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Potent inhibition of HIV-1 gene expression and TAT-mediated apoptosis in human T cells by novel mono- and multitarget anti-TAT/Rev/Env ribozymes and a general purpose RNA-cleaving DNA-enzyme

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Abstract

One of the hallmarks of progression of HIV-1/AIDS is the rapid depletion of CD4 + T cells that is known to occur at the late stages of the disease when usually X4 tropic HIV-1 predominates. Besides direct killing of T lymphocytes, HIV-1 infection leads to extensive apoptosis of naïve/uninfected bystander T cells, which is predominantly mediated by HIV-1 TAT protein. Therefore, reduction of HIV-1 TAT protein is likely to reduce substantially the pathogenesis associated with HIV-1 infection. We designed two non-GUX hammerhead ribozymes (Rzs) and a Di-Rz by placing them in direct tandem. These were targeted against the most conserved second exon of HIV-1 TAT/Rev/Env region. Although very impressive *in vitro* cleavage of the target RNA by the two hammerhead Rzs was obtained under standard conditions of cleavage, only one of them was active under simulated physiological conditions. Sequence-specific cleavage by the Di-Rz was most efficient under standard conditions. Cleavage reactions carried out under simulated physiological conditions by the Di-Rz, however, indicated that both mono-Rzs were functional. We also assembled a 10–23 catalytic motif containing general purpose RNA-cleaving DNA-enzyme (Dz) against the same region, which cleaved the target RNA very efficiently. Both Rzs and Dz showed not only potent inhibition of HIV-1 gene expression but also showed remarkable protection against HIV-1 TAT protein-mediated apoptosis in Jurkat T cells. Possible implications of these findings are discussed.

Keywords: HIV; Catalytic nucleic acids; Apoptosis; Gene therapy

1. Introduction

Human T lymphocytes and macrophages are the two major cell types that support HIV-1 replication. The infection is usually initiated by macrophage tropic (R5) HIV-1 that uses the 7-transmembrane-G-coupled protein receptor-CCR5 and CD4 to gain entry into a susceptible cell (usually Langerhans cells or macrophages). During the advanced stages of the disease, T cell tropic virus (X4) that uses the chemokine recetor CXCR4 present on CD4 + T cells usually predominates (Berger et al., 1999). The regulatory protein TAT of HIV-1 plays a crucial role in viral replication cycle (Stevenson, 2003) and is a powerful transcriptional transactivator of gene expression that binds to a well defined stem-loop structure called TAR present at the 5′-ends of all HIV-1 transcripts (Dingwall et al., 1990). TAT is encoded by two exons, the first exon is sufficient for transcriptional activation (Jeang et al., 1993) and the second exon can independently exert antiviral effects on T cells (Neuveut and Jeang, 1996) and macrophages (Neuveut et al., 2003). Although HIV-1 TAT protein lacks the signal sequence, it is readily detected in culture supernatants of cells infected with the virus (Chang et al., 1997) and in the serum of HIV-1 infected individuals (Xiao et al., 2000). Extracellular HIV-1 TAT protein is efficiently taken up by several mammalian cell lines including T cells using clathrin-mediated endocytosis (Vendeville et al., 2004). It is also capable of regulating cytokine gene expression (Westendorp et al., 1994), causing immune cell-hyperactivation (Ott et al., 1997) and promoting growth of Kaposi's sarcoma cells (Yen-Moore et al., 2000). It has been earlier demonstrated quite convincingly that TAT secreted by an infected cell is able to induce apoptosis in neighboring by-stander cells (Purvis et al., 1995) and that massive depletion of CD4 + T cells, that eventu-

Abbreviations: Rz, ribozyme; Dz, DNA-enzyme; RE, restriction enzyme

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ally results in the loss of immune competence, is due to apoptosis (Meyaard et al., 1992; Fauci, 1993). Exogenous application of TAT was shown to up-regulate the expression of Fas ligand mRNA in macrophages that led to CD4-cross-linking induced death (Cohen et al., 1999). Besides modulating these reactions, TAT seems to exert a global change in variety of key cell functions (Chun et al., 1998; Lohr et al., 2003; Flores et al., 1999; Weissman et al., 1998). HIV-1 mediated apoptosis has recently been shown to involve microtubular network and mitochondrial pathways (Giacca, 2005).

Therefore, HIV-1 TAT is an attractive antiviral target for interfering with HIV-1 gene expression. Several earlier antiviral approaches including ribozymes, siRNAs and DNA-enzymes (Coburn and Cullen, 2002; Rossi, 1999; Morris and Rossi, 2004; Akkina et al., 2003; Banerjea et al., 2003, 2004, 2005; Chakraborti and Banerjea, 2003; Jacque et al., 2002) have been used against HIV-1 genes but none of these studies addressed the question whether the target cells could be protected against HIV-1 TAT protein-mediated apoptosis. We, therefore, designed non-GUX hammerhead Rzs and a 10–23 catalytic motif containing DNA-enzyme (Santoro and Joyce, 1997) that selectively cleaved the target RNA that possessed TAT/Rev sequences, down-regulated HIV-1 TAT gene expression and the apoptosis caused by its action in Jurkat T cells.

2. Experimental

2.1. Materials

THP-1 or HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) and 10% FBS. THP-1 cells were grown in suspension and stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 h (100 nM) as described earlier (Fitzgerald et al., 2000). These cells matured into macrophages and were used for virus challenge experiments. Macrophages were also derived from human peripheral blood by subjecting the Ficoll-hypaque population of mononuclear cells to plastic adherence according to standard procedures (Banerjea et al., 1981). They were >90% positive for CD14 antigen by FACS analysis (data not shown). Plasmid DNAs, pNL4-3 (Adachi et al., 1986), pSV-Tat72 and pNL4-3.LucR^-E^- (Luciferase gene was inserted into the Nef region of pNL4-3) were obtained from the NIH AIDS repository and were grown according to standard procedures. The supercoiled plasmid DNAs were purified on a Qiagen (Qiagen, GmbH, Germany) column before transfection using Lipofectin reagent (GIBCO/BRL, MD).

2.2. Cloning of target gene and in vitro transcription

Target gene containing second exons of Tat, Rev including the Env region was obtained by PCR amplification of a 63-nt fragment from pNL4-3 HIV-1 DNA (Adachi et al., 1986) (Fig. 1, panels A and B). The following primers were synthesized:

(1) sense terminal primer: 5′-CACCATTATCGTTTCAG-3′;
(2) antisense terminal primer: 5′-CTATTCCTTCGGGCCTG-3′.

The PCR amplified product was cloned into a T-tailed vector pTargeT^{TM} (Promega Corporation, Madison, WI), which placed the target sequence (substrate) under the influence of T7 as well as the CMV promoter (Fig. 1, panel B). This expression vector carries the human cytomegalovirus (CMV) immediate–early enhancer/promoter region to enable constitutive expression of cloned genes in mammalian cells. The T7 promoter allows the *in vitro* generation of transcripts in the presence of T7 RNA polymerase according to the manufacturer's instructions. Plasmid containing the target gene was linearized with *Not*I restriction enzyme present in the polylinker region of the vector and transcribed in the presence of labeled UTP using T7 RNA polymerase (Promega Corporation, Madison, WI) according to the manufacturer's instructions.

2.3. Selection of cleavage sites in target RNA

The cleavage sites against which the Rzs and a DNA-enzyme were designed are shown in Fig. 1, panel C and in more detail in Fig. 2. Rz-8366 was targeted against UUC, Rz-8377 against CUC (position 8366 and 8377 of pNL4-3 DNA), while Dz-8381 possessing a 10–23 catalytic motif was targeted to cleave between A and U residues.

2.4. Cloning of ribozymes

The strategy to clone Rz was essentially the same as described before by us (Goila and Banerjea, 2001; Shahi et al., 2001). Briefly, an oligonucleotide containing the hammerhead catalytic motif of the Rz that was flanked on either side by 8 nt complementary to the target RNA was synthesized chemically (Fig. 2, panel A and B). The Rz-encoding oligonucleotide was amplified by PCR using specific terminal primers. The following primers were synthesized: (1) Rz-oligonucleotide-8377 5′-GGGATT-GG**CTGATGAGTCCGTGAGGACGAA**AGGTGGGT-3′.

(a) sense terminal primer: 5′-GGGATTGGCTGATGA-3′;
(b) antisense terminal primer: 5′-ACCCACCTTTCGTCC-3′.

In the similar manner Rz-8366 was constructed by designing the following primers: (1) Rz-oligonucleotide-8366 5′-GTG-GGTCT**CTGATGAGTCCGTGAGGACGAA**AAACGATA.

(a) sense terminal primer: 5′-GTGGGTCTCTGATG;
(b) antisense terminal primer: 5′-TATCGTTTTTCGTCCTC.

The catalytic hammerhead motif in Rz-encoding oligonucleotide is shown in bold letters.

The PCR amplified product was cloned into a T-tailed vector, pGEM-T (Promega Corporation, Madison, WI). Rz-8377 was also cloned in pTarget^{TM} vector, which placed the Rz downstream of the T7 and CMV promoters. Di-Rz was assembled in the same manner by joining the two Rzs in tandem in the correct orientation by placing Rz-8366 downstream of Rz-8377 (Fig. 2, panel C). Dz-8381 containing a 10–23 catalytic domain with seven bases long flanking sequences complementary to the target RNA (Fig. 2, panel D) was synthesized. Design of 10–23 catalytic motif containing Dz was the same as originally described

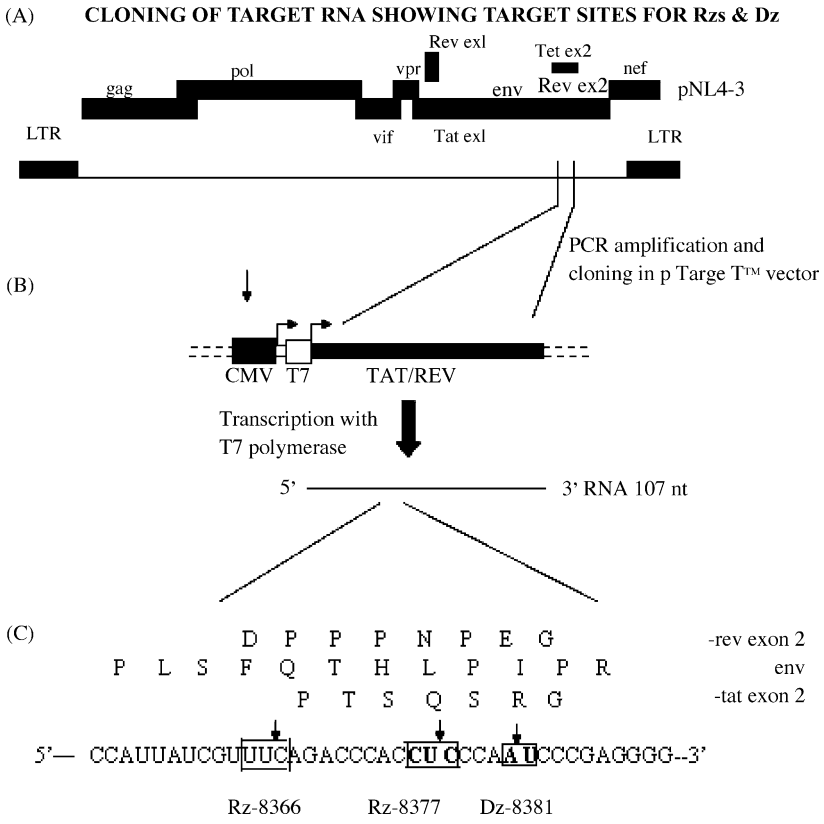

Fig. 1. Panel A: Genomic structure of HIV-1 is shown with all the open reading frames for structural (Gag, Pol and Env) and regulatory genes (TAT, Rev and Nef) in the infectious clone of HIV-1, pNL4-3 (Adachi et al., 1986). Panel B: The region that was PCR amplified from the infectious clone, which contained the second exon of Tat and Rev and a portion of Env open reading frame. The PCR amplified product was cloned into pTargeT^{TM} vector (Promega Corporation Madison, WI) downstream of the CMV and T7 promoter. Panel C: The target sequence against which the Rzs and Dz were designed. The corresponding amino acids in all the three reading frames are also shown by a single letter code and the target sequences for the Rzs and Dz are shown in bold letters. Arrows indicate cleavage sites for Rz and Dz.

by Santoro and Joyce (1997). The cleavage is expected to take place after the A nucleotide (shown by an arrow). A mutant Dz with same target specificity was synthesized by substituting a C with G residue (panel E, shown by an arrow) in the 10–23 catalytic motif and was used as a control. This mutant Dz showed no cleavage activity even in the presence of 50 mM $MgCl_2$ (data not shown).

2.5. Construction of Dz-8381 and Dz-8381Mt

The Dz-8381 containing a 10–23 catalytic domain with seven bases long flanking sequences complementary to the target RNA (Fig. 2) was synthesized. The cleavage is expected to take place after the A nucleotide (shown by an arrow). A mutant DNA-enzyme with the same target specificity was synthesized by substituting C with a G residue in the 10–23 catalytic motif and was used as a control. This mutant showed no cleavage activity even in the presence of 50 mM $MgCl_2$ (data not shown).

2.6. In vitro transcription

Ribozyme encoding pGEM-T vectors were linearized at their 3′-ends with appropriate restriction enzymes (REs) (Fig. 3, panel A) and transcribed in the presence of labeled UTP using either T7 or SP6 RNA polymerase (Promega corporation, Madison, WI) according to the instructions given by the manufacturer and as described earlier (Banerjea and Joklik, 1990). The authenticity of the Rz clones was confirmed by sequencing the supercoiled plasmid DNA using either terminal primers or SP6/T7 primers.

2.7. In vitro cleavage reaction

For cleavage reactions, equimolar concentrations (100 pmoles) of ^{32}P-labeled substrate RNA and unlabeled Dz or Rz were mixed in 10 μl of 50 mM Tris–HCl, pH 7.5 containing 10 mM $MgCl_2$ (standard condition). Cleavage reaction was allowed to continue for 1 h at 37 °C and the products were subjected to gel analysis as described before (Goila and Banerjea, 2001; Shahi et al., 2001) using the mini-protein gel system from Bio-Rad. Radioactive bands were visualized by autoradiography of the dried gel. Cleavage reactions were also carried at simulated physiological conditions (50 mM Tris–HCl pH 7.5, 150 mM KCl, 2 mM $MgCl_2$ and 37 °C) (Santoro and Joyce, 1997).

2.8. Kinetic analysis

Kinetic parameters of Rz and Dz were determined by terminating cleavage reactions at varying time points under

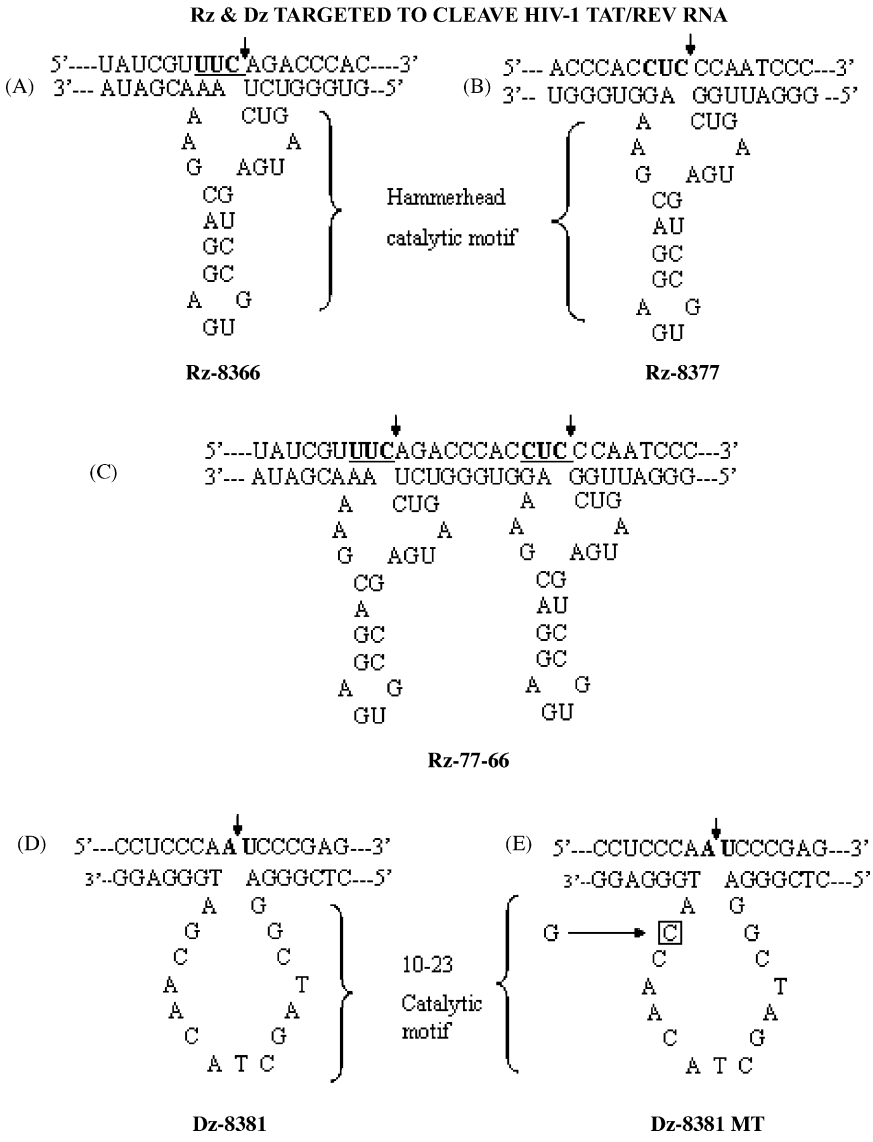

Fig. 2. The Rzs and Dz with their target sequences are shown. The Rz-8366 was designed against the target sequence UUC (shown in bold letters) (panel A); Rz-8377 against CUC (panel B); Rz-77-66 (Di-Rz) against the same two target sequences and the Dz-8381 against AU dinucleotide (in bold letters). All the Rzs possessed the conserved hammerhead catalytic motif, whereas the Dz possessed the 10–23 catalytic motif. Seven bases on either side of the target site were synthesized leaving the A nucleotide unpaired in the 10–23 motif containing Dz (panel D). A single base mutation G to C (boxed) in the catalytic motif was synthesized that was referred to as Dz-8381MT (panel E).

enzyme saturating conditions as described by us earlier (Goila and Banerjea, 2001) and others (Santoro and Joyce, 1997). The cleaved RNA fragments were quantified with the help of a densitometer (GS-710 Calibrated Imaging Densitometer, Bio-Rad) and the kinetic parameters were calculated from a Lineweaver–Burk's plot.

2.9. Toxicity studies with Rz and Dz

Cell lines, HeLa and THP-1, or human peripheral blood derived macrophages were grown in six-well plates (1×10^6 per well) and incubated with 0.5 or 1.0 μg of Rz, Dz-8381 or Dz-8381Mt in plain medium in the presence or absence of Lipofectin (GIBCO/BRL, MD) for 2 h and then incubated in the presence of medium + 10% fetal bovine serum for 24 h. Viability of the cells was checked by trypan-blue dye exclusion test. No toxicity was observed under these experimental conditions.

2.10. HIV-1 inhibition experiments

THP-1 cells or human peripheral blood derived macrophages were co-transfected with wild-type infectious clone of HIV-1, pNL 4-3 and Dz-8381 or Rz DNA constructs (1 μg each in 1 ml) in six-well plates. Six days post transfection, cell supernatant was collected and centrifuged at 2000 × *g* to remove cell debris and then further centrifuged at 40,000 × *g* for 1 h to pellet the virus particles. The virus pellet was then lysed with NP-40 and the amounts of p24 antigen were determined by using p24 ELISA kit (Orgenon Teknika Corporation).

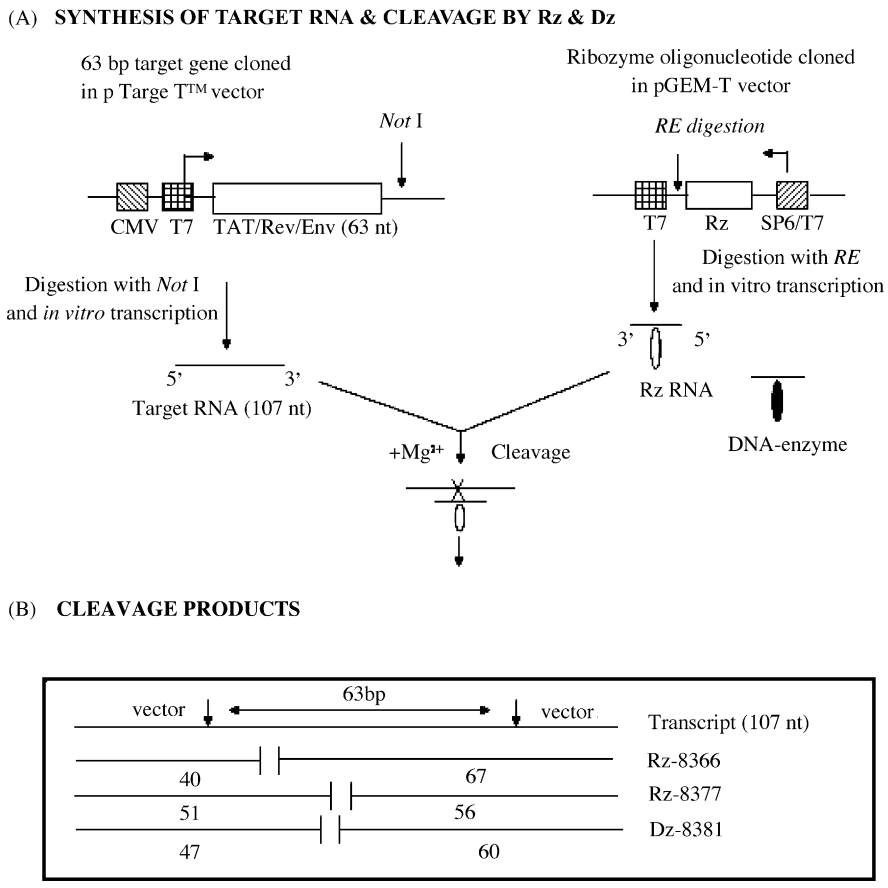

Fig. 3. Panel A: The plasmid encoding the HIV-1 Tat/Rev/Env (substrate) was linearized with *Not*I restriction enzyme and transcribed using T7 RNA polymerase to yield uniformly labeled 107 nt long transcript. Rz encoding plasmids were linearized at their 3′-ends with appropriate restriction enzymes (REs) and transcribed using either SP6 or T7 RNA polymerase (Promega). Equimolar amounts (100 pmoles) of unlabeled Rz or Dz and ^{32}P-labeled substrate RNA were mixed for carrying out cleavage reaction, as described earlier. Panel B: The expected cleavage pattern resulting after cleavage of the same target RNA by each Rz and Dz.

2.11. Inhibition of HIV-1 gene expression by ribozyme and DNA-enzyme

PMA-stimulated adherent population of THP-1 cells (humans macrophages) were employed for evaluating the bio-efficacy of the Rzs and Dz. Infectious HIV-1 clone pNL4-3 was used to transfect PMA-stimulated THP-1 cells. Six days post-transfection, total RNA was isolated using TRIZOL reagent (GIBCO/BRL). About 1 μg of total RNA was used to estimate the levels of HIV-1 Tat RNA (179 nt long) and the control RNA (β-actin) by RT-PCR techniques using the Access RT-PCR kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. The following set of primers were used for amplifying HIV-1 Tat specific RNA:

(1) forward primer (5851–5870) CTAGAGCCCTGGAAGCATCC;
(2) reverse primer (6007–6030) GCTTGATGAGTCTGACTGTTCTG.

Human β-actin-specific primers (Stratagene, La Jolla, CA, Catalog #302010) were used for estimating the control RNA that was 636 nt long.

2.12. Inhibition of TAT-mediated apoptosis in T cells

The protocol used for studying TAT-mediated apoptosis was the same as described by Li et al. (1995), using Jurkat cells. Briefly, Jurkat T cells were co-transfected with pSVTat72 plasmid DNA (1.0 μg) and 1.0 μg each of Rz-8377 construct or Dz-8381. Transfection was allowed to proceed for 6 h and then replaced with complete medium (DMEM + 10% fetal bovine serum). After overnight incubation (12 h) the cells were grown in the presence of 0.1% FBS serum for 48 h. Rz and Dz treated cells were then stained for apoptosis using DeadEnd colorimetric TUNEL system (Promega Corporation, Madison, WI) according to the manufacturer's instructions. This assay measures nuclear DNA fragmentation by end-labeling the DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP nick-end labeling) assay. Biotinylated nucleotides are incorporated at the 3′-end of the DNA using the enzyme terminal deoxynucleotidyl tranferase (TdT). Horseradish peroxidase-labeled streptavidin then reacts specifically with biotinylated nucleotides, which are detected by a color reaction using DAB chromogen. Using this procedure, apoptotic nuclei are stained dark brown.

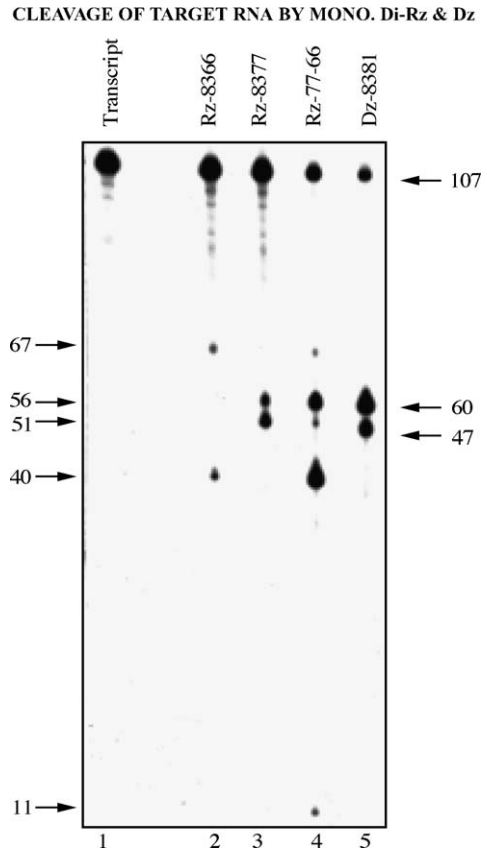

Fig. 4. *In vitro* cleavage of synthetic 107 bases long HIV-1 TAT/Rev/Env RNA. Labeled target RNA was subjected to cleavage with Rzs and Dz. Lane 1 shows the 107 bases long labeled substrate RNA, as described earlier. When equimolar amounts (100 pmoles) of unlabeled Rz or Dz and substrate RNA was mixed in the presence of 10 mM $MgCl_2$, specific cleavage products could be seen by Rz-8366 (lane 2); Rz-8377 (lane 3); Di-Rz or Rz-77-66 (lane 4) and Dz (lane 5). Note the significant reduction of the substrate RNA (107 nt) in lane 4 and 5 after treatment with Di-Rz and Dz, respectively. Dz-8381MT failed to show any cleavage products under these conditions (data not shown).

3. Results

3.1. Sequence-specific cleavage activities of Rzs and Dz

The labeled transcript (107 nt long, Fig. 4, lane 1) was subjected to *in vitro* cleavage by adding equimolar amounts (100 pmoles) of Rz or Dz (cold) in presence of $MgCl_2$ (10 mM). Both Rzs cleaved the target RNA in a specific manner with varying efficiencies (Fig. 4, lanes 2 and 3), Rz-8377 being the more efficient. Extremely efficient cleavage of the target RNA was observed with Di-Rz (77–66). This Di-Rz retained the cleavage specificity of the two mono-Rzs. The appearance of an 11 base-long RNA fragment was also seen after cleavage (lane 4) reaction. This fragment could only appear if the target RNA was cleaved at both the target sites either simultaneously or sequentially. Specific cleavage products were also observed for Dz-8381 and its efficiency was almost equivalent to Di-Rz (lane 5). Mutant version of the Dz (Dz-8381MT) failed to cleave the target RNA completely under identical conditions (data not shown).

3.2. Effect of $MgCl_2$ on cleavage efficiency

In presence of increasing concentrations of $MgCl_2$ (indicated at the top of each lane) a dose-dependent increase in the cleavage products could be observed with both Rzs and Dz (Fig. 5, panels A–D). The most important observation was that Rz-8377, Di-Rz and Dz-8381 could cleave the target RNA in the presence of 1 mM $MgCl_2$ in the cleavage buffer (lane 2, panels B–D). All, except for Rz-8366, showed significant cleavage activities at micromolar levels of Mg++ (data not shown). Almost 70–80% cleavage of the target RNA was observed by Rz-8377, Di-Rz and Dz-8381 in the presence of 20 mM $MgCl_2$ (lane 6, panel B–D) and the same pattern is reflected in the Fig. 5E (representative data from two independent experiments).

3.3. Cleavage under simulated physiological condition by Rzs and Dz: Di-Rz exhibits novel cleavage activity

All the three Rzs and the Dz were tested for their ability to cleave the same target RNA under simulated physiological condition as described in Section 2.1, and the results are shown in Fig. 6. Lane 1 (panels A–D) shows the synthesis of uniformly labeled target RNA as described earlier, and lane 2 depicts the cleavage reaction carried out in the absence of Mg++. Note that no cleavage was observed if the Mg++ was omitted (lane 2, panels A–D). Lane 3 (all panels) shows the cleavage obtained with the indicated catalytic Rz or Dz. Rz-8366 failed to show any cleavage (panel A); Rz-8377 showed the expected cleaved fragments; Di-Rz showed all the cleaved products as observed in Fig. 4, lane 4. Dz also showed the expected cleavage products. We conclude that except for Rz-8366, all other Rzs and Dz were functional under simulated physiological conditions.

3.4. Intracellular inhibition of HIV-1 gene expression by DNA-enzymes

We selected Rz-8377, Di-Rz and a Dz-8377 for evaluating their bio-efficacy because of their superior *in vitro* cleavage activity. They were co-transfected along with the infectious HIV-1 DNA-pNL4-3 (1 μg for 1×10^6 THP-1 cells in 1 ml) and incubated for 6 days. The supernatant was collected and the levels of p24 antigen were determined by ELISA. Dz-8381Mt that failed to cleave the target RNA completely, served as an important control. HIV-1 p24 levels from three independent experiments are shown in Fig. 7A. A dose-dependent decrease in HIV-1 gene expression was observed with Rz-8377, Di-Rz and Dz-8381 when compared with the amounts of p24 antigen released in the supernatant by pNL4-3 transfected cells (taken as 100%). When they were used in 0.5 μg amounts, all of them showed varying extent of reduction (25–80%) in the amounts of p24 antigen, while at 1.0 μg concentration, a significant inhibition of almost 80% was observed. Cells transfected with mutant Dz (1 μg) showed no significant reduction in HIV-1 gene expression. RT-PCR-based estimation of HIV-1 TAT RNA of treated cells exhibited similar pattern of specific reduction in a dose-dependent manner (Fig. 7B). Both Rz-8377 and Dz-8381

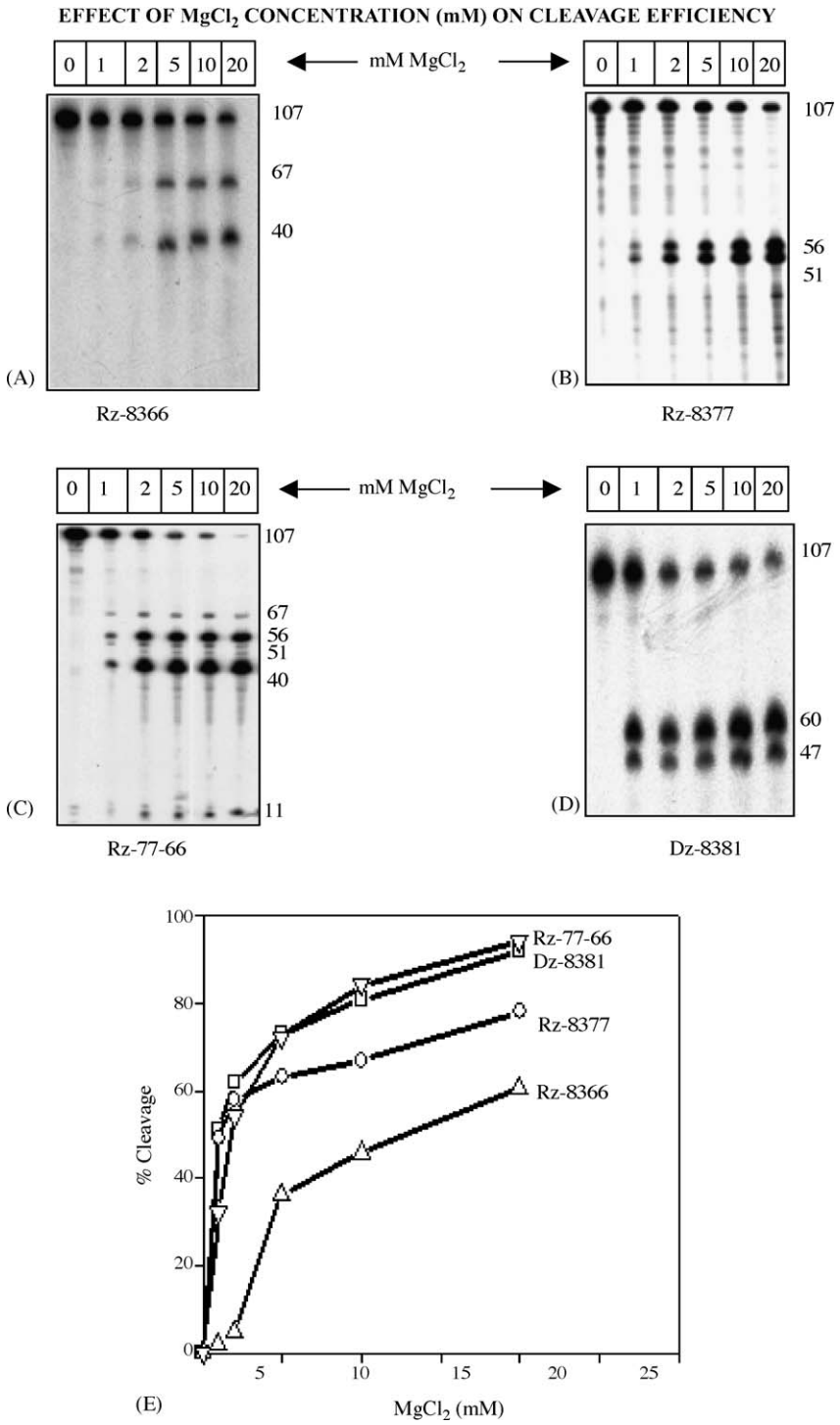

Fig. 5. Effect of varying concentrations of $MgCl_2$ on the cleavage efficiency. Panel A shows cleavage of target RNA by Rz-8366; Rz-8377 (panel B); Rz-77-66 or Di-Rz (panel C); and Dz-8381 (panel D) under varying $MgCl_2$ concentrations (indicated at the top of each lane). No cleavage was observed if the $MgCl_2$ was omitted from the reaction (lane 1 of all the panels). Specific cleavage products could be observed by both Rz and Dz in the presence of increasing amounts of $MgCl_2$. Note significant cleavage by Rz-8377, Rz-77-66 and Dz in presence of 1 mM $MgCl_2$ concentration which is close to physiological levels in humans. The extent of cleavage obtained at varying concentration of $MgCl_2$ is shown in panel E (representative of two separate experiments). This was obtained by calculating the remaining radioactivity of the substrate after the cleavage reaction. Note that Rz-77-66 (Di-Rz) and Dz cleaved the target RNA with equal efficiency.

showed about a three- to four-fold specific reduction in the levels of TAT RNA (compare with the lane that received only pNL4-3) when 0.5 μg amounts of either Rz or Dz was used. When this dose was increased by two-fold (1 μg), about six- to eight-fold reduction in TAT RNA was observed. On the contrary, cells that received 1 μg of mutant Dz-8381 showed no reduction in the levels of TAT RNA. The levels of control RNA were essentially the same in all the corresponding lanes. We conclude that Rz-

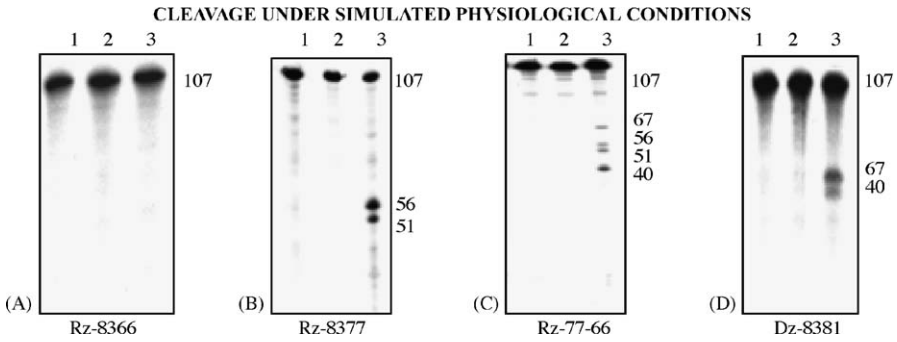

Fig. 6. Cleavage under simulated physiological conditions (150 mM KCl, pH 7.5, 37 °C, 2 mM $MgCl_2$). The labeled target RNA was subjected to cleavage by Rzs or Dz under simulated physiological conditions. Lane 1 in all the panels A–D shows the synthesis of correct size substrate RNA (107 nt) as described earlier. In the complete absence of $MgCl_2$ in the cleavage buffer, no cleavage was observed by both Rz and Dz (lane 2, all panels). Lane 3 (all panels) shows cleavage under simulated physiological conditions. Specific cleavage products could be observed with Rz-8377, Rz-77-66 and Dz-8381 but Rz-8366 failed to show any cleavage products under these conditions.

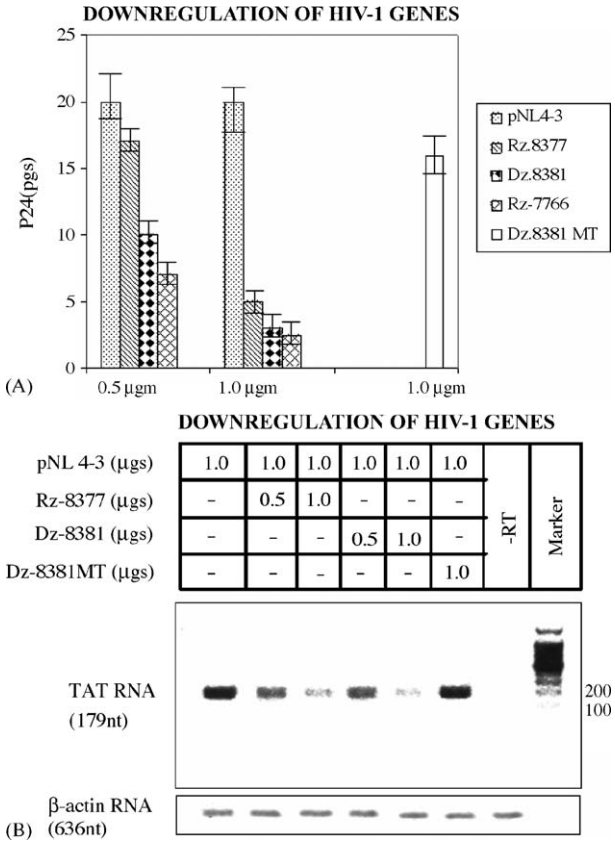

Fig. 7. (A) Downregulation of HIV-1 genes. PMA-stimulated THP-1 cells were grown in six-well plates and transfected with 1 μg of pNL4-3 DNA in the presence of either 0.5 or 1 μg either Rz or Dz using Lipofectin (GIBCO/BRL). Six days post-transfection, supernatants were assayed for p24 antigen by ELISA. The cells transfected with pNL4-3 alone was taken as 100% (control). Rz-8377, Di-Rz and Dz-8381 interfered significantly with the amounts of p24 antigen production. Note that both Rz and Dz showed a dose-dependent protection against HIV-1 challenge. Inhibition was most efficient with Di-Rz, followed by Dz-8381 and then Rz-8377 (mean ± S.D. from three experiments). Mutant Dz failed to show any significant inhibition. (B) RT-PCR was carried out for estimating the levels of Tat/Rev RNA from Rz or Dz treated cells as described earlier. Dose-dependent decrease the levels of Tat/Rev RNA could be seen with Rz-8377 and Dz-8381. Mutant Dz failed to shown any reduction (compare with lane that received pNL4-3 alone). No TAT/REV specific amplification was observed in the absence of RT. Control RNA in all the corresponding lanes showed no reduction.

8377 and Dz-8381 were able to down-regulate the expression of HIV-1 TAT RNA specifically. Rz-8366 treated cells showed only 10–20% inhibition and this correlated well with its *in vitro* cleavage efficiency (data not shown). Level of inhibition by Di-Rz was slightly better than for Dz (data not shown). No TAT RNA specific band was amplified in the absence of RT. The levels of house keeping RNA (control) remained essentially unchanged in all the corresponding lanes. We conclude that Rz-8377, Di-Rz and Dz-8381 are able to specifically down regulate HIV-1 gene expression.

3.5. Inhibition of Tat-mediated apoptosis in Jurkat cells

Jurkat cells were co-transfected with pSVTat72 and Dz-8381 or Rz-8377 in a 1:1 ratio as described earlier. The cells were grown under serum-starved conditions for 48 h and after transfection with indicated Rz or Dz the cells were examined for apoptosis. As a control, cells were also exposed to 0.3% H_2O_2 for 15 min to induce apoptosis. More than 90% of the cells showed apoptosis under these conditions. Reporter gene (pSV-β-gal, Promega) containing plasmid was used to ensure uniform transfection efficiency of the cells. As reported earlier (Li et al., 1995), we observed that Jurkat cells when transfected with HIV-1 TAT-encoding DNA, showed extensive apoptosis when compared to cells alone (as detected by blue stained cells, Fig. 8, panel B). Control cells treated with equivalent amounts of lipofectin did not show any apoptosis (panel A). HIV-1 TAT encoding plasmid induced apoptosis that was comparable to apoptosis induced by H_2O_2 (data not shown), which was earlier shown to be a potent inducer of apoptosis in T cells (Li et al., 1995). When equivalent amounts of Dz or Rz-8377 (1.0 μg each for 1×10^6 cells) was added, the number of cells exhibiting apoptosis was significantly reduced (about 80–90% reduction). The mutant Dz-8381MT, which was used as a control showed apoptosis comparable to cells transfected with pSVTAT72 Tat alone. Di-Rz treated cells also showed impressive inhibition of apoptosis that was comparable to Dz-8377 (data not shown). We conclude that Rz and Dz treated cells show specific protection against TAT-mediated apoptosis.

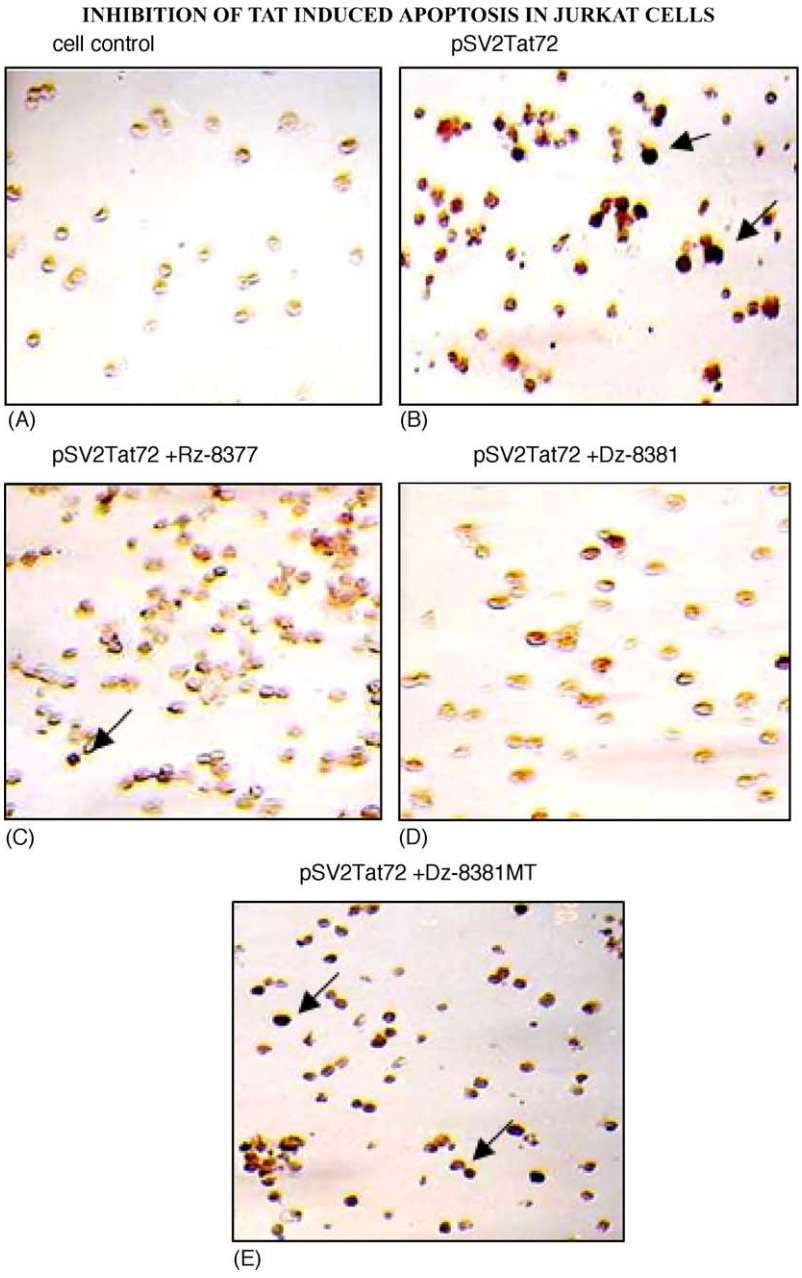

Fig. 8. Inhibition of TAT-mediated apoptosis in Jurkat T cells. Jurkat T cells were co-transfected with HIV-1 TAT expressing plasmid (pSV-Tat) and Rz-8377 or Dz-8381 (1 μg each). The cells were grown in serum-starved conditions and treated cells were stained for apoptosis, as described in Section 2.1. Arrows indicate apoptotic nuclei that stained dark blue. Panel A shows the cell control (no apoptosis). Panels B–D show cells transfected with pSV-Tat72 alone, pCMV-Tat72 + Rz-8377 and pSV-Tat72 + Dz-8381, respectively. Both the Rz and Dz significantly inhibited TAT-induced apoptosis (compare number of blue-stained cells with panel B). The mutant of the same Dz (Dz-8381MT) (panel E) treated cells on the other hand, showed apoptosis comparable to that observed with cells transfected with pCMV-Tat alone (panel B). Di-Rz was just as effective as Dz-8381 in its ability to inhibit apoptosis (data not shown).

4. Discussion

Several *in vitro* and *in vivo* studies suggest that HIV-1 TAT protein is the major determinant for causing programmed cell death or apoptosis among uninfected cells. Apoptotic signals generated as a result of virus infection must be transmitted to uninfected cells because large scale T cell destruction is observed in HIV-1-infected individuals even when only 1 in 10,000 lymphocytes are infected (Li et al., 1995). HIV-1 TAT protein was earlier shown to be secreted by the infected cells, which could exert biological activity on uninfected cells. Therefore, in the present study, we chose an earlier established model of HIV-1 TAT-mediated apoptosis of Jurkat-T cells (Li et al., 1995) to examine the efficacy of the Rz/Dz-mediated antiviral approach. Towards this end, we constructed two monohammerhead Rzs, a Di-Rz by joining them in tandem, and a 10–23 catalytic motif containing Dz that were targeted against a short stretch of genomic RNA. It is important to mention that this region shows extensive overlap of multiple exons (Tat, Rev, Env) and therefore these Rzs or Dz will have the potential to cleave

more than one target RNA of HIV-1. It is noteworthy that the second exon of TAT is highly conserved and is important for *in vivo* replication of HIV-1 (Smith et al., 2003). This is an important feature in the design of our catalytic nucleic acids because it not only can down regulate HIV-1 gene expression but also has potential to delay the appearance of escape mutants. Both Rz and Dz exhibited sequence-specific cleavage activities under variety of experimental conditions and kinetic properties (K_m/K_{cat}) were very similar to the ones reported earlier for other Rzs or Dzs by Santoro and Joyce (1997) and us (Goila and Banerjea, 2001). Increased cleavage with Di-Rz under both experimental conditions (standard and physiological) was most likely due to the melting of secondary structure after successful hybridization of one Rz that facilitated more efficient Watson–Crick base pairing of target RNA by the second Rz. This also explains why under simulated physiological conditions, cleavage products specific for both mono-Rzs were observed, despite the failure of Rz-8366 alone. Di-Rz treated cells when challenged with HIV-1, showed more potent inhibition than compared to mono-Rzs alone and was close to what was achieved with Dz treated cells. This observation correlates well with the *in vitro* cleavage efficiencies of each Rz or Dz. We recently observed significant synergistic effect when Rz-8377 and Dz-8381 were combined (data not shown).

Although the inhibition of the TAT-mediated apoptosis in Jurkat-T cells can be attributed to the sole action of HIV-1 TAT protein, inhibition of virus gene expression could also be due to additive knock down effects of Tat, Rev and Env genes of HIV-1 in our experiments. Also it was clear from our RT-PCR data that both Rz- and Dz-treated cells showed dose-dependent inhibition of intracellular HIV-1 TAT RNA synthesis, which correlated with the inhibition of HIV-1 TAT-mediated apoptosis. Rz or Dz treatment of cells most likely resulted in degradation of multiply spliced 2 kb RNA (encodes TAT, Rev), which included the apoptosis causing glutamine-rich region (Campbell et al., 2004).

While there are several reports of hammerhead Rz being used for achieving inhibition of gene expression (Rossi, 1999; Akkina et al., 2003), there are relatively few studies where catalytic DNA molecules with a 10–23 motif have been exploited. This Dz not only exhibited *in vitro* sequence-specific cleavage activities but also showed impressive inhibition of apoptosis, which was equivalent to what was observed with either Rz-8377- or Di-Rz-(data not shown) treated cells. It is possible to deliver Dz directly into human macrophages in the absence of charged lipid molecules by attaching multiple G residues as shown earlier (Unwalla and Banerjea, 2001). On the contrary, the disabled version of the same Dz (which serves as an antisense DNA control), showed no protection against apoptosis. In this connection it is important to point out that both Rz-8377 and Di-Rz and Dz-8381 retained cleavage activities in simulated physiological conditions even at submillimolar concentrations of Mg++ (data not shown). Dzs that retained cleavage activities under simulated physiological conditions were earlier reported to be more efficacious (Dash and Banerjea, 2004). While the Rzs could be delivered via several viral vectors including lentiviral vectors (Akkina et al., 2003; Banerjea et al., 2003, 2004) to the target cells, catalytic DNA molecules can also be expressed in a mammalian cell using single-stranded DNA expression vectors (Chen, 2002).

In summary, we have identified a potent mono-Rz, a Di-Rz with novel cleavage properties and a Dz that cleaves the HIV-1 TAT/Rev target RNA in a sequence-specific manner which inhibits not only HIV-1 gene expression but also decreases HIV-1 TAT-mediated apoptosis in macrophages.

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References

Adachi, A., Glendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabsom, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59, 284–291.

Akkina, R., Banerjea, A., Bai, J., Anderson, J., Li, M.J., Rossi, J.J., 2003. siRNAs, ribozymes and RNA decoys in modeling stem cell-based gene therapy for HIV/AIDS. Anticancer Res. 23, 1997–2005.

Banerjea, A.C., Joklik, W.K., 1990. Reovirus protein sigma 1 translated in vitro, as well as truncated derivatives of it that lack up to two-thirds of its C-terminal portion, exists as two major tetrameric molecular species that differ in electrophoretic mobility. Virology 179, 460–462.

Banerjea, A.C., Kedarnath, N., Gore, M.M., Dayaraj, C., Jamkar, A.V., Ghosh, S.N., 1981. Interferon (type α and γ) production by fresh and cryopreserved human mononuclear cells. Arch. Virol. 69, 91–94.

Banerjea, A., Li, M.J., Bauer, G., Remling, L., Lee, N.S., Rossi, J.J., Akkina, R., 2003. Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34+ progenitor cell-derived macrophages. Mol. Ther. 8, 62–71.

Banerjea, A.C., Chakraborti, S., Unwalla, H., Goila, R., Basu, S., Dash, B.C., Sriram, B., Paramasivam, N., Viswanathan, S., 2005. Potential therapeutic application of DNA enzymes and siRNAs against viral and cellular genes. In: Khachigian, L.M. (Ed.), Synthetic Nucleic Acids as Inhibitors of Gene Expression: Mechanisms, Applications, and Therapeutic Implications. CRC Press, Florida, USA, pp. 115–134.

Banerjea, A., Li, M.J., Remling, L., Rossi, J., Akkina, R., 2004. Lentiviral transduction of Tar decoy and CCR5 ribozyme into CD34 + progenitor cells and derivation of HIV-1 resistant T cells and macrophages. AIDS Res. Ther. 1, 2.

Berger, E.A., Murphy, P.M., Farber, J.M., 1999. Chemokine receptors as HIV-1 coreceptor: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. 17, 657–700.

Campbell, G.R., Pasquier, E., Watkins, J., Bourgarel-Rey, V., Peyrot, V., Esquieu, D., Barbier, P., de Mareuil, J., Braguer, D., Kaleebu, P., Yirrel, D.L., Loret, E.P., 2004. The glutamine-rich region of the HIV-1 Tat protein is involved in T-cell apoptosis. J. Biol. Chem. 279, 48197–48204.

Chakraborti, S., Banerjea, A.C., 2003. Inhibition of HIV-1 gene expression by novel DNA enzymes targeted to cleave HIV-1 TAR RNA: potential effectiveness against all HIV-1 isolates. Mol. Ther. 7, 817–826.

Chang, H.C., Samaniego, F., Nair, B.C., Buonaguro, L., Ensoli, B., 1997. HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. AIDS 11, 1421–1431.

Chen, Y., 2002. A novel single-stranded DNA expression vector. Expert Opin. Biol. Ther. 2, 735–740.

Chun, R.F., Semmes, O.J., Neuveut, C., Jeang, K.T., 1998. Modulation of Sp1 phosphorylation by human immunodeficiency virus type 1 Tat. J. Virol. 72, 2615–2629.

Coburn, G.A., Cullen, B.R., 2002. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol. 76, 9225–9231.

Cohen, S.S., Li, C., Ding, L., Cao, Y., Pardee, A.B., Shevacch, E.M., Cohen, D.I., 1999. Pronounced acute immunosuppression in vivo mediated by HIV-1 Tat challenge. Proc. Natl. Acad. Sci. U.S.A. 96, 10842–10847.

Dash, B.C., Banerjea, A.C., 2004. Sequence specific cleavage activities of DNA enzymes targeted against HIV-1 Gag and Nef regions. Oligonucleotides 14, 41–47.

Dingwall, C., Ernberg, I., Gait, M.J., Green, S.M., Heaphy, S., Karn, J., Lowe, A.D., Singh, M., Skinner, M.A., 1990. HIV-1 tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. EMBO. J. 9, 4145–4153.

Fauci, A.S., 1993. Multifactorial nature of human immunodeficiency virus disease: implications for therapy. Science 262, 1011–1018.

Fitzgerald, M.L., Moore, K.J., Freeman, M.W., Reed, G.L., 2000. Lipopolysaccharide induces scavenger receptor A expression in mouse macrophages: a divergent response relative to human THP-1 monocyte/macarophages. J. Immunol. 164, 2692–2700.

Flores, O., Lee, G., Kessler, J., Miller, M., Schlief, W., Tomassini, J., Hazuda, D., 1999. Host-cell positive transcription elongation factor b kinase activity is essential and limiting for HIV type 1 replication. Proc. Natl. Acad. Sci. U.S.A. 96, 7208–7213.

Giacca, M., 2005. HIV-1 Tat, apoptosis and the mitochondria: a tubulin link? Retrovirology 2, 7.

Goila, R., Banerjea, A.C., 2001. Inhibition of hepatitis B virus X gene expression by novel DNA enzymes. Biochem. J. 353, 701–708.

Jacque, J.M., Triques, K., Stevenson, M., 2002. Modulation of HIV-1 replication by RNA interference. Nature 418, 435–438.

Jeang, K.T., Berkhout, B., Dropulic, B., 1993. Effects of integration and replication on transcription of the HIV-1 long terminal repeat. J. Biol. Chem. 268, 24940–24949.

Li, C.J., Friedman, D.J., Wang, C., Metelev, V., Pardee, A.B., 1995. Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. Science 268, 429–431.

Lohr, M., Kibler, K.V., Zachary, I., Jeang, K.T., Selwood, D.L., 2003. Small HIV-1-Tat peptides inhibit HIV-1 replication in cultured T-cells. Biochem. Biophys. Res. Commun. 300, 609–613.

Meyaard, L., Otto, S.A., Jonker, R.R., Mijnster, K.J., Keet, R.P., Miedema, F., 1992. Programmed death of T cells in HIV-1 infection. Science 257, 217–219.

Morris, K.V., Rossi, J.J., 2004. Anti-HIV-1 gene expressing lentiviral vectors as an adjunctive therapy for HIV-1 infection. Curr. HIV Res. 2, 185–191.

Neuveut, C., Jeang, K.T., 1996. Recombinant human immunodeficiency virus type 1 genomes with tat unconstrained by overlapping reading frames reveal residues in Tat important for replication in tissue culture. J. Virol. 70, 5572–5581.

Neuveut, C., Scoggins, R.M., Camerini, D., Markham, R.B., Jeang, K.T., 2003. Requirement for the second coding exon of Tat in the optimal replication of macrophage-tropic HIV-1. J. Biomed. Sci. 10, 651–660.

Ott, M., Emiliani, S., Van Lint, S., Herbein, G., Lovett, J., Chirmule, N., McCloskey, T., Pahwa, S., Verdin, E., 1997. Immune hyperactivation of HIV-1-infected T cells mediated by Tat and CD28 pathway. Science 275, 1481–1485.

Purvis, S.F., Jacobberger, J.W., Sramkoski, R.M., Patki, A.H., Lederman, M.M., 1995. HIV type 1 Tat protein induces apoptosis and death in Jurkat cells. AIDS Res. Hum. Retroviruses 11, 443–450.

Rossi, J.J., 1999. The application of ribozymes to HIV-1 infection. Curr. Opin. Mol. Ther. 1, 316–322.

Santoro, S.W., Joyce, G.F., 1997. A general purpose RNA-cleaving DNA-enzyme. Proc. Natl. Acad. Sci. U.S.A. 94, 4262–4266.

Shahi, S., Shanmugasundaram, G.K., Banerjea, A.C., 2001. Ribozymes that cleave reovirus genome S1 also protects cells from pathogenesis caused by reovirus infection. Proc. Natl. Acad. Sci. U.S.A. 98, 4101–4106.

Smith, S.M., Pentlicky, S., Klase, Z., Singh, M., Neuveut, C., Lu, C.Y., Reitz Jr., M.S., Yarchoan, R., Marx, P.A., Jeang, K.T., 2003. An in vivo replication-important function in the second coding exon of Tat is constrained against mutation despite cytotoxic T lymphocyte selection. J. Biol. Chem. 278, 44816–44825.

Stevenson, M., 2003. HIV-1 pathogenesis. Nat. Med. 9, 853–860.

Unwalla, H., Banerjea, A.C., 2001. Inhibition of HIV-1 gene expression by novel macrophage-tropic DNA-enzymes targeted to cleave HIV-1 TAT/Rev RNA. Biochem. J. 357, 147–155.

Vendeville, A., Rayne, F., Bonhoure, A., Bettache, N., Montcourrier, P., Beaumelle, B., 2004. HIV-1 Tat enters T cells using coated pits before translocating from acidified endosomes and eliciting biological responses. Mol. Biol. Cell 15, 2347–2360.

Weissman, J.D., Brown, J.A., Howcroft, T.K., Hwang, J., Chawla, A., Roche, P.A., Schiltz, L., Nakatani, Y., Singer, D.S., 1998. HIV-1 tat binds TAFII250 and repress TAFII250-dependent transcription of major histocompatibility class 1 genes. Proc. Natl. Acad. Sci. U.S.A. 95, 11601–11606.

Westendorp, M.O., Li-Weber, M., Frank, R.W., Krammer, P.H., 1994. Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. J. Virol. 68, 4177–4185.

Xiao, H., Neuveut, C., Tiffany, H.L., Benkirane, M., Rich, E.A., Murphy, P.M., Jeang, K.T., 2000. Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. Proc. Natl. Acad. Sci. U.S.A. 97, 11466–11471.

Yen-Moore, A., Hudnall, S.D., Rady, P.L., Wagner, R.F., Moore, T.O., Memar, O., Hughes, T.K., Tyring, S.K., 2000. Differential expression of the HHV-8 vGCR cellular homolog gene in AIDS-associated and classic Kaposi's sarcoma: potential role of HIV-1 Tat. Virology 267, 247–251.