Oligonucleotide-mediated retroviral RNase H activation leads to reduced HIV-1 titer in patient-derived plasma

Jochen Heinrich*, Srikanth Mathur*, Alexey A. Matskevich* and Karin Moelling

Background: The retroviral RNase H is essential for viral replication. This component has not yet been extensively studied for antiviral therapy. It can be activated by an oligodeoxynucleotide (ODN) resulting in self-destruction of the virions.

Objective: To examine antiviral potential of ODN in clinical samples using plasma of HIV-1-infected patients.

Design: Plasma of 19 HIV-1-infected patients from Zurich and 10 HIV-1 isolates from Africa and drug-resistant strains were processed for ex-vivo treatment.

Methods: Cell-free virions were treated with ODN in the plasma and HIV RNA was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Furthermore, infectivity of the treated virions was tested on primary human peripheral blood mononuclear cells.

Results: Cell-free virions in plasma contained significantly less intact HIV RNA upon treatment with ODN ($P = 0.0004$), and their infectivity was decreased 52-fold ($P = 0.0004$). In 39% of the Zurich samples, infectivity was reduced more than 10-fold, in 33% more than 100-fold, and in 28% more than 1000-fold. Also, the isolates from Africa exhibited a 63-fold reduction in infectivity ($P = 0.0069$) with 80% of the isolates responding more than 10-fold, 40% more than 100-fold, and 10% more than 1000-fold.

Conclusion: Significant reduction of plasma HIV RNA levels and infectivity of treated virions was achieved on the basis of induced self-destruction of HIV observed with clinical samples. Reduction of viral load *ex vivo* was designed as model for potential effects *in vivo*. Premature activation rather than inhibition of a viral enzyme could be a model strategy for future antiretroviral control.

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Keywords: antiretroviral strategy, HIV, oligodeoxynucleotides, patients, RNase H activation

Introduction

The retroviral RNase H is part of the reverse transcriptase (RT). The RT/RNase H is located inside the virus particles and is carried into the cell during infection. During RT-mediated reverse transcription RNA–DNA hybrids are generated. The RNase H removes the RNA in the hybrids, but leaves the polypurine tract (PPT) RNA intact. The PPT is required as primer for initiation of second-strand DNA synthesis [1]. Previous studies with HIV have demonstrated that an oligodeoxynucleotide (ODN) A that specifically binds the PPT of HIV-1, forms a local RNA–DNA hybrid that mimics a natural replication intermediate [2–5]. This hybrid activates

Institute of Medical Virology, University of Zurich, Zurich, Switzerland.
Correspondence to Professor Dr Karin Moelling, Institute of Medical Virology, University of Zurich, Gloriastrasse 30, 8006 Zurich, Switzerland.
Tel: +41 44 634 19 29; fax: +41 44 634 49 17; e-mail: moelling@immv.uzh.ch
* J.H., S.M., and A.A.M. contributed equally to the writing of the article.
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RNA hydrolysis by the HIV RT/RNase H [6] and destroys the viral RNA template before DNA synthesis [3,4]. The partially double-stranded hairpin loop-structured 54 mer ODN A inhibits HIV-1 replication in infected cells, including drug-resistant strains of HIV [2–4]. Furthermore, as the RT-associated RNase H is associated with virus particles [7], it can be activated outside of the cell and this renders the virus noninfectious [5,8]. The effect of ODN A is sequence-specific [4], and a partially double-stranded ODN is more strongly inhibitory than a single-stranded antisense (as) ODN [4,5].

An ODN also showed in-vivo efficacy against a murine oncogenic retrovirus [8]. The virus causes splenomegaly in mice and we were able to demonstrate reduction of virus load in the blood and of disease progression. However, a murine oncogenic retrovirus does not reflect the situation of primary blood-borne HIV. We designed this study to be as close to the human situation, it builds on the unique property that virus can be targeted before entering a cell. It therefore intervenes earlier than the inhibitors of RT, which only works inside of a cell. The RNase H can also be activated early during infection of a cell and prevents DNA provirus formation [2,3].

Here we demonstrate that HIV particles present in the plasma of infected individuals from Zurich and HIV field isolates from Africa including two drug-resistant strains can be treated before entering a cell with an ODN for activation of the viral RNase H and self-destruction of the virus. Statistically significant reduction of viral titers was observed in all cases. Premature activation of an enzyme instead of its inhibition may be an approach worth pursuing further. Reduction of viral load *ex vivo* may be a model for the in-vivo situation.

Methods

Patients and HIV isolates

We obtained HIV-infected blood from 19 patients from the Zurich University Hospital with a broad range of history, HAART, ART, or no therapies, high or low viral loads, and CD4 cell counts (see Fig. 3a). Furthermore, we obtained HIV-1 field isolates from Africa Uganda-92 (Ug92), Uganda-93 (Ug93), Rwanda (Rw), Malawi (Mw), HIV-1 ELI, HIV-1 LAI/BRU, HIV-1 BaL, HIV-1 IIIB as well as strains resistant to the nucleoside analogue AZT (Azt) and the protease inhibitor Saquinavir (Saqi) from the National Institute of Health (NIH) AIDS Research and Reference Reagent Program (ARRRP) [9].

Plasma, primary peripheral blood mononuclear cells, and HIV

Plasma was extracted from 10 ml of blood of HIV-1-infected patients (available through the Department of Infectious Diseases of the University Hospital, University of Zurich, Zurich, Switzerland) using the BD Vacutainer CPT method (Becton Dickinson, Franklin Lakes, New Jersey, USA). Primary peripheral blood mononuclear cells (pPBMCs) and plasma were extracted from fresh blood obtained from healthy donors (available through the Blood Donation Center, Zurich, Switzerland) using Ficoll Paque Plus methods (GE Health Technologies, USA). Sucrose-purified virions from patient 8898 used for cleavage assays were a kind gift from Dr J. Boeni from the Swiss National Center for Retroviruses, Zurich, Switzerland.

Oligodeoxynucleotides

The ODN A [10] consists of a 25 mer antisense and a 25 mer passenger strand connected by four thymidines (Fig. 1a). ODN Sc [4], which has the same length and nucleotide composition as ODN A but a randomized sequence of both strands, served as a control for nonspecific activity of ODN A (Fig. 1a). The ODNs were phosphorothioated at each end (three bases) and in the T4 linker. The ODNs were purchased from Operon (Germany) or Integrated DNA Technology (USA).

RNA isolation

Viral RNA was isolated from plasma [11] or cell supernatants using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's directions.

Determination of HIV RNA by qRT-PCR

HIV-1 RNA levels were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using RNA extracted from plasma or cell culture supernatants as indicated in the legends. Isolated viral RNA was reverse transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) and qRT-PCR was then performed using the Taqman Universal PCR master mix and the ABI 7300 instrument from Applied Biosystems. Location of the PCR primers and probes on the HIV-1 genome are schematically indicated in Fig. 1b. Coordinates for the primers and probes refer to HIV Gene Bank accession number 9629357. Sequences were gag forward primer, 5′-GCAGCCATGCAAATGTTAAAAGAG-3′; gag reverse primer, TCCCCTTGGTTCTCTCATCTGG [12]; FAM-TAMRA-gag probe, TCTATCCCATTCTG-CAGCTTCCTCATT; UTR forward primer, CAAT-GACTTACAAGGCAGCTGTAGA; UTR reverse primer, TTAGCAGAACTACACACCAGGGC; FAM-TAMRA-UTR probe, TTCACTCCCAAAGAAGA-CAAGATATCCTTGAT. Sequences for nef-readout and cycling conditions were published previously [4,5]. All primers and the gag probe were purchased from Microsynth (Switzerland), the nef and UTR probes from Applied Biosystems. The results are presented in the figures as HIV RNA corresponding to absolute numbers of copies per assay unless otherwise stated.

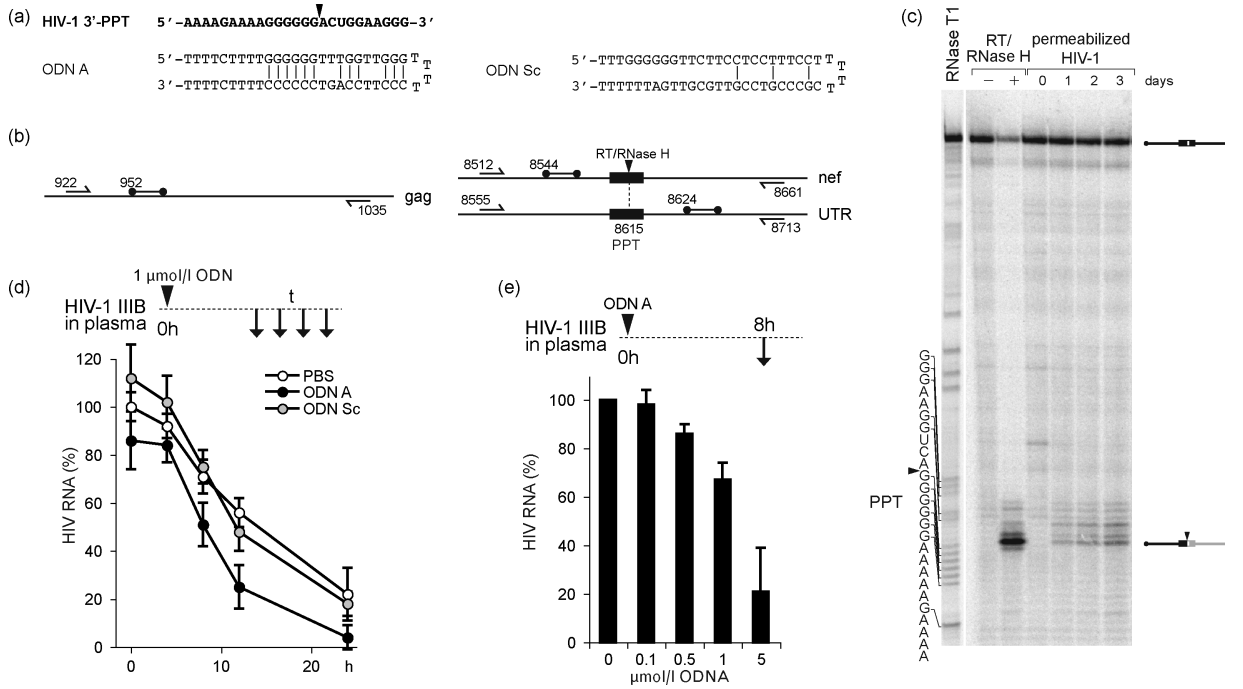

Fig. 1. Oligodeoxynucleotide A mediated degradation of HIV RNA. (a) Sequences of the extended polypurine tract (PPT) region of HIV-1 and oligodeoxynucleotide (ODNs). The cleavage site by RT/RNase H is indicated by an arrowhead, vertical bars indicate base pairing. (b) Detection of HIV-1 by qRT-PCR. The primers (one-sided arrows) and probes (lines with closed circles), and coordinates on the HIV-1 reference sequence (Gene Bank accession number 9629357) as well as the PPT (black box) and the RT/RNase H cleavage site are shown schematically. The primer pairs for detection were designated as gag, nef, and UTR. (c) Cleavage of HIV-PPT RNA by RT/RNase H. Radioactively labeled (dot) HIV PPT-RNA annealed to oligonucleotide was incubated with sucrose gradient-purified virions of a patient, permeabilized with 0.1% NP-40, for the indicated time at 37°C or without (–) or with (+) recombinant RT/RNase H for 30 min. In parallel, HIV PPT-RNA, partially digested with RNase T1, was used as a marker. PPT is indicated by a black bar interrupted by the RNase H cleavage site. (d, e) Assays for virion-associated RNA levels. (d) Time dependence. HIV-1 IIIB supplemented with human healthy serum was treated with 1 µmol/l ODN A, ODN Sc, or phosphate-buffered saline (PBS) (arrow head on dotted line) for the indicated times at 37°C. The viral RNA was extracted (arrows) and quantified by qRT-PCR. The mean values of three independent experiments are shown. (e) Dose dependence. HIV-1 IIIB virions were incubated with the indicated concentrations of ODN A for 8 h at 37°C and the amount of RNA measured by qRT-PCR.

Determination of HIV RNA in virions

HIV-1 virions, derived from 20 µl of patient plasma, were incubated with different concentrations of ODNs. At indicated time points, viral RNA was purified and the amount of undigested RNA was quantified by qRT-PCR using two sets of primers covering the PPT region of the HIV genome, either nef or UTR. Samples (3 µl) obtained from the NIH were diluted to 20 µl in RPMI containing 20% FBS and treated similarly. qRT-PCR was performed using nef primers.

Test for infectivity of cell-free HIV on primary peripheral blood mononuclear cells

One hundred microliters of HIV-containing plasma from patients (Zurich isolates) or 5 µl of the African isolates were treated with either 1 or 5 µmol/l of ODNs for 1 or 4 h. One million phytohemagglutinin (PHA)-activated PBMCs [13] were infected with pretreated HIV-1 virions and grown in culture at 37°C for 3 days in a total volume of 5 ml of RPMI-1640 with 20% FBS in the presence of interleukin 2 (IL-2, 20 U/ml; Zeptometrix, USA). At day 3, 3 ml of RPMI-1640, 20% FBS, and IL-2 were added. At day 7 HIV RNA levels were analyzed in the supernatants using gag-specific qRT-PCR (Fig. 1b). For the experiments with passaged materials, the equivalents of approximately 2×10^4 HIV RNA copies were used.

DNA sequencing

RNA isolated from HIV-1-infected patients and cell culture supernatants was reverse transcribed using random primers and High-Capacity cDNA Archive Kit (Applied Biosystems) and the cDNA was sequenced in the HIV-1 PPT-flanking genomic regions using forward primer CCTCAGGTACCTTTAAGACCAATGAC and reverse primer CCCCTGGCCCTGGTGTGTA. Thermal cycle sequencing was performed on a thermocycler (Touchgene from Genius, USA) for 25 cycles with either the forward or reverse primer using 10 ng of DNA templates. Purified sequencing products were run on an automated ABI 3100 genetic analyzer (Applied Biosystems). Sequences of both strands were

analyzed and edited individually using the software package Sequencer 4.7 (Gene-Codes, USA), assembled as contigs with Sequence Assembler (Applied Biosystems) and subsequently aligned through Clustal-W algorithm of MacVector 7.1 software program (Accerlerys, USA).

Cleavage reactions

HIV-PPT-RNA was transcribed *in vitro*, dephosphorylated, and 5′-labeled as described [5]. Cleavage with virus-associated RT/RNase H in virions was performed with a patient isolate, purified with a sucrose-step gradient, and permeabilized. HIV virions were from patient 8898 (3×10^7 copies/ml). Ten nanomol/l HIV-PPT-RNA and 50 nmol/l oligonucleotide in RT buffer [5] containing 100 ng/ml carrier RNA and 0.1% NP-40 were annealed (2 min 90°C, 10 min 37°C), supplemented with 0.2 U/μl RNasin and incubated for the indicated times at 37°C. The control cleavage was performed with 0.1 U/μl RT/RNase H (GE Healthcare) for 30 min. The samples were proteinase K-treated, phenolized, precipitated, and subjected to electrophoresis in 10% polyacrylamide containing 8 mol/l urea, together with a marker, HIV-PPT-RNA partially digested with RNase T_1 (Ambion, USA) as described by the manufacturer.

Statistical analysis

Samples from several patients were excluded from the analysis for individual experiments, when HIV RNA levels were not detectable in the presence of phosphate-buffered saline (PBS) (four samples) or unusually high (one sample). In the analysis of Zurich virions, some samples were not tested for ODN Sc control, but only for PBS control (six samples). In case of undetectable HIV RNA levels upon treatment with ODN A or ODN Sc, the values were set to the detection limit of 1 copy per assay. Viral RNA levels after treatment were compared using the Wilcoxon signed-rank test using nontransformed HIV RNA levels in copies per assay. To address the problem of multiple comparisons, a Bonferroni correction was performed and thus *P* values smaller than 0.017 are considered statistically significant. SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analyses.

Results

Experimental design and targeting the RNase H in virions

We are using ODNs to target the RNase H in HIV particles. The ODNs are designed to bind to the PPT of HIV-1 IIIB. As controls, we used a scrambled ODN Sc [4] or buffer PBS. The sequences of the PPT and ODNs are shown in Fig. 1a. ODN A effects on HIV were analyzed by measuring HIV RNA levels by quantitative RT-PCR (qRT-PCR) using primers flanking the PPT, designated as nef or UTR, close to the cleavage site to avoid other effects on the RNA. Because this is a highly variable region, we used two primer pairs. The more conserved gag (gag) primers are similar to those used in routine diagnostics tests (Fig. 1b).

In order to verify whether virus-associated RT/RNase H can be activated by an oligonucleotide to degrade PPT-containing RNA, in-vitro transcribed radioactively end-labeled HIV-PPT RNA together with the oligonucleotide was allowed to form a local hybrid as substrate for the RNase H and incubated with permeabilized virions from a sucrose gradient-purified patient-derived HIV preparation. The cleavage site of the viral RT/RNase H was verified with recombinant RT/RNase H and proven to correspond to the natural cleavage site, 5′ of the ACU sequence (Fig. 1c). Furthermore, in a separate study using lysate supplementation assays, we have shown that overexpression of only wild-type RT/RNaseH but not of a mutant deficient in its RNase H activity leads to enhanced antiviral activity of ODN A (Kwok *et al.*, unpublished results).

In order to establish the conditions required for treatment of HIV in blood, we isolated plasma from the blood of healthy donors, spiked it with the cell culture-adapted strain HIV-1 IIIB, and treated it with ODNs for up to 24 h *in vitro*. The amount of nonhydrolyzed RNA was determined by quantitative RT-PCR using primers flanking the PPT and shown to be significantly reduced with time and dose (Fig. 1d and e).

We used HIV-containing plasma from patient 13 and one field isolate from Rwanda, for which sufficient material was available, to establish the conditions with two concentrations of ODNs and various times (4, 8, 12, 16, and 24 h; Fig. 2a and b). We chose 8 h and 5 μmol/l ODNs for further experiments, as these conditions were sufficient to cause degradation of HIV RNA.

We then tested the samples for their infectivity as a second independent read-out, which correlates with viral RNA loads [5], by treating a sample from Zurich patient 3 and HIV-1 BaL strain with ODN A for 1 and 4 h, infecting normal human pPBMCs and culturing them for 7 days (Fig. 2c and d). The amount of HIV-1 RNA in the supernatants was reduced 4 h postincubation with 5 μmol/l ODN A as determined by qRT-PCR using the conserved gag primers. These conditions were further used.

Reduction of viral loads in plasma

HIV-1-containing blood samples of patients from Zurich with different histories were analyzed in this study immediately after isolation. The samples were supplied with information on plasma viral loads and $CD4^+$ cell counts determined prior to this study (Fig. 3a, top). We also analyzed well characterized HIV-1 African primary

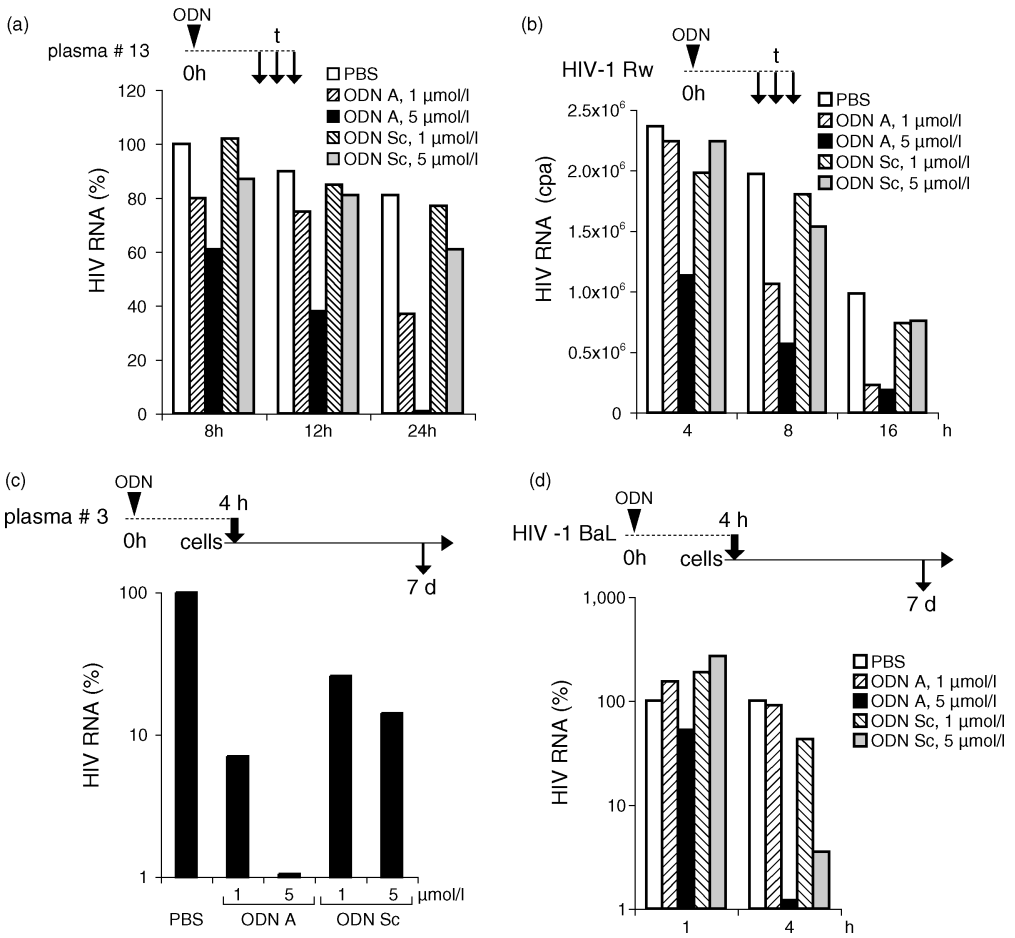

Fig. 2. Oligodeoxynucleotide A mediated degradation of HIV RNA in plasma samples and field isolates. (a, b) Assays for virion-associated RNA levels with time and dose dependence. HIV-1-containing plasma of Zurich patient 13 (a) or field isolate from Rwanda (b) were treated with ODNs at 37°C as indicated and the levels of viral RNA were measured by qRT-PCR. Oligodeoxynucleotide (ODN) Sc or phosphate-buffered saline (PBS) served as controls. (c, d) Assays for infectivity of plasma and virions. HIV-containing plasma of patient 3 (c) or HIV-1 BaL strain (d) was incubated with ODNs as indicated at 37°C for 4 h (patient 3) and 1 and 4 h (HIV-1 BaL). The plasma was then used for infection (thick arrow) of primary human healthy pPBMCs. After 7 days (7d), viral RNA was extracted from the supernatant of infected cells and HIV RNA levels were measured by qRT-PCR.

field isolates and strains resistant against the protease inhibitor Saquinavir (Saqi) or the nucleoside analogue AZT (Azt) from the AIDS Reagent Program (Fig. 3a, bottom). These samples differed from Zurich isolates, as they were supplied after passaging in primary PBMCs. Therefore, we present them as a separate group. The sequences of the HIV-1 PPT regions of few selected patients and field strains were determined and are presented in Fig. 3b.

We tested for ODN-mediated degradation of HIV RNA in plasma samples using qRT-PCR with nef or UTR primers. The amount of nonhydrolyzed viral RNA in blood samples from Zurich and African field isolates of this and the following experiments are shown as median in a box plot (Fig. 3c). Treatment of Zurich samples with ODN A led to reduction of viral RNA levels more than two-fold in 94% and more than 10-fold in 25% of the cases in comparison to PBS. Similar results were obtained with African field isolates and drug-resistant strains (Tables 1 and 2).

Inhibition of viral infectivity of plasma

We then tested the samples for infectivity of ODN-treated HIV-1 virions by measuring HIV RNA levels using the conserved gag primers. The infectivity of all samples of the plasma from patients from Zurich, African field isolates, and drug-resistant strains was analyzed and showed a strong antiviral effect, ranging from two-fold to more than 1000-fold (Fig. 3c and Tables 1 and 2).

In order to examine the antiviral effect of ODNs in the absence of blood factors and differences in viral titers, we used the supernatants from these infected cell cultures (first passage), matched the viral inputs to identical RNA copy numbers, treated them with ODNs and infected primary human pPBMCs. The supernatants (second passage) were analyzed after 7 days, showing even more

(a)

Zurich isolates

Patient ID	HIV-1 RNA	$CD4^+$	Therapy
1	9.0×10^3	491	+
2	5.7×10^3	402	+
3	6.5×10^4	276	+
4	2.0×10^2	354	-
5	3.8×10^4	376	-
6	1.1×10^5	161	-
7	6.1×10^3	434	-
8	1.4×10^5	529	-
9	8.0×10^4	453	-
10	2.0×10^4	336	-
11	1.3×10^5	330	+
12	9.0×10^4	448	-
13	7.8×10^4	566	-
14	9.5×10^5	202	-
15	3.9×10^5	36	+
16	2.1×10^4	453	-
17	9.2×10^4	291	-
18	1.1×10^4	36	-
19	5.6×10^4	453	-

African isolates

Isolate	HIV-1 RNA	Therapy
Ug92	1.0×10^5	n.a.
Ug93	2.2×10^5	n.a.
Rw	4.9×10^9	n.a.
Mw	2.1×10^5	n.a.
LAI	3.9×10^4	n.a.
ELI	1.6×10^6	n.a.
BaL	2.4×10^7	n.a.
Azt	2.7×10^9	+
Saqi	2.5×10^9	+
SIV hu	1.1×10^5	n.a.

(b)

HIV	Sequence
5	TTCT AGGGGAAAAGGGGGGACTGGATGGG
8	TTTA AAAGTAAAAGGGGGGACTGGATGGG
14	TTTC AAGAGAAAAGGGGGGACTGGATGGG
15	TTCA AAAGCAAAAGGGGGGACTGGAAGGG
16	TTTT AAAAGTAAAGGGGGGACTGGAAGGG
17	TTTT AAAAGTAAAGGGGGGACTGGAAGGG
18	TTTA AAAGTAAAAGGGGGGACTGGAAGGG
Ug92	TTTT AAAAGAAAAGGGGGGACTGGAAGGG
Ug93	TTTA AAAAGAAAAGGGGGGACTGGAAGGG
Rw92	TTTT AAAAGAAAAGGGGGGACTGGAAGGG
Mw	TTTA AAAGGAAAAGGGGGGACTGGAAGGG
LAI	TTTT AAAAGAAAAGGGGGGACTGGAAGGG
BaL	TTTT AAGAAAAAAGGGGGGACTGGAAGGG
ELI	TTTT AAAGGAAAAGGGGGGACTGGAAGGG
IIIB	TTTT AAAAGAAAAGGGGGGACTGGAAGGG

extended PPT

(c)

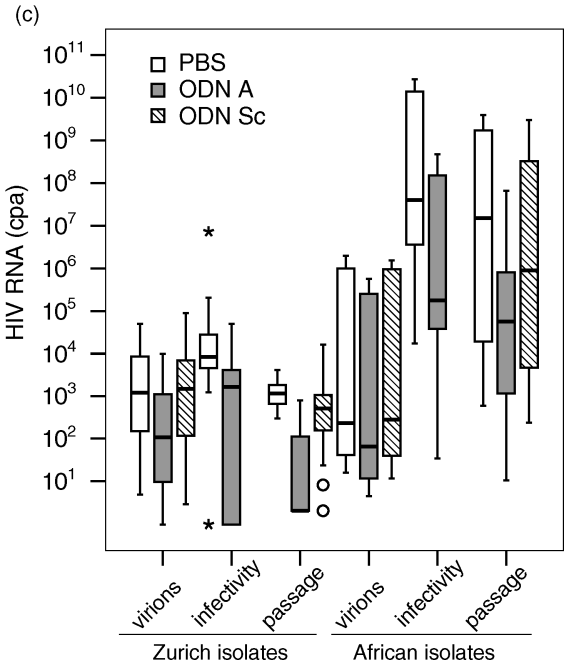

Fig. 3. Overview of Zurich patients, African, and drug-resistant isolates, and analysis of oligodeoxynucleotide A mediated effects. (a) (Top) Characteristics of patients from Zurich infected with HIV-1. HIV-1 plasma loads (copies/ml) measured by COBAS Taqman assay (Roche, Switzerland), $CD4^+$ cell count (cells/ml) and antiretroviral therapy status of the patients was monitored prior to enrollment. (Bottom) African isolates and drug-resistant strains were obtained from the ARRRP through the NIH (n.a., not available). (b) Sequences of the PPTs of HIV from selected Zurich patients and from African and drug-resistant isolates. The HIV-1IIIB extended PPT is indicated by a horizontal line with the RT/RNase H cleavage site (arrow head). Differences to the PPT are underlined. (c) Box-plot presentation of HIV RNA levels in copies per assay after treatment with phosphate-buffered saline (PBS), oligodeoxynucleotide (ODN) A, or ODN Sc (see also Table 1). Black bars represent the median, boxes the quartile, and lines the whisker. Asterisks and circles represent outliers. These data were used for the nonparametric Wilcoxon signed-rank test.

pronounced the ODN A-mediated inhibition (Fig. 3c and Tables 1 and 2).

Discussion

We demonstrate that the viral load in HIV-infected plasma samples from patients can be reduced by activation of the viral RNase H by an ODN. This leads to self-destruction of the virus particles. All ODN A-treated samples had a statistically significant decrease of the mean value of viral RNA compared to the control ODN Sc-treated and PBS-treated groups. The infectivity of treated virions was reduced on average 52–63-fold (Fig. 3c and Table 1). Seventy-five percentage of all isolates showed a more than two-fold reduction of infectivity, 54% more than 10-fold, and 21% more than 1000-fold reduction (Table 2). A combination of all experiments analyzed by a nonparametric test

Table 1. Analysis of the response of HIV-1 isolates to oligodeoxynucleotide A treatment.

Experiment	Treatment	*n*	HIV RNA in copies per assay					Fold reduction by ODN A	*P* values for treatment with ODN A
			Mean	SD	Median	Minimum	Maximum		
Zurich									
Virions	PBS	16	9.2×10^3	1.7×10^4	1.6×10^3	4.0×10^0	5.0×10^4	7.5	0.00044
	ODN A	16	1.2×10^3	2.5×10^3	1.1×10^2	1.0×10^0	1.0×10^4		
	ODN Sc	10	1.1×10^4	2.8×10^4	1.5×10^3	2.0×10^0	9.0×10^4	9.2	0.02842
Infectivity	PBS	17	4.5×10^5	1.8×10^6	8.5×10^3	1.0×10^0	7.3×10^6	52.1	0.00044
	ODN A	17	8.7×10^3	1.6×10^4	1.7×10^3	1.0×10^0	5.0×10^4		
Passage	PBS	19	1.5×10^3	1.1×10^3	1.2×10^3	3.0×10^2	4.1×10^3	12.6	0.00013
	ODN A	19	1.2×10^2	2.2×10^2	1.0×10^0	1.0×10^0	8.1×10^2		
	ODN Sc	19	6.2×10^2	5.8×10^2	5.2×10^2	1.0×10^0	2.2×10^3	5.2	0.00028
African									
Virions	PBS	10	4.1×10^5	7.0×10^5	3.6×10^2	1.6×10^1	2.0×10^6	3.3	0.00506
	ODN A	10	1.2×10^5	2.1×10^5	8.4×10^1	3.6×10^0	5.7×10^5		
	ODN Sc	10	3.6×10^5	5.8×10^5	4.2×10^2	1.1×10^1	1.5×10^6	3.0	0.00506
Infectivity	PBS	10	6.3×10^9	1.0×10^{10}	4.1×10^7	1.7×10^4	2.7×10^{10}	62.5	0.00691
	ODN A	10	1.0×10^8	1.8×10^8	2.1×10^5	3.4×10^1	4.7×10^8		
Passage	PBS	10	1.0×10^9	1.5×10^9	1.9×10^7	6.0×10^2	3.9×10^9	133.1	0.00506
	ODN A	10	7.8×10^6	2.1×10^7	7.3×10^4	9.8×10^0	6.6×10^7		
	ODN Sc	10	5.1×10^8	1.0×10^9	9.1×10^5	2.4×10^2	3.0×10^9	64.8	0.00506

Virion-associated RNA (virions), infectivity of treated primary virions (infectivity), and passaged virions (passage) following treatment with oligodeoxynucleotide (ODN) A or negative controls phosphate-buffered saline (PBS) or ODN Sc were measured by qRT-PCR. The total number of patients tested (*n*) is indicated. HIV RNA levels after treatment of Zurich and African HIV isolates are given as arithmetic mean of HIV RNA in number of copies per assay. SD, median, minimum, and maximum values are shown. Fold reduction by ODN A treatment is the ratio of mean values HIV RNA from PBS-treated or ODN Sc-treated samples and ODN A-treated samples. *P* values compare HIV RNA levels of ODN A treated with PBS-treated or ODN Sc treated groups and were obtained by the nonparametric Wilcoxon signed-rank test. *P* values less than 0.017 are considered statistically significant (n.d., not done; n.a., not available).

with Bonferroni post-hoc test correction (Fig. 3c) revealed a significant decrease not only in viral load but also in variability. The variations may in part be due to the presence of blood-borne components, the status of the patient, the sequences and fitness of the viruses, and so on. All virus samples of patients with or without a history of antiviral therapy were susceptible to ODN A treatment. A second passage with standardized viral inputs strongly increased the specificity and confirmed the antiviral activity of ODN A to be statistically significant (Fig. 3c and Tables 1 and 2).

We have previously shown that multidrug-resistant mutants in the RT can be inhibited by the ODN A-assisted activation of the RNase H [3], which we confirm here with two natural drug-resistant isolates. We noticed the mutations in the 5′ part of the PPT (Fig. 2b) and do not know their contribution to the inhibitory effect of ODN A, yet the isolates were still susceptible to inhibition by ODN A. We showed previously that three mismatches out of 25 between PPT and ODN sequences reduced the inhibitory effect [4]. Some mismatches were determined here (Fig. 3b). Matched ODNs may

Table 2. Analysis of responders.

Isolates	*n*	Percentage of responders to ODN A treatment versus PBS for different ranges of fold reduction			
		>2	>10	>100	>1000
Zurich isolates					
Virions	16	94	25	13	0
Infectivity	16	67	39	33	28
Passage	18	95	63	58	32
African isolates					
Virions	10	100	0	0	0
Infectivity	10	90	80	40	10
Passage	10	100	90	40	20
All isolates					
Virions	26	96	15	8	0
Infectivity	26	75	54	36	21
Passage	28	97	72	52	28

The percentage of responders to oligodeoxynucleotide (ODN) A was calculated for the indicated fold reductions in comparison to phosphate-buffered saline (PBS). The total number of tested samples is indicated (*n*).

therefore enhance the antiviral efficiencies and combinations of ODNs are feasible against a population of mutant viruses.

Furthermore, our unpublished data indicate that in tissue culture, serial passage of HIV-1 with ODN A did not result in mutations in the PPT during 11 passages, and in the RNase H-coding regions of the HIV genome, whereas escape mutants appeared after four passages when control drugs such as forscarnet were used (data not shown).

We previously demonstrated in-vivo efficacy of ODN against a murine retrovirus, the spleen focus-forming virus (SFFV), in mice [8]. The ODN was modified to match the PPT sequence of SFFV and resembles ODN A. Chronically infected mice exhibited either transient or long-term reductions of virus titer depending on the regimen of treatment. Treatment prior to, during, or shortly after infection reduced the viral load in the blood and can delay disease progression, increase survival rates, and prevent viral infection [8]. However, SFFV is a cancer virus with significantly different biological properties than HIV; yet, in both cases, reduction in viral load was observed.

The ODN A used here to target the PPT is directed against the extended PPT covering the ACU site, the natural cleavage site of the RNAse H during replication, and 3′ sequences (see Fig. 1a). The sequencing results demonstrate that the G tract and the sequences 3′ of the ACU site are highly conserved, whereas the 5′ A rich region was more variable (Fig. 3b).

Although the antiviral potential of different forms of ODNs have been extensively studied (reviewed in [14,15]), the ODN A utilized in our experiments may have special properties, as the target strand as well as the second strand of the ODN A are rich in Gs, which may form G tetrads [16]. The second strand may confer higher stability to the ODN A compared to single-stranded antisense strands [4,5]. Furthermore, phosphorothioated modifications present at the termini and in the linker region of the ODNs may help protect against nuclease digestion and increase binding to serum proteins. Other modifications might further improve the antiviral activity of ODN A by changing its stability, uptake, transport, and so on. The properties of ODN A and virus in the blood cannot easily be extrapolated from in-vitro conditions. A better understanding of the mechanism of the antiviral activity of ODN A regarding different aspects of its action would be helpful for further development. Furthermore, the dose, regimen, and toxicity have to be determined. We consider these ex-vivo studies as a model for in-vivo effects in the blood stream.

The fact that the ODN can induce retroviral RT/RNase H-mediated cleavage of HIV outside of cells rendering the virions noninfectious suggests some possibilities for future applications, such as prevention of mother-to-child or sexual transmission, the two major routes of spread of HIV in the Third World. Also, a bridging therapy, allowing multidrug-resistant viruses to become sensitive again, could be envisaged. Our approach suggests that it is feasible to activate a viral enzyme for self-destruction of the virus rather than designing inhibitors.

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