Selective Inhibition of HIV-1 Reverse Transcriptase (HIV-1 RT) RNase H by Small RNA Hairpins and Dumbbells

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We present here the design of a novel class of RNA inhibitors of the RNase H domain of HIV-1 RT, a ribonuclease activity that is essential for viral replication in vivo. Specifically, we show that small RNA hairpins and dumbbells can selectively inhibit the RNase H activity of HIV-1 RT without affecting other cellular RNases H (e.g., E. coli *and human RNase H). These results suggest that the inhibitors do not interact with the nucleic acid binding site of RT RNase H, as this region should be well conserved among the various enzymes. The most potent inhibitors displayed IC_{50} values in the 3–8 μM range. Remarkably, the DNA polymerase activity, an intrinsic property of HIV RT, was not inhibited by the hairpin and dumbbell aptamers, a property not previously observed for any nucleic acid aptamer directed against RT RNase H. The results described here suggest a noncompetitive binding mechanism, as outlined in the differential inhibitory characteristics of each of the nucleic acid aptamers against the bacterial, human, and viral RNase H homologues.*

Introduction

Reverse transcriptase (RT) plays a central role in the life cycle of the human immunodeficiency virus (HIV),[1] and has been a prominent target in the development of antiviral agents that suppress HIV.[1–3] Current anti-HIV therapeutics (e.g., nucleoside and non-nucleoside reverse transcriptase inhibitors) are directed against the polymerase activity of HIV-1 reverse transcriptase (HIV-1 RT). Unfortunately, prolonged clinical use of these compounds gives rise to drug-resistant forms of HIV-RT, prompting widespread research to generate new anti-HIV-1 RT drugs.

HIV-1 RT has three distinct enzymatic activities: namely, an RNA-dependent DNA polymerase activity, a DNA-dependent DNA polymerase activity, and an RNase H activity. The last of these cleaves the viral RNA strand of a DNA/RNA heteroduplex intermediate, thereby permitting the viral DNA to be released and invade the host cell's genetic material. A search for alternative treatments and more specific antiviral agents has led to a relatively new class of RT inhibitors termed the oligonucleotide reverse transcriptase inhibitors (ONRTIs).[4–7]

The pioneering work by Gold and co-workers led to the discovery of RNA pseudoknots with very potent inhibition profiles.[6] The RNA ligands were selected from a randomized pool of RNA molecules by high-affinity binding to the target protein by the use of SELEX (**S**ystematic **E**volution of **L**igands by **E**xponential **E**nrichment).[7] These RNA pseudoknots, or "aptamers", inhibit HIV polymerase activity at the low nanomolar range by binding competitively to the primer–template junction of the enzyme.[6, 8] In fact, most of the oligonucleotide inhibitors shown to inhibit RT activity do so by competitively inhibiting the polymerase function of the enzyme, without specificity for its RNase H domain.[9–11]

Andreola et al. have described 35-nt aptamers, based on a G-quartet motif, that inhibit both the RNase H and the DNA polymerase activities of HIV-1 RT equally well ($IC_{50} = 500$ nM for both activities) and therefore show poor discrimination between these two sub-domains of the multimeric enzyme.[9] The premise of these aptamers and their selection by SELEX relies upon the presence of sequences rich in guanosine content that are capable of forming G-tetrads prior to enzyme interaction and subsequent inhibition. Other investigators have reported potent aptamers and some of the selected sequences inhibit HIV-1 activity in human cell culture.[12]

There are several other published examples of HIV-1 RT inhibitors that block *both* the DNA polymerase and the RNase H activities. Among these is *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH), studied by Parniak and co-

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 DOI: 10.1002/cbic.200300831 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

workers.[13] Illimaquinone, a natural product of marine origin, inhibits the RNase H activity of HIV-1 RT while also hindering the RNase H functionality of HIV-2 RT, MLV-RT, and *Escherichia coli* [14, 15] Most recently, a diketo acid, 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid, has been shown to bind the RNase H domain selectively and to inhibit it with an $IC_{50} = 3.2\ \mu M$, with only a modest effect on DNA polymerization ($IC_{50} > 50\ \mu M$).[16]

In pursuit of novel antiviral agents, our laboratory has initiated the examination of nucleic acid duplexes as inhibitors of RNase H.[17] Initial investigations revealed that 18 base-pair RNA/RNA and 2′,5′-RNA/RNA duplexes inhibited RNase H by competitively suppressing binding of the DNA/RNA substrate to HIV-1 RT.[17] Although high-affinity binding was observed in vitro, the bimolecular natures of such complexes make them difficult to develop into effective therapeutics since they would not remain in their duplex state following administration. The RNA/RNA and RNA/2′,5′-RNA duplexes also hindered *E. coli* RNase H activity, suggesting that such duplexes may undesirably obstruct cellular RNase H function. Furthermore, the presence of free termini makes them exceedingly susceptible to degradation by ubiquitous cellular nucleases, predominantly of the 3′-exonuclease type.[18]

We demonstrate here that small RNA hairpins and dumbbells can selectively inhibit the RNase H activity of HIV-1 RT without affecting other cellular RNases H (e.g., *E. coli* and human RNase H). Importantly, we find that these RNA hairpin and dumbbell duplexes do not disrupt the DNA polymerase activity, a property not previously observed for any nucleic acid aptamer directed against RT RNase H.

Results and Discussion

For rapid generation of RNA molecules that are able to inhibit HIV-1 RNase H activity, we used solid-phase synthesis to prepare a series of RNA hairpins and dumbbells.[19] Specifically, we used a combinatorial-type approach, in which solid-support beads are split during chain assembly to generate a library of structurally diverse hairpins (see Supporting Information).[20, 21] The RNA dumbbell **9** was synthesized by chemical ligation by following slight modifications of recently published procedures.[22] Representative hairpin structures are listed in Table 1. These small RNAs retain specific helical (A- or B-form) conformations and the hairpins have displayed ample resistance against a variety of nucleases.[23] Compounds **1**, **2**, **4**, and **6** contain the highly stabilizing 3′,5′-linked rUUCG tetraloop structure extensively studied by Tinoco, Varani, and co-workers.[24, 25] The tetraloop residues in compounds **3** and **7** are connected by 2′,5′-phosphodiester linkages and collectively fold into a distinct rigid structure that is unlike the native tetraloop.[26] The nicked dumbbell **8** exhibited biphasic thermal melting profiles, consistent with the presence of nicked double hairpin structures with diverse sequence composition. Thermal dissociation of the ligated dumbbell **9** (and hairpins) displayed a cooperative, unimolecular transition, indicating that cyclization had been realized in the case of the dumbbell RNA (Table 1).

Table 1. *Potency (IC_{50})[a] and melting temperature (T_m)[b] of hairpin and dumbbell inhibitors in enzymatic assays.*

	Code	Oligonucleotide[c]	IC_{50} (μM)	T_m [°C]
1	R_4RR_4	U U U C A G G -5′ C G G U C C -3′	25.8	71.8
2	R_4RR_4	U U U C A G G -5′ C G U C C -3′ G	26.2	45.2
3	R_4RR_4	U U U C A G G -5′ C G G U C C -3′	69.3	68.9
4	D_4RD_4	U U c a g g -5′ C g t c c -3′ G	≫100	54.6
5	R_4DR_4	u u C A G G -5′ c G U C C -3′ g	≫100	63.4
6	R_6RR_6	U U C A G G U G -5′ C G G U C C A C -3′	7.8	n.d.[d]
7	R_6RR_6	U U C A G G U G -5′ C G G U C C A C -3′	29.7	n.d.[d]
8	nicked dumbbell	U G U C A G G t * t U U U G C C G U C C A A A A A C U G G	40.4	43.0, 76.6
9	ligated dumbbell	U G U C A G G t t U U U G C C G U C C A A A A A C U G G	3.3	87.0

[a] IC_{50} values (± 1 μM) are the average of 2 – 3 independent measurements, each of which was calculated from a plot of the outstanding undegraded 5′-[^{32}P]-RNA versus the concentration of RNA hairpin or dumbbell oligonucleotide. [b] T_m values (± 0.5 °C) for nicked and ligated dumbbells represent the average of three successive runs (buffer: 10 mM Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.0). [c] RNA residues are represented by capital letters, whereas small letters indicate DNA residues. Capital underlined letters represent 2′,5′-RNA residues (e.g. *UC* = 5′-$U_{2'p5'}C_{2'p}$). The triangle represents a nicked stem (i.e., no linkage exists between the neighboring T residues, resulting in open 5′-OH and 3′-monophosphate ends). [d] n.d. = not determined.

A systematic study of the inhibitory properties against HIV-1 RNase H (Figure 1), resulted in the identification of hairpins **1** and **6** among the most potent (Table 1, $IC_{50} = 8 – 26\ \mu M$). Substitution of the RNA tetraloop with a more flexible DNA loop (d-uucg; hairpin **5**) completely abolished activity, suggesting that HIV-1 RT distinguishes and recognizes the unusually folded and rigid 3′,5′-rUUCG loop structure[24, 25] as a signal for binding to its substrate. In fact, base mutations within the loop region (UUCG to UACG) of the RNA hairpins completely abolished hairpin activity.

Replacement of the native loop structure with a 2′,5′-linked rUUCG loop leads to inhibitor **3**, with an approximately threefold

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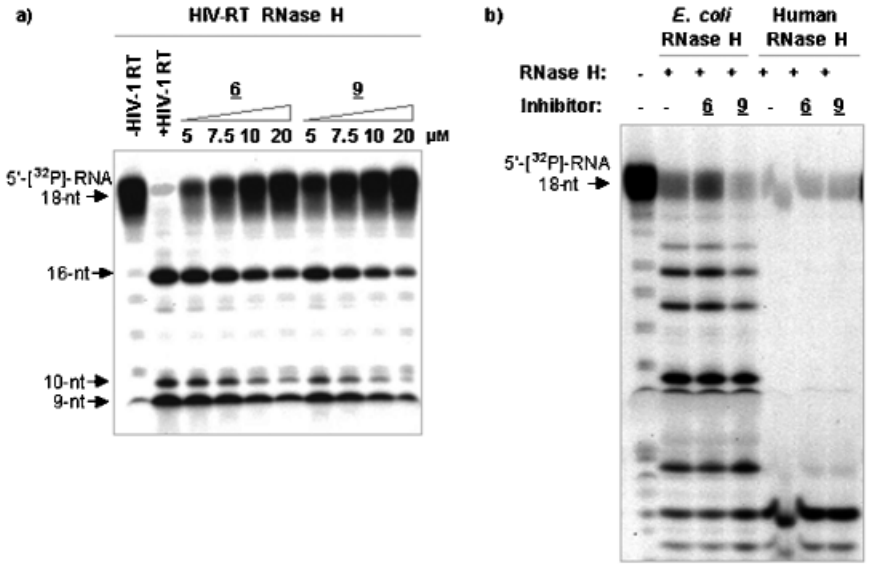

Figure 1. *a) Inhibition of HIV-1 RT RNase H (p66/p51 heterodimer) by hairpin (**6**) and dumbbell aptamer (**9**). b) Specificity of inhibition detected by RNA aptamers.*

decrease in potency in relation to compound **1** (Table 1; IC_{50} = 69 μM), signifying that while the 2′,5′-UUCG loop is well tolerated, the enzyme attains more favorable interactions with the native tetraloop structure (see also **6** vs. **7**; Table 1). Further evidence suggesting that HIV RT RNase H interacts with the loop arises from mutation experiments conducted within the 2′,5′-UUCG loop. For example, mutation of the 2′,5′-UUCG loop to 2′,5′-UACG completely abolishes inhibitory activity. This mutation would be expected to disrupt loop conformation because its position is central to *intraloop* tertiary folding.[26]

No decrease in potency was observed upon replacement of the native 3′,5′-internucleotide linkages in the *stem* of hairpin **1** with 2′,5′-phosphodiester linkages to produce the corresponding hairpin analogue **2** (Table 1, **1** vs. **2**). This is consistent with the observation that **1** and **2** each display a global A-type helical arrangement (CD data; not shown), which has been shown to be the preferred helical geometry for effective RNase H binding.[17, 27] Consistent with the notion that the activity of these inhibitors is dependent on helical conformation is the observation that hairpin **4**, in which the stem is a B-form DNA duplex and the loop is 3′,5′-UUCG, displays no activity (Table 1).

The degree of inhibition appeared to correlate directly with stem length as well. Specifically, when the stem was composed of six base pairs (hairpin **6**) rather than four base pairs (hairpin **1**) a threefold enhancement in potency was evident (Table 1). This suggests that longer RNA/RNA duplexes are better accommodated in the RNase H domain of HIV-1 RT. Incorporation of a second stabilizing loop motif by the creation of a "double hairpin" (i.e., "nicked dumbbell" **8**) structure did not increase the inhibitory potency; rather, biological activity was severely compromised (Table 1; compare **1** to **8**). Contrarily, the ligated dumbbell **9**, which also contained two UUCG loop motifs, was the most potent of all the molecules studied (Table 1, IC_{50} = 3.3 μM and Figure 1 a). From these observations, we propose that HIV-RT requires only one loop structure for ample recognition and binding, but a longer and uninterrupted stem region is requisite for grasping and positioning of the substrate within its binding domain. Regardless, the second loop motif of the dumbbell may play an essential role in vivo, by stabilizing the structure against ever-prominent exonucleases present in the biological milieu.

At a low concentration of inhibitors (i.e., 5 μM), neither the nicked form of the dumbbell (i.e., **8**) nor the most potent short RNA hairpin (**1**) displayed any inhibitory activity (Figure 2). Conversely, both the longer RNA hairpin (**6**) and the ligated RNA dumbbell (**9**) effectively inhibited the HIV-1 RT RNase H-mediated degradation of the RNA strand (Figure 2 a). Nonetheless, the activity of the RNA dumbbell **9** maintained nearly double the potency of hairpin **6** (Figure 2 b).

In an effort to verify the specificity of HIV-1 RT RNase H inhibition and to

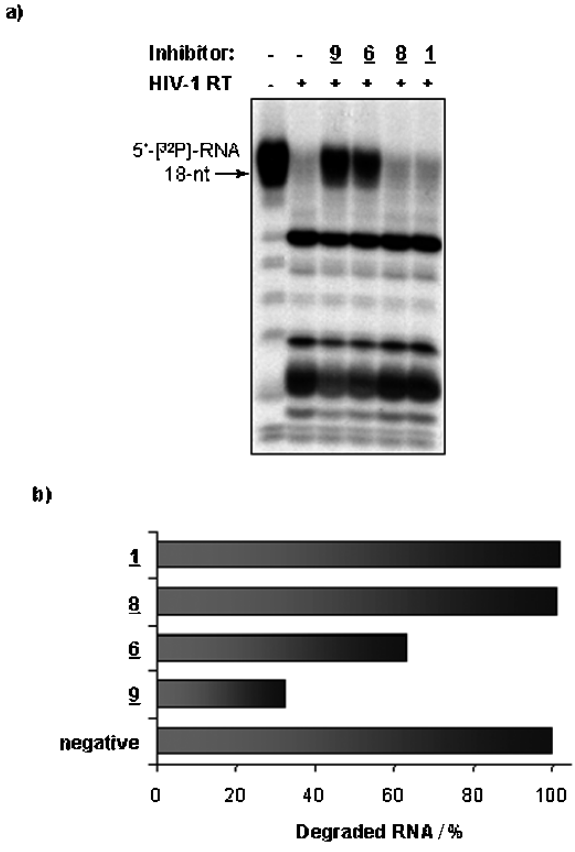

Figure 2. *a) Autoradiogram comparing the RNase H inhibitory potency of hairpin RNAs **6** and **1** with that of nicked (**8**) and RNA dumbbell (**9**) at low inhibitor concentration (5 μM). HIV-1 RT was incubated with a 5′-[32P]-RNA/DNA heteroduplex substrate (see Experimental) and was supplemented with 5 μM of cold hairpins, nicked dumbbell, or ligated dumbbell. b) Chart demonstrating the inhibitory potency of hairpin and dumbbell RNAs at low inhibitor concentration (5 μM). The % degraded RNA was determined by densitometric quantitation of the remaining radiolabeled RNA in the autoradiogram (Panel a).*

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substantiate the potential use of hairpin and dumbbell structures as anti-HIV agents, we tested their ability to inhibit both bacterial and mammalian homologues of RNase H. The same 5′-[^{32}P]-RNA/DNA heteroduplex that was used in the inhibition profiles above was utilized in this assay. Duplexes were incubated with *E. coli* RNase H or human RNase H (type II) either in the absence or in the presence of the hairpin (**6**) or ligated RNA dumbbell (**9**) inhibitors (Figure 1 b). RNase H inhibition was monitored by comparing the amount of undegraded RNA present in the reaction to that in the absence of any inhibitor. Neither the RNA hairpin (**6**) nor the dumbbell (**9**) structures had any effect on the bacterial or human RNase H-mediated degradation of the RNA template strand, indicating an effect remarkably specific toward the retroviral RNase H domain (Figure 1 b).

In order to exclude the possibility that the RNA structures were acting as polymerase inhibitors and indirectly affecting the RNase H function of RT, the compounds were tested directly against the DNA polymerase activity of the enzyme. The effects on both the RNA-dependent and DNA-dependent polymerase reactions were tested with either a 5′-[^{32}P]-DNA primer/RNA template or a 5′-[^{32}P]-DNA primer/DNA template complex (Figure 3). Indeed, the results obtained indicated that neither the DNA-dependent nor the RNA-dependent DNA polymerase functionalities of HIV-1 RT was compromised in the presence of either hairpin (**1**, **2, 6**; data not shown), or nicked and ligated forms of the dumbbells (**8**, **9**; Figure 3). These results indicate that the RNA hairpin and dumbbell molecules do indeed act as aptamers toward HIV-1 RT and, most importantly, they inhibit its RNase H functionality without any consequence on its polymerase activity.

Finally, to confirm that the RNA aptamers were binding specifically to the RNase H domain of HIV-RT, we conducted a UV crosslinking experiment between the most potent hairpin (**6**) or dumbbell (**9**) and either the HIV-1 RT heterodimer (p66/p51), containing both the DNA polymerase and RNase H domains, or the homodimer (p51/p51) consisting of only a functional DNA polymerase domain. The p66 monomeric subunit of HIV-1 RT is proteolytically processed to form both a p51 and p15 subunit, resulting in an RNase H-deficient product.[28] In the virion particle, p66 is always found in stable association with the p51 subunit, and it is this resultant heterodimeric entity that displays the full functionality of the HIV-1 reverse transcriptase.[29, 30] By taking advantage of the natural photoreactivity of the RNA bases at 254 nm, it may be possible to form a stable, localized complex (i.e., crosslink) between the RNA dumbbell aptamers and the RNase H domain located in the C-terminal portion of the p66 subunit. Generally, when a covalent crosslink is formed between an enzyme and its oligonucleotide substrate, the complex exhibits an altered and retarded electrophoretic mobility in relation to its unbound state. In the case of hairpin **6**, the oligonucleotide was radioactively labeled at the 5′-terminus so that a complex could easily be visualized by autoradiography (Figure 4 a, b). However, in the case of the RNA dumbbell **9**, both the 5′- and the 3′-termini of the molecule are engaged in a circularized structure, so introduction of a terminal radiolabel was not feasible. In this case, complex formation was detected by monitoring of the change in the electrophoretic mobility of the protein subunits themselves on a denaturing SDS gel, followed by staining of the protein complex (Figure 5). The results clearly demonstrate that the hairpin or ligated RNA dumbbell do not form a covalent complex with the p51/p51 homodimer, which lacks the RNase H

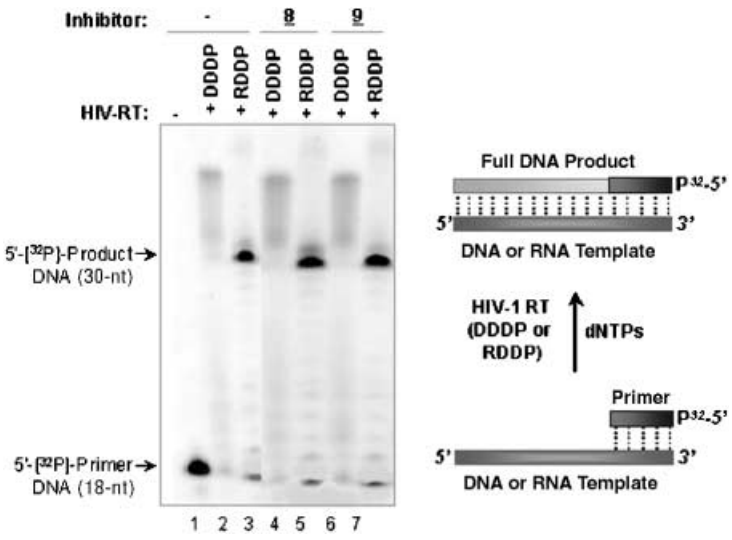

Figure 3. *Inhibition of DNA synthesis catalyzed by HIV-1 RT DNA-dependent (DDDP) or RNA-dependent (RDDP) DNA polymerase activity by nicked (**8**) and ligated (**9**) RNA dumbbells. Polymerization reactions were conducted for 15 minutes both in the absence (−) and in the presence (+) of cold RNA dumbbells. In the absence of inhibitor, a new, full-length DNA oligonucleotide product was formed both from the RNA primer (RDDP activity; lane 3) and from the DNA primer (DDDP activity; lane 2). Owing to its higher mass to charge ratio, the newly synthesized DNA complement (30 nucleotides) exhibits a retarded electrophoretic mobility relative to its corresponding 18-nucleotide primer on a denaturing gel. In all cases, the band corresponding to the full-length complementary DNA product synthesized by the DDDP activity was highly diffused.*

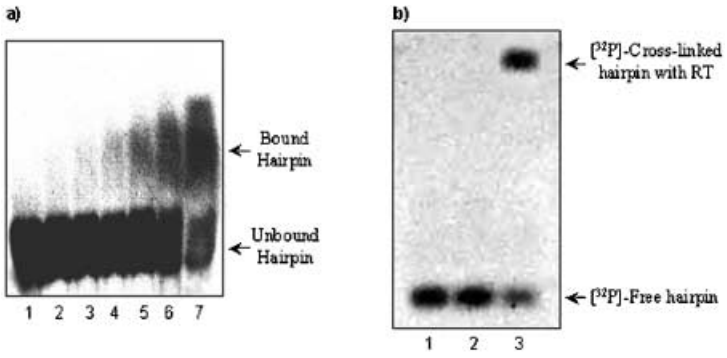

Figure 4. *a) Gel-shift binding assay to determine formation of [HIV-1 RT]/[hairpin **6**] complex. Binding of HIV-1 RT to [^{32}P]-labeled-hairpin **6** was carried out in the presence of increasing concentration of the p66/p51 RT. Lane 1: control lane with no enzyme present. Lanes 2 – 7: binding of hairpin to HIV-1 RT [at amounts of 1 ng (lane 2), 2 ng (lane 3), 5 ng (lane 4), 10 ng (lane 5), 20 ng (lane 6), and 100 ng (lane 7)]. The gel autoradiograph was exposed to an X-ray film. b) UV crosslinking experiment with hairpin **6** and HIV-1 RT. Lane 1: hairpin **6** alone. Lane 2: p51/p51 HIV-1 RT homodimer and hairpin **6**. Lane 3: p66/p51 HIV-1 RT heterodimer and hairpin **6**.*

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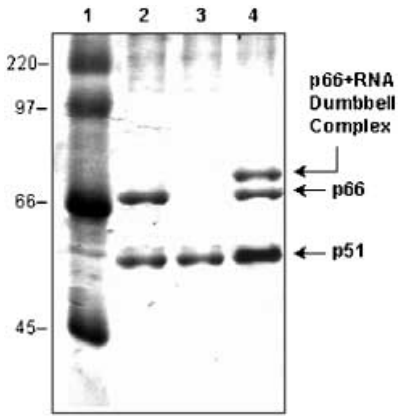

Figure 5. *UV crosslinking analysis of HIV-1 RT with ligated RNA dumbbell* ***9****. Lane 1: molecular weight markers (in KDa). Lane 2: HIV-1 RT p66/p51 heterodimer (no dumbbell inhibitor). Lane 3: HIV-1 RT p51/p51 homodimer (only polymerase domain—no RNase H domain) irradiated with UV in the presence of RNA dumbbell* ***9****. Lane 4: HIV-1 RT p66/p51 heterodimer (both polymerase domain and RNase H domain) irradiated with UV in the presence of RNA dumbbell* ***9****.*

binding domain (Figure 4b, lane 2 and Figure 5, lane 3). Conversely, aggregate formation with the p66/p51 heterodimeric species was manifest, as evidenced by the presence of a more slowly migrating product complex on the SDS-PAGE (Figure 4b, lane 3 and Figure 5, lane 4). Furthermore, the covalent complex formed between the p66 subunit and the RNA dumbbell **9** displayed a molecular weight consistent with the expected value of about 75 KDa. These findings clearly ascertain that **6** and **9** do not bind the DNA polymerase region of HIV-1 RT, and are instead highly specific toward the RNase H domain of the enzyme.

At present, we do not know exactly how our compounds inhibit HIV RT RNase H, but we suspect that inhibition does not arise from a competitive effect. This is because the polymerase and RNase H domains in HIV RT overlap, so it is conceivable that an inhibitor binding to this region of the enzyme might block both activities. Secondly, the fact that the hairpins do not inhibit *E. coli* and human RNase HII implies an allosteric or noncompetitive effect on HIV-RT RNase H activity. This is a reasonable assumption, since *E. coli* and HIV RNase H are close homologues and lack a dsRNA binding domain. To elaborate, the intrinsic binding affinity of these enzymes towards RNA duplexes follows the order: human $>$ *E. coli* $=$ HIV.[17, 31] The complete absence of inhibition for both *E. coli* and human homologues at the concentrations of hairpin/dumbbell used here (i.e., 25-fold higher concentration of aptamer vs. IC_{50} with HIV RT), however, indirectly suggests that the inhibitor does not interact with the nucleic acid binding site of RT RNase H, as this region should be well conserved among the various enzymes. This observation is even more profound given that the human enzyme generally binds A-form duplexes more tightly, exhibiting a 10- to 20-fold greater affinity than the bacterial enzyme.[31] Thus, if the inhibition were to arise from a competitive effect, then one would expect the human enzyme to be most susceptible, perhaps even more so than that observed with the retroviral counterpart. We propose that the loop rather than the A-form helical arrangement is the main determinant of inhibition by our hairpin and dumbbell constructs, and this is consistent with a high inhibitory profile for nucleic acid structures containing RNA vs. DNA loops linking the helical stems. Furthermore, the results described here suggest a noncompetitive binding mechanism, as outlined in the differential inhibitory characteristics of each of the nucleic acid aptamers against the bacterial, human, and viral RNase H homologues.

Conclusion

In summary, we have designed highly specific RNA-based inhibitors of HIV-1 RT that are capable of selectively hampering the RNase H-mediated activity of the retroviral enzyme. The most potent construct, a ligated RNA dumbbell consisting of a 10-base-pair stem and two flanking rUUCG loop motifs, demonstrated potent inhibition of RNase H with an IC_{50} in the 3 μM range, without any consequence on the RT's polymerase activity. Preliminary nuclease stability assays on the hairpin inhibitors support the notion that they are highly stabilized against cellular nucleases; this makes them good candidates for in vivo studies. Importantly, the RNA inhibitors did not inflict any effect on mammalian RNase H activity (human RNase H type II); this signifies that such compounds should not interfere with cellular RNase H function were they developed into clinically useful anti-HIV agents.

Experimental Section

RNA aptamers and enzymes: Hairpins and dumbbell oligonucleotides were synthesized by standard phosphoramidite chemistry by published protocols,[19, 21, 32] and full details will be published elsewhere.[20] The p66- and p51-kDA subunits of HIV-RT were prepared by cloning into a pBAD/HisB prokaryotic expression vector (Invitrogen) between the XhoI and HindIII sites of the plasmid. The RT p66/p51 heterodimers and p51/p51 homodimers were purified as described previously by Fletcher and co-workers.[33]

UV melting denaturation measurements: T_m values for nicked and ligated dumbbells (Table 1) were obtained as described previously[32] and represent the average of three successive runs and are within $\pm 0.5\,^{\circ}C$. The buffer used was 10 mM Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.0.

Inhibition of the RNase H activity of HIV-1 reverse transcriptase: The hybrid substrate for RNase H was prepared by labeling the 5′-hydroxy termini of the RNA sequence 5′-GAU CUG AGC CUG GGA G-CU-3′ by the transfer of ^{32}P from [γ-^{32}P]-ATP in a reaction catalyzed by bacteriophage T4 polynucleotide kinase. This labeled RNA was annealed to its complementary unlabeled DNA sequence, 5′-AGC TCC CAG GCT CAG ATC-3′, to form the [^{32}P]-RNA/DNA hybrid substrate. Variable amounts of cold RNA hairpins and nicked and ligated RNA dumbbells were pre-incubated in 10 μL of 50 mM Tris-HCl (pH 8.0), containing 60 mM KCl, 2.5 mM $MgCl_2$, and 1.5 nM p51/p66 heterodimeric RT at 37 °C for 15 minutes. The reactions were initiated by the addition of [^{32}P]-RNA/DNA hybrid duplex substrate (50 nM final concentration), and the individual assay tubes were incubated for a further 15 min at 37 °C. An equal volume of gel loading dye (98% deionized formamide containing 10 mM EDTA, 1 mg mL^{-1} bromophenol blue, and 1 mg mL^{-1} xylene cyanol) was added to the samples, and the reaction products were denatured by heating at 100 °C for 5 minutes. The degradation products were

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resolved on a 16% (19:1 crosslinking of acrylamide/bis-acrylamide) polyacrylamide sequencing gel (7 M urea) and visualized by autoradiography. The extent of cleavage of the 18-nt RNA portion of the RNA/DNA hybrid was determined quantitatively by densitometric analysis (UN-SCAN-IT software, Silk Scientific) of the disappearance of the full-length RNA and/or the appearance of any smaller degradation products. The IC_{50} values for RNA aptamer inhibition of HIV-RT associated RNase H activity is the amount of aptamer molecule required to inhibit the HIV-1 RT RNase H mediated degradation of a substrate DNA/RNA hybrid by 50%, and were calculated from plots of the residual undegraded 5′-[^{32}P]-RNA versus dumbbell concentration.

***E. coli* and human RNase H inhibition assays**: RNA aptamer molecules were tested for their ability to inhibit either the *E. coli* or human (type II) RNase H activities. RNase H-mediated degradation assays were supplemented with 60 μM of cold RNA aptamers under conditions identical to those used for HIV-RT RNase H. The degradation products were quantified from the autoradiogram by use of the UN-SCAN-IT software program.

UV crosslinking of RNA aptamers to HIV-1 RT: Homodimeric (p51/p51) and heterodimeric (p66/p51) HIV-1 RT enzymes (500 ng) were incubated with RNA hairpin **6** or ligated RNA dumbbell **9** (50 pmol) in 50 mM Tris (pH 7.8), 50 mM KCl, and 5 mM $MgCl_2$ for 30 min at 37 °C. The reaction mixtures were placed on ice and irradiated with a handheld UV light ($\lambda = 254$ nm) for 15 min. Samples were denatured by addition of 2× sample loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 125 mM Tris, pH 6.8, and bromophenol blue) and heated at 100 °C for 5 min. Protein complexes were partitioned on a 12% SDS-PAGE gel run at constant voltage (160 V). The ^{32}P-RNA hairpin – protein complexes were visualized by autoradiography, whereas dumbbell – protein complexes were visualized by staining as follows. Firstly, the gel was fixed with fixing solution (12% (w/v) trichloroacetic acid, 3.5 (w/v) 5-sulfosalicylic acid) for 30 min and then stained with Coomassie® Brilliant Blue G/perchloric acid solution (0.04% (w/v) Brilliant Blue G in 3.5% (w/v) perchloric acid); Sigma – Aldrich) for 60 min and finally rinsed with distilled water. Complexes were run alongside molecular weight markers consisting of ovalbumin (45 KDa), bovine serum albumin (66 KDa), phosphorylase B (97 KDa), and myosin (220 KDa).

Gel-shift binding assay: The binding of hairpin **6** to HIV-1 RT was measured by electrophoretic mobility shift assay (native conditions). The 5′-[^{32}P]-labeled hairpin was heated to 95 °C and then left to anneal slowly at room temperature for one hour. The binding reaction assays consisted of 50 mM Tris/HCl, pH 7.8, 50 mM KCl, 10% glycerol, various amounts of HIV-1 RT and 0.3 pmol of the labeled annealed hairpin in a final volume of 25 μL. The reaction mixtures were incubated for 20 min at room temperature, and then analyzed on a 6% polyacrylamide (non-denaturing) gel in 25 mM Tris/glycine/1 mM EDTA, pH 8.0 at 160 V for about 1 hr. The gel autoradiograph was exposed to an X-ray film. Complex formation between the [^{32}P]-labeled hairpin and HIV-1 RT was detected (with increasing enzyme concentration) from the apparent retarded electrophoretic mobility of the hairpin as a result of its association with the enzyme.

Inhibition of HIV-1 reverse transcriptase DNA polymerase activity: The assays described below were performed in order to determine whether the inhibition of HIV-1 RT RNase H activity by RNA dumbbells was specific to this domain of the protein, without inhibition of its DNA-dependent or RNA-dependent DNA polymerase activity (i.e., HIV-1 RT-mediated synthesis of DNA).

(a) RNA-dependent DNA polymerase activity assay: The unlabeled, 30-nt RNA template, 5′-AUC UCU AGC AGA GGC GCC CGA ACA GGG ACA-3′ (threefold molar excess), was annealed to a 5′-[^{32}P]-end labeled complementary DNA primer—5′-TGT CCC TGT TCG GGC GCC-3′—in a separate reaction vessel. The RNA aptamers (80 μM) were preincubated with the enzyme at room temperature for 20 min prior to reaction. Polymerase reactions were carried out in a 10 μL volume, containing 50 mM Tris-HCl (pH 8.0), 60 mM KCl, and 2.5 mM $MgCl_2$. The reaction was initiated by the addition of RNA template/5′-[^{32}P]-DNA primer complex and deoxynucleotide triphosphates (dNTPs, 200 nM final concentration of each) and incubated at 37 °C for 15 minutes. The polymerase activity was deactivated by the addition of an equal volume of formamide loading dye (98% deionized formamide containing 10 mM EDTA, 1 mg mL^{-1} bromophenol blue, and 1 mg mL^{-1} xylene cyanol) and denatured by heating at 100 °C for 5 minutes prior to gel analysis (16%, 7 M urea). The gel was visualized by autoradiography and the amount of DNA synthesized was quantified by densitometric analysis by use of the UN-SCAN-IT software program.

(b) DNA-dependent DNA polymerase activity assay: The ability for RNA aptamers to inhibit DNA synthesis from a DNA template stand was assessed under conditions similar to those used above in the RNA-dependent DNA polymerase assay. The 5′-[^{32}P]-DNA primer above was annealed to a threefold molar excess of DNA template, 5′-ATC TCT AGC AGA GGC GCC CGA ACA GGG ACA-3′. All other conditions for polymerization and analysis were identical to those described above.

Acknowledgements

This work was supported by a grant from the National Sciences and Engineering Research Council of Canada. R.N.H. and S.C. gratefully acknowledge financial support from NSERC (Canada) and FCAR (Québec) in the form of postgraduate scholarships. We thank Maria Mangos for thoughtful criticisms and suggestions, and for proofreading the manuscript.

Keywords: antiviral agents · HIV · reverse transcriptase · RNA structures

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Received: November 25, 2003 [F 831]

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