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In vitro and in vivo cleavage of HIV-1 RNA by new SOFA-HDV ribozymes and their potential to inhibit viral replication

Sébastien Lainé,[1,2,4,6] Robert J. Scarborough,[1,2] Dominique Lévesque,[5] Ludovic Didierlaurent,[6] Kaitlin J. Soye,[1,2,†] Marylène Mougel,[6] Jean-Pierre Perreault[5] and Anne Gatignol[1-3,*]

[1]Virus-Cell Interactions Laboratory; Lady Davis Institute for Medical Research; [2]Department of Microbiology & Immunology and [3]Experimental Medicine; McGill University; Montréal; [4]Laboratoire de Microbiologie Fondamentale et Pathogénicité; CNRS UMR 5234; Université de Bordeaux 2; Bordeaux, France; [5]RNA Group/Groupe ARN; Département de Biochimie; Université de Sherbrooke; Sherbrooke, Québec Canada; [6]UMI; UMII; CNRS UMR 5236; CPBS; Montpellier, France

[†]Current address: Montreal General Hospital Research Institute; Montréal, QC Canada

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RNA-based compounds are promising agents to inactivate viruses. New specific hepatitis delta virus (HDV)-derived ribozymes are natural molecules that can be engineered to specifically target a viral RNA. We have designed specific on-off adaptor (SOFA)-HDV ribozymes targeting the *tat* and *rev* sequences of the human immunodeficiency virus type 1 (HIV-1) RNA. We show that the SOFA-HDV ribozymes cleave their RNA target in vitro. They inhibit the Tat-mediated trans-activation of HIV-1 from 62% to 86% in different assays. In vivo, the amount of HIV RNA was decreased by 60 and 86% with two distinct ribozymes, which indicates that the inhibition of HIV production is directly correlated to the decline in spliced and unspliced viral RNAs. These SOFA-HDV-ribozymes inhibited the expression and the viral production of four HIV-1 strains, indicating an extended potential to act on multiple HIV variants. In HEK 293T and HeLa cells transfected with pNL4-3 and the SOFA-HDV-ribozymes, the reduced RNA levels consequently decreased the Gag protein expression in the cell and virus production in the supernatant. When transfected before HIV-1 infection, the ribozymes prevented the incoming virus from being expressed. The ribozymes inhibited HIV production up to 90% when transfected in combination with the HIV protease inhibitor Atazanavir. Our results strongly suggest that SOFA-HDV ribozymes have a great potential to target HIV-1 and to be used as therapeutic agents in combination therapy.

Background

HIV-1 expression starts with transcription from the viral DNA integrated into the host chromosome. The basal transcriptional activity of HIV is very low and the viral Tat protein and host factors increase the transcription of the viral genome. Tat acts through a cis acting RNA enhancer, the trans-activation response element located in the R region of the long terminal repeat (LTR).[1-3] The HIV-1 RNA then undergoes complex multiple splicing to produce mRNAs for the regulatory/accessory and structural proteins. In the early phase, HIV mRNA is multiply spliced to produce several splice variants ranging from 1.8 to 2 kb in size. They are mainly polycistronic, but produce preferentially Tat, Rev or Nef depending on the splice acceptor site used.[4,5] These mRNAs are constitutively exported to the cytoplasm and translated. In the late phase, in the presence of Rev, unspliced and incompletely spliced RNAs (4 kb) are exported to the cytoplasm to produce Gag, Pol, Env, Vif, Vpr and Vpu (**Fig. 1A**). Rev and cellular factors work in concert to bring these RNAs to the cytoplasm for translation.[6]

Inhibiting gene expression to affect viral replication can be achieved by RNA-based methods including molecular decoys, antisense RNAs, ribozymes (RNA enzymes; Rzs) and small interfering (si) RNAs.[7-9] Rzs are catalytic RNAs that can be designed to specifically base-pair with and cleave an RNA target in trans. The ability of Rzs to specifically recognize and catalyze the cleavage of RNA substrates triggered their use as therapeutic tools for the inactivation of RNA viruses.[10,11] They provide an interesting alternative to the siRNA approach and could be used in combination therapies.[12] Hammerhead and hairpin Rzs are currently in clinical trials against HIV, expressed from murine retroviral or lentiviral vectors.[13-15] Therefore, Rzs appear to have a great potential for further development of a gene-inactivation system aiming to control HIV propagation.

Among the different Rzs, the hepatitis delta virus (HDV) Rz is one of the rare examples derived from an RNA species found in human cells. It therefore has a natural ability to function in the presence of human proteins and offers several advantages.[10,16] The HDV Rz is fully active at physiological concentrations of magnesium in human cells and possesses a long half-life regardless of

*Correspondence to: Anne Gatignol; Email: anne.gatignol@mcgill.ca
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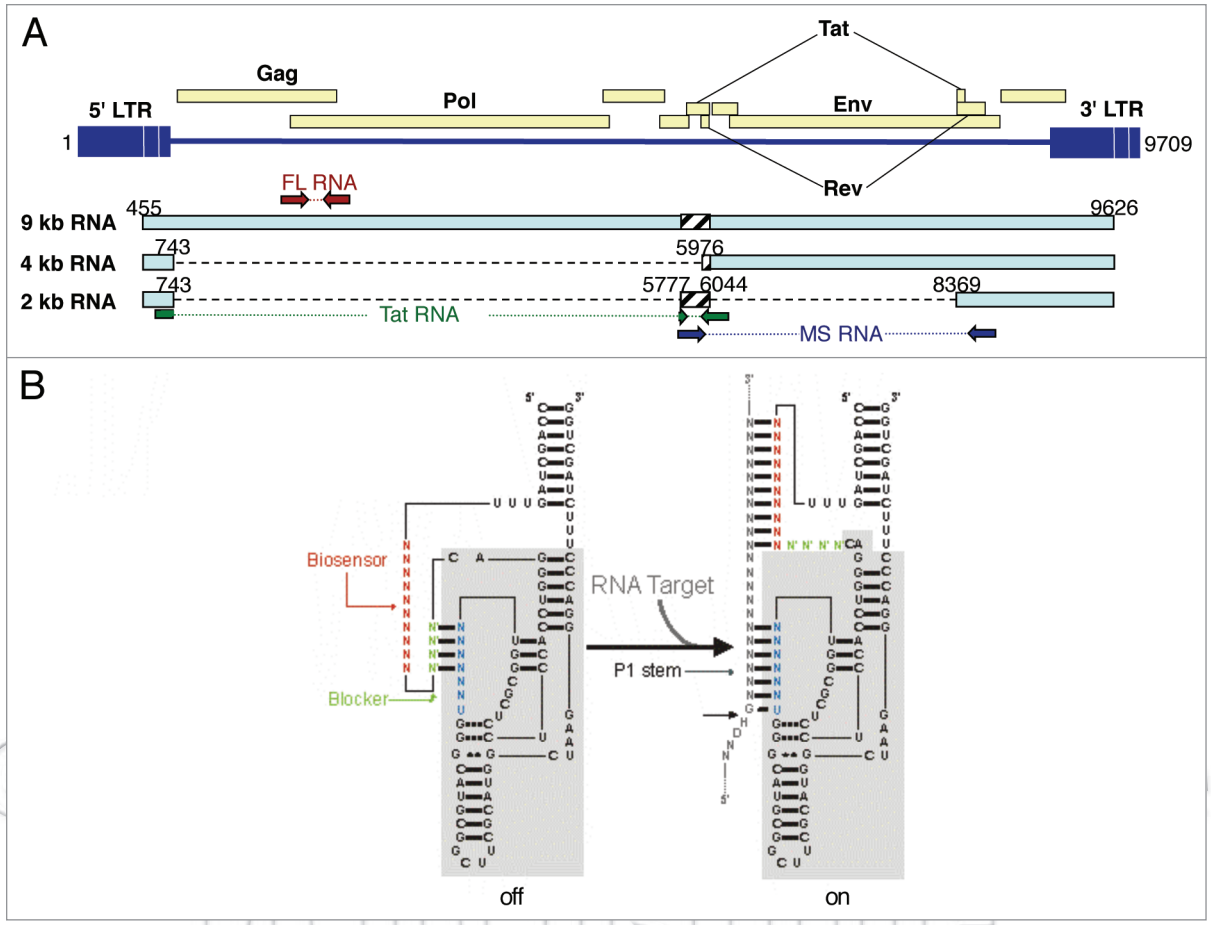

Figure 1. Schematic representation of HIV RNA and HDV Rz. (A) Structure of HIV RNAs targeted by the Rzs. The 9 kb RNA represents the full length (FL) genomic RNA. The 4 kb and the 2 kb RNAs represent the singly spliced and the multiply-spliced (MS) RNA species, respectively. The regions targeted by the HDV Rzs in the Tat and Rev reading frames are indicated on mRNA as hatched boxes. Numbers refer to the pNL4-3 strain (GenBank accession number M19921). The indicated splice junctions for the 4 and 2 kb RNAs refer to the main RNA species coding for the Env and Tat RNAs respectively.[4] Arrows show the location of the primers used in qRT-PCR for the FL, MS and Tat RNAs. (B) Secondary structure and nucleotide sequence of the SOFA-HDV Rzs used in this study. Both the *off* and *on* conformations are illustrated. The gray section indicates the original HDV Rz. The biosensor, blocker and P1 stem of the Rz are indicated in red, green and blue, respectively. A spacer of 5 nt is shown on the substrate in the *on* conformation.

both the cell line tested and the means of transfection used. This increased stability is likely due to the absence of single-stranded regions at both the 5' and 3' extremities of this RNA.[17,18] The potential of HDV Rzs to cleave in trans was demonstrated in vitro and in vivo targeting various natural RNA species.[19-23]

The target recognition mechanism of the HDV Rz is dependent on the formation of the P1 stem that is composed of only 7 base pairs (bp) between the Rz and the substrate (**Fig. 1B**). It is estimated that a minimum of 15 or 16 bp stretch is required to avoid off target effects on the human transcriptome.[24] To increase target specificity, a Specific *On*/*oFf* Adaptor (SOFA) was incorporated into the original version of the HDV Rz yielding an improved version of Rz (namely SOFA-HDV Rz).[25,26] The SOFA module switches the cleavage activity from *off* to *on* solely in the presence of the appropriate substrate (**Fig. 1B**). Initially, the substrate binding site forms a short duplex with an inserted sequence element (the blocker), putting the SOFA-HDV Rz under an inactive conformation (*off* state). This increases the energetic barrier for non-specific base-pairing interactions with the substrate binding site (i.e., HIV RNA), thus reducing the potential for off-target cleavages. A second inserted sequence element (the biosensor) extends base-pairing with the substrate to favor binding of the genuine substrate, and formation of this duplex concomitantly results in disruption of the short duplex involving the blocker sequence. In other words, upon the addition of the appropriate substrate, the SOFA biosensor sequence binds to the complementary sequence of the substrate releasing the Rz into an active conformation (*on* state), and subsequently, the base-pairing of the P1 stem between the substrate and the Rz takes place and the cleavage occurs.[25,26] Analysis of the cleavage activity using a large collection of substrate and SOFA-HDV Rz mutants provided evidence as to the roles of each domain and gave hints for design optimization.[26] This arrangement greatly diminishes non-specific effects, providing a tool with significant potential for the development of a gene-inactivation system in human cells.[10]

In the present study, SOFA-HDV Rzs have been designed to target specifically the mRNAs coding for HIV-1 Tat and Rev regulatory proteins. These RNA sequences are present on the

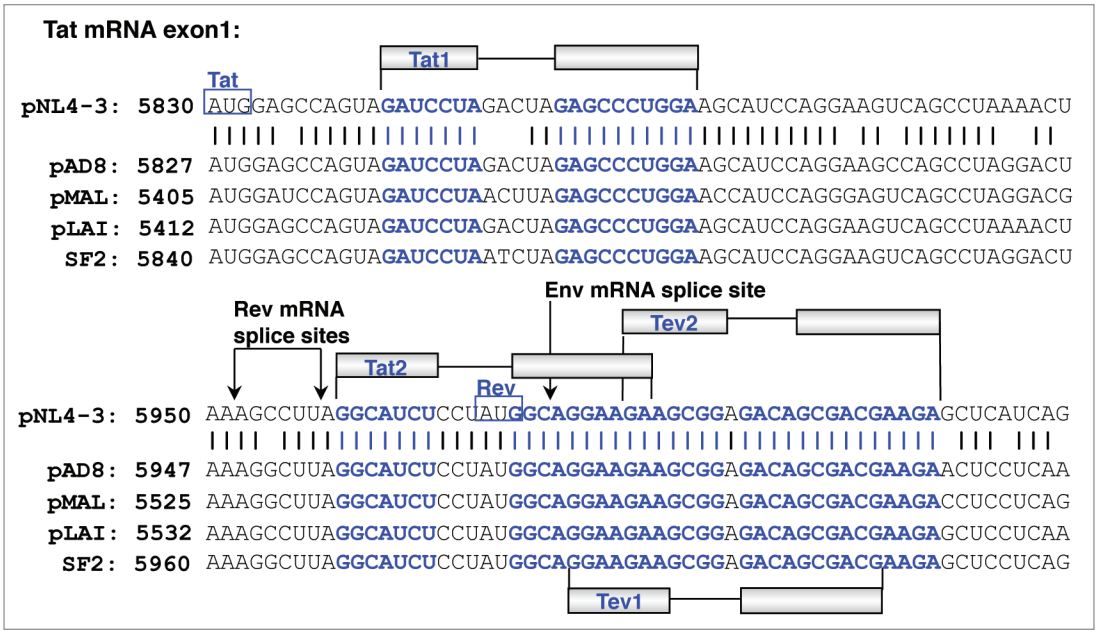

Figure 2. Sequences targeted by the HDV Rzs in the various HIV-1 strains. The left part of each Rz represents the P1 stem. The right part represents the binding stem. SOFA-HDV-RzTat1, -RzTat2, -RzTev1 and -RzTev2 are shown on their target sequences in the first exon of the 2 kb mRNAs. Tat and Rev start and stop codons are indicated when present. Rev and Env major mRNA splice junctions[4] are indicated by an arrow. Vertical bars indicate a conserved sequence in all strains. Sequences included in the different HDV-Rz are represented in bold. Numbers are according to GenBank accession number K02013 for pLAI, AF004394 for pAD8, K02007 for SF2 and K03456 for pMAL.

multiply-spliced 2 kb RNAs that code for Tat and Rev, some also target the singly-spliced RNAs and all target the genomic RNA (**Fig. 1A**). We show that these SOFA-HDV Rzs cleave their targets in vitro and in vivo and are effective inhibitors of HIV-1 expression and virion production in cell culture alone and in combination with an HIV-1 protease inhibitor.

Results

Design of SOFA-HDV Rzs targeting HIV-1 Tat and Rev mRNA. Initially, SOFA-HDV Rzs were designed to target regions of HIV RNA in the Tat and Rev reading frames because they will cleave spliced and unspliced viral RNAs (**Figs. 1A and 2**). Several constraints were considered to maximize their efficacy. First, conservation estimates were made using alignments of all available complete Clade B HIV sequences in the Los Alamos HIV database (www.hiv.lanl.gov). Each nucleotide in the selected target sites was found to be >87% conserved compared to the corresponding nucleotide in the strains used to design the Rzs (total of 234 sequences). Only sequences that were identical in the five different HIV strains used in the experiments were selected (**Fig. 2**). Two of these strains, pNL4-3,[27] and pLAI[28] use CXCR4 (X4) as a coreceptor and can infect lymphocytes. Two other strains, pMAL[28] and pAD8,[29] use CCR5 (R5) as a coreceptor and can infect monocyte-macrophages and lymphocytic cell lines in which CCR5 has been added.[30] The sequences in Tat were also identical in the SF2 strain[31] present on our pCMV1-Tat plasmid.[32] This consideration increases the chances to target a wide range of viruses. Second, constraints of specific sequence features known to be important for efficient cleavage by the Rz domain were considered:[10,16] (1) The first base pair of the P1 stem between the substrate and the Rz must be a GU Wobble base pair; (2) The nucleotide in position -1 compared to the cleavage site cannot be a G as this results in an uncleavable substrate; and (3) It is important to avoid the presence of two consecutive pyrimidines in position -1 and -2 as this impairs the cleavage efficiency. Taking into consideration the various constraints, two Rzs against Tat (SOFA-HDV-RzTat1-2) and two others against Tat and Rev (SOFA-HDV-RzTev1-2) coding sequences were designed to target different regions of the 2 kb mRNAs encoding Tat and Rev. Because HIV spliced sequences are naturally included in the pre-RNA molecule, all SOFA-HDV-Rzs target the full-length (FL) HIV RNA in addition to specific spliced viral RNAs (**Figs. 1A and 2**). The biosensor, blocker and P1 sequences were unique for each Rz while the core of the Rz was conserved (**Table 1**). The spacer domain, was always of the same length (5 nucleotides).

To investigate if these resulting SOFA-HDV Rzs offer enough substrate specificity to limit the potential off-target effect, bioinformatics analysis on the human cDNA database was performed using the RiboSubstrate software.[33] The closest potential human mRNA substrate showed more than 1 mismatch and 2 Wobble base pairs, which should be sufficient to prevent any off target cleavage activity (data not shown).

SOFA-HDV Rzs cleave RNA in vitro in Tat and Rev sequences. The cleavage activity of all four SOFA-HDV Rzs was first assessed in vitro. The corresponding DNA sequences of each Rz were cloned into the pmδRz-X vector[25] for expression in both mammalian cells and in vitro transcription with the inserted T7 promoter. The SOFA-HDV Rzs and radioactive Tat and Rev substrates were produced by in vitro transcription. In vitro

Table 1. Sequences of biosensor, blocker and P1 regions that target HIV-1 Tat and Rev mRNA on the SOFA-HDV ribozymes

SOFA-HDV Rz	BIOSENSOR	BLOCKER	P1
Tat1	5'-UCC AGG GCU C-3'	5'-CCU A-3'	5'-UAG GAU U-3'
Tat2	5'-UGU UCC UGC C-3'	5'-AUC U-3'	5'-AGA UGC U-3'
Tev1	5'-CGU CGC UGU C-3'	5'-AGA A-3'	5'-UUC UUC U-3'
Tev2	5'-UCU UCG UCG C-3'	5'-GCG G-3'	5'-CCG CUU U-3'

cleavage assays were performed under single turnover conditions ([Rz]>>[S]). A typical autoradiogram of the cleavage reaction products performed with the SOFA-HDV Rzs targeting the Tat mRNA is illustrated in **Figure 3A**. The four Rzs cleaved specifically the Tat mRNA substrate yielding cleavage products of the expected size. In that case, the level of cleavage varied between 27% for the SOFA-HDV-RzTat2 and 86% for the SOFA-HDV-RzTev1. Indeed, this latter Rz exhibited an outstanding level of cleavage under the condition tested (**Fig. 3A**). In the case of the two SOFA-HDV-Rzs targeting the Rev sequence, SOFA-HDV-RzTev1 also showed an excellent 73% cleavage efficiency, whereas SOFA-HDV-RzTev2 exhibited a 20% cleavage on a Rev RNA located in exon 1 (**Fig. 3B**). These results suggest an overall good to excellent efficiency of cleavage for the four Rzs targeting the first exon of the doubly-spliced RNAs.

The SOFA-HDV Rzs against Tat mRNA inhibit HIV-1 trans-activation. We next wanted to verify if the SOFA-HDV Rzs that target Tat mRNA express their activity in cells. By using the SOFA-HDV Rz plasmids and a trans-activation assay based on Tat-induced luciferase expression from the HIV-1 promoter,[34] we analyzed if the SOFA-HDV Rzs induced a loss of Tat function (**Fig. 4A**). In conditions where Tat activated the HIV-1 LTR fifty- to one hundred-fold, the inhibition induced by the SOFA-HDV Rzs that target Tat ranged from 40 to 50% compared to the control SOFA-HDV Rz (lanes 2–5). This result is consistent with the in vitro cleavage efficiency for the SOFA-HDV-RzTat1-2 and -RzTev2. Compared to the in vitro cleavage, SOFA-HDV-RzTev1 had a lower efficiency than expected, which may be due to a lower accessibility of the 3' end of the SF2 Tat mRNA used in this assay (**Fig. 4A**).

In the aforesaid system, Tat is much more expressed by the CMV promoter than in the viral context where Tat controls its own expression by an autoregulatory loop. To circumvent this limitation, we measured the Rz activities on Tat mRNA directly produced from the virus. We co-transfected HeLa-P4 cells[35] with the different SOFA-HDV Rzs and HIV molecular clone pNL4-3 (**Fig. 4B**). These cells have an integrated HIV-LTR-β-gal plasmid, which can be trans-activated by the Tat protein produced by the viral RNA, resulting in β-gal activation. In this assay, SOFA-HDV-RzTat1, -RzTat2 and -RzTev1 induced 61, 78 and 73% reduction respectively. SOFA-HDV-RzTev2 was the most active with 86% reduction, therefore showing a much higher activity in cells compared to the in vitro assay (lanes 2–5).

SOFA-HDV Rzs against Tat and Rev sequences cleave HIV genomic and spliced RNA in vivo. As several SOFA-HDV Rzs showed a very good efficiency in RNA cleavage as well as in

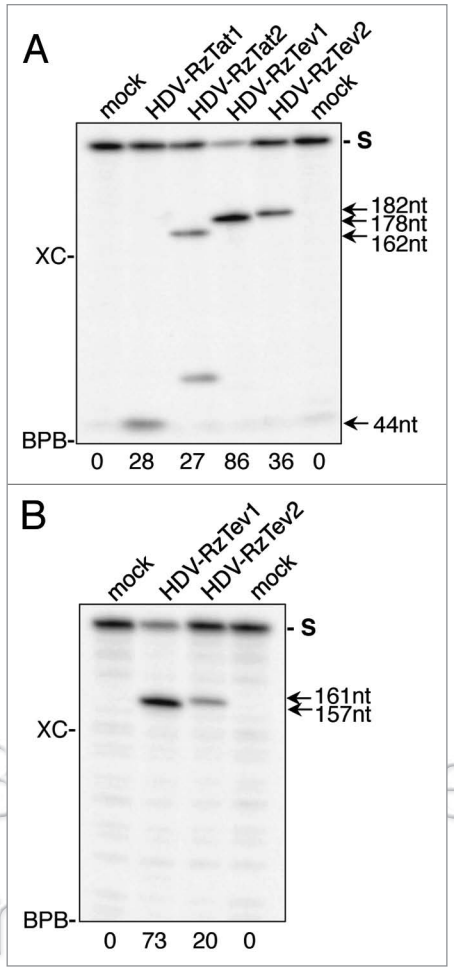

Figure 3. Autoradiograms of in vitro cleavage of Tat and Rev RNA species by SOFA-HDV Rzs. In vitro cleavage assays were performed using 5'-^{32}P-labeled transcripts incubated with each Rz under single turnover conditions and migrated on 5% polyacrylamide gels and autoradiographed. (A) Cleavage reactions of the Tat mRNA. Tat RNA was used as substrate. (B) Cleavage reactions of the Rev mRNA. Rev RNA was used as substrate. On the left of the gels are the migration positions of the xylene cyanol (XC) and bromophenol blue (BPB). On the right of the gels are the positions of the substrates and products. Below the gels are the percentages of cleavage for each lane. The autoradiograms shown are representative experiments.

functional assays, we next wanted to verify if they were able to cleave the viral RNAs when expressed from the virus and consequently inhibit viral production. To do so, HeLa P4 cells were co-transfected with HIV pNL4-3 and with SOFA-HDV-RzTev1-2. A representative experiment is shown in **Figure 5**. The activity of the SOFA-HDV Rzs was quantified by the β-gal activity induced by the Tat protein (**Fig. 5A**). In a duplicated assay, the supernatants were analyzed for viral production by p24 ELISA (**Fig. 5B**). In both assays, SOFA-HDV-RzTev1 and -RzTev2 induced an 80% reduction in viral expression and production. The levels of the HIV transcripts were determined from the total RNA extracted from the cells. The viral RNAs were then analyzed by RT-qPCR using primers that can specifically detect the full-length RNA (FL), the multiply-spliced RNAs (MS) or the Tat

RNA (Tat) (**Fig. 1A**). Quantitations show 73 to 86% and 44 to 66% RNA decrease for SOFA-HDV-RzTev1 and -RzTev2 respectively compared to the control Rz (**Fig. 5C**). For both Rzs, the maximum efficacy was shown on the Tat mRNA followed by the FL and the MS RNAs. These results indicate an excellent correlation between the reduction in viral production and in vivo RNA cleavage.

SOFA-HDV Rzs inhibit HIV-1 protein and progeny virus production of different HIV strains. The SOFA-HDV Rzs were designed to be active against five HIV strains. Consequently, the next step was to verify and compare their activity against the four available HIV molecular clones (**Fig. 6**). SOFA-HDV-RzTat1-2 and -RzTev1-2 were tested in HeLa P4 cells against pNL4-3, pMAL, pAD8 and pLAI and assayed for β-gal activity (**Fig. 6A**). In this assay, the Rzs were found active on all strains and SOFA-HDV-RzTev1-2 showed the highest inhibition of expression. To measure the virus production, the cell supernatants were tested by p24 ELISA (**Fig. 6B**). In this assay, SOFA-HDV-RzTev1-2 showed an extremely high activity, up to 96% inhibition against pMAL and pAD8. Overall, the results show that these Rzs have a high inhibitory potential against various viral strains.

SOFA-HDV Rzs decrease HIV-1 protein production and inhibit virion production in different cells. To further determine to which extent RNA cleavage by the Rzs affects viral protein expression, a similar assay was performed in HEK 293T and in HeLa cells co-transfected with pNL4-3 and the different SOFA-HDV Rzs (**Fig. 7**). Similarly to previous assays, when cells were transfected with HDV-Rz and the virus production quantified by RT assay in the cell supernatant, we found a consistent 68–78% reduction in RT activity for all SOFA-HDV-Rzs in HEK 293T and in HeLa cells compared to the control Rz (**Fig. 7A and B**, top, dark bars). The expression of viral proteins was reduced in HEK 293T cells but showed some variations as seen by the level of $p55^{GAG}$ and consequently of the $p24^{CA}$ up to an undetectable level with SOFA-HDV-RzTat2 (**Fig. 7A** and bottom). In HeLa cells the results were similar with a reduction in $p55^{GAG}$ polyprotein and a very low level of the processed intermediates and $p24^{CA}$ (**Fig. 7B** and bottom). In addition, increasing the amount of the transfected HDV-Rz in HeLa cells resulted in a 93–96% inhibition of viral production as quantified by RT assay (**Fig. 7B** and top, white bars). These results show that the RNA cleavage mediated by the HDV-Rzs consequently inhibits HIV protein synthesis and viral production in different cell types.

SOFA-HDV Rzs protect cells against HIV-1 expression after viral infection. To determine the activity of the Rzs in the context of HIV infection, we transfected HeLa-P4 cells with the four active SOFA-HDV Rzs and infected the cells 24 h later with infectious LAI virus (**Fig. 8**). β-gal assay on cell extracts 48 h post-transfection showed that the SOFA-HDV Rzs were able to inhibit the effects from incoming virus particles. Tat-activated β-gal expression was reduced by 83% when SOFA-HDV-RzTat2 and -RzTev1 were present in the cell before the infection. SOFA-HDV-RzTev2 showed the best protection with a 92% reduction. These results demonstrate that SOFA-HDV Rzs can act against viral RNA production after viral infection, which reflects more physiological conditions.

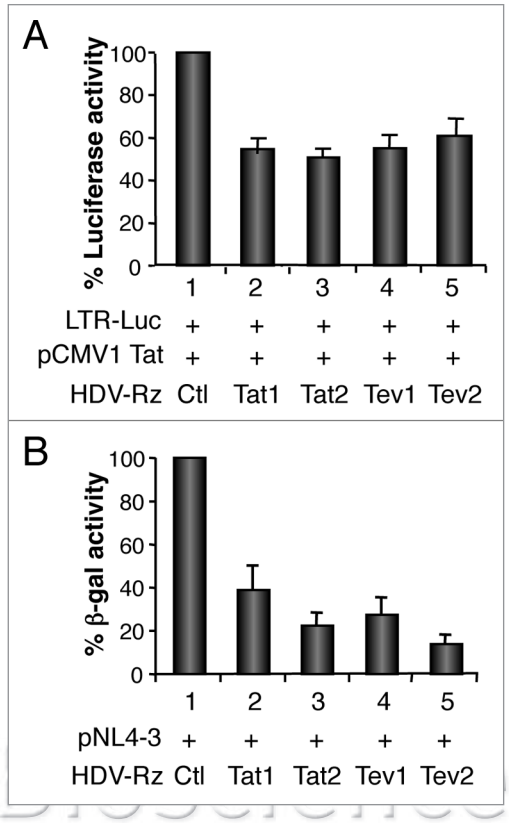

Figure 4. SOFA-HDV Rzs against Tat mRNA inhibit HIV-1 trans-activation. (A) Inhibition of HIV-1 trans-activation in a LTR-Luciferase assay. HeLa cells were transfected with 0.05 μg of pLTR-Luc, 0.01 μg of pCMV1Tat (lanes 1–7) and 2 μg of SOFA-HDV-Rz expressing an unrelated sequence (Ctl, lane 1) or targeting Tat mRNA as indicated (lanes 2–5). The luciferase expression is normalized to 100% with the Ctl Rz. Luciferase activity is the ratio between the luciferase level in the presence of the SOFA-HDV-Tat-Rev-Rz versus Ctl. Each value is the average of 6 independent experiments ±SEM. (B) Inhibition of Tat-induced LTR-β-galactosidase activity. HeLa-P4 cells were transfected with 0.05 μg of pNL4-3 (lanes 1–5) and with 3 μg of SOFA-HDV-Rz-Ctl (lane 1) or targeting Tat and Rev mRNAs as indicated (lanes 2–5). β-gal activity is the ratio between the β-gal level in the presence of the HDV-Rz versus Ctl. Each value is the average of 2 independent experiments ±SEM.

SOFA-HDV Rzs have an additive activity with an anti-protease compound. Current strategies against HIV-1 use combination therapies. We next tested if the SOFA-HDV-Rzs will have additional activity if used in combination with an HIV protease inhibitor Atazanavir (ATZ). Since the ATZ acts to inhibit Gag and GagPol processing, both in the cell and in the released virions, its activity effectively decreases the amount of active reverse transcriptase present in the supernatants. By using different concentrations of ATZ, we found that 1 μM reduced viral production by 70% in the absence of Rz. In the presence of the active HDV-RzTev1-2, ATZ was able to provide an additive effect on the observed Rz inhibition up 90% compared to the control (**Fig. 9**). These results show the potential of the SOFA-HDV-Rzs to be used in combination with small molecule inhibitors to provide an additive effect against HIV-1.

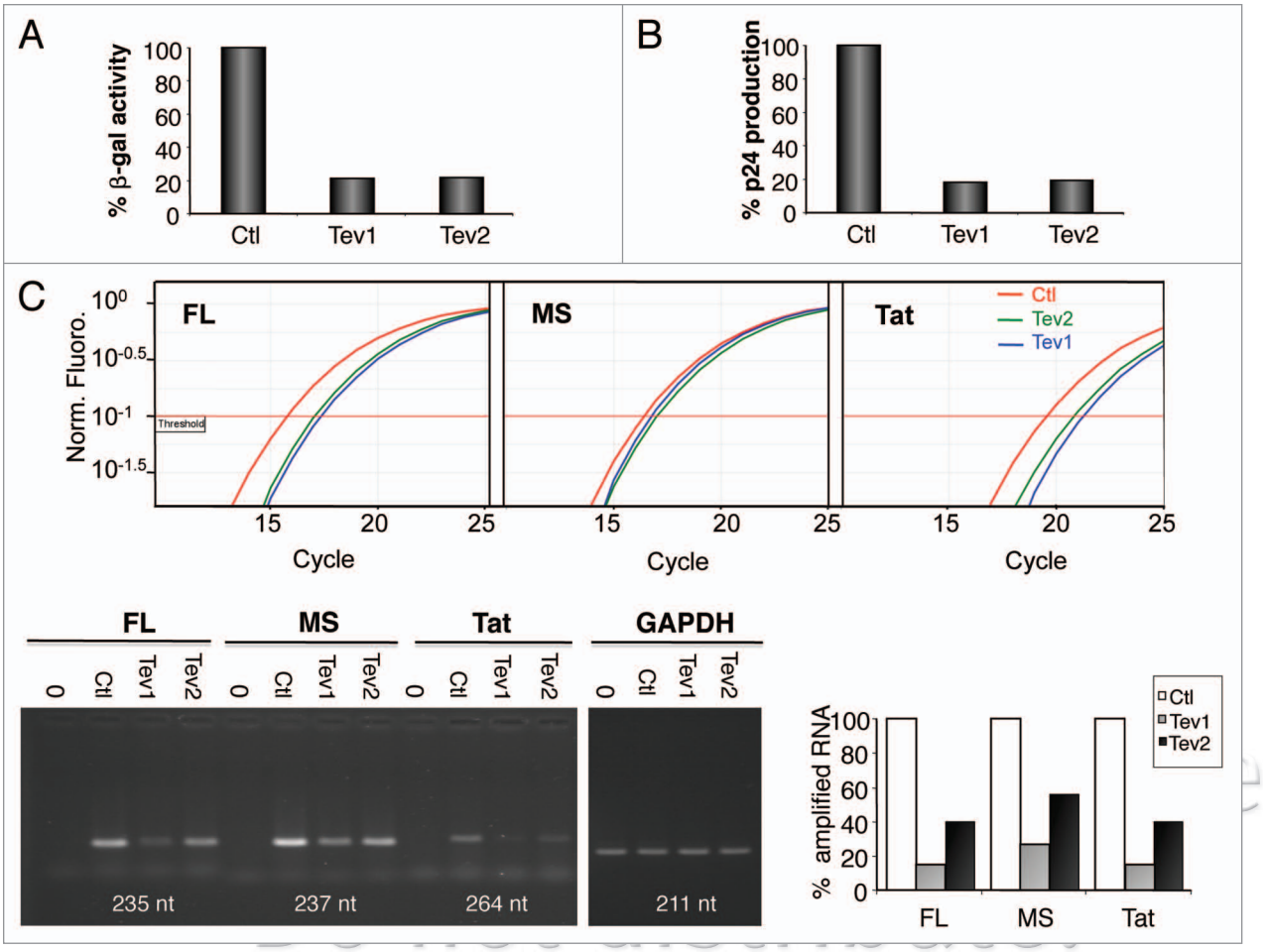

Figure 5. SOFA-HDV Rzs against Tat and Rev sequences mediate in vivo cleavage of HIV-1 mRNAs. HeLa-P4 cells were transfected with 0.05 μg of pNL4-3 and with 3 μg of SOFA-HDV-RzCtl, -RzTev1 or -RzTev2 as indicated. Cells were assayed for β-gal activity, whereas duplicates were lysed for RNA analysis. P24 ELISA was performed on the cell supernatant. β-gal, p24 ELISA and RNA levels represent the values from the same experiment among 4. (A) Inhibition of HIV-Tat-mediated β-gal activity by SOFA-HDV-RzTev1 and -RzTev2. β-gal activity is the ratio between the β-gal level in the presence of the HDV-Rz versus Ctl. (B) Inhibition of HIV-1 virus production by SOFA-HDV-RzTev1 and -RzTev2. HIV-1 pNL4-3 virus production was measured by p24 ELISA in the cell supernatant. Percentage of p24 amount is the ratio between the p24 amount in the presence of the HDV-Rz versus Ctl. (C) Analysis of viral RNA targets in HIV-1 producer cells (Top). Levels of full-length RNA (FL), multiply-spliced RNA (MS), Tat transcripts and GAPDH mRNA were determined by RT-qPCR in cells transfected with SOFA-HDV RzCtl (Ctl), -RzTev1 (Tev1) or -RzTev2 (Tev2) and with HIV-1 pNL4-3. Quantitative PCR plots are given for FL, MS and Tat amplifications. The horizontal red line indicates the threshold determined by the RotorGene software (Bottom left). Analysis of qPCR products by visualization on agarose gel. Sizes of amplicons are indicated. (Bottom right) Copy numbers are given for 2 x 10^4 GAPDH copies and normalized to a value of 100% for the HDV-RzCtl.

Discussion

The constant progression of the AIDS epidemic requires the development of new molecules and the improvement of previous compounds to obtain a sustained inhibition of HIV replication. This is likely to be achieved by the combination of multiple molecules acting on different targets. The current treatments act on RT, protease, integrase and entry, but thus far none target the viral RNA directly. Several molecules targeting the HIV RNA have been used to decrease HIV replication in cell culture,[12,36,37] in animal models[38] and some of them are in clinical trials for their use in gene therapy.[13,14,39] The experience with these compounds has provided several orientations and possible improvements including: (1) New compounds are always needed as leads for improvement; (2) The active compounds need improvements to reach several logs of viral inhibition; (3) The molecules need to be used in combination therapy against different targets; and (4) In vivo stability for long-term expression is required, to name only these examples.[14,15,39,40]

siRNAs have received much attention during the last years and their use against infectious agents is widely studied both in cell culture and in vivo using various methods.[41] These studies and numerous possible improvements are promising, but their use in patients will likely require their use in combination therapy to avoid viral escape.[7,42-44] Various Rzs have been tested in vitro and in vivo.[12,37,45,46] New SOFA-HDV Rzs have been developed and have shown a high stability in mammalian cells and a strong efficacy against various targets like the murine subtilisin

pro-convertase 2 and the human Pax5-B transcriptional factor mRNAs.[21,23] Therefore, they represent a new class of molecules with potential to inhibit viruses both by their novelty and as an improvement of the current molecules targeting RNA. As an RNA virus, HIV is a target of choice because Rzs may target both the incoming RNA in newly infected cells and the RNA produced during the replicating cycle.[15,47]

The design of SOFA-HDV Rzs should correspond to criteria of new compounds with improved efficacy and stability. The in vitro activity of the computer-designed SOFA-HDV-Rzs shows that new active compounds have been obtained (**Figs. 1–3**). The assays based on Tat activity (**Fig. 4**) led to several conclusions. First, the efficacy between the different Rzs shows a correlation but not a strict parallel between in vitro cleavage and cellular assays. This indicates that in vitro cleavage is an excellent indication of in vivo activity that may vary in intensity depending on the cellular environment. Second, the β-gal assay showed a better inhibition compared to the luciferase assay likely due to the autoregulated virally-encoded Tat RNA. We cannot exclude that Tat RNA from the pNL4-3 clone may be reached better than the sequence from the SF2 strain in pCMV1-Tat due to differences in secondary structure.

The ability to function in different cell types is an important parameter for efficacy. Differences in the activity between the various active Rzs on virus production after transfection of HeLa-P4 or HeLa cells (**Figs. 4–6 and 7B**) as well as HEK 293T cells (**Fig. 7A**) show that SOFA-HDV-RzTev1-2 are the most active in the first settings whereas -RzTat2 is the best in the second context. This difference likely reflects variations in the cleavage intensity in different cellular environments but may be also partially ascribed to the different detection methods. Results with a hammerhead Rz, showed that the Rzs which were effective in co-transfection experiments, were also effective against HIV infection of a T cell line stably expressing them.[48] Future comparisons of the efficiencies of our active HDV-Rzs in primary lymphocytes and monocytes as well as in viral cellular reservoirs will show to which extent the cellular context influences their activity.

In the cellular context, Tat activity or viral replication could be influenced by parameters other than RNA cleavage. We demonstrate here a direct correlation between Tat activity shown by β-gal expression, viral production analyzed by p24 ELISA and in vivo RNA cleavage (**Fig. 5**). This result strongly suggests that both SOFA-HDV-RzTev1-2 affect viral replication by directly mediating RNA cleavage and degradation, which consequently affects viral expression. They also affect the genomic RNA by direct cleavage and via decreased Tat expression (**Fig. 5C**) and could give rise to defective particles with reduced infectivity. Their activity on genomic viral RNA as well as the protection of cells from viral infection (**Fig. 8**) suggests that they also have the potential to decrease virion production during natural infection.

By acting on viral RNA at different steps of HIV replication, the SOFA-HDV Rzs affect the expression and the production of several HIV strains (**Fig. 6**), which represents the first step towards the discovery of other Rzs that will target many primary isolates and will offer the possibility to treat many patients with the same molecule. The moderate differences in activity on the viral strains may be due to variations of multiple parameters including basal expression, Tat activity, viral kinetics and accessibility to the targeted RNA influenced by the surrounding sequence.

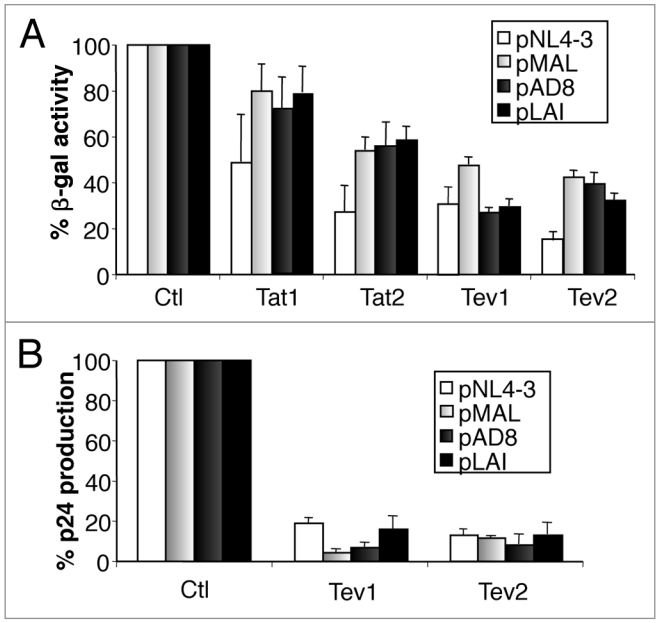

Figure 6. SOFA-HDV Rzs against Tat and Rev inhibit HIV-1 production of different strains. HeLa-P4 cells were transfected with 0.05 μg of pNL4-3 (white bars), pMAL (light grey), pAD8 (dark grey) or pLAI (black) and with 3 μg of SOFA-HDV-Rz-Ctl, RzTat1-2 or -RzTev1-2 as indicated. (A) Inhibition of HIV Tat-mediated β-gal activity by SOFA-HDV-RzTat1-2 and -RzTev1-2. β-gal activity is the ratio between the β-gal level in the presence of the HDV-Rz versus Ctl. (B) Inhibition of HIV-1 production by SOFA-HDV-RzTev1-2 in the cell supernatant. HIV-1 production was measured by p24 ELISA in the cell supernatant. % p24 expression is the ratio between the p24 amount in the presence of the HDV-Rz versus Ctl. Each value is the average of 4 independent experiments ±SEM.

Considering that combination therapy is required to treat HIV, the additive effect of the HDV-Rzs and the HIV protease inhibitor ATZ shows that they could be used with the current drug regimen to obtain a sustained inhibition of HIV-1 replication (**Fig. 9**). In addition, the observed 90% decrease in RT suggests that the use of stronger promoters and additional combinations will further increase Rz concentration and overall efficacy against the virus. Together, the data presented here and the great potential of future delivery methods[13,41,49,50] suggest that SOFA-HDV Rzs constitute a very promising new avenue to control HIV replication.

Materials and Methods

Plasmids. Rzs were cloned in *EcoRV* linearized pmδRz-X plasmids.[25] This vector is a modified version of pcDNA3 (Invitrogen) in which the cloned Rzs are followed by a polyadenylation signal. Each Rz was constructed by PCR using primer sense 5'-GGG CCA GCT AGT TT (N_{10}; Biosensor) (N_4; Blocker stem) CAG GGT CCA CCT CCT CGC GGT (N_7; P1 stem) GGG CAT CCG TTC GCG-3' and primer antisense 5'-CCA GCT AGA AAG GGT CCC TTA GCC ATC CGC GAA CGG ATG CCC-3' where the "N_{10}", "N_4" and "N_7" regions contain specific

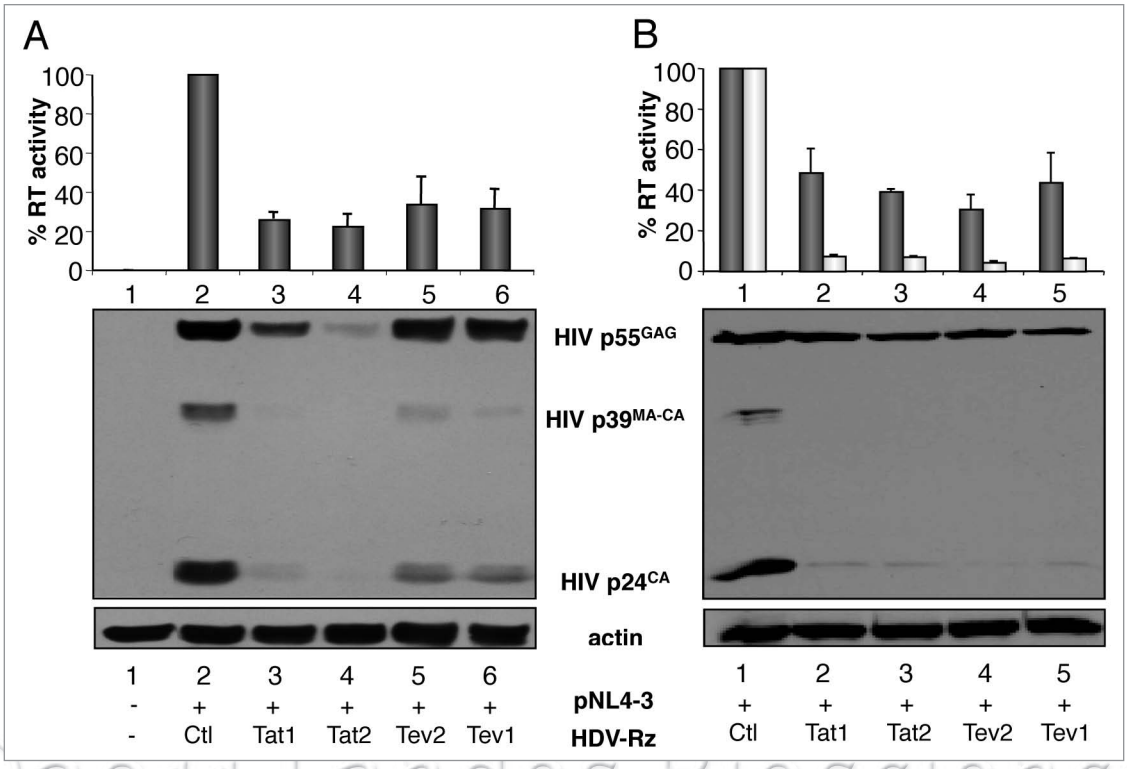

Figure 7. SOFA-HDV Rzs against Tat and Rev sequences inhibit HIV-1 production in different cells. (A) Activity in HEK 293T cells. HEK 293T cells were mock transfected (lane 1) or transfected with 0.5 μg of pNL4-3 provirus (lanes 2–6) and 1.5 μg of SOFA-HDV-RzCtl, RzTat1-2 or RzTev1-2 as indicated (top). RT activity in cell supernatants. Each value represents the average of 5 independent experiments ±SEM (bottom). HIV protein expression in cell lysates. 100 μg of cell lysates were resolved by a 10% SDS-PAGE and analyzed by immunoblotting with an antibody against HIV-1 p24 and exposed for 15 sec or against actin and exposed for 30 sec. The blot is a representative experiment among 2 independent assays. (B) Activity in HeLa cells. HeLa cells were transfected with 0.1 μg of pNL4-3 provirus (lanes 1–5) and 1.5 (dark grey) or 3 (light grey) μg of SOFA-HDV-Rz expressing the Ctl sequence or targeting Tat and Rev mRNA sequences as indicated (top). RT activity in cell supernatants. RT activity was calculated as indicated in the methods. Each value represents the average of 3 independent experiments normalized as a percentage of the SOFA-HDV-Rz-Ctl value ±SEM (bottom). HIV protein expression in cell lysates. 100 μg of cell lysates were analyzed as in (A). The blot is a representative experiment among 2 independent assays.

sequences for each SOFA-HDV Rz (**Table 1**). The PCR was carried out for 10 cycles, each consisting of 1 min at 94°C, 1 min at 50°C and 1 min at 76°C. PCR products containing the Rz sequence were then inserted in *EcoRV* digested pmδRz-X, sequenced and called pcDNA3-SOFA-HDV-Rz. pcDNA3-SOFA-HDV-Rz-Ctl is the control in which no specific sequence has been inserted.

The T7-Tat plasmid was made by inserting the T7 promoter into the *ClaI* site of pCMV1-Tat (HIV-1 strain SF2),[32] using the annealed sense 5'-CGA TGG TTC CAA GGT AAT ACG ACT CAC TAT AGG GAT-3' and antisense 5'-CGA TCC CTA TAG TGA GTC GTA TTA CCC TGG AAC CAT-3' oligonucleotides. The plasmid was linearized by *Sal* I digestion for in vitro transcription.

The T7-Rev1 plasmid was constructed by PCR amplification from pNL4-3. The PCR fragments were cloned into the pcDNA3.1 expression vector (Invitrogen) using *Hind*III and *Xba*I restriction sites. The sense: 5'-TGA AAG CTT GAG AGC AAG AAA TGG AGC CAG TAG AT-3' and the antisense: 5'-GCG CGC TCT AGA ACT ATT GCT ATT ATT ATT GCT ACT AC-3' primers were used. The plasmid was linearized by *Xba* I digestion for in vitro transcription.

In vitro transcription. In vitro transcription reactions were carried out at 37°C for 2 h in a final volume of 100 μl containing 200 pmol of DNA template and 15 U of RNA Guard (Amersham Biosciences) and the previously described reagents.[20] They were labeled at their 5' end with [γ^{32}P] ATP (New England Nuclear). The transcripts were then DNase treated, ethanol precipitated and purified on denaturing 5–10% polyacrylamide gels using 50 mM Tris-borate (pH 7.5), 8 M urea and 1 mM EDTA solution as running buffer and formamide dye (95% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol) as loading buffer. The reaction products were visualized by UV shadowing and the bands corresponding to the correct sizes excised, eluted, precipitated and quantified by spectroscopy at 260 nm.[20]

In vitro SOFA-HDV Rz assays. The reactions were carried out under single turnover conditions in which the [Rz]>>[S]. Trace amounts of the 5'-^{32}P-labelled Tat and Rev transcripts (~10,000 cpm, 0.01 pmol) were mixed with 1 μM of the appropriate SOFA-HDV Rz and 50 mM of Tris-HCl (pH 7.5) to a total volume of 9 μl and incubated at 37°C for 5 min. $MgCl_2$ was then added to a final concentration of 10 mM for a 37°C incubation for 2 h. The reactions were stopped by the addition

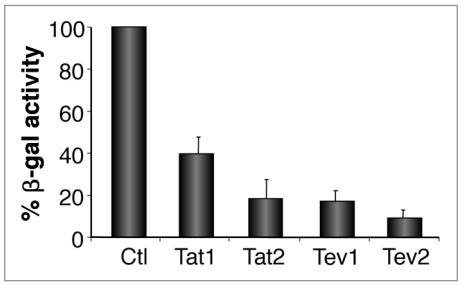

Figure 8. SOFA-HDV Rzs protect cells against HIV-1 expression after viral infection. HeLa-P4 cells were transfected with 3 μg of SOFA-HDV-Rz-Ctl, -RzTat1-2 or -RzTev1-2 as indicated. 24 h post-transfection, they were infected with 200 μl of viral supernatant containing 25,000 pLAI particles. β-gal activity is the ratio between the β-gal level in the presence of the SOFA-HDV Rz versus Ctl. Each value is the average of 3 independent experiments ±SEM.

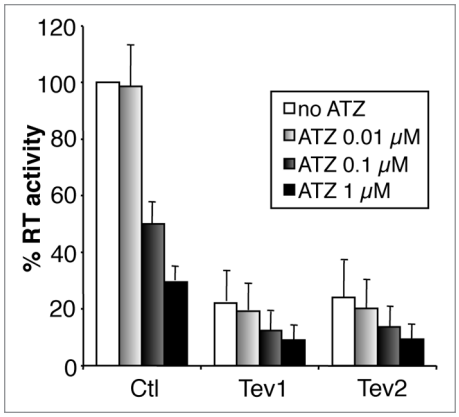

Figure 9. SOFA-HDV Rzs have an additive activity with Atazanavir (ATZ). HeLa cells were transfected with 0.1 μg of pNL4-3 provirus and 1.5 μg of SOFA-HDV-Rz-Ctl or -RzTev1-2 as indicated in cell medium containing no (white bars), 0.01 μM (light grey), 0.1 μM (dark grey) or 1 μM (black) ATZ. RT activity was calculated as a percentage of the value with HDV-Rz Ctl and no ATZ. Each value represents the average of 3 independent experiments ±SEM.

of 10 μl of formamide dye. The reactions were fractionated by denaturing 5% polyacrylamide gel electrophesis. The gels were analyzed with a radioanalytic scanner.

Cells and transfections. HeLa (ATCC), HeLa-P4,[35] and HEK 293T (ATCC) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). For luciferase assays, HeLa cells were transfected, lysed and analyzed for luminescence as described in reference 51. Viral inhibition was assayed on HeLa P4 cells expressing receptors CD4 and CXCR4, and carrying the stably integrated lacZ gene under the control of HIV-1. HeLa P4 cells were transfected with the SOFA-HDV Rzs using lipofectamin (Invitrogen). They were transfected with HIV molecular clones or infected with HIV particles as indicated. 48 h post-transfection or infection, p24 ELISA was performed on the cell supernatant and beta-galactosidase (β-gal) assay on the cell extract. HEK 293T and HeLa cells were plated in 6-well plates and transfected with TransIT Reagent (Mirus) as described in reference 52, followed by reverse transcriptase (RT) assay on the cell supernatant and immunobloting of the cell extract. When indicated, Atazanavir (NIAID AIDS reagents Catalog # 10003) was added in the culture medium.

β-galactosidase assays. The β-gal assays were performed as previously described in reference 53, with the following modifications. HeLa-P4 cells were transfected in 24-well plates, with pcDNA3-SOFA-HDV-Rz and pNL4-3, pLAI, pAD8 or pMAL. When indicated, they were transfected with pcDNA3-SOFA-HDV-Rz and infected 24 h later with pLAI virus particles. 48 h post-transfection, cells were washed 4 times with 1x PBS. 200 μl of reaction buffer 50 mM Tris-HCl pH 8.0, 100 mM β-Mercaptoethanol, 0.05% Triton X-100 and 5 mM 4-methyl umbelliferyl glucuronide (4-MUG; Sigma) was added in each well and incubated for 3 h at 37°C. The reactions were then analyzed using a fluorimeter (Applied Biosystems).

p24 ELISA and RT assay. 200 μl of the supernatant of HIV-transfected HeLa-P4 cells was analyzed 48 h after transfection by p24 ELISA (PerkinElmer) according to the manufacturer's instructions. The supernatant of transfected HEK 293T and HeLa cells were analyzed for virus production by RT assay as described in reference 54. 5 μl of viral supernatant in 50 μl of supplemented RT cocktail was incubated at 37°C for 2 h. 5 μl of each reaction mixture were spotted onto DEAE filter paper (Whatman). The membranes were washed and counted as described in reference 54.

Immunoblotting. Cell lysates were prepared, separated and transferred for immunoblotting as described in reference 55. The membrane was blocked for 1 h in 5% nonfat milk and 0.05% Tris-buffer saline Tween 20 (TBST).[56] The membranes were incubated overnight at 4°C with mouse monoclonal anti-HIV p24 antibody at a 1/1,000 dilution or with mouse anti-actin at a 1/10,000 dilution.[54] After five washes, the membranes were incubated with peroxidase-conjugated secondary goat anti-mouse (Amersham) for 1 h at room temperature at a 1:5,000 dilution. The bands were visualized as previously in reference 54.

RNA extraction and quantitative analysis. RNA extractions from cells were performed as previously described in reference 57 and 58. Briefly, cellular RNA was extracted from cell pellets with Tri-Reagent (MRC) according to the manufacturer's instructions. 15 μg of RNA was treated with 2 U of RNase-free DNase (RQ1, Promega) in presence of 60 U of RNaseOUT (Invitrogen) for 25 min at 37°C. RNA concentrations were determined by measuring absorbance at 260 nm. RT reactions were performed with 2 μg of cellular RNA samples and oligo dT was used as the RT-primer for all viral and GAPDH mRNAs as described in reference 57 and 58. Therefore, one RT-dT reaction allows the quantitative PCR (qPCR) analysis of the different full-length (FL), multiply spliced (MS), Tat and GAPDH cDNA species.[59] A 1/60 aliquot of the RT-dT reaction was used per qPCR assay. qPCR were achieved with SYBR Green Kit (Roche) and using the RotorGene systems (Labgene). A standard curve was generated from 50 to 500,000 copies of pNL4-3 plasmid. Each RT-PCR assay was accompanied by controls without reverse transcriptase that showed DNA contamination levels less than 0.1% of the HIV RNA. Systematically, cellular GAPDH mRNA level was

Table 2. Sequences of primers used in qRT-PCR

Primer	Sequences	RNA Target
sense HIV1306	5'-TCA GCA TTA TCA GAA GGA GCC ACC-3'	FL
antisense HIV1541	5'-TCA TCC ATC CTA TTT GTT CCT GAA G-3'	FL
sense HIV729	5'-GAG GGG CGG CGA CTG AAT T-3'	Tat
antisense HIV6025	5'-GAT GAG TCT GAC TGT TCT GAT GAG-3'	Tat
sense HIV5967	5'-CTA TGG CAG GAA GAA GCG GAG-3'	MS
antisense HIV8527	5'-CAA GCG GTG GTA GCT GAA GAG-3'	MS
sense GAPDH721	5'-GCT CAC TGG CAT GGC CTT CCG TGT-3'	GAPDH
antisense GAPDH931	5'-TGG AGG AGT GGG TGT CGC TGT TGA-3'	GAPDH

Numbers correspond to position of the 5' end of primer in pNL4-3 sequence.

quantitated for normalization. The oligonucleotides used for real time PCR are listed in **Table 2**.

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References

1. Bannwarth S, Gatignol A. HIV-1 TAR RNA: the target of molecular interactions between the virus and its host. Curr HIV Res 2005; 3:61-71.
2. Brady J, Kashanchi F. Tat gets the "green" light on transcription initiation. Retrovirology 2005; 2:69.
3. Gatignol A. Transcription of HIV: Tat and cellular chromatin. Adv Pharmacol 2007; 55:137-59.
4. Purcell DF, Martin MA. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication and infectivity. J Virol 1993; 67:6365-78.
5. Schwartz S, Felber BK, Benko DM, Fenyo EM, Pavlakis GN. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J Virol 1990; 64:2519-29.
6. McLaren M, Marsh K, Cochrane A. Modulating HIV-1 RNA processing and utilization. Front Biosci 2008; 13:5693-707.
7. Berkhout B, ter Brake O. Towards a durable RNAi gene therapy for HIV-AIDS. Expert Opin Biol Ther 2009; 9:161-70.
8. Nazari R, Joshi S. HIV-1 gene therapy at pre-integration and provirus DNA levels. Curr Gene Ther 2009; 9:20-5.
9. Scherer LJ, Rossi JJ. Approaches for the sequence-specific knockdown of mRNA. Nat Biotechnol 2003; 21:1457-65.
10. Asif-Ullah M, Levesque M, Robichaud G, Perreault JP. Development of ribozyme-based gene-inactivations; the example of the hepatitis delta virus ribozyme. Curr Gene Ther 2007; 7:205-16.
11. Puerta-Fernandez E, Romero-Lopez C, Barroso-delJesus A, Berzal-Herranz A. Ribozymes: recent advances in the development of RNA tools. FEMS Microbiol Rev 2003; 27:75-97.
12. Akkina R, Banerjea A, Bai J, Anderson J, Li MJ, Rossi J. siRNAs, ribozymes and RNA decoys in modeling stem cell-based gene therapy for HIV/AIDS. Anticancer Res 2003; 23:1997-2005.
13. Mitsuyasu RT, Merigan TC, Carr A, Zack JA, Winters MA, Workman C, et al. Phase 2 gene therapy trial of an anti-HIV ribozyme in autologous CD34$^+$ cells. Nat Med 2009; 15:285-92.
14. Rossi JJ, June CH, Kohn DB. Genetic therapies against HIV. Nat Biotechnol 2007; 25:1444-54.
15. von Laer D, Hasselmann S, Hasselmann K. Gene therapy for HIV infection: what does it need to make it work? J Gene Med 2006; 8:658-67.
16. Bergeron LJ, Ouellet J, Perreault JP. Ribozyme-based gene-inactivation systems require a fine comprehension of their substrate specificities; the case of delta ribozyme. Curr Med Chem 2003; 10:2589-97.
17. Lévesque D, Choufani S, Perreault JP. Delta ribozyme benefits from a good stability in vitro that becomes outstanding in vivo. RNA 2002; 8:464-77.
18. Wu HN, Lin YJ, Lin FP, Makino S, Chang MF, Lai MM. Human hepatitis delta virus RNA subfragments contain an autocleavage activity. Proc Natl Acad Sci USA 1989; 86:1831-5.
19. Al-Anouti F, Ananvoranich S. Comparative analysis of antisense RNA, double-stranded RNA and delta ribozyme-mediated gene regulation in *Toxoplasma gondii*. Antisense Nucleic Acid Drug Dev 2002; 12:275-81.
20. Bergeron LJ, Perreault JP. Development and comparison of procedures for the selection of delta ribozyme cleavage sites within the hepatitis B virus. Nucleic Acids Res 2002; 30:4682-91.
21. D'Anjou F, Bergeron LJ, Larbi NB, Fournier I, Salzet M, Perreault JP, et al. Silencing of SPC2 expression using an engineered delta ribozyme in the mouse betaTC-3 endocrine cell line. J Biol Chem 2004; 279:14232-9.
22. Fiola K, Perreault JP, Cousineau B. Gene targeting in the Gram-Positive bacterium *Lactococcus lactis*, using various delta ribozymes. Appl Environ Microbiol 2006; 72:869-79.
23. Robichaud GA, Perreault JP, Ouellette RJ. Development of an isoform-specific gene suppression system: the study of the human Pax-5B transcriptional element. Nucleic Acids Res 2008; 36:4609-20.
24. Peracchi A. Prospects for antiviral ribozymes and deoxyribozymes. Rev Med Virol 2004; 14:47-64.
25. Bergeron LJ, Perreault JP. Target-dependent on/off switch increases ribozyme fidelity. Nucleic Acids Res 2005; 33:1240-8.
26. Bergeron LJ, Reymond C, Perreault JP. Functional characterization of the SOFA delta ribozyme. RNA 2005; 11:1858-68.
27. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, et al. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 1986; 59:284-91.
28. Peden K, Emerman M, Montagnier L. Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL and HIV-1ELI. Virology 1991; 185:661-72.
29. Theodore TS, Englund G, Buckler-White A, Buckler CE, Martin MA, Peden KW. Construction and characterization of a stable full-length macrophage-tropic HIV type 1 molecular clone that directs the production of high titers of progeny virions. AIDS Res Hum Retroviruses 1996; 12:191-4.
30. Peden KW, Farber JM. Coreceptors for human immunodeficiency virus and simian immunodeficiency virus. Adv Pharmacol 2000; 48:409-78.

31. Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM, Oshiro LS. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 1984; 225:840-2.
32. Gatignol A, Buckler-White A, Berkhout B, Jeang KT. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. Science 1991; 251:1597-600.
33. Lucier JF, Bergeron LJ, Briere FP, Ouellette R, Elela SA, Perreault JP. RiboSubstrates: a web application addressing the cleavage specificities of ribozymes in designated genomes. BMC Bioinformatics 2006; 7:480.
34. Daher A, Longuet M, Dorin D, Bois F, Segeral E, Bannwarth S, et al. Two dimerization domains in the trans-activation response RNA-binding protein (TRBP) individually reverse the protein kinase R inhibition of HIV-1 long terminal repeat expression. J Biol Chem 2001; 276:33899-905.
35. Charneau P, Mirambeau G, Roux P, Paulous S, Buc H, Clavel F. HIV-1 reverse transcription. A termination step at the center of the genome. J Mol Biol 1994; 241:651-62.
36. Ding SF, Lombardi R, Nazari R, Joshi S. A combination anti-HIV-1 gene therapy approach using a single transcription unit that expresses antisense, decoy and sense RNAs and trans-dominant negative mutant Gag and Env proteins. Front Biosci 2002; 7:15-28.
37. Li MJ, Bauer G, Michienzi A, Yee JK, Lee NS, Kim J, et al. Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. Mol Ther 2003; 8:196-206.
38. Braun SE, Johnson RP. Setting the stage for bench-to-bedside movement of anti-HIV RNA inhibitors-gene therapy for AIDS in macaques. Front Biosci 2006; 11:838-51.
39. Scherer L, Rossi JJ, Weinberg MS. Progress and prospects: RNA-based therapies for treatment of HIV infection. Gene Ther 2007; 14:1057-64.
40. Reyes-Darias JA, Sanchez-Luque FJ, Berzal-Herranz A. Inhibition of HIV-1 replication by RNA-based strategies. Curr HIV Res 2008; 6:500-14.
41. Lanao JM, Briones E, Colino CI. Recent advances in delivery systems for anti-HIV1 therapy. J Drug Target 2007; 15:21-36.
42. Boden D, Pusch O, Lee F, Tucker L, Ramratnam B. Human immunodeficiency virus type 1 escape from RNA interference. J Virol 2003; 77:11531-5.
43. Leonard JN, Shah PS, Burnett JC, Schaffer DV. HIV evades RNA interference directed at TAR by an indirect compensatory mechanism. Cell Host Microbe 2008; 4:484-94.
44. Westerhout EM, Ooms M, Vink M, Das AT, Berkhout B. HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. Nucleic Acids Res 2005; 33:796-804.
45. Bai Y, Trang P, Li H, Kim K, Zhou T, Liu F. Effective inhibition in animals of viral pathogenesis by a ribozyme derived from RNase P catalytic RNA. Proc Natl Acad Sci USA 2008; 105:10919-24.
46. Nazari R, Ma XZ, Joshi S. Inhibition of human immunodeficiency virus-1 entry using vectors expressing a multimeric hammerhead ribozyme targeting the CCR5 mRNA. J Gen Virol 2008; 89:2252-61.
47. Hotchkiss G, Maijgren-Steffensson C, Ahrlund-Richter L. Efficacy and mode of action of hammerhead and hairpin ribozymes against various HIV-1 target sites. Mol Ther 2004; 10:172-80.
48. Unwalla HJ, Li H, Li SY, Abad D, Rossi JJ. Use of a U16 snoRNA-containing ribozyme library to identify ribozyme targets in HIV-1. Mol Ther 2008; 16:1113-9.
49. Li M, Rossi JJ. Lentiviral vector delivery of siRNA and shRNA encoding genes into cultured and primary hematopoietic cells. Methods Mol Biol 2008; 433:287-99.
50. Zhou J, Swiderski P, Li H, Zhang J, Neff CP, Akkina R, et al. Selection, characterization and application of new RNA HIV gp 120 aptamers for facile delivery of Dicer substrate siRNAs into HIV infected cells. Nucleic Acids Res 2009; 37:3094-109.
51. Daher A, Laraki G, Singh M, Melendez-Peña CE, Bannwarth S, Peters AH, et al. TRBP control of PACT-induced phosphorylation of PKR is reversed by stress. Mol Cell Biol 2009; 29:254-65.
52. Daniels SM, Melendez-Peña CE, Scarborough RJ, Daher A, Christensen HS, El Far M, et al. Characterization of the TRBP domain required for Dicer interaction and function in RNA interference. BMC Mol Biol 2009; 10:38.
53. Metifiot M, Faure A, Guyonnet-Duperat V, Bellecave P, Litvak S, Ventura M, et al. Cellular uptake of ODNs in HIV-1 human-infected cells: a role for viral particles in DNA delivery? Oligonucleotides 2007; 17:151-65.
54. Clerzius G, Gélinas JF, Daher A, Bonnet M, Meurs EF, Gatignol A. ADAR1 interacts with PKR during human immunodeficiency virus infection of lymphocytes and contributes to viral replication. J Virol 2009; 83:10119-28.
55. Laraki G, Clerzius G, Daher A, Melendez-Peña C, Daniels S, Gatignol A. Interactions between the double-stranded RNA-binding proteins TRBP and PACT define the Medipal domain that mediates protein-protein interactions. RNA Biol 2008; 5:92-103.
56. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press 1989.
57. Houzet L, Battini JL, Bernard E, Thibert V, Mougel M. A new retroelement constituted by a natural alternatively spliced RNA of murine replication-competent retroviruses. EMBO J 2003; 22:4866-75.
58. Smagulova F, Maurel S, Morichaud Z, Devaux C, Mougel M, Houzet L. The highly structured encapsidation signal of MuLV RNA is involved in the nuclear export of its unspliced RNA. J Mol Biol 2005; 354:1118-28.
59. Houzet L, Paillart JC, Smagulova F, Maurel S, Morichaud Z, Marquet R, et al. HIV controls the selective packaging of genomic, spliced viral and cellular RNAs into virions through different mechanisms. Nucleic Acids Res 2007; 35:2695-704.