Inhibition of HIV-1 replication by RNA targeted against the LTR region

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Objective: The use of small RNA molecules able to effect gene inactivation has emerged as a powerful method of gene therapy. These small inhibitory RNAs are widely used for silencing malignant cellular and viral genes. We have assayed a series of inhibitory RNAs named catalytic antisense RNAs, consisting of a catalytic domain, hairpin or hammerhead ribozyme, and an antisense domain. The aim of the present study was to evaluate the effect of these inhibitory RNAs on HIV-1 replication.

Methods: A series of expression vectors has been constructed for the intracellular synthesis of inhibitory RNAs, differing in the promoter that drives their synthesis. These inhibitory RNAs were designed to act at two possible cleavage sites in the long terminal repeat (LTR) region and the TAR domain was chosen as a target for the antisense domain. We have evaluated the effects of different inhibitory RNAs in HIV replication via changes in p24 antigen levels. Mobility shift assays have been used to check the binding capacity of inhibitory RNAs.

Results: Catalytic antisense RNA designed to target the LTR region of HIV-1 inhibited viral replication in an eukaryotic cell environment by more than 90%. The conventional hairpin and hammerhead ribozymes, however, failed to inhibit viral replication.

Conclusions: The data provide preliminary evidence of a new class of inhibitory RNAs that can be used to block HIV replication. The results clearly show the importance of the *ex vivo* antisense effect in the inhibition achieved. A good correlation was found between the *in vitro* binding efficiency of the inhibitor RNA to the HIV-1 LTR and the inhibition of viral replication.

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Introduction

The generation of RNA-based molecular tools with applications in biotechnology and medicine is currently of great interest. Allosteric ribozymes have emerged as powerful tools for the production of biosensors [1,2], antisense RNAs and RNA decoys have been successfully used for silencing cellular and viral genes [3–7], ribozymes have been designed to specifically cleave or repair RNA substrates (reviewed in [8]), and small inhibitory (si)RNAs are now routinely used for knocking out genes in various organisms (reviewed in [9]).

HIV-1 RNAs have been favorite targets in the development of inhibitory RNA. Ribozymes [10–12], antisense RNAs [13–15], RNA decoys [6,16–18] and siRNAs [19,20] have all been designed to specifically target different viral genes, as well as cellular genes

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necessary for viral replication [21]. Current HIV-1 therapy is based on the use of a combination of different antiviral agents aimed at reducing plasma viral load and improving patient quality of life [22–24]. The same principle is adhered to in RNA-based antiviral approaches. A strategy to overcome the great variability of the virus is to combine the use of different ribozymes with different specificities [25,26]. Some authors have also described the effectiveness of combining different inhibitor RNA to fight HIV-1 infection [20,27].

The HIV-long terminal repeat (LTR) region plays an important role in the viral cycle [28], and so it is often chosen as a target for inhibitory RNAs [14,29–31]. The LTR region acts as a promoter of viral transcription and has been described as a regulatory checkpoint for controlling different leader functions through changes in its conformation [32].

Our group developed and characterized *in vitro* the catalytic antisense RNAs [33,34], a new class of inhibitory RNAs. These are hybrid molecules composed of a catalytic motif (hairpin or hammerhead ribozymes) and a stem–loop antisense motif. The latter, complementary to a stem–loop domain present in the corresponding substrate molecule, is covalently linked to the 3′ end of the ribozyme. Catalytic antisense RNAs combine two inhibitory features: they bind efficiently to the substrate RNA (antisense effect) and catalyze its specific cleavage. We have demonstrated *in vitro* the ability of the catalytic antisense RNAs to cleave HIV-1 LTR RNA, using TAR as an anchoring site [34]. The inhibitory RNAs used consisted of a TAR complementary domain, named αTAR, linked to a hairpin (HP) or hammerhead (HH) ribozyme specifically designed to cleave the LTR region at positions +113 and +159. These two sites were previously shown to be effective targets for these catalytic motifs [29,35]; we demonstrated that the catalytic antisense RNAs cleave the LTR region more efficiently than conventional ribozymes at either of these positions [33,34]. Moreover, they showed improved substrate binding and gained better access to their target sequences inside folded RNA. Here we provide preliminary evidence of a new class of inhibitory RNAs. We report that these catalytic antisense RNAs achieve up to 90% inhibition of HIV-1 replication in eukaryotic cells, as measured by reductions in p24 levels.

Materials and methods

Construction of the anti-LTR catalytic antisense RNAs expression vectors

CMV-derived vectors

CMV-derived vectors were obtained by cloning the catalytic antisense RNA coding sequences between the *Eco*RI and *Xba*I sites of the pcDNA3 vector (InvitroGen). This vector contains the cytomegalovirus (CMV) promoter as well as the BGH polyA sequence. The inhibitory RNA coding sequences were obtained from the previously described pG3HP113αTAR, pG3HP159αTAR, pG3HH113αTAR and pG3HH159αTAR vectors [34]. The vectors in this series were named pcHP113αTAR, pcHP159αTAR, pcHH113αTAR and pcHH159αTAR.

tRNA-derived vectors

tRNAval-derived vectors were obtained by cloning the catalytic antisense RNA coding sequences between the *Kpn*I and *Eco*RV sites of the pUC-tRNA/KE vector [36], kindly provided by Dr. K Taira (University of Tokyo). This vector contains the tRNAval promoter as well as a linker sequence that ensures appropriate transcript folding for ribozyme activity and localization [36]. Inhibitory RNA coding sequences were obtained by PCR amplification from the CMV-derived vectors using primers 5′-HP113U6 (5′-ATATGCGGTACCACAACAAGAAGGCAACCA-3′), 5′-HP159U6 (5′-ATATGCGGTACCCACACTAGAAGCAAACCA-3′), 5′-HH113U6 (5′-ATATGCGGTACCACAACACTGATGAGTCCG-3′) or 5′-HH159U6 (5′-ATATGCGGTACCCACACTCTGATGAGTCCG-3′) and 3′-RzstRNA (5′-ATATGCGATATCGGATCCGGGTCTCTCTG-3′; restriction sites underlined).

U6-derived vectors

The pU6 vector was obtained by cloning the U6 promoter sequence [37] into the *Bgl*II and *Dra*III sites of pcDNA3. The U6 gene cassette used for its expression contains the sequence upstream of the human U6 gene from positions −1 to −265, the U6 RNA sequence from nucleotides +1 to + 27, and a 3′ stem as a transcript terminator. This vector ensures a high level of transcription, nuclear localization, and high intracellular stability [37]. The restriction sites used for cloning the inhibitory RNA into the pU6 vector were *Kpn*I and *Apa*I. pU6-derived vectors were therefore obtained by cloning different inhibitory RNA coding sequences between the *Kpn*I and *Apa*I restriction sites. Catalytic antisense RNAs coding sequences were obtained by amplifying the CMV-derived vectors with the already described primers 5′-HP113U6, 5′-HP159U6, 5′-HH113U6 or 3′-HH159U6, and 3′-RzsU6 (5′-ATATGCGGGCCCGGATCCGGGTCTCTCTG-3′; site underlined is *Apa*I). The vectors obtained were named pU6HP113αTAR, pU6HP159αTAR, pU6HH113αTAR and pU6HH159αTAR. The αTAR coding sequence present in vectors pU6HP159αTAR and pU6HH113αTAR was eliminated to yield the pU6HP159 and pU6HH113 control vectors. These were used to determine the intracellular activity of conventional HP159 and HH113 ribozymes. Vectors pU6HP159mutαTAR and pU6HH113mutαTAR, used for the expression of the inactive catalytic antisense RNA, were obtained by cloning the inactive ribozyme coding sequences between the *Kpn*I and *Bgl*II sites of pU6HP159αTAR and pU6HH

113αTAR respectively. The HP159mut coding sequence was obtained by the annealing and extension [33] of the primer pair 5′-HP159U6 and 3′-HPmut (5′-CAAGATCTTACCAGGAGATGTACCACGACTTA TA CGTCGTGTGTTT-3′; mutations introduced to avoid catalytic activity are underlined). The annealing and extension of primers 5′-HH113mutU6 (5′-GGGTAC-CACAACACTAATGAGTCCGTGAGG-3′) and 3′-HH113mutU6 (5′-CAAGATCTTGCCCGTTTCGT CCTCACGGACTC-3′) was undertaken to obtain the HH113mut coding sequence.

To obtain the pU6αTAR vector, the αTAR coding sequence was obtained by the annealing and extension of the primer pair 5′αTARU6 (5′-CGGGTTCCCTAGT TAGCCAGAGAGCTCCCAGGCTCAAATCTGGTC TAACCAGAGAGACCCGGGCC-3′) and 3′αTARU6 (5′-CGGGTCTCTCTGGTTAGACCAGATTTGAG CCTGGGAGCTCTCTGGCTAACTAGGGAACCC GGTAC-3′). Oligonucleotides were previously phosphorylated with T4-polynucleotide kinase (New England Biolabs) and the resulting product (which already has *Kpn*I–*Apa*I compatible ends) cloned into the pU6 vector.

Determination of inhibitory RNA intracellular activity

The U87-CD4-CXCR4 glioma cell line [38] was used to assay the activity of the inhibitory RNA. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 50 000 U/l penicillin and 50 mg/l streptomycin. Cells (1×10^5) were co-transfected with 0.5 μg inhibitory RNA-expressing vector, and 1 μg pNL4-3 proviral DNA, using the LipofectAMINE-2000 reagent (Life Technologies, London, UK). Controls were performed by transfecting 0.5 μg of the empty vector and 1 μg of the pNL4-3 plasmid. Five days after transfection, HIV-1 replication in control cells, and in cells expressing the inhibitory RNAs, was evaluated by determining p24 release. This was done in duplicate using a commercial p24 antigen enzyme-linked inmunosorbent assay kit (Innogenetics, Madrid, Spain). All assays were performed twice. To calculate the inhibitory effect of the different inhibitory RNA, the amount of p24 antigen determined for the control sample (empty vector-transfected) was normalized to 100, and the results for test samples converted to a corresponding percentage. All calculations were made using data from at least three replicates.

In vitro binding assays

Catalytic antisense RNAs and LTR substrate RNA were obtained by *in vitro* transcription as previously described [34]. The αTAR coding sequence was obtained by PCR amplification of the plasmid pU6HP159αTAR with the oligonucleotides P1-5′-TAR(-10) (5′-GCGAATTC TAATACGACTCACTATAGGGGGTTCCCTAGT TAGCC-3′) and P2-3′-TAR(-10) (5′-GCGGATCCGG GTCTCTCTGGTTAGA-3′). The resulting product, which already has the T7 promoter (underlined), was used as a template for the *in vitro* transcription reaction to yield the αTAR RNA.

Electrophoretic mobility shift assays were performed in 5% (w/v) TBE native polyacrylamide gels at 4°C. The dissociation constant (*K*d) of the different inhibitory RNA–LTR complexes was deduced from the shift of 2 nM [^{32}P]-5′ end-labeled LTR RNA in the presence of increasing concentrations of the inhibitory RNAs. Different RNA molecules were separately renatured by incubating in binding buffer (Tris–HCl 50 mM pH 7.5, 10 mM $MgCl_2$) at 65°C for 10 min, and at 37°C for 10 min. Reactions were initiated by combining both molecules and performed on ice to avoid ribozyme cleavage activity. Complex formation was quantified using a Storm 820 instrument (Amersham Biosciences). The *K*d was extracted from data point fitting using SigmaPlot 8.0 software, according to the equation $y = ax / b + x$, where y represents the percentage of complex formation, x is the inhibitor concentration, a represents the maximum percentage of complex, and b corresponds to the dissociation constant (*K*d).

Results

Intracellular activity of the anti-LTR catalytic antisense RNAs

We previously described the capacity of the anti-LTR catalytic antisense RNAs, HH113αTAR, HH159αTAR, HP113αTAR and HP159αTAR ([33,34]; Fig. 1) to cleave LTR RNA *in vitro*. Both the catalytic and the antisense RNA domains of the hybrid inhibitory molecule target the HIV-1 LTR; the catalytic motif is designed against positions 113 (HH113 and HP113) and 159 (HH159 and HP159), and the TAR domain is the target of the antisense motif (αTAR). TAR acts as an anchoring site of the inhibitory RNA by binding to the complementary stem–loop motif (antisense domain αTAR) in the substrate molecule. The target sequences were selected based on their high conservation among different HIV isolates, as well as on the defined substrate sequence requirements of the ribozymes [39–41].

To assay the intracellular activity of the catalytic antisense RNAs, we generated series of constructs in which the synthesis of these RNAs was driven by pol II or pol III promoters. Different expression vectors based on RNA polymerase II (pol II) or III (pol III) promoters are widely used to achieve optimum levels of small RNA molecules in the appropriate intracellular compartment [42]. We chose the CMV immediate early promoter as the pol II promoter, and the tRNA and U6 promoters as pol III promoters. The tRNA promoter sequence is the tRNAval sequence described for the intracellular expression of hammerhead ribozymes [36], whereas

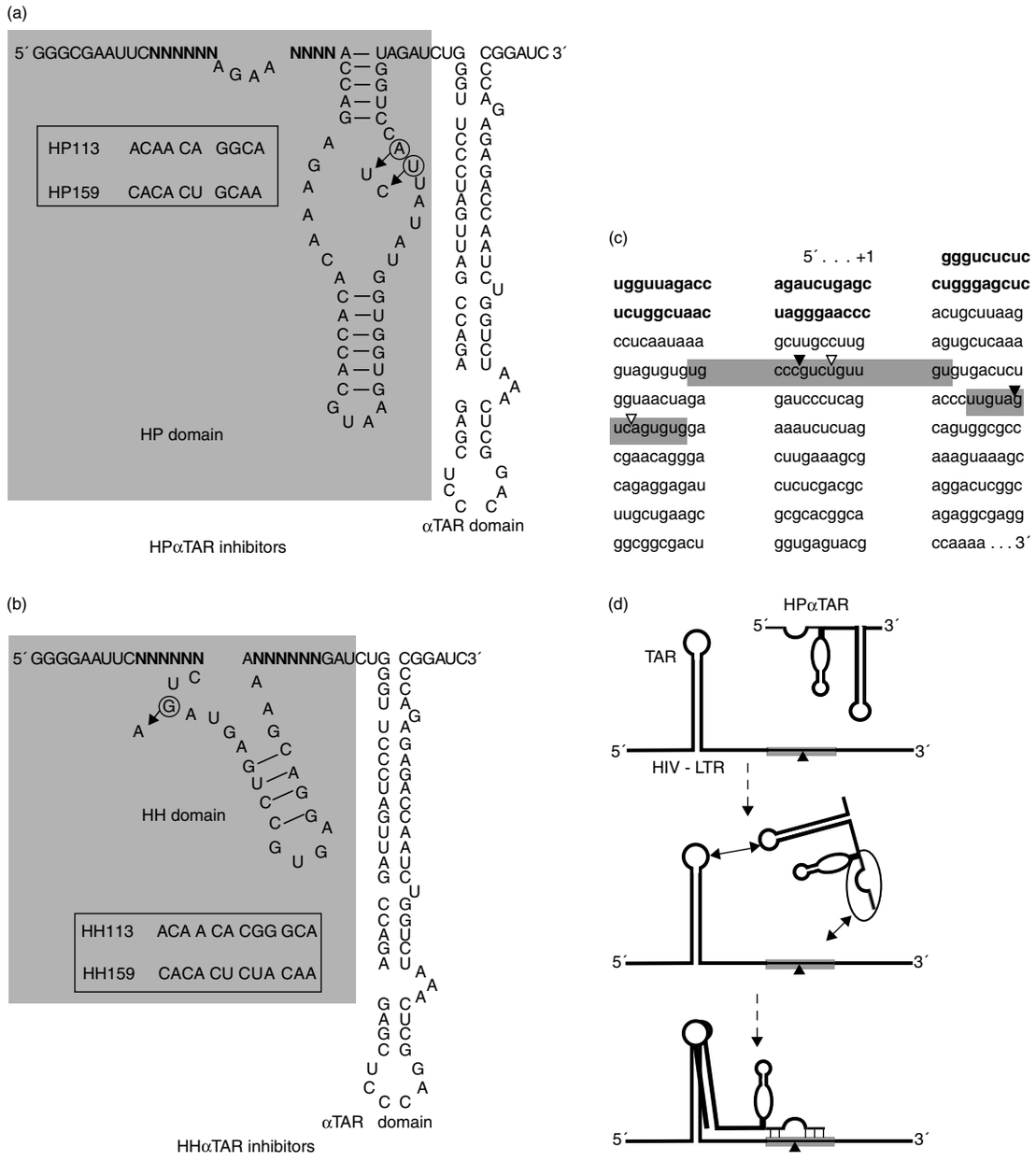

Fig. 1. Anti-LTR catalytic antisense RNAs. (a) Sequence and secondary structure model for the anti-LTR hairpin-derived catalytic antisense RNAs (HP). The catalytic domain (shadowed) is derived from the (−)sTRSV hairpin ribozyme. The antisense domain is the TAR complementary stem–loop domain (αTAR). Nucleotides in the substrate binding arms of the catalytic domains are shown with bold NS. The specific sequences used for targeting positions 113 (HP113) and 159 (HP159) in the HIV-1 LTR region are also shown boxed. The encircled nucleotides were mutated to generate catalytically inactive molecules. (b) Anti-LTR hammerhead-derived catalytic antisense RNAs (HH). The catalytic domain (shadowed) is derived from the (+)sTRSV hammerhead ribozyme. The αTAR RNA is the antisense domain. Bold nucleotides are those corresponding to the hammerhead substrate binding arms. Sequences for targeting the LTR RNA in positions 113 (HH113) and 159 (HH159) are shown boxed. The encircled nucleotides were mutated to generate catalytically inactive molecules. (c) Nucleotide sequence of NL4.3 HIV-1 strain. Nucleotides from +1 to 305 are shown. Bold letters correspond to TAR domain. Shadowed nucleotides are the substrate target sites 113 and 159 recognized by the ribozymes. Arrowheads indicate the cleavage site of the hairpin catalytic domains (solid) and the hammerhead ones (empty). (d) Schematic representation of the hypothesized action of the catalytic antisense RNAs. Potential pathway of interactions between the catalytic antisense RNA and the LTR substrate molecule is outlined. Note that the molecules are not drawn to scale. Substrate binding domain of the catalytic domain is encircled. Arrowhead indicates the site of cleavage. Grey box represents target sequences within the substrate RNA (113 or 159 in this work).

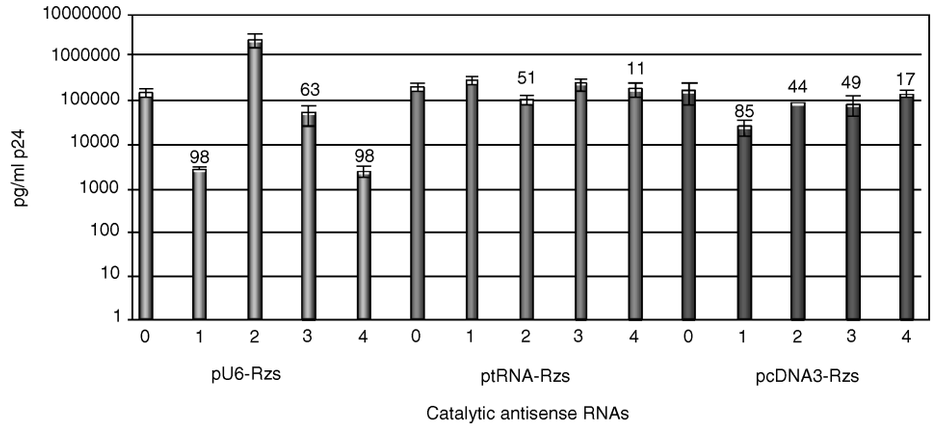

Fig. 2. Intracellular activity of catalytic antisense RNAs. Antigen p24 levels obtained on day 5 after co-transfection of pNL4-3 and the corresponding inhibitor-expressing vector in U87-CD4-CXCR4 cells. The promoter used in each case is indicated. Numbers represent the different inhibitor RNA coding sequences tested. 0: empty vector; 1: HH113αTAR; 2: HH159αTAR; 3: HP113αTAR; 4: HP159αTAR. The percentage of inhibition achieved, normalized with respect to the value obtained for the control, is shown above each bar. Values represent the mean ± SD of two experiments.

the U6 promoter construct is a modification of that described by Engenkel et al. [37] (see Materials and methods). Thus, three different plasmids were generated for each of the catalytic antisense RNAs to be assayed (see Materials and methods).

The 12 inhibitory RNA-expressing constructs were challenged with the pNL4-3 plasmid containing HIV-1 NL4-3 proviral DNA. Different plasmids were co-transfected with pNL4-3 into U87-CD4-CXCR4 mammalian cells (see Materials and methods). Transfection with plasmid pNL4-3 allowed a normal viral replication cycle. The ability of the different RNAs to inhibit HIV-1 expression was measured in terms of their capacity to reduce p24 production (Fig. 2). Day 5 was the optimum time for measuring p24 levels. The inhibitory effect could be maintained in cells cultured for up to 6 days after transfection (data not shown). The catalytic antisense RNAs synthesized under the control of the U6 promoter resulted in the highest rates of inhibition. A reduction of almost two orders of magnitude in p24 levels was achieved with HH113αTAR and HP159αTAR compared to the control. No significant inhibition was obtained with the RNAs synthesized from the tRNA or CMV promoter constructs, with the exception of the HH113αTAR molecule synthesized under the control of the CMV promoter, which caused an 80% reduction in p24 levels.

Inhibitory contribution of the catalytic and antisense domains

To analyze further the contribution of each inhibitory domain to the inhibition shown by the two most active molecules (HH113αTAR and HP159αTAR), we assayed a series of RNA molecules derived from each. Plasmids encoding the conventional hammerhead and hairpin ribozymes (HH113 and HP159), as well as catalytically non-active derivatives (HH113mutαTAR and HP159mutαTAR; [33,34]; Fig. 1) were constructed under the control of the U6 promoter. The ability of these molecules to inhibit viral replication, plus that of the antisense domain (αTAR) by itself, was tested as described above (Fig. 3). No obvious defects were observed at day 5 in cells transfected only with plasmids encoding the inhibitory RNAs. Interestingly, neither the HH113 nor the HP159 ribozymes showed any significant inhibition of replication, showing that conventional ribozymes fail to reduce viral replication under these conditions. For the HH113-derived catalytic antisense RNAs, the active molecule (HH113αTAR) showed appreciable activity, causing some 70% inhibition. Surprisingly, the best results were obtained with the molecule whose catalytic activity had been abolished (HH113mutαTAR). This molecule showed a reduction in p24 levels of $> 90\%$. With respect to the catalytic antisense RNAs derived from the HP159 ribozyme, the best results were obtained with the active molecule (HP159αTAR)-with $> 90\%$ reduction in p24 production. The activity of the HP159mutαTAR molecule was quite significant as well (nearly 80%). These results indicate that the intracellular inhibitory capacity of these molecules is due mainly to an antisense effect, and is therefore independent of the molecule's catalytic activity. The αTAR molecule itself, however, was unable to reduce viral replication under the same conditions (Fig. 3), demonstrating that the ribozyme domain – active or inactive – in the catalytic antisense RNAs is required for inhibition to be achieved.

In vitro binding of the catalytic antisense RNAs

The intracellular results show that the inhibition achieved by these chimeric molecules is owed mainly to an antisense effect. The surprising results obtained with the HH113mutαTAR molecule led us to examine its

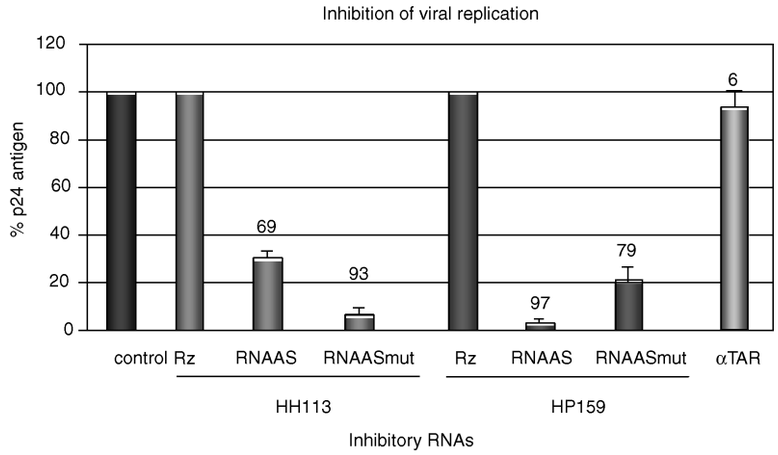

Fig. 3. Inhibition of HIV replication by U6-driven catalytic antisense RNAs. Inhibition of viral replication obtained by different HH113- and HP159-derived catalytic antisense RNAs. The percentage inhibition achieved, normalized with respect to the value obtained for the control, is shown above each bar. Values are the mean ± SD of at least three independent experiments. Rz, conventional ribozyme; RNAAS, catalytic antisense RNA; RNAASmut, mutated catalytic antisense RNA.

inhibitory features further. This molecule has the same sequence as HH113αTAR, except that the nucleotide G5 in the hammerhead core is mutated to A (Fig. 1b) to impede cleavage activity. This residue has been shown essential for ribozyme activity, and it seems to be involved in the folding of the ribozyme after binding to the substrate [43]. HH113mutαTAR-RNA was unable to cleave the LTR RNA *in vitro* (data not shown). To analyze its binding capacity, mobility shift assays were performed (Fig. 4) and the results compared with those obtained for the active molecule HH113αTAR. Reactions were performed on ice to avoid the cleavage activity of the HH113αTAR molecule. Under these conditions, HH113mutαTAR showed a higher binding efficiency than the corresponding active molecule (Table 1). The binding activity of the HP159αTAR and HP159mutαTAR molecules was also compared, and the results show that the active molecule binds the LTR RNA more efficiently than the catalytically null molecule (Table 1). The binding of the antisense domain αTAR molecule to the target LTR was also assayed, and showed a very poor binding rate (Table 1). Therefore, under these conditions, the molecules that bind to LTR RNA most efficiently are those whose intracellular inhibitory effect is highest.

Discussion

This work shows the ability of catalytic antisense RNAs to inhibit HIV-1 replication in mammalian cells. These molecules showed greater inhibition of p24 production than their ribozyme motif on its own. This agrees with previous *in vitro* characterization studies [34] in which these inhibitory molecules processed the LTR RNA more efficiently than the catalytic motifs alone, validating this strategy for optimizating ribozyme design. The results in Fig. 2 correlate well with the *in vitro* cleavage efficiencies obtained; HP159αTAR and HH113αTAR were the most efficient inhibitors in cells. We cannot conclude whether the inhibitor RNAs are acting on 3′ or 5′ LTR. In the above earlier studies [34], we showed that the antisense motif promoted effective anchorage of the inhibitory molecule to the substrate, allowing its catalytic part to efficiently reach its target site inside folded RNA molecules. In this work, assaying the inhibition of viral replication inside cells, a strong inhibitory antisense effect was seen. Such an effect might indicate that the binding of the antisense domain to TAR may also interfere with the biologically important roles of TAR (e.g., binding of Tat protein to the TAR motif, challenging the transactivation

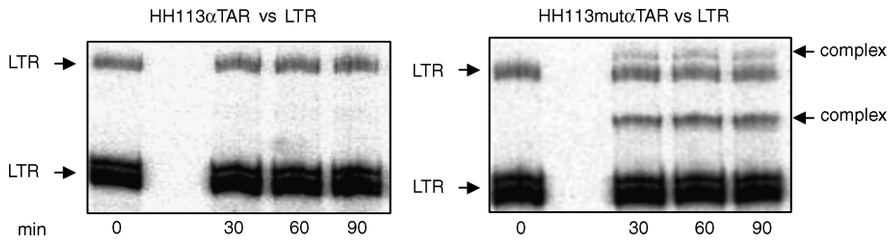

Fig. 4. Electrophoretic mobility shift assays. Labeled LTR RNA was incubated with unlabeled inhibitor RNAs at 4°C for 90 min. Aliquots were removed at the indicated time intervals. The figure shows the binding between LTR RNA and HH113-derived catalytic antisense RNAs. Complexes, shown by arrows, migrate less than the LTR RNA.

Table 1. K_d values for the different catalytic antisense RNAs.

	HH113αTAR	HH113mutαTAR	HP159αTAR	HP159mutαTAR	αTAR
K_d (nM)[a]	n.d.[b]	34.0331 ± 6.47	53.2208 ± 2.1837	n.d	n.d

[a]Values are the means of at least four independent trials ± SD.
[b]n.d. not determined: concentrations up to 800 nM were tested but complex formation was not appreciated.

of transcription). It has been reported that U6 promoter leads to nuclear localization of the resulting RNAs [37,44], whereas the other promoters assayed (tRNA and CMV) lead to preferentially cytoplasmic localization of the transcripts [36,42]. The observed inhibition differences might be explained by the different subcellular localization of the inhibitory RNAs. This may suggest a post-integration inhibitory effect achieved by RNAs synthesized under the control of the U6 promoter, but we cannot rule out any other possibility.

A comparison of the inhibitory features of the catalytically active and inactive RNA molecules shows that the intracellular inhibitory capacity of these molecules is independent of their catalytic action. With respect to the HH113-derived catalytic antisense RNAs, HH113mutαTAR caused more than a 90% reduction in p24 levels. The active molecule (HH13αTAR) showed substantially lower inhibitory activity, although it was still very significant (Fig. 3). Such differences might be explained by the different binding behavior of these active and inactive variants. The molecule HH113mutαTAR was able to bind to the substrate more efficiently than the HH113αTAR molecule. This result correlates well with the data obtained from comparisons of the binding of the HP159-derived catalytic antisense RNAs (Fig. 4, Table 1), suggesting that the inhibitory capacity of the catalytic antisense RNAs *ex vivo* is determined by their ability to bind to HIV RNA. Although all the RNAs used have the same αTAR domain as the antisense motif, their antisense effect was different depending on their overall structure—the inhibitory effect of each therefore differed. Thus, the inhibition achieved seems to be an intrinsic feature of the complete RNA molecule rather than being owed to any specific domain. This is further supported by the fact that none of the ribozymes used, nor the αTAR on its own, showed any significant inhibitory activity.

Studies performed with natural antisense RNA systems show that antisense RNAs with complementarity to their target sequences in the stem–loop domain can establish kissing-complexes, but do not go on to form more stable complexes [45]. Complementary nucleotides in single-stranded regions adjacent to the stem–loop are required for these more stable complexes to form [45]. In our system, the αTAR molecule shows complementarity only to the TAR stem–loop, making the formation of a stable complex more difficult. Nevertheless, the catalytic antisense RNAs (active or inactive) were able to bind to the LTR region through the stem–loop domain and the substrate-binding domain of the ribozyme, which might facilitate progress from the initial complex towards a more stable one. This is supported by the data obtained from the binding assays. Such binding increases their antisense inhibitory capacity and their *ex vivo* anti-HIV-1 activity. Further analysis is required to confirm this mechanism.

Conclusion

This work provides preliminary evidence of a new class of inhibitory RNAs. It shows the inhibition of HIV replication exerted by catalytic antisense RNA targeted against the LTR region. Further, the *ex vivo* capacity of these RNAs to block viral replication correlates well with their ability to bind the HIV substrate *in vitro*. These results may help establish general rules for the design of new antiviral-in particular anti-HIV-1-agents based on RNA molecules.

Acknowledgments

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