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Design of modified U1i molecules against HIV-1 RNA

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ABSTRACT

Several gene therapeutic approaches have been proposed to add to current antiretroviral therapy against HIV-1. U1 interference (U1i) is a promising new gene therapy tool that targets mRNAs with modified U1 snRNAs. For efficient inhibition, the 3′-terminal exon of pre-mRNAs must be recognized by the modified U1 snRNA. Subsequent interaction between the U1-associated 70K protein and poly(A) polymerase leads to inhibition of polyadenylation and consequently degradation of the pre-mRNA. We designed 14 new U1i inhibitors against HIV-1 mRNA regions that are 100% complementary to at least 70% of HIV-1 sequences listed in the HIV database. All U1i inhibitors were tested transiently in HIV-1 production assays as well as luciferase reporter experiments and three candidates were examined further in stably lentivirus-transduced T cell lines. We identified U1i-J that targets the region encoding the NF-κB binding sites as the most effective inhibitor that substantially reduced viral protein expression. The potency of J is determined in part by the presence of a duplicated target within the HIV-1 mRNA. The stably transduced SupT1 T cells were challenged with HIV-1 but no antiviral effect was detected. U1i inhibitors can be potent suppressors of HIV-1 production in transient assays but further optimization of this antiviral approach is needed.

1. Introduction

Since the identification of HIV-1 infection as the cause of AIDS in 1983 (Barré-Sinoussi et al., 1983), numerous antiretroviral drugs have been developed to suppress viral replication. Unfortunately, the virus may eventually acquire resistance against these drugs. Thus, new drugs and novel therapeutic strategies are needed, including gene therapy applications that may durably protect the lymphocyte population against HIV-1. Blood T cells or CD34+ hematopoietic stem cells can be stably transduced with transgenes that express antiviral molecules, including shRNAs, RNA aptamers, decoys, ribozymes, or antisense RNA (Ter Brake et al., 2008; Mitsuyasu et al., 2009; Lu et al., 2004b). This study deals with the design and testing of novel antiviral transcripts that are based on modified U1 snRNP (U1 small nuclear ribonucleoprotein) complexes.

The U1 snRNP plays a critical role in the mRNA splicing process. The active U1 snRNP comprises a single RNA molecule and 10 proteins, seven Sm proteins and the three U1-associated proteins U1-A, U1-70K, and U1-C (Rossi et al., 1996; Will and Luhrmann, 1997; Query et al., 1989; Scherly et al., 1989, 1990). The splice donor site of a pre-mRNA is recognized by the complementary 5′-end of the 164-nt U1 snRNA (U1 small nuclear RNA) that binds at the exon/intron border (Robberson et al., 1990). Recently, modified U1 snRNPs were designed to suppress the expression of specific mRNAs (Abad et al., 2010; Beckley et al., 2001; Liu et al., 2004; Kato et al., 2002; Jankowska et al., 2008; Sajic et al., 2007; Li et al., 2005). Such modifications of the 5′-end of the U1 snRNA (position 2–11) can redirect the U1 snRNP to the 3′-terminal exon of a specific pre-mRNA target. Interaction between the U1-70K protein and the poly(A) polymerase complex was shown to block pre-mRNA polyadenylation, which is an obligatory RNA processing step for nearly all eukaryotic mRNAs (Gunderson et al., 1998). The targeted mRNA is subsequently degraded, thus blocking gene expression at the post-transcriptional level.

Several rules for optimal U1i design have been previously formulated (Fortes et al., 2003; Abad et al., 2008). For instance, it is critical to target the 3′-terminal exon and to avoid highly structured regions in the pre-mRNA. Strong sequence specificity was demonstrated as a single nucleotide mismatch between the U1i molecule and the targeted mRNA (position 3–8) abolishes the inhibitory efficacy (Abad et al., 2008). This sequence specificity is an obvious therapeutic advantage as it reduces the risk of off-target effects on other mRNAs. In contrast to RNAi inhibitors, no

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saturation effects have been described so far (Fortes et al., 2003; Koornneef et al., 2011). U1i molecules directed against the HIV-1 RNA genome were recently described and strong transient inhibition of viral gene expression was observed, but only slight inhibition of virus replication was shown (Sajic et al., 2007). We set out to design 14 new antiviral U1i molecules that were extensively tested in various cell culture experiments and in lentivirus-transduced CD4+ T cells to study the impact on HIV-1 replication.

2. Materials and methods

2.1. Plasmid constructs

Plasmids expressing U1i inhibitors were cloned by ligation of base-paired oligonucleotides (Sigma, St. Louis, MD) containing the target sequences described in Table 1 into the Bcl1-Bgl2 site of pGEM-U1inWT (Abad et al., 2010). This plasmid contains the U1 snRNA sequence flanked by U1 promoter and terminator, inserted into the BamH1 site of pGEM3. Note that the U1 snRNA gene expressed from this plasmid contains four point mutations to distinguish them from the endogenous U1 snRNA. The resulting U1 snRNA is identical in functionality to endogenous U1 snRNA (Beckley et al., 2001). To generate the pMSCV-puro plasmids that express the U1i inhibitors, the U1 expression cassette, consisting of the U1 promoter, the U1 snRNA sequence and the unique termination sequence were obtained from the pGEM3-based plasmids by digestion with Hinc2 and EcoR1 and inserted into the same sites of pMSCV-puro resulting in pU1i vectors (Clontech, Madison, WI).

To generate lentiviral vectors expressing U1i inhibitors, the U1i cassettes were cloned into the lentiviral vector JS1 (pRRLcpptpgkgfppreSsin) (Seppen et al., 2002). pMSCV plasmids containing J, C, S and U1wt were digested with Xho1 and Stu1 (blunt end cutter) and cloned into JS1 upon restriction with EcoR5 (blunt end cutter) and Xho1.

To construct a Renilla reporter plasmid with the entire HIV-1 target region, the HIV-1 molecular clone pLAI (GenBank No. K02013) served as template for PCR amplification of a 1.7 kb 3′-terminal exon fragment (Fig. 1, L) with oligonucleotides 8462_Xba1 5′-GC*TCTAGA*TATCGTTTCAGACCCACCTC-3′ and 10174_Not1 5′-ATAAGAAT*GCGGCCGC*ACCCAACTGATCTTCAGC-3′ (restriction sites are indicated in italics). The HIV-1 fragment was digested with Xba1 and Not1 and cloned into pRL-SV40 (Promega, Madison, WI) to yield the reporter construct pRL-L.

To test for the sequence specificity of J, five pRL-S reporter variants with modified J target sequences were constructed by annealing two complementary oligonucleotides: J-wt_fw 5′-*CTAGA*GGGACTTTCCGCTGGGGACTTTCC*GC*-3′ and J-wt_rev 5′- *GGCCGC*GGAAAGTCCCCAGCGGAAAGTCCC*T*-3′, J-mut1_fw 5′-*CTAGA*GG**C**A**C**ATTCCGCTGGGGACTTTCC*GC*-3′ and J-mut1_rev 5′- *GGCCGC*GGAAAGTCCCCAGCGGAA**T**GT**G**CC*T*-3′, J-mut2_fw 5′- *CTAGA*GGGACTTTCCGCTGG**C**AC**A**TTCC*GC*-3′ and J-mut2_rev 5′- *GGCCGC*GGAA**T**GT**G**CCCAGCGGAAAGTCCC*T*-3′, J-mut1 + 2_fw 5′- *CTAGA*GG**C**A**C**ATTCCGCTGG**C**AC**A**TTCC*GC*-3′ and J-mut1 + 2_rev 5′- *GGCCGC*GGAA**T**GT**G**CCCAGCGGAA**T**GT**G**CC*T*-3′, J-spacer_fw 5′- *CTAGA*GGGACTTTCCGC**GGTACCGCTAGC**TGGGGACTTTCC*GC*-3′ and J-spacer_rev 5′-*GGCCGC*GGAAAGTCCCCA**GCTAGCGGTACC**GCGGAAAGTCC*CT*-3′. J-target sites are underlined, restriction sites are marked in italics, and mutations or additional nucleotides are indicated in bold. All oligonucleotides were synthesized by Eurogentec (Seraing, Belgium). The matching oligonucleotides were annealed and the DNA fragments were cloned into pRL-SV40 upon digestion with Xba1 and Not1.

For investigations of the NF-κB sites in the HIV-1 promoter, the doxycycline-inducible HIV-1 LTR constructs pLTR2ΔtetO-luc and pLTR2ΔtetOΔNFκB-luc were used that express Firefly luciferase from the LTR-2ΔtetO promoter derived from the HIV-rtTA molecular clone. They contain or miss the two NF-κB sites (Marzio et al., 2001, 2002; Zhou et al., 2006; Das et al., 2011).

All constructs were verified by sequencing using the BigDye terminator cycle sequencing kit (ABI, Forster City, CA).

2.2. Cell culture and DNA transfection

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum (FCS, Hydrobond, Escondido, CA) and minimal essential medium nonessential amino acids (DMEM/10% FCS). The SupT1 cell line was maintained in Advanced RPMI (Gibco, Carlsbad, CA) supplemented with L-glutamine, 1% FCS, 30 U/ml penicillin and 30 μg/ml streptomycin. All cells were kept at 37 °C and 5% CO_2.

Table 1
U1i inhibitors of HIV-1 RNA.

U1i inhibitor	HIV-1 RNA target sequence[a]	Target position[b]	Sequence conservation[c]	Inhibition of	
				CA-p24 production[d]	Renilla reporter expression[d]
A	GAAGAAGAAG	8017–8026	85	–	+
BD	UCUGGGACGA	8084–8093	76	++	+
	UCUGGGACGc[e]	8169–8177			
B	UGGGACGACC	8086–8095	75	+	+
C	UUGAGAGACU	8128–8137	84	++	–
D	CUUCUGGGAC	8167–8176	77	–	++
E	GGGGUGGGAA	8181–8190	80	–	++
F	UAAGACAGGG	8357–8366	77	–	–
G	AGUGGUCAAA	8400–8409	76	–	+
H	GUAGGAGCAG	8477–8486	81	–	+
I	GGGACUGGAA	8674–8683	81	–	++
S	GAAGGGCUAAU	8681–8691	81	–	++
J	GGGACUUUCC	9028–9037	84	+++	+++
	GGGACUUUCC	9042–9051			
K1	CCUCAGAUGCU	9091–9101	72	–	++
K2	AUGCUGCAUAU	9097–9107	72	–	++
3	UUUGCUAUAA	8379–8388	56	++	++

[a] According to the HIV-1 molecular clone pLAI.
[b] Position in the LAI viral RNA genome.
[c] Percentage of HIV-1 sequences in the HIV Los Alamos database 100% identical to the target sequence.
[d] Inhibition in transient co-transfection experiments: +++ = strong inhibition, ++ = medium inhibition, + = low inhibition, – = no inhibition.
[e] Mismatch between U1i molecule and target RNA is underlined.

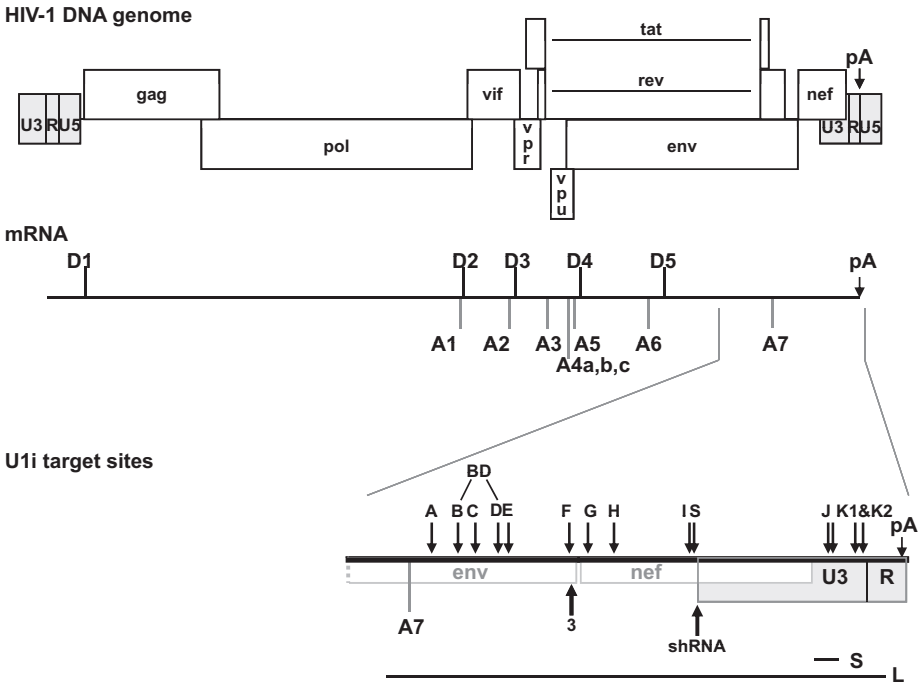

Fig. 1. Target sites of anti-HIV U1i molecules. Depicted is the HIV-1 LAI proviral genome (upper panel), the unspliced mRNA with main splice donor sites (D1-5) and splice acceptor sites (A1-7) (middle panel), and the anti-HIV U1i target sites (A-K2, and 3) (lower panel). shRNA indicates the target site of the control shRNA nef, L and S designate the reporter regions for pRL-L and pRL-S. pA marks the position of the polyadenylation signal.

For transfections with Lipofectamine 2000 (Invitrogen) cells were seeded in DMEM without antibiotics. Transient transfections were performed in the 24-well format, seeding 1.5×10^5 cells per well one day prior to transfection. Lipofectamine 2000 was used for all transfections according the Manufacturer's instructions. All transfections were carried out in duplicate or triplicate and the procedure was repeated once or twice. For the HIV-1 inhibition assay, pU1i-1, -3, -5, -7, and -12 were first titrated (10, 50, 75, 100, and 150 ng) with 250 ng pLAI and 1 ng pRL-CMV (Promega). To test the U1i molecules for HIV-1 inhibition, 250 ng HIV-1 pLAI, 1 ng pRL-CMV, and 75 ng pU1wt or pU1i variants or 25 ng pSuper-shNef were used. Supernatants and cells were harvested after 48 h. HIV-1 production was determined via CA-p24 ELISA in the supernatant, Renilla expression was determined in cell extracts. Cells were lysed in 1x Renilla Lysis Buffer (Promega) for 30 min at room temperature. The extract was used to determine the protein expression using the Renilla Luciferase Assay System (Promega) according to the Manufacturer's instructions. The CA-p24 data were normalized for Renilla expression and all transfection experiments were corrected with Factor Correction as previously described (Ruijter et al., 2006).

For luciferase reporter inhibition assays, 100 ng pRL-L, 75 ng pU1wt or pU1i variants and 50 ng pGL3 (Promega) that expresses Firefly luciferase were co-transfected. Renilla and Firefly luciferase expression was measured 48 h later. To test the effect of pRL-L amount on the Firefly expression in the presence of U1i inhibitors, 293T cells were transfected with 200 ng pGL3, 75 ng pU1wt or pU1i-variants and either 0.2, 1, or 10 ng pRL-L. Both luciferase expression levels were measured 48 h post transfection. To read out dual luciferase expression, cells were lysed in 1× Passive Lysis Buffer (Promega) and both luciferase activities were determined using the Dual-Luciferase® Reporter Assay System (Promega). The Renilla data were normalized for Firefly expression. To determine the specificity of U1i gene repression, 100 ng pRL-S variants or non-modified pRL-SV40 were co-transfected with 50 ng pU1wt or pU1i-J, and 1 ng pGL3. Cells were lysed 48 h later and dual luciferases were determined as described above.

To exclude the possibility that U1i inhibitors work also on DNA, a LTR reporter system was applied that harbored the J target site in its promoter region but not in the transcribed pre-mRNA. 20 ng pLTR2ΔtetO-luc (pLTR+) or pLTR2ΔtetOΔNFκB-luc (pLTR-), 0.4 ng prtTA, and 50 ng pU1wt or pU1i-J were co-transfected. pLTR+ contains two NF-κB sites, pLTR- lacks these two sites in the promoter region and serves therefore as negative control (Das et al., 2011). This system is only residually active and 1000 ng/ml doxycycline was added to fully activate gene transcription. Dual luciferase expression was determined 48 h post-transfection. The Firefly expression was normalized for the Renilla expression as previously described (Zhou et al., 2006).

The lentiviral JS1 constructs harboring the U1 cassettes were tested for their inhibitory activity in a transient transfection assay. 293T cells were transfected with 100 ng pRL-L, 50 ng pGL3 and 50 ng of either JS1-J, JS1-C, JS1-S, empty JS1, pU1i-J (positive control) or pU1wt following the Lipofectamine protocol. Cells were harvested after 48 h and Renilla and Firefly expression was determined.

2.3. Lentivirus production and T cell transduction

293T cells were seeded in 6-well-plate format one day prior to transfection to reach a 70% confluence for lentivirus production. A mixture of 950 ng lentiviral plasmid JS1-U1i variants, unmodified JS1 or JS1-pol1, expressing shRNA pol1 (Ter Brake et al., 2006), 600 ng pSYNGP, 330 ng pVSVg, and 250 ng pRSV-rev were transfected using Lipofectamine 2000 as previously described (Liu et al., 2009). The titer was determined via titration on SupT1 cells, scoring the percentage of GFP-positive cells by FACS as described previously (Liu et al., 2009). Then, a multiplicity of infection (MOI) of 0.15 was used for transduction (Ter Brake et al., 2006). Four days later, cells were sorted for GFP-expression by fluorescence-activated cell sorting.

2.4. RNA isolation and real-time PCR

Total RNA was isolated using TRI reagent following the manufacturer's instructions (Sigma). 2 μg of total RNA was DNase treated (Invitrogen) and reverse-transcribed with MMLV reverse transcriptase (Promega). U1 snRNA expression was quantified by real-time PCR (BioRad, Berkeley, CA) with primers designed to detect exogenous U1 snRNA (U1Fw: 5′-GATCTCATAGTTCCATGGCAGGGGAGATACCAT-3′ and U1Rev: 5′-CGAGTTTGGCACATTTGGCC-3′) or endogenous U1 (EndoU1Fw: 5′-GATCTCATAGTTCCATGGCAGGGGAGATACCAT-3′ and EndoU1Rev: 5′-CGAGTTTCCCACATTTGGGG-3′) and normalized to human actin expression (Act Fw 5′-AGCCTCGCCTTTGCCGA-3′ and mAct Rv 5′-CTGGTGCCTGGGGCG-3′). Oligo hybridization was performed at 60 °C and each cycle of amplification was done at 72 °C for 25 s.

2.5. HIV-1 challenge experiments

HIV-1 virus stock was produced in 293T cells, of which 6×10^5 were seeded in a T75 flask one day prior to transfection. 40 μg HIV-1 pLAI was transfected following the Lipofectamine 2000 protocol and supernatant was harvested at 48 h post transfection. Cells were removed by centrifugation (4000*g*) and the supernatant was aliquoted and stored at −80 °C. The HIV-1 LAI virus stock was quantified for CA-p24 by ELISA measurement.

HIV-1 replication assays were performed in the 24-well-plate format. A total of 2×10^5 transduced SupT1 cells were seeded per well and infected with 0.5 and 0.05 ng of CA-p24 HIV-1 LAI. Supernatant was sampled three times a week, and cells were passaged twice a week. HIV-1 replication was monitored using light microscopy by the appearance of cytopathic effects (syncytia) and quantified by measuring CA-p24 in the supernatant by ELISA.

2.6. Statistical analysis

To test significant differences in the transfection experiments, the unpaired t-test (two-tailed) was applied, using GraphPad Prism 5 Software.

3. Results

3.1. Design of anti-HIV U1i inhibitors

The U1i mechanism has been described as a potent tool to trigger degradation of mRNAs of interest and thereby inhibit protein expression. The targets could be of cellular origin or exogenous infectious agents such as HIV-1 (Abad et al., 2010; Jankowska et al., 2008; Sajic et al., 2007). Our aim was to identify U1i molecules that can potently suppress HIV-1 replication, to compare their activity with well-known antiviral shRNA molecules, and to study the mechanism of U1i-mediated inhibition of gene expression.

The new antiviral U1 snRNAs were designed according to the design rules proposed by Fortes et al. (Fortes et al., 2003; Abad et al., 2008): (I) the U1i molecules must target the 3′-terminal exon of the targeted mRNAs; (II) the 5′ sequence of the U1 snRNA that is complementary to the target mRNA should be optimally 10 or 11 nucleotides in length; (III) we actively looked for repeated target sequences as the level of inhibition is increased if multiple targets are present within the 3′-terminal exon; (IV) structured regions of the mRNAs should be avoided. In total, we designed 14 new U1i inhibitors with HIV-1 target complementarities of 10 or 11 nucleotides that were distributed across the 3′-terminal exon (Table 1, Fig. 1). For comparison, we also included U1i-3 which was described previously (Sajic et al., 2007). Although HIV-1 encodes numerous alternatively spliced transcripts, they all share this 3′-terminal exon that contains part of the env and the complete nef open reading frame (Purcell and Martin, 1993; Cochrane et al., 2006). An additional selection criterion concerns the conservation of the target sequence among virus strains. We obviously prefer to target as many HIV-1 strains as possible, and targets were selected based on the highest sequence conservation in the env–nef coding sequences (Table 1). In evolutionary terms, these targets may also avoid the selection of escape HIV-1 variants if indeed no or not much sequence variation is allowed. Therefore, the selected U1i inhibitors were 100% complementary to at least 70% of up to 1496 complete HIV-1 sequences in the Los Alamos database (<http://www.hiv.lanl.gov>) (Table 1).

The U1i molecules target sequences in the 3′-terminal domain of the env gene (A–F, and positive control 3), the nef gene (G–I, and S), and non-coding regions in the U3 segment of the 3′-LTR (J, K1, and K2) (Fig. 1). BD overlaps with the binding site for B, and it has a partial match (1 mismatch) with a sequence that overlaps with target D. In fact, according to Abad et al., this terminal mismatch (Table 1, underlined), does not frustrate U1i action (Abad et al., 2008). S was designed to compare inhibition by the U1i and RNAi mechanisms as the target site of the well-characterized shRNA nef contains the same 10 nucleotide sequence (Ter Brake et al., 2006). Inhibitor J recognizes a tandem target separated by only four nucleotides. The K1 and K2 target sites overlap by five nucleotides. As a negative control, we included an anti-Notch1 U1i molecule (Abad et al., 2010). Notch is a transmembrane receptor that is involved in signal transduction and its knock-down should not affect HIV-1 production.

3.2. Transient inhibition of HIV-1 gene expression

To test the antiviral potency of the U1i molecules, we established a co-transfection assay of the molecular HIV-1 clone pLAI with the pU1i expression vectors. We determined the optimal ratio of the molecular HIV-1 clone pLAI, pU1i vectors and pRL-CMV. Good inhibition could be observed with 75 ng/well pU1i expression plasmid (data not shown). Among the positive control constructs (Sajic et al., 2007), inhibitor 3 turned out to be the best HIV-1 inhibitor and was therefore selected to serve as positive control.

Thereafter, the potencies of the new U1i inhibitors were studied. The U1i action was measured by knock-down of CA-p24 production (Fig. 2A, middle panel). We also measured Renilla expression as transfection control (Fig. 2A, lower panel). These two values were used to calculate the normalized virus production levels (Fig. 2A, upper panel). Results obtained with the control plasmid expressing U1wt were set at 100% and all results of the anti-HIV U1i molecules were compared to it (Fig. 2A). Most potent inhibition of HIV-1 gene expression to merely 3% was observed for J. Two other U1i molecules demonstrated good inhibition: C and BD suppressed viral protein production to approximately 40%. Thus, the two U1i variants with tandem target sites in HIV-1 mRNA, BD and J, are among the three potent U1i candidates. U1i-3 and shRNA nef repressed virus production to 30% and 14%, respectively. The negative control U1i-Notch did not influence HIV-1 production. In general, the Renilla values did not vary significantly among the samples.

We next performed titration experiments with the best inhibitor J versus the negative control U1wt and the positive control 3 in the 1–150 ng concentration range. J proved its robustness with up to 46-fold higher inhibitory capacity than U1i-3 (Fig. 2B). All transfected cultures were monitored via light microscopy and in general, we observed cytotoxic effect for the U1i inhibitors at 150 ng DNA/well.

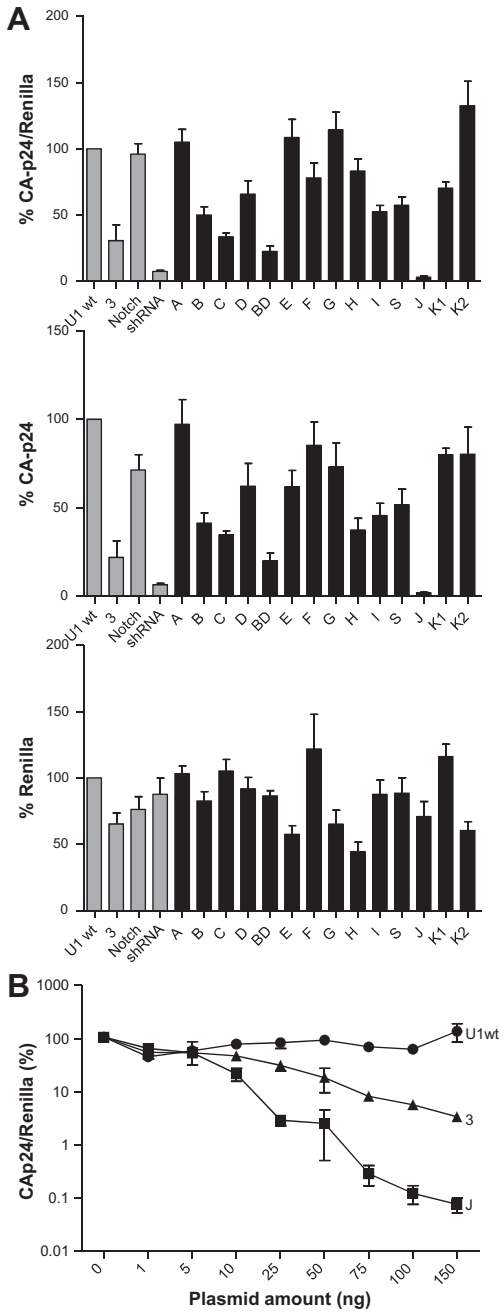

Fig. 2. Activity of U1i inhibitors in HIV-1 production assays. 293T cells were transfected with the HIV-1 molecular clone pLAI, pU1i-variants and pRL-CMV. After 48 h, CA-p24 and Renilla expression levels were determined and CA-p24 values were normalized for Renilla expression. The standard error of the mean (SEM) is indicated. (A) Activity of 14 new U1i molecules (A-K2) compared to wildtype U1 snRNA expressing plasmid (U1wt), which served as negative control and was set at 100%, pU1i-3 (3) is the positive control, pU1i-Notch (Notch) served as additional negative control. Controls are marked in grey, results of the new U1 snRNAs in black. The shRNA nef (shRNA) expressed from pSuper was included for comparison of the activities of U1i molecules to a potent RNAi inhibitor. The data are presented separately by CA-p24 (middle panel) and Renilla expression (lower panel) and normalized (CA-p24/Renilla, upper panel). B) Titration of U1wt, 3, and J from 0 to 150 ng pU1wt or pU1i-variants, the culture without pU1(i) plasmids was set at 100%. U1i-J inhibition was significantly stronger ($p < 0.05$) compared to U1i-3 with 10 ng plasmid input and this difference was more pronounced at higher plasmid input being very significant ($p < 0.01$) with 25–100 ng and extremely significant ($p < 0.001$) with 150 ng of plasmid. Differences between U1wt and U1i-J were significant with 150 ng plasmid input, and extremely significant with plasmid amounts of 10–100 ng. Also differences between U1wt and U1i-3 were significant with 25 and 150 ng, very significant with 75 ng plasmid input and extremely significant using 50 and 100 ng of plasmid.

3.3. Transient inhibition of luciferase reporters

HIV-1 gene expression is a highly complicated process that includes regulation at diverse levels, including transcription, splicing and translation. Therefore, the U1i inhibitors were tested with an artificial and less complex reporter assay. We designed a simplified Renilla luciferase reporter system pRL-L with the HIV-1 3′-terminal exon (Fig. 3A). A Firefly luciferase expression plasmid pGL3 served as transfection control and Renilla data were normalized for Firefly expression. We evaluated different concentrations of pU1i plasmids, pRL-L and pGL3 (data not shown), and inhibitor 3 was approved as positive control.

The complete set of U1i molecules and controls were tested in co-transfection with pRL-L. U1wt served as negative control and normalized Renilla values were set at 100%. Again, J exhibited the strongest potency with a reduction of protein expression to 8%, molecules D, BD, and K1 to around 34% (Fig. 3B, upper panel). Both C and the positive control 3 showed only weak inhibition of around 60%.

However, inspection of the individual Renilla and Firefly values showed that the Firefly control was also significantly affected by U1i expression (Fig. 3B, lower panels). Apparently, the inhibition pattern of the U1i constructs on Firefly luciferase resembles the impact on Renilla expression. This unexpected result could not be avoided by the usage of less pRL-L (data not shown). We found a suppression of Renilla expression to 30% or less for D, BD, E, I, S, and J (Fig. 3B, middle panel). The negative control Notch also inhibited Renilla (27%) and Firefly expression (26%). These results raise doubts about the specificity of U1i action.

3.4. J activity reveals major nonspecific effects

To investigate sequence-specificity of U1i action we selected the most potent inhibitor J that has a tandem target site in HIV-1 and then generated pRL-S reporters (Fig. 4A). We introduced inactivating mutations in the 5′ and/or 3′-target site. More precisely, point mutations were introduced at position 3 and 6 of the 10-nucleotide target sequence because it has been demonstrated that even a single mutation at position 3–8 decreases U1i action dramatically, and two mutations lead to a complete loss of function (Abad et al., 2008). In addition, we inserted 12 nucleotides between the two targets that increase the spacer from four to 16 nucleotides to test whether close proximity of the two target sites contributes to potent inhibition (Fig. 4A). We deliberately tested the effects on the Renilla-HIV reporter without Firefly control.

Each of the reporters was tested in combination with J or U1wt. Results with U1wt were set at 100% and J is compared to it for each reporter variant separately (Fig. 4B). Optimal J-mediated inhibition of Renilla expression was observed with both targets in the wildtype configuration with a Renilla knock-down to 10% (Fig. 4B). Insertion of the spacer did not exert an impact. Destruction of the first or the second target site partially abrogated the Renilla knock-down to around 18% (Fig. 4B), and mutations in both target sites further diminished Renilla knock-down to 26%. These reductions are statistically significant compared to the J effect on the wildtype target region (Fig. 4B). However, it was expected that mutating both target sites will lead to a complete loss of inhibition. Similar to the results obtained with pRLmut1+2, J also repressed Renilla expression of the non-modified pRL(−) plasmid to 29% even though this plasmid lacks the J target sites (Fig. 4B). These results with pRL(−) and pRLmut1+2 led us to conclude that there is a major level of nonspecific repression of Renilla expression of almost 70%. In contrast, the sequence-specific contribution of J to inhibit the Renilla expression seems negligible. These results corroborate the presence of a major nonspecific component in U1i action.

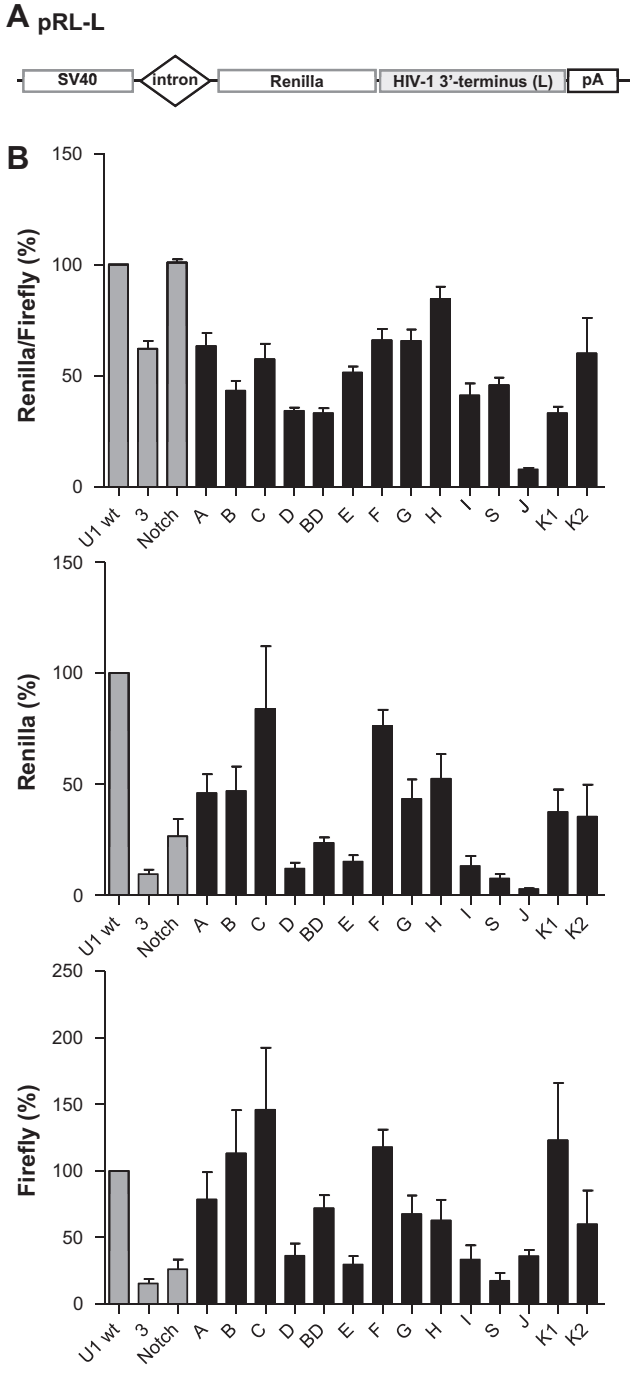

Fig. 3. Activity of U1i molecules in a dual luciferase reporter assay. (A) pRL-L encoding the 1.7 kb target sequence for 14 new U1i molecules. SV40 = SV40 promoter; pA = polyadenylation signal. (B) pRL-L, pGL3 and pU1i-variants were co-transfected into 293T cells. 48 h post-transfection, Renilla and Firefly expression levels were determined. The data are blotted separately for normalized Renilla/Firefly results, only Renilla and only Firefly measurements. U1wt served as negative control and was set at 100%, Notch was a negative control, U1i-3 was included as positive control, all controls are marked in grey. The SEM is provided.

3.5. J does not affect the HIV-1 promoter activity via the NF-κB sites

J targets a tandem sequence near the 3′-end of the HIV-1 mRNA (Fig. 1). At the DNA level, the targets encompass the two NF-κB sites that act as a transcription factor binding site in the 5′-LTR promoter. It was shown previously that besides their significant splicing function, U1 snRNAs can also regulate transcriptional initiation via interaction with TFIIH (Kwek et al., 2002). We therefore wanted to exclude that the strong J activity is induced by sequence-specific interaction of the modified U1 snRNA with the DNA promoter. In analogy, the RNA-targeting RNAi mechanism may also have such a DNA-phase that is known as transcriptional gene silencing (Suzuki et al., 2005; Morris et al., 2004). To investigate this possibility for J, we used an LTR-luciferase reporter with (pLTR+) or without (pLTR-) NF-κB sites in the LTR promoter (Fig. 5A). This modified LTR promoter system contains tetO elements that require the rtTA transcriptional activator protein and doxycycline for activation (Zhou et al., 2006).

The effect of J on Firefly luciferase expression was measured in a co-transfection assay and U1wt served as negative control. The data were normalized for Renilla expression from the co-transfected pRL-CMV plasmid. U1i-J did not have a significant effect on either the pLTR+ or the pLTR- reporter (Fig 5B). We detected only very low levels of luciferase expression without doxycycline, but also this basal promoter activity was not affected by J. We can therefore exclude any antiviral effects of J on DNA target sequences present in the LTR promoter.

3.6. HIV-1 replicates in lentiviral-transduced U1i expressing cells

We generated lentiviral vectors with the U1i cassettes for stable transduction of the SupT1 T cell line (J with high, C with intermediate and S with low potency, and wt U1 control) (Liu et al., 2009; Seppen et al., 2002). We first confirmed the activities of these JS1-U1i DNA constructs in co-transfections with the Renilla reporter construct pRL-L, which revealed similar activities as observed in Fig. 3B (results not shown). We next transduced SupT1 cells with the lentiviral vectors at a multiplicity of infection (MOI) of 0.15 to ensure the establishment of a single integrated vector copy per transduced cell. The GFP expression cassette in JS1 allowed the transduced cells to be sorted by FACS.

The transduced and sorted cells were challenged with two doses of virus. Rapid virus replication was observed in all U1i-transduced SupT1 cells, comparable to the virus kinetics observed with control cells transduced with the empty JS1 vector (Fig. 6A). Real-time PCR was used to determine the level of exogenous U1 snRNA expression in the stably transfected cells (Fig. 6B). We measured a 34- to 145-fold lower expression of exogenous versus endogenous U1 snRNAs (Fig. 6B). Previous quantitative real-time PCR experiments indicated a similar expression level of exogenous and endogenous U1 snRNAs in transiently transfected cells that gave a robust U1i inhibition of targeted mRNAs (data not shown). It can be assumed that in transiently transfected cells, several copies of the U1i inhibitor are necessary per cell to yield potent mRNA suppression. This implies further that a single copy of the U1i gene is apparently not sufficient to reach inhibiting levels and to prevent the spread of HIV-1. In contrast, a single copy of a potent antiviral shRNA cassette does efficiently suppress HIV-1 replication (Fig. 6A) (Ter Brake et al., 2006).

4. Discussion

Several RNA-based inhibitors against HIV-1 are being tested in a pre-clinical or clinical setting for durable gene therapy (Ter Brake et al., 2009; DiGiusto et al., 2010; Banerjea et al., 2003; Anderson et al., 2007; Levine et al., 2006; Lu et al., 2004a). Each method has some specific restrictions, such as saturation of important cellular pathways, cellular toxicity, or sub-optimal antiviral potency. The U1i mechanism was recently proposed as an alternative tool for effective anti-HIV gene therapy that could be combined synergistically with RNAi-based antiviral constructs (Abad et al., 2010; Koornneef et al., 2011; Sajic et al., 2007). U1 snRNAs were modified

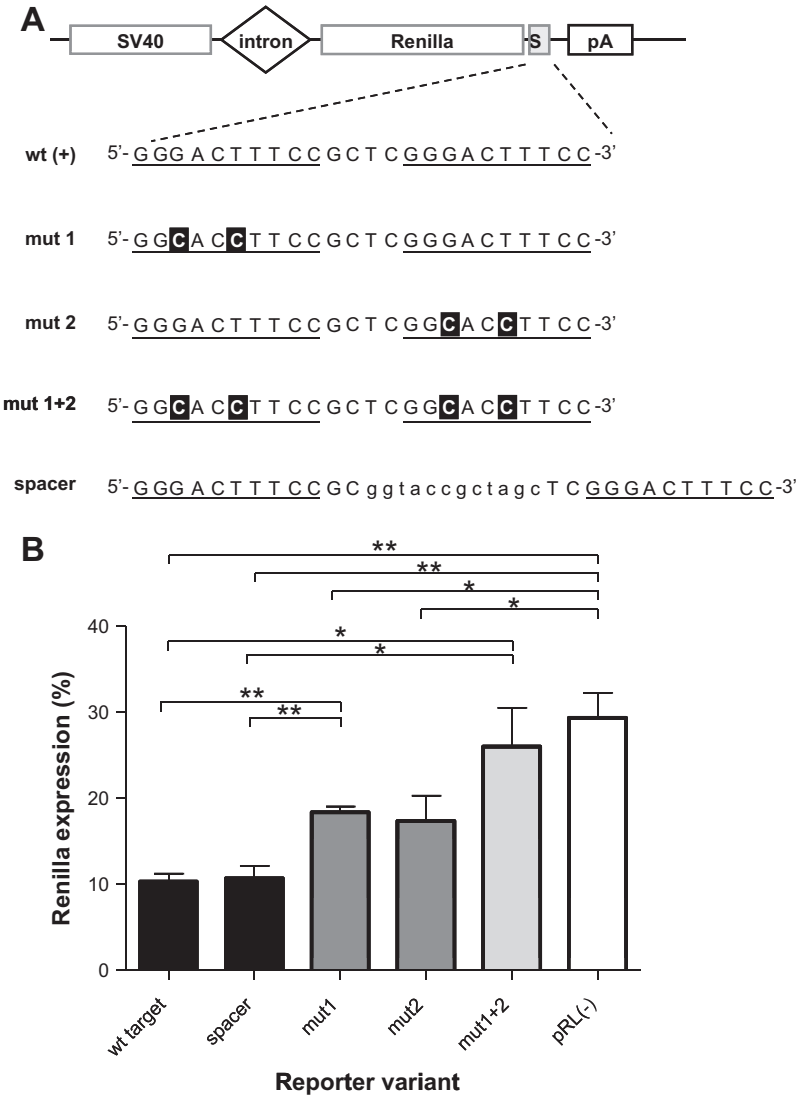

Fig. 4. Test of sequence specificity for J. (A) Renilla luciferase reporter variants pRL-S encoding wildtype or mutated versions of the target sequence of J. The 10 nt target sequence is underlined, mutations are indicated in black boxes with white letters. SV40 = SV40 promoter, pA = polyadenylation signal. (B) pU1wt or pU1i-J were co-transfected with each pRL-S variant or unmodified pRL-SV40. After 48 h the Renilla luciferase expression was determined. Renilla expression upon U1wt transfection was set at 100% for each reporter variant. In relation to that, the Renilla expression with J co-transfection is displayed. pRL-wt contains the wild-type configuration of U1i target site, pRLmut1, pRLmut2, and pRLmut1+ 2 harbor point mutations in the first, second or both target sites, respectively, pRLspacer contains 12 additional nucleotides. Unmodified pRL(−) harbors no J target site. The SEM and *p*-values are depicted ($^{*}p < 0.05$, $^{**}p < 0.01$).

to be complementary to the 3′-terminal exon of HIV-1 pre-mRNAs. Interference of the U1-70K protein with polyadenylation leads to premature degradation of the targeted mRNA.

Fourteen U1i inhibitors were designed and tested for antiviral activity in HIV-1 production and with dual reporter constructs. We have identified the U1i inhibitor J as a potent HIV-1 suppressor in these two assay systems. The robust antiviral efficacy of J is linked to the presence of tandem target sites in the U3 region of HIV-1 mRNA. Mutating one of the two target sites abolished incrementally the J-specific knock-down of the Renilla reporter expression. This is in accordance with previous findings that U1i inhibitors with more than a single target site achieve higher levels of inhibition than single-target inhibitors (Fortes et al., 2003). A complete loss of J-specific inhibition was observed when both target sites were mutated. We speculate that J would be an ideal antiviral because HIV-1 cannot easily escape as two positions need to be mutated independently. In addition, the J target sites are well conserved sequences that constitute the NF-κB binding sites in the 5′-LTR promoter (Jeeninga et al., 2000; Van Opijnen et al., 2004). In a control experiment, we excluded any U1i effects at the transcription level with LTR-reporter constructs.

Remarkably, we observed adverse effects for most U1i constructs on the SV40-driven Firefly luciferase control vector pGL3, without a matching target sequence. In contrast, the U1i constructs did not influence expression of the CMV-driven Renilla control construct and did also not alter Firefly expression controlled by a HIV-1 LTR promoter. Therefore, J and probably other U1i inhibitors could target transcription from SV40 promoters by an unknown mechanism. Alternatively, the two SV40 promoters of the pRL-SV40 and pGL3 plasmids interfere with each other, but such an effect has never been reported before (Abad et al., 2008, 2010). Modified U1 snRNAs were previously applied to restore normal exon usage of mutated pre-mRNAs (Pinotti et al., 2008; Tanner et al., 2009). Thus, modified U1 snRNAs can in principle also contribute to recognition of splice donor sites and we cannot exclude that adverse effects might be induced by inaccurate splicing

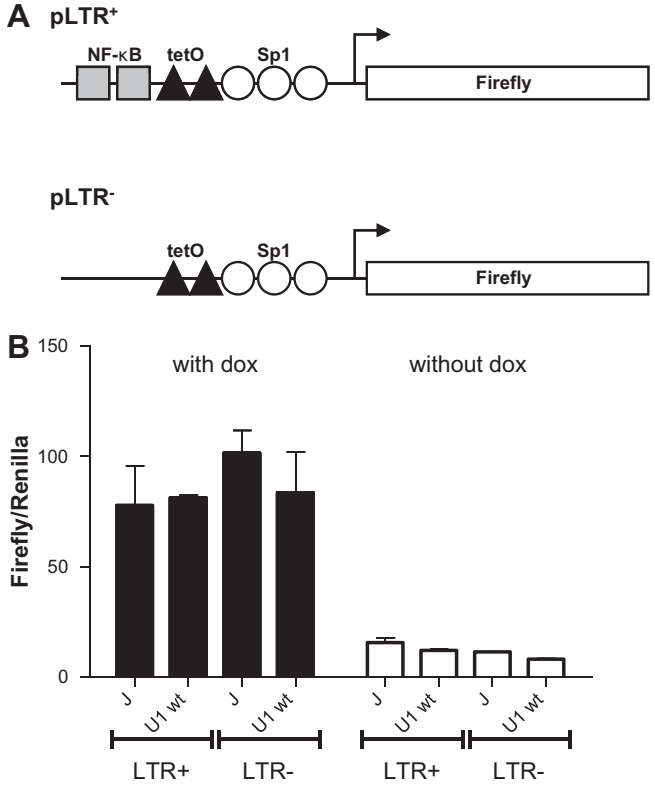

Fig. 5. No impact on HIV-1 DNA promoter sequence by J. (A) Firefly reporter constructs with promoter regions enclosing three SP1 sites and two tetO boxes. pLTR+ additionally contains two NF-κB sites, pLTR- lacks NF-κB sites. The J target sequences are identical with the NF-κB sites of the LTR promoter. (B) 293T cells were co-transfected with the Firefly luciferase reporters pLTR+ or pLTR-, pU1wt or pU1i-J and pRL-CMV. At 48 h post transfection, Firefly and Renilla luciferase expression were determined, and Firefly expression was normalized for Renilla expression. The experiments were conducted in the presence (black bars) or in absence of doxycycline (white bars).

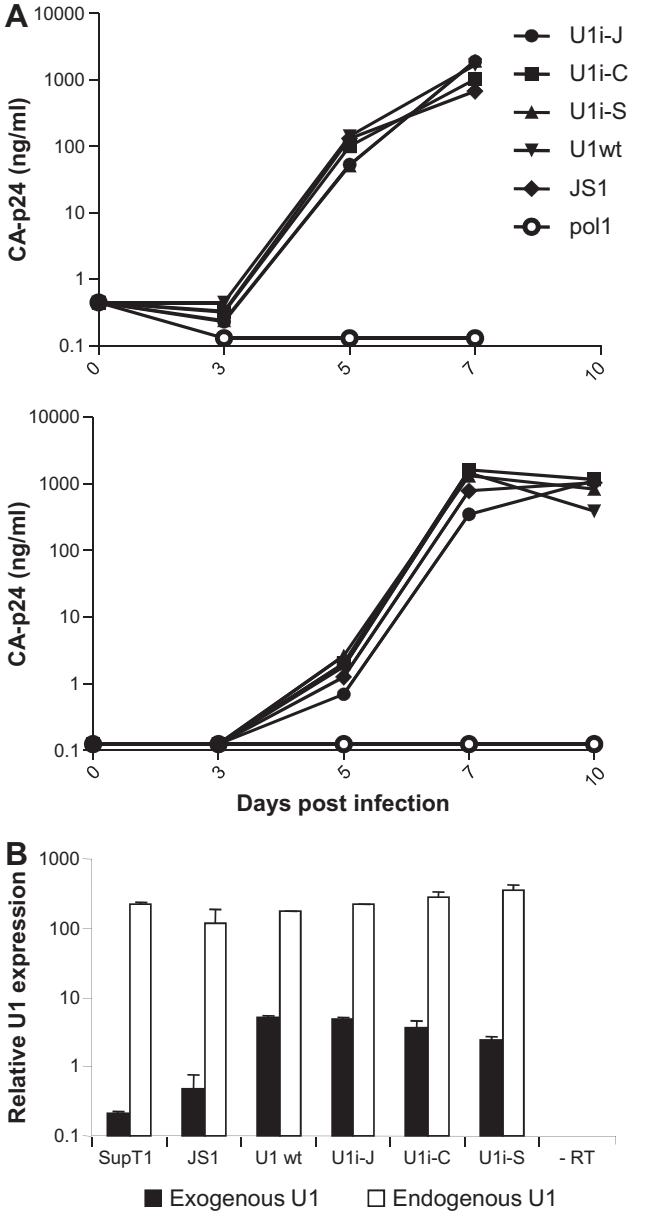

Fig. 6. HIV-1 replication in T cells expressing U1i molecules. SupT1 T cells were transduced at a low multiplicity of infection (MOI) of 0.15 with lentiviral vector JS1 expressing J, C, S, U1wt, or shRNA pol1. As negative control served SupT1 cells transduced with empty JS1. Transduced cells were sorted twice via live FACS for GFP expression. (A) The cell lines were infected with 0.5 ng (upper panel) and 0.05 ng (lower panel) CA-p24 HIV-1 LAI and virus replication was monitored for 10 days by measuring CA-p24. One representative experiment out of three independent infection assays is shown. (B) Real-time PCR measurements of modified, exogenously (black) and endogenously (white) expressed U1 snRNAs. Total RNA from non-transduced (SupT1) and transduced cells (JS1, U1wt, U1i-J, U1i-C, U1i-S) was isolated, reverse transcribed and the expression of endogenous and exogenous U1 snRNAs was quantified and related to actin mRNA expression. The samples SupT1 and JS1 did not contain modified U1 snRNAs and the expression levels were below the experimental cut-off. U1 snRNA expression was undetectable in the – RT (reverse transcriptase) control.

events. Recently, Mandal et al., successfully used modified U1 snRNAs to alter the preferential usage of 5′ splice sites in the HIV-1 mRNA and thereby decreased virus production and replication (Mandal et al., 2010).

Despite these unexplained reporter issues, we decided to move the J inhibitor forward towards stably transduced T cells and HIV-1 replication studies. We used a lentiviral vector for stable transduction of T cells with the U1i cassette. Our RNAi gene therapy approaches are based on one lentiviral integration per cell, and in cell culture experiments provide a durably potent protection against HIV-1 replication (Ter Brake et al., 2006, 2008; Ter Brake and Berkhout, 2008). Unfortunately, this approach was not successful for U1i inhibition, and only the control shRNA was strong enough to suppress HIV-1. Our results suggest that the expression level of the exogenous U1 snRNAs in stably transduced SupT1 cells is too low for inhibition of HIV expression. In contrast to our findings, previous studies reported on long-term inhibition via U1i. There, cells were either transfected and puromycin or G418-selected, or transduced with adeno associated viruses (AAVs) (Abad et al., 2010; Koornneef et al., 2011; Sajic et al., 2007). In contrast to the lentiviral approach, the selection-based methods allow multiple transgene copies per cells and may even select for cells with numerous U1i constructs.

In conclusion, we showed that U1i inhibitors can be potent suppressors of HIV-1 production in transient assays and we identified one interesting candidate. However, the application of U1i as an anti-HIV gene therapy will need further studies.

Disclosure statement

The authors declare no conflict of interest.

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