Inhibition of HIV-1 gene expression by novel macrophage-tropic DNA enzymes targeted to cleave HIV-1 TAT/Rev RNA

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Many regions of the HIV-1 genome have been targeted in earlier studies by RNA-cleaving DNA enzymes possessing the 10–23 catalytic motif, and efficient inhibition of HIV-1 gene expression was reported. All these studies employed charged synthetic lipids to introduce the catalytic DNA into the mammalian cells, which severely limits its practical application and usefulness in vivo. Taking advantage of the ability of G residues to interact directly with the scavenger receptors on the macrophages, we synthesized a DNA enzyme 5970 that contained 10 G residues at the 3' end. With the aim of improving the intracellular stability of the DNA enzyme 5970, we added two short stretches of stem-loop structures that were 12 bases long on either side of the DNA enzyme 5970. DNA enzyme 5970 without the poly-G tracts cleaved the synthetic RNA of HIV-1 TAT/Rev, two important regulatory proteins of HIV, very efficiently in a sequence-specific manner. Addition of 10 G residues at the 3' end of the DNA enzyme affected the cleavage efficiency only marginally whereas the same DNA enzyme with stem-loop structures on either end was significantly less efficient. The DNA enzyme with the poly-G tract at its 3' end was taken up specifically by a human macrophage-specific cell line directly in the absence of Lipofectin and was also able to inhibit HIV-1 gene expression in a transient-expression system as well as when challenged with the virus. The potential applications of these novel macrophage-tropic DNA enzymes are discussed.

Key words: gene silencing, gene therapy, HIV-1 replication.

INTRODUCTION

General-purpose RNA-cleaving DNA enzymes with the catalytic motifs 10–23 and 10–17 were reported by Santoro and Joyce [1] by in vitro selection from a library of DNA sequences using a short HIV-1 target RNA. Since then a number of groups, including ours, have reported sequence-specific cleavage of a variety of target RNAs including HIV-1 RNA [2–6]. All of these studies involved the use of charged lipid molecules to introduce the DNA enzyme into mammalian cells that were subsequently challenged with the virus. Although encouraging results were obtained in tissue-culture experiments, the generous use of charged lipid molecules to facilitate entry of a foreign DNA presents a practical problem besides exerting toxic effects to mammalian cells. Clearly there is great need to develop novel DNA-enzyme delivery systems that work in the absence of charged lipid molecules.

It is now fairly well established that macrophage-tropic R5 viruses initiate infection using CCR5 chemokine receptors, which are present mainly on macrophages and Langerhans cells [7]. This receptor is also involved in transmission, as individuals with a homozygous deletion of 32 bp, corresponding to the second extracellular loop of the seven-transmembrane G-protein-coupled receptor, show strong protection against HIV-1 infection [8–10]. Macrophages are also considered to be reservoirs for HIV-1 by many investigators. Given the important role of macrophages in the early establishment of HIV-1 infection and in the pathogenesis of HIV-1, it would be most appropriate to target macrophages specifically. Earlier studies aimed at specific interference of target-gene expression exploited the affinity of G residues at the end of antisense constructs for scavenger receptors present on macrophages [11,12]. Such G-rich oligonucleotides are involved in the formation of G-tetradis that are sufficient to be recognized by the scavenger receptors and for subsequent uptake. Prasad et al. [13] showed that replication of vesicular stomatitis virus in macrophages could be inhibited using this strategy. This property was exploited by us in this study by designing a DNA enzyme that was targeted to cleave HIV-1 TAT/Rev RNA corresponding to the region where exons 1 of HIV-1 TAT and Rev overlap. We show specific cleavage of HIV-1 TAT/Rev RNA by DNA enzyme 5970 (Dz-5970) alone and by Dz-5970 that possessed 10 G residues at the 3' end (henceforth referred to as poly-G-Dz-5970) and show specific interference of HIV-1 gene expression in a human macrophage cell line in the presence and, importantly, the absence of charged lipid molecules. We also investigated the cleavage potential and bioefficacy of Dz-5970 that contained a short (12 nt) stem-loop structure at either end (henceforth referred to as SL-Dz-5970) with the hope that this modification may afford stability against intracellular degradation by nucleases. Here, we describe in detail the comparison of these novel DNA enzymes with respect to their cleavage potentials and compare their abilities to interfere with the expression of HIV-1 genes.

MATERIALS AND METHODS

Cell lines

Three cell lines (human macrophage THP-1, Cos-1 and HeLa) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 5% fetal bovine serum (Hyclone). THP-1 cells...
were grown in suspension and then, after stimulation with PMA (100 nM) for 24 h as described in [14], were grown in 75 cm² tissue-culture flasks (Becton Dickinson) as monolayers. These cells matured into macrophages and were used for viral-challenge experiments.

**Cloning of HIV-1 TAT/Rev under simian virus 40/T7 promoter**

A DNA fragment encoding the target site was excised by digesting plasmid pNL4-3 with EcoRI (nt 5746; for nucleotide positions see [15]) and HindIII (nt 6026) and subcloned into the polylinker region of the modified pSG5 expression vector (Clontech, Palo Alto, CA, U.S.A.) to yield plasmid pSG1-TAT/Rev (Figure 1B). When linearized with HindIII and subjected to *in vitro* transcription using T7 RNA polymerase (Promega), a 280 nt transcript was expected (Figure 1C). The predicted secondary structure of this region of the target RNA has been exploited by others to design ribozymes [16]. We selected the Dz-5970 target site (position 5970 in the target RNA), as it is present in the predicted loop region (Figure 1D), providing easy access for the Dz-5970 to hybridize on a sequence-specific basis.

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**Figure 1** DNA enzymes against TAT or TAT/Rev RNA

(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 [15].

(B) The region that was excised from the infectious clone [EcoRI (E)–HindIII (H) fragment] that contained exons 1 of TAT and Rev and subcloned into a modified pSG5 vector downstream of the eukaryotic simian virus 40 and T7 promoter. (C) The strategy used to generate an *in vitro* transcript of 280 nt using T7 RNA polymerase. (D) The predicted secondary structure of the TAT/Rev RNA that consists of several stem-loop structures. The target-site AUG for the Dz-5970 is shown by arrows.
Macrophage-specific DNA enzyme against HIV-1

Construction of DNA enzymes

All the RNA-cleaving DNA enzymes possessed the 10–23 catalytic motif. The target site was carefully selected with the hope that HIV-1 Tat as well as Rev could be targeted (Figure 1A). This target site is present in the loop region of the predicted secondary structure of the TAT RNA (Figure 1D). The overall strategy for synthesizing the DNA enzyme was the same as described originally by Santoro and Joyce [1] and described by us recently [17–20]. Briefly, seven bases on either side of the target RNA were synthesized that were complementary to the target RNA. A 10–23 catalytic motif was provided to achieve catalytic cleavage of the target RNA. Two modifications were introduced into the Dz-5970. In one, an extra 10 G residues were added to the 3' end of Dz-5970 (poly-G-Dz-5970; Figure 2B); the other modification was the addition of a short stem-loop structure (12 nt) at the 5' and 3' ends of the Dz-5970 (SL-Dz-5970; Figure 2B). A mutant Dz-5970 (Dz-5970MT) was also synthesized that possessed a single mutation in the catalytic motif (G to C, shown by a box in Figure 2A).

In vitro cleavage reaction

Equimolar concentrations (100 pmol each) of the [γ-32P]UTP-labelled substrate RNA and unlabelled DNA enzymes were mixed in 10 μl of 50 mM Tris/HCl, pH 7.5, containing various amounts of MgCl₂ (final concentration, 10 or 20 mM) and incubated at 37°C for 2 h. The cleavage products were resolved by electrophoresis on a 7% polyacrylamide/7 M urea gel as described previously by us [19].

Labelling of oligonucleotides and cellular uptake

Dz-5970, poly-G-Dz-5970 and SL-Dz-5970 were labelled with [γ-32P]ATP (DuPont/NEN, specific activity 2000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.) and purified on an RPN 5757 column (Amersham Pharmacia Biotech). Approximately equal amounts of radioactively labelled (≈ 80000 c.p.m.) DNA enzymes in a final volume of 1 ml were added to cell monolayers in six-well plates (Nunc) in the presence or absence of Lipofectin (Gibco BRL, Gaithersburg, MD, U.S.A.) for various periods of time. Before harvesting, the cells were washed five times with PBS and radioactivity was determined by standard techniques. Poly-G-Dz-5970 was also end-labelled with 5'-iodoacetamidofluorescein (5-IAF) using a 5'-oligolabelling fluorescence kit (Amersham Pharmacia Biotech) and added to stimulated THP-1 cells. At various time points the cells were washed thrice with PBS and then fixed in 0.1% paraformaldehyde. Samples were analysed on an Elite-ESP flow cytometer (Coulter Electronics, Hialech, FL, U.S.A.) and the data were analysed with FlowJo software (Treestar, San Carlos, CA, U.S.A.). The percentage of fluorescent cells was determined at different time points according to standard procedures.
Inhibition of HIV-1 gene expression by DNA enzymes

A PMA-stimulated adherent population of human macrophage THP-1 cells were employed for evaluating the bio-efficacy of the DNA enzymes. Infectious HIV-1 clone pNL4-3 [15] was used to transfect PMA-stimulated THP-1 cells in both the presence and absence of Lipofectin. Using Trizol reagent (Gibco BRL), total RNA was isolated from cells that had been transfected for 6 days and divided into two equal portions. One set was used to estimate the levels of HIV-1 TAT RNA (179 nt long) and the other set for estimating the control RNA (β-actin) by reverse transcriptase (RT)-PCR techniques using a kit from Promega. The following set of primers were used for amplifying HIV-1 TAT-specific RNA: forward primer (nt 5851–5870), 5′-CTAGAGCCCTGGAAGCATCC-3′; reverse primer (nt 6007–6030), 5′-GCTTGTAGTGCTGACTGTTCTG-3′. Human β-actin-specific primers (Stratagene, La Jolla, CA, U.S.A.; catalogue no. 302010) were used for estimating the control RNA, which was 636 nt long. Supernatants that were pooled from DNA-enzyme-treated and -untreated cells were also used to estimate the levels of HIV-1 TAT RNA (179 nt long) and the other set for estimating the control RNA (β-actin) by reverse transcriptase (RT)-PCR techniques using a kit from Promega. The following set of primers were used for amplifying HIV-1 TAT-specific RNA: forward primer (nt 5851–5870), 5′-CTAGAGCCCTGGAAGCATCC-3′; reverse primer (nt 6007–6030), 5′-GCTTGTAGTGCTGACTGTTCTG-3′. Human β-actin-specific primers (Stratagene, La Jolla, CA, U.S.A.; catalogue no. 302010) were used for estimating the control RNA, which was 636 nt long. Supernatants that were pooled from DNA-enzyme-treated and -untreated cells were also used to estimate the levels of RT using a non-radioactive assay kit from Boehringer Mannheim.
Specific uptake of fluorescence-tagged poly-G-Dz-5970

When 5-IAF-tagged Dz-5970 and poly-G-Dz-5970 (50 pmol each) were applied to stimulated THP-1 cells, only the latter DNA enzyme showed a significant increase in cells that scored positive by FACS analysis (Figure 5), as described in the Materials and methods section. Dz-5970 probably shows background levels of uptake that decreased by 48 h (results not shown). We conclude from this study that poly-G-Dz-5970 is taken up with greater efficiency by human macrophages.

Reduction of HIV-1 TAT RNA by poly-G-Dz-5970 in human macrophage cell line in the absence of Lipofectin

An infectious clone of HIV-1 DNA, pNL4-3, was co-transfected with equal amounts of the four DNA enzymes in the presence of Lipofectin. In addition, poly-G-Dz-5970 was also co-transfected in the absence of Lipofectin. Total RNA was isolated and the levels of HIV-1 TAT RNA and control RNA (β-actin, 636 nt) were estimated by RT-PCR techniques. Figure 6 (lane 1) shows a TAT-specific amplified product (179 bases) when stimulated THP-1 cells were transfected with pNL4-3 DNA. Dz-5970 interfered with the expression of the Tat gene very efficiently (> 10 fold; Figure 6, lane 2). Poly-G-Dz-5970 (1 μg)-treated cells in the presence (Figure 6, lane 3) and absence (Figure 6, lane 4) of Lipofectin reduced the target-gene expression by 10- and 8-fold respectively (compare Figure 6, lane 1 with lanes 3 and 4). Under similar conditions, both SL-Dz-5970 (Figure 6, lane 5) and Dz-5970MT (Figure 6, lane 6) failed to interfere with the gene expression significantly (less than 2-fold reduction). In contrast, RNA levels of the β-actin housekeeping gene remained essentially unchanged (Figure 6, lower panel), suggesting specific anti-viral effects of the DNA enzymes. TAT cDNA was not amplified from THP-1 cells that were transfected with pNL4-3 in the absence of RT (Figure 6, lane 7).

Inhibition of LTR-mediated reporter-gene expression by DNA enzymes

We wanted to compare the efficacy of three DNA enzymes (Dz-5970, poly-G-Dz-5970 and SL-Dz-5970) in HeLa cells, which require the use of Lipofectin for the introduction of DNA enzymes into mammalian cells. Equivalent amounts (1 μg each) of pNL.Luc. and DNA enzymes were used in the presence of 10 μl of Lipofectin for 1 × 10⁶ cells and the results are shown in Figure 7(A). The amount of luciferase detected after transfecting pNL.Luc. was taken to be 100 %. Both Dz-5970 and poly-G-Dz-5970 interfered very significantly with reporter-gene expression.
Figure 4 Uptake of poly-G-Dz-5970 by mammalian cell lines

PMA-stimulated THP-1 and HeLa cells were grown as monolayers in six-well plates. Poly-G-Dz-5970 (A), Dz-5970 (B) and SL-Dz-5970 (C) were labelled with [\(^{32}\)P]-ATP and purified. Radioactive DNA enzymes (\(\approx 80,000\) c.p.m.) were added directly to the monolayer with (right-hand panels) or without (left-hand panels) Lipofectin and incubated for various periods of time. Cells were washed three times with PBS and the radioactivity was determined by standard techniques. In the complete absence of Lipofectin, only poly-G-Dz-5970 showed an increase in uptake with longer incubation (A1) by THP-1 cells, but not in HeLa cells. No specific uptake of Dz-5970 (B1) or SL-Dz-5970 (C1) was observed in either cell line without Lipofectin. As expected, all three DNA enzymes showed an increase in the cellular uptake in the presence of Lipofectin (A2–C2).

Figure 5 Specific uptake of 5-IAF-labelled poly-G-Dz-5970 by THP-1 cells stimulated by PMA

5-IAF-labelled poly-G-Dz-5970 and Dz-5970 were applied to PMA-stimulated THP-1 cells in a six-well plate as described in the Materials and methods section, and the percentage of fluorescent cells analysed by FACS. Significantly more cells were labelled with the fluorescent material when poly-G-Dz-5970 was used.

Figure 6 Down-regulation of HIV-1 TAT mRNA by poly-G-Dz-5970 in THP-1 cells transfected with pNL4-3

PMA-stimulated cells were grown in six-well plates and transfected with 1 \(\mu\)g of pNL4-3 DNA in the presence of equivalent amounts of DNA enzymes using Lipofectin. After transfection (6 days), the levels of HIV-1 TAT and control (\(\beta\)-actin) RNA were estimated as described in the Materials and methods section. As expected, pNL4-3-transfected cells showed prominent HIV-1 TAT-specific amplification (lane 1). Dz-5970-treated cells showed a reduction in TAT-specific RNA (lane 2). Poly-G-Dz-5970-treated cells, with (lane 3) or without (lane 4) Lipofectin, showed interference with the expression of TAT RNA. In comparison, both SL-Dz-5970 (lane 5) and Dz-5970MT (lane 6) failed to reduce the levels of HIV-1 TAT RNA under the same conditions. No TAT-specific amplification was detected from pNL4-3-transfected cells if RT was omitted (lane 7). The inhibition of expression observed with Dz-5970 and poly-G-Dz-5970 was specific, as the levels of control RNA remained unchanged (bottom panel). Lane 8 shows the sizes of the standard DNA markers (PCR markers; Promega).

Down-regulation of HIV-1 proteins by DNA enzymes

When transfected into PMA-stimulated THP-1 cells, the infectious HIV-1 clone pNL4-3 is able to generate infectious virus, as has been reported for many other mammalian cells [15]. The experimental protocol was the same as followed for an earlier experiment (see Figure 6), with the exception that the incubation period after transfection of stimulated THP-1 cells was 6 days. HIV-1 gag-pol particles were recovered from the pooled supernatants as described earlier [21] and the amount of RT was determined using an ELISA kit (non-radioactive; Boehringer Mannheim, Indianapolis, IN, U.S.A.). The values were compared with that of pNL4-3-transfected cells (Figure 7B), which was taken to be 100\% (control). Both Dz-5970 and poly-G-Dz-5970
Figure 7 Down-regulation of HIV-1-specific genes by DNA enzymes

(A) LTR-mediated reporter-gene expression. This experiment was the same as in Figure 6, except that the plasmid pNL.Luc was transfected into HeLa cells and cell lysates were prepared 48 h after transfection to determine the amount of luciferase. Cells treated with 1 μg of the reporter-gene plasmid were taken as 100% (control; column 1). Cells treated with equivalent amounts of Dz-5970 (column 2) and poly-G-Dz-5970 (column 3) showed very significant reductions in reporter-gene expression. On the contrary, SL-Dz-5970 (column 4) as well as the Dz-5970MT (column 5) showed insignificant reduction. (B) HIV-1 RT assay. PMA-stimulated cells were transfected for 6 days with pNL4-3 DNA and DNA enzymes in equal amounts. Supernatants were pooled and HIV-1 gag-pol particles were collected and the amount of RT was determined as described in the Materials and methods section. Cells transfected with pNL4-3 alone were taken as 100% (control; column 1). Dz-5970 (column 2) and poly-G-Dz-5970 (with and without Lipofectin, columns 3 and 4) interfered significantly with the amounts of RT detected in the particles. On the contrary, SL-Dz-5970 (column 5) and Dz-5970MT (column 6) had no effect. All values are means ± S.D. from three independent experiments.

Figure 8 Down-regulation of HIV-1 TAT mRNA by poly-G-Dz-5970 in THP-1 cells infected with the III B strain of HIV-1

PMA-stimulated cells were grown in six-well plates and infected with the III B strain of HIV (subtype B) using DEAE-dextran as described in the Materials and methods section. Cells were harvested 3 days after transfection and the levels of HIV-1 TAT as well as control β-actin RNA were determined by RT-PCR using specific primers (see text). As expected, a prominent HIV-1 TAT RNA band was detected in cells that received the virus alone (lane 1). On the contrary, the cells transfected with poly-G-Dz-5970 (1 μg) showed a significant reduction in HIV-1 TAT RNA (lane 2). Virus-infected cells did not show any signal specific for HIV-1 TAT if the RT was omitted from the reaction (lane 3). This DNA-enzyme-mediated inhibition was specific because the levels of control RNA in the corresponding lanes remained more or less unchanged. Lane 4 shows the sizes of standard DNA marker (PCR markers: Promega).

HIV-1 TAT RNA is reduced in THP-1 cells infected with the HIV-1 III B strain

The HIV-1 III B strain was grown in HeLa-CD4 cells and the dose of the virus for 1×10⁶ THP-1 cells was standardized according to the method described by Paik et al. [21]. The infection was carried out using DEAE-dextran as described in [23]. Cells were harvested 3 days after infection and total RNA was isolated for the purpose of simultaneous detection of HIV-1 TAT and control (β-actin) RNA. As expected, the cells that were infected with the III B virus strain showed a prominent band specific for TAT RNA (Figure 8, lane 1). This band was not detected when RT was omitted from the PCR (Figure 8, lane 3). Poly-G-Dz-5970-treated THP-1 cells interfered very significantly with the expression of HIV-1 TAT RNA (Figure 8, lane 2) and a more than 10-fold reduction was observed (compare Figure 8, lanes 2 and 1). The uninfected cells showed no HIV-1 TAT specific amplification (results not shown).

DISCUSSION

We report for the first time the feasibility of using poly-G-Dz-5970 for the purpose of specifically down-regulating the most important HIV-1 regulatory proteins, namely TAT and Rev, in human macrophages in the absence of any charged lipid molecules. Studies using anti-retroviral therapies have shown any significant reduction of RT activity. Both these DNA enzymes were, as expected, completely inactive in the absence of Lipofectin (results not shown). Thus the difference in RT activity between poly-G-Dz-5970 with and without Lipofectin is about 20%. It must be mentioned that, in this macrophage cell line, replication-competent virus is generated that can initiate multiple rounds of infection. We usually found peak RT production to be 4 or 5 days after transfection [21].
clearly that it is extremely difficult to get rid of HIV-1 totally and that it reappears once treatment is stopped. This is because HIV-1 resides in macrophages/Langerhans cells and continues to release the virus without lysis. Our macrophage-tropic DNA enzymes could potentially be combined with other known anti-retroviral therapies to target these cells. It may also be possible to restore immune competence to the affected macrophages by selectively down-regulating HIV-1 replication in them. We have shown clearly that addition of 10 G residues at the 3’ end of a DNA enzyme containing the 10–23 catalytic domain allowed the retention of significant cleavage activity besides bestowing the unique ability of being taken up specifically by human macrophages. A slight reduction in the cleavage potential of poly-G-Dz-5970 is expected, as the G residues create the potential to form additional secondary structures that can affect its binding with the target RNA. We have not explored the minimum number of G residues that would be required for it to maintain its ability to be taken up by macrophages. It depends upon the ability of formation of quadruplex structures with the G nucleotides [11]. It may be possible to still retain the specificity for macrophages by reducing the G residues to five, as has been reported elsewhere [11]. This not only reduces the cost of the synthesis of oligonucleotides but might also increase the efficiency of cleavage, as in principle fewer secondary structures could be generated. DNA enzymes lacking the G residues at their ends showed no evidence of cellular uptake in the absence of Lipofectin. In presence of Lipofectin, as expected, the three DNA enzymes (Dz-5970, poly-G-Dz-5970 and SL-Dz-5970) showed increased uptake by HeLa and Cos-1 cells. Poly-G-Dz-5970 in the presence of Lipofectin showed significantly higher uptake in THP-1 cells 6 h post-transfection. This is expected as it involves Lipofectin-dependent and -independent (through scavenger receptors) uptake mechanisms.

Furthermore, adding stem-loop structures at both ends of the DNA enzyme not only affected its in vitro cleavage potential adversely but also failed to interfere with the intracellular expression of HIV-1 genes. The amount of reduction in HIV-1 TAT RNA, in both the presence and absence of Lipofectin in THP-1 cells, was very similar. The reduction was due to the catalytic nature of the DNA enzyme because Dz-5970MT failed to interfere with the expression of HIV-1 TAT RNA. This reduction was specific because the levels of control RNA (β-actin) remained unchanged. Interestingly, in the presence of Lipofectin in HeLa cells, DNA enzymes with and without poly-G tracts showed very efficient interference with the levels of reporter-gene expression, with poly-G-Dz-5970 being only slightly less efficient. When comparing the production of structural protein, RT (Figure 7B), with reporter-gene activity (Figure 7A), it seems that the level of interference was not very high in the former case. In connection with this it is important to mention that the reporter-gene activity was studied in HeLa cells that were transfected for only 48 h with pNL.Luc., whereas the structural protein RT was estimated in a macrophage cell line from the pooled supernatant collected over a period of 6 days, which might allow for multiple rounds of replication by HIV-1, as the input DNA enzyme may have been degraded by then. This was necessary to allow the detection of structural proteins by ELISA. Here also, Dz-5970MT and SL-Dz-5970 failed to interfere with the expression of HIV-1-specific genes.

Whether PMA-stimulated THP-1 cells that were treated with poly-G-Dz-5970 in the absence of Lipofectin could be protected against HIV-1 challenge was studied by infecting THP-1 cells with the III B strain of HIV-1. Poly-G-Dz-5970-treated cells showed remarkable protection 3 days after the infection with HIV-1, which was specific because the amounts of β-actin remained unchanged among all the corresponding lysates. It is possible that this protection is transient in nature and may require the continuous presence of this DNA enzyme to down-regulate HIV-1 replication.

In summary, we show that by simply adding poly-G tracts at the 3’ end of Dz-5970, the enzyme can be taken up specifically by human macrophages without significantly altering its catalytic ability to cleave the target RNA in a sequence-specific manner. We also show that such a novel DNA enzyme possesses potent anti-HIV-1 activity and shows significant protection when challenged with HIV.

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Macrophage-specific DNA enzyme against HIV-1


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