catalytic activities with IC_{50} values in the 0.7–2.3 μM range. SAR studies showed that C-7-modified and *O*-methylated tropolones were inactive and confirmed the hypothesis that active derivatives inhibit HIV-1 RNaseH activity by binding divalent metal ions in the active site. Importantly, HIV-1 RDDP inhibition by 3,7-dihydroxytropolones was also reported to be very sensitive to Mg^{2+} concentration and it was proposed that metal in the DNA polymerase catalytic site is required for binding, and that these compounds compete for Mg^{2+} chelation. 3,7-dihydroxytropolones, however, were found to be cytotoxic [27].

A series of manicol derivatives substituted at its alkene moiety were synthesized and demonstrated to inhibit HIV-1 RNaseH activity in the submicromolar range, with the most potent derivative, 5,7-dihydroxy-2-(1-hydroxy-1-methyl-2-piperidin-1-yl-ethyl)-9-methyl-1,2,3,4-tetrahydrobenzocyclohepten-6-one (compound **8**, Figure 3), exhibiting an IC_{50} value of 0.8 μM , an EC_{50} value of 10 μM and minimal cytotoxicity at 50 μM [28]. A 2.70 Å crystal structure resolution of HIV-1 RT containing an NNRTI and manicol showed that the carbonyl oxygen and both hydroxyls of the tropolone ring coordinate the divalent cations in a manner similar to that previously observed in the RT- β -thujaplicinol structure. However, unlike β -thujaplicinol, manicol was shown to form extensive contacts with the imidazole ring of His539, including contacts with interatomic distances of under 3 Å. Modeling studies further indicated that a number of additional interactions with His539 could be achieved by introducing other groups into manicol. Notably, two manicol analogs inhibited the RNA-dependent DNA polymerase activity, suggesting they may be capable of occupying a second site on HIV-1 RT [28].

Hydroxypyrimidines

Combined electronic analysis of *N*-hydroxyimides, DKAs and hydroxytropolones in the context of dual metal chelation indicated that a doubly-anionic species may be necessary for effective coordination of both Mg^{2+} ions by RNaseH active site inhibitors. However, given their intrinsic problems of low stability under aqueous conditions, pyrimidinol carboxylic acids, previously reported as stable HIV IN [29] and HCV polymerase inhibitors [30], were selected as a scaffold for synthesis of new RNaseH inhibitors [31]. Among this series, the most interesting compound was the di-substituted analog 2-(3,4-dichlorobenzyl)-5,6-dihydroxypyrimidine-4-carboxylic acid (**9**; Figure 4) that selectively inhibited RNaseH activity with an IC_{50} value of 1.2 μM [31]. Preincubating enzyme with inhibitor/ Mg^{2+} prior to adding the RNA/DNA substrate significantly quenched the initial burst of catalysis. However, RNaseH activity more closely resembled the inhibitor-free conditions when Mg^{2+} was absent during preincubation. In conjunction with a similar order-of-addition analysis of β -thujaplicinol, the work of Kirschberg *et al.* supported a metal-dependent mechanism of action for hydroxypyrimidines [31]. Crystal analysis also showed that the divalent cation

activating the water molecule for nucleophilic attack is coordinated by the two phenolic oxygen atoms of **9**. In addition, binding between the C2 aromatic substituent and the imidazole ring of His539 was observed, consistent with the improved potency observed with the methylene linker [31,32]. None of the reported pyrimidinol carboxylic acids, however, displayed antiviral activity *in vivo* [31], which could be ascribed to low cell permeability or high protein binding. In a second report, the *N*-hydroxyquinazolinedione analog 3-hydroxy-6-(phenylsulfonyl)quinazo-line-2,4(1*H*,3*H*)-dione (**10**; Figure 4) was found to inhibit HIV-1 RNaseH activity with an IC₅₀ value of 0.2 μM and be inactive on human H1 RNaseH [32]. The 3-hydroxy-*H*-quinazoline-2,4-dione scaffold of **10** was also co-crystallized with a truncated HIV-1 RNaseH domain (p15-Ec) and demonstrated to coordinate the divalent ions the same manner as **9**. However, in contrast to compound **9**, the phenyl substituent of **10** interacted with the imidazole group of the His539 side chain with a parallel displacement (with two π-ring systems lying parallel to each other when **10** is bound to RNaseH) [32].

Naphthyridinones

The naphthyridine pharmacophore was originally described as an effective HIV-1 IN inhibitor by coordinating divalent ions at the active site [33], and subsequently shown to inhibit HIV-1 RNaseH by Merck Research Laboratories [34]. The naphthyridine derivative ethyl 1,4-dihydroxy-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate (MK1, **11**; Figure 4) inhibited HIV-1 RNaseH *in vitro* with an IC₅₀ value of 0.11 μM and HIV-1 replication with an EC₅₀ value of 2.8 μM, displaying minimal cytotoxicity at 50 μM [34]. A 2.8 Å resolution crystal structure revealed direct binding of MK1 to the RNaseH active site in a single orientation chelating the two cations and making additional interactions with Gly444, Ser499, Ala538, His539, Val552 and Ser553 [34]. Interestingly, crystal studies also showed that another derivative, methyl 6-(4-(diethylamino)phenoxy)-1,4-dihydroxy-2-oxo-1,2,3,4-tetrahydro-1,8-naphthyridine-3-carboxylate (MK3, **12**; Figure 4) (IC₅₀ and EC₅₀ = 0.22 and 9.0 μM, respectively), bound to the enzyme distant from RNaseH site, interacting with Leu100, Val108, Tyr181, Tyr183, Asp186, Leu187, Lys223, Phe227, Leu228, Trp229 and Leu234, which are adjacent to the NNRTI pocket previously identified as the target of hydrazones [34,35].

Additional SAR studies on 1-hydroxy-1,8-naphthyridin-2(1*H*)-one derivatives lead to 4-((4'-(aminomethyl)-[1,1'-biphenyl]-4-yl) methyl)-1-hydroxy-1,8-naphthyridin-2(1*H*)-one (**13**; Figure 4) that inhibited HIV-1 RNaseH activity with an IC₅₀ value of 45 nM, and the RT-associated RDDP and IN activities with IC₅₀ values of 13 and 24 μM, respectively [36]. In single-cycle infectivity assay, **13** inhibited replication of wt and IN inhibitor-resistant HIV-1 with an EC₅₀ value of 0.19 μM and showed a selective index of 17, while it was inactive in multicycle viral replication assays due to high toxicity [36].

Nitrofuran-2-carboxylic acid carbamoylmethyl ester derivatives

Screening of 20,000 low-molecular-weight compounds representing key structural features of three million chemicals identified derivatives of 5-nitro-furan-2-carboxylic acid carbamoylmethyl ester (NAMCE) as RNaseH inhibitors [37]. 5-nitro-furan-2-carboxylic acid adamantan-1-carbamoylmethylester (**14**; Figure 5) and 5-nitro-furan-2-carboxylic acid

[[4-(4-bromo-phenyl)-thiazol-2-yl]-(tetrahydrofuran-2-ylmethyl)-carbamoyl]-methyl ester (**15**; Figure 5) inhibited HIV-1 RNaseH activity with IC₅₀ values of 30 and 27 μ M, respectively, did not inhibit *E. coli* RNaseH, and were poorly active on human RNaseH1. Cell-culture studies showed that **15** inhibited HIV-1 replication with an EC₅₀ value of 25 μ M [37]. Docking studies indicated that the nitrofuranyl group of **14** chelates the two Mg²⁺ ions and orients toward His539, possibly to form a hydrogen bond, while its carbonyl oxygen atom in the amide group may interact with the side chain of Ser553 and its adamantyl group can make hydrophobic contacts with the Lys550 side chain. For **15**, in addition to the above interactions, a nitrogen atom in the thiazole group was proposed to interact with Asp549 by hydrogen bonding [37].

Three series of NAMCE analogs were subsequently synthesized by the following strategies:

- Conversion of the hydrophobic substituent bound to the amide group to other substitutes;
- Conversion of the nitrofuranyl ring to several other chemical structures;
- Conversion of the ester linkage to an amide bond [38].

While most analogs designed according to the first strategy exhibited a similar potency as the lead compounds **14** and **15**, 2-([4-acetoxybenzyl][tert-butyl]amino)-2-oxoethyl 5-nitrofuranyl-2-carboxylate (**16**; Figure 5) inhibited HIV-1 RNaseH with an IC₅₀ value of 0.9 μ M. Analogs designed according to the second and third strategies were mainly inactive. SAR studies showed that:

- The connection of the nitrofuranyl group and the hydrophobic moiety is essential for activity and that its conversion into an amide bond results in loss of activity;
- The hydrophobic moiety connecting to carbonyl carbon could be substituted without loss of activity;
- A nitrofuranyl core is indispensable potency [38].

Molecular modeling studies suggested that NAMCE derivatives occupy the RNaseH active site with their oxygen atoms coordinating the divalent metal ions [38].

Thiocarbamates & triazoles

High-throughput screening efforts at Wyeth Research identified thiocarbamates and triazoles as inhibitors of HIV-1 RNaseH [39]. 2-((2,4-dichlorophenyl)amino)-2-oxoethyl dibenzyl carbamodithioate (**17**; Figure 6) inhibited HIV-1 RNaseH activity with an IC₅₀ of 5 μ M and virus replication with an EC₅₀ of 1.3 μ M, and a selectivity index >100 [39]. Among triazole derivatives, 4-benzyl-3-(benzylthio)-5-(thiophen-2-yl)-4*H*-1,2,4-triazole (**18**; Figure 6) inhibited HIV-1 RNaseH activity with an IC₅₀ of <2 μ M, viral replication with an EC₅₀ of 4 μ M and a selectivity index >130 [39]. Time of addition studies confirmed that these compounds acted at the level of reverse transcription.

Allosteric RNaseH inhibitors

Hydrazones

The first hydrazone derivative with anti-RT activity was *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH, **19**; Figure 7) [40]. BBNH was demonstrated to inhibit both enzymatic activities of HIV-1 RT *in vitro* with IC₅₀ values in the low micromolar range (IC₅₀ = 0.8–3.4 μM), depending on the template:primer used in the assay [40]. BBNH was also active on mutant RTs resistant to NNRTIs and inhibited HIV-1 replication in infected cells. Considering its chelation properties, it was hypothesized that Mg²⁺ coordination was the main mechanism of action for RNaseH inhibition, while allosteric inhibition of RNA-dependent DNA polymerase activity was proposed. According to this hypothesis, BBNH exerted its multitarget inhibition by binding to spatially separate sites in the DNA polymerase and RNaseH domains. The interaction between hydrazones and the RNaseH active site was predicted by molecular modeling, involving π – π stacking between the naphthyl ring and the Tyr501 of the p66 subunit [41]. Site-directed mutagenesis showed that BBNH was sixfold less potent on a Tyr501Trp mutant and completely inactive on Tyr501Arg RT [41]. Further derivatization yielded 4-chlorophenylhydrazone of mesoxalic acid (CPHM, **20**; Figure 7) that likewise inhibited DNA strand transfer reaction by specifically targeting RNaseH (IC₅₀ = 2.2 μM) [42]. CPHM has two carboxylic groups, partially supporting a chelation-related mechanism of action. Interestingly, while CPHM inhibited *E. coli* RNaseH activity, no inhibition of the RNaseH activity of murine or avian RTs was detected.

Substituting the BBNH naphthyl ring with a phenyl ring produced (4-*t*-butylbenzoyl)-2-hydroxy-1-salicylyl hydrazone (BBSH, compound **21**, Figure 7), where the π – π stacking interaction with Tyr501 was lost: consequently, the compound failed to inactivate RNaseH function, but retained its ability to inhibit DNA polymerase activity (IC₅₀ = 4.7 μM) [43]. Moreover, BBNH and BBSH binding were shown to affect the interaction of the p66 and p51 HIV-1 RT subunits, decreasing the Gibbs free energy of dimer destabilization by 3.7–3.8 kcal mol^{–1} [43]. Therefore, *N*-acylhydrazones were proposed to bind to RT at a site overlapping, but distinct from, the NNRTI binding site, and this binding was suggested to affect dimer stability. This hypothesis was confirmed when derivative (E)-3,4-dihydroxy-*N*-((2-hydroxynaphthalen-1-yl)methylene)benzohydrazide (DHBNH, **22**; Figure 7) was co-crystallized with HIV-1 RT and shown to bind to a pocket >50 Å from the RNaseH active site and distinct from the NNRTI-binding site, interacting with active site residues Asp186, Tyr188 and DNA polymerase primer grip residue Trp229 [35]. DHBNH inhibited HIV-1 RNaseH activity noncompetitively with an IC₅₀ value of 0.5 μM [35]. Notably, hydrazone derivatives with bulky substitutions at the *para* position of DHBNH benzoyl ring more effectively inhibited DNA polymerase activity while retaining equivalent RNaseH inhibition ability. This finding was correlated to the possible access of the bulky substitution to the NNRTI-binding pocket [35]. However, a second binding site close to the RNaseH active site was not excluded, since RNaseH inhibition was fully retained even in presence of saturated concentration of nevirapine that should prevent DHBNH binding.

Further molecular docking studies on a series of hydrazone analogs proposed a new binding-mode in which the compound trihydroxybenzoyl naphthylhydrazone (THBNH, **23**; Figure 7) should fill a large pocket formed between RT and the RNA/DNA hybrid substrate, establishing hydrogen bonds with Asn418 and the RNA:DNA substrate and hydrophobic interactions with p66 residues Tyr405, Trp406 and Lys424, as well as p51 residues Pro420, Pro421 and Val423 [44]. Since these residues are close and partially involved in positioning of the RNA primer, hydrazone binding to such a pocket could affect RNaseH primer grip efficacy in RNaseH functioning. Moreover, when the DHBNH naphthyl ring system was replaced with a flexible and extended biphenyl system possessing a carboxylate moiety in the distal phenyl ring, retaining a 3,4,5-trihydroxybenzoyl group in the acylidrazide portion, the analog (E)-3'-((2-(3,4,5-trihydroxybenzoyl)hydrazono)methyl)-[1,1'-biphenyl]-4-carboxylic acid (BHMP07, compound **24**, Figure 7) inhibited both DNA polymerase and RNaseH activity with IC₅₀ values of 0.3 and 0.2 μM, respectively [45]. Notably, deleting one of the three hydroxyl groups (BHMP03, **25**; Figure 7) led to selective inhibition of RNaseH function (IC₅₀ = 0.4 μM). NMR studies showed the interaction of BHMP07 with the RNaseH domain was Mg²⁺ independent, involving residues in the substrate handle region (Asp499, Ala502, Ile505, Ile506 and Ile526) [45]. RNaseH activity of mutant Ala502Phe and Ala502Gly RTs was substantially resistant to BHMP07, BHMP03 and DHBNH [45]. NMR analysis on the interaction between BHMP07 and full-length RT confirmed binding to a site located between the RNaseH active site and the region encompassing helices B and D [13] of the RNaseH domain [46]. DHBNH was also successfully used in virtual screening to identify new scaffolds able to allosterically inhibit both DNA polymerase and RNaseH functions [47,48]. The hydrazonoinдолin-2-one derivative identified (E)-3-(2-(4-(3,4-dihydroxyphenyl)-thiazol-2-yl)hydrazono)indolin-2-one (compound **26**, Figure 6) was shown to inhibit both RNaseH and RDDP activities with IC₅₀ values of 2.0 and 1.4 μM, respectively. Notably, **26** retained significant potency against NNRTI-resistant RT mutants and was not cytotoxic, even though it failed to inhibit virus replication [47,48].

Anthraquinones

Screening of plant natural metabolites amide identified a new series of emodin [49] and alizarin anthraquinone derivatives [50,51] that inhibited both HIV-1 RT and HCV RNA polymerase [15]. Interestingly, compound 1-acetoxy-9,10-dioxo-9,10-dihydroanthracen-2-yl-4-bromobenzoate (KNA-53, **27**; FIGURE 7) inhibited both RNaseH and RNA-dependent DNA polymerase activity of wt and Lys103Asn RTs (IC₅₀ = 22 and 9 μM, respectively) but only the RNaseH function of Tyr181Cys RT (IC₅₀ = 22 μM). Mode of action and molecular dynamic simulation studies suggested a putative anthraquinone binding site in the pocket originally reported for hydrazone derivatives [35,50]. Consequently, it has been hypothesized that anthraquinone binding to RT may influence the manner through which the RNA:DNA hybrid is positioned in the nucleic acid binding cleft, possibly modifying its trajectory toward the RNaseH catalytic site [50].

Naphthalene sulfonic derivatives

Naphthalene sulfonic acid derivatives were first reported to preferentially inhibit the RT-associated DNA polymerase activity (i.e., IC₅₀ = 1–4 μM vs 15–50 μM for RNaseH activity) [52]. Further derivatization yielded (4-hydroxy-7-[[[5-hydroxy-6-[(4

cinnamylphenyl]azo]-7-sulfo-2-naphthalenyl]amino]-carbonyl]amino]-3-[(4-cinnamylphenyl)]azo (KM-1, **28**; Figure 7) that inhibited both RNA-dependent DNA polymerase and RNaseH activities with IC₅₀ values of 24–100 nM [53]. KM-1 was subsequently shown to interfere with nucleic acid binding, suggesting it functioned by distorting RT conformation, thereby mis-aligning the RNA:DNA hybrid at the active site and weakening its binding affinity [54].

Vinylogous ureas & thienopyrimidinones

High-throughput screening of natural and synthetic compounds at the National Cancer Institute identified the vinylogous ureas 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[b]thiophene-3-carboxamide (**29**; Figure 8; IC₅₀ = 2.0 μM) and *N*-[3-(aminocarbonyl)-4,5-dimethyl-2-thienyl]-2-furancarboxamide (**30**; Figure 8; IC₅₀ = 3.2 μM) as modestly potent inhibitors of the HIV-1 and HIV-2 RNases H [55]. In the absence of a crystal structure, protein footprinting implicated p51 residue Cys280 in inhibitor binding, suggesting these compounds occupied a site between the p51 thumb subdomain and the p66 RNaseH domain. Since: first, p51 thumb residues Cys280–Thr290 and p66 RNaseH residues Pro537–Glu546 constitute ~33% of the buried surface at the p51–p66 dimer interface [56,57], and second, x-ray crystallography [6] indicates that binding of the RNA/DNA hybrid is accompanied by movement of the p51 thumb and p66 RNaseH domain as a single unit (Figure 8), **29** and **30** most likely inhibit the subdomain/subunit flexibility that is an integral component of catalysis. Additional structure–activity studies identified the cyclized thienopyrimidinone, 5,6-dimethyl-2-(4-nitrophenyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one (**31**; Figure 8) with sub-micromolar activity *in vitro* (IC₅₀ = 0.85 μM).

To more precisely determine the binding site for this class of RNaseH inhibitor, horizontal alanine scanning between p51 thumb residues Lys275 and Thr286 was combined with a limited vertical scan of Cys280. These studies revealed that enzymes harboring Val276Ala and Arg284Ala were approximately eightfold more sensitive to **31** (IC₅₀ = 0.13 and 0.14 μM, respectively), while p51 mutants Cys280Ala and Thr286Ala were highly resistant (IC₅₀ > 50 μM and 28.2 μM, respectively) [58]. Cys280 vertical scanning mutants were also highly resistant, implicating p51 α-helix I as the site of inhibitor binding. At the same time, the observation that a single Cys280 mutation conferred drug resistance raised the possibility that the equivalent mutation might be easily selected in culture. In order to address this possibility, a panel of drug-resistant and -sensitive recombinant enzymes was created to screen additional thienopyrimidinones with a goal of identifying compounds that were active against drug-resistant variants. This strategy identified 2-(3,4-dihydroxyphenyl)-5,6-dimethylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**32**; Figure 8), a catechol derivative with equal activity against wild type RT (IC₅₀ = 0.26 μM) and the Cys280Ala variant (IC₅₀ = 0.32 μM) [58]. An interesting feature of thienopyrimidinone-derived RNaseH inhibitors is their ability to destabilize HIV-1 RT in both the absence and presence of the nucleic acid substrate, evidenced by a drop in the melting temperature of the enzyme by as much as 5°C [59]. This is in contrast to active site RNaseH inhibitors, which increase the melting temperature by 2–3°C. Last, our recent studies have indicated that while 3,4-dihydroxyphenyl is critical for activity, the thiophene ring can be replaced with an isosteric benzene ring without loss of activity (**33**; Figure 8; IC₅₀ = 0.41 μM) [MASAOKA T *ET AL.*, UNPUBLISHED DATA].

Although there have been toxicity concerns with catechol-containing drugs, based on the ability of the catechol-derived semiquinone radical to participate in production of a superoxide anion radical, the use of apomorphine and entacapone to treat Parkinson's disease, as well as the benzazepine derivative fenoldopam to treat hypertension [60] illustrates it is possible to overcome such toxicity.

Future perspective

Although progress of RNaseH inhibitors towards clinical trials has been challenging, the notion of targeting this critical RT-associated activity should not be dismissed. While the ease that active site inhibitors can be displaced by the nucleic acid substrate is disappointing for further development of this structural class, vinyllogous ureas and thienopyrimidinones provide encouraging evidence that allosteric inhibition of RNaseH activity could be achieved. Indeed, continuing in this direction would have the obvious advantage of avoiding inhibition of related host enzymes that belong to the polynucleotidyl phosphotransferase superfamily, prominent among which is human RNaseH, an enzyme that is essential for removing ribonucleotides that have been mis-incorporated into DNA [61]. The recent crystal structure of HIV-1 RT containing an NNRTI and an RNA/DNA hybrid has clearly shown the necessity for enzyme flexibility in accommodating and processing the RNA/DNA replication intermediate [6]. Thus, targeting small-molecule binding pockets in and around the RNaseH domain with a goal of altering subdomain and subunit motion should be given further consideration. Finally, recent studies by Tavis *et al.* [62] have shown that drug-discovery strategies aimed at other viruses of clinical significance, such as hepatitis B virus can be guided by, and gain from, our experience with HIV-1 RNaseH.

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Financial & competing interests disclosure

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Key Terms

Ribonuclease H:

An activity of the retroviral reverse transcriptase responsible for degrading RNA of the RNA/DNA hybrid produced by reverse transcription.

Reverse transcriptase:

The retroviral enzyme responsible for converting the ssRNA genome into dsDNA.

Nucleoside reverse transcriptase inhibitor:

Incorporated into the growing DNA chain at the DNA polymerase active site but cannot be extended due to a 3' deoxyribose modification.

Non-nucleoside reverse transcriptase inhibitor:

Binds to HIV-1 reverse transcriptase outside the DNA polymerase active site to cause a conformational change that is incompatible with catalysis.

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Executive summary

Reverse transcriptase-associated ribonuclease H & its role in HIV-1 replication

- Ribonuclease H activity nonspecifically degrades RNA of the HIV RNA/DNA replication intermediate.
- RNaseH activity is also required for accurate removal of the (–) and (+) strand RNA primers.
- RNaseH activity is indispensable for HIV replication.

Active site RNaseH inhibitors

- The pharmacophore of most RNaseH active site inhibitors suggests they function by chelating the divalent metal required for catalysis.
- Order-of-addition experiments suggest RNaseH active site inhibitors are ineffective against the enzyme–substrate complex.
- Cellular enzymes of the polynucleotidylphosphotransferase family present a major obstacle when developing active site RNaseH inhibitors.

Allosteric RNaseH inhibitors

- Non-nucleoside reverse transcriptase inhibitors in clinical use illustrate that regions outside the DNA polymerase and RNaseH active sites can be targeted.
- Small molecules binding to the RNaseH domain could alter trajectory of the nucleic acid substrate as it enters the active site.
- RNaseH domain residues contribute significantly to the buried surface at the reverse transcriptase dimer interface, suggesting this as a target for allosteric inhibition.

Figure 1. p66/p51 HIV-1 reverse transcriptase containing an RNA/DNA hybrid.

(A) Fingers, palm, thumb and connection subdomains are color coded blue, red, green and yellow, respectively, with the darker and lighter colors representing the p66 and p51 subunits, respectively. The p66 C-terminal RNaseH domain is depicted in gold. RNA and DNA strands of the hybrid are depicted as magenta and sand-colored spheres, respectively. (B) Close-up of the p66 RNaseH domain containing portions of the RNA/DNA hybrid described by Lapkouski *et al.* [6]. Structural elements have been outlined, and catalytic residues (cyan) are: D¹: Asp498; D²: Asp549; D³: Asp443; E: Glu478. RNA and DNA strands of the RNA/DNA hybrid are depicted in red and blue, respectively. RNaseH: Ribonuclease H.

Figure 2.
N-hydroxyimide and diketo acid-derived HIV-1 ribonuclease H inhibitors.

Figure 3.
Natural product (5 & 6) and synthetic (7 & 8) active site α -hydroxytropolone-derived HIV-1 ribonuclease H inhibitors.

Figure 4.
Hydroxypyrimidine and naphthyridinone-derived HIV-1 ribonuclease H active site inhibitors.

Figure 5. Nitrofuran-2-carboxylic acid carbamoylmethylester-derived HIV-1 ribonuclease H inhibitors.

Figure 6.
Thiocarbamate and triazole-derived HIV-1 ribonuclease H active site inhibitors.

Figure 7.
Hydrazones, anthraquinones and naphthalene sulfonic derivatives as allosteric inhibitors of HIV-1 ribonuclease H activity.

Figure 8. Allosteric inhibition of HIV-1 ribonuclease H activity by vinylogous ureas and thienopyrimidinone and benzopyrimidinone derivatives.

The central cartoon depicts the p66 C-terminal RNaseH domain (gold, with active site carboxylates in magenta) supported by the p51 thumb subdomain (green). α -helix I, proposed as the binding site for compounds **29–33** by a combination of protein footprinting and site-directed mutagenesis, is depicted in yellow. RNaseH: Ribonuclease H.