Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1751–1761
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

The use of a new in vitro reaction substrate reproducing both U3 and U5 regions of the HIV-1 3′-ends increases the correlation between the in vitro and in vivo effects of the HIV-1 integrase inhibitors

Enzo Tramontano*, Loredana Onidi, Francesca Esposito, Roberta Badas, Paolo La Colla

Department of Sciences and Biomedical Technologies, University of Cagliari, Cittadella Universitaria SS554, 09142 Monserrato, Cagliari, Italy

Received 22 April 2003; accepted 20 January 2004

Abstract

Human Immunodeficiency Virus type 1 (HIV-1) integrase (IN) is an attractive target for the development of new antiviral therapies. Recently, several HIV-1 recombinant IN (rIN) in vitro inhibitors have been described. However, the great majority of them failed to block the virus replication in cell-based assays, suggesting the inadequacy of the in vitro assay systems used for inhibitor screening. To improve these systems, we designed a 40^{mer} duplex DNA reaction substrate consisting of recognition sequences from both U3 and U5 HIV-1 long terminal repeat (LTR) termini. The HIV-1 rIN was able to catalyze its enzyme activities recognizing both ends of the 40^{mer} dsDNA. Using this substrate we assayed the effects on rIN catalysis of different classes of compounds which inhibit the HIV-1 rIN in vitro when the reaction substrate is the standard 21^{mer} U5 dsDNA, and that are either active or inactive on the HIV-1 replication. We also compared the efficacy of these compounds when added to the reaction before or after the formation of the rIN–dsDNA complex. In this system, the enzyme preincubation with the two-ended 40^{mer} dsDNA before the addition of the compounds allowed a strong correlation between the effects of hydroxylated aromatics derivatives on rIN activity in cell-free assays and their effects on viral replication in cell-culture assays. This increase in drug selectivity of the rIN in vitro assay was explored by investigating whether it was due to the length of the 40^{mer}, longer than the standard 21^{mer}, or to presence of both viral ends, versus only one viral end. To this purpose we designed four 40^{mer} oligonucleotides containing either only one viral end or two-repetitive ends, finding that the architecture of the rIN–dsDNA complex and its compound susceptibility is significantly influenced by the sequence of the dsDNA substrate.
© 2004 Elsevier Inc. All rights reserved.

Keywords: HIV-1; Integrase; Viral inhibitors; Hydroxylated aromatics; Flavonoids; Integrase assay

1. Introduction

Retrovirus multiplication requires the integration of a DNA copy of the viral RNA genome into a chromosome of the host cell. This essential step in the retrovirus growth cycle is accomplished by the viral-coded integrase (IN) which brings close the ends of the viral linear DNA and introduces them into the cellular DNA at a distance, in the case of the Human Immunodeficiency Virus type 1 (HIV-1), of five nucleotides from each other [1]. The HIV-1 IN acts as the main component of a large nucleoprotein complex, termed preintegration complex (PIC), which contains both viral and cellular constituents. Viral constituents are the linear DNA and the reverse transcriptase, matrix, vpr and nucleocapsid proteins, cellular constituents are the barrier to auto-integration factor (BAF) and the high-mobility group [HMG-I(Y)] protein [1,2].

The overall integration process proceeds through three phases. In the first, which takes place in the cytoplasm and is termed 3′-processing, a dinucleotide (GT) is cleaved from each 3′-ends of the linear viral DNA adjacent to a conserved CA dinucleotide. In the second, which takes

* Corresponding author. Tel.: +39-070-6754205; fax: +39-070-6754210.
E-mail address: tramon@unica.it (E. Tramontano).

Abbreviations: HIV-1, Human Immunodeficiency Virus type 1; rIN, recombinant integrase; PIC, preintegration complex; LTR, long terminal repeat; ATA, aurintricarboxylic acid; RDS1158, 2-(3,4-dihydroxybenzyl)-6-(3,4-dihydroxybenzylidene)cyclohexanone; L-731,988, 4-[1-(4-fluorobenzyl)-4-iodopyrrole-2-yl]-2,4-diketobutanoic acid; RDS1473, 4-oxo-3-(3,4,5-trihydroxybenzyl)-5-(3,4,5-trihydroxybenzylidene)cyclohexanecarboxylic acid; IC_{50}, inhibitor concentration required to reduce enzyme activity by 50%; EC_{50}, inhibitor concentration required to achieve 50% protection of MT-4 cells from the HIV-1-induced cytopathogenicity; CC_{50}, inhibitor concentration required to reduce MT-4 cell viability by 50%

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.01.015

place in the nucleus and is termed strand-transfer, the exposed 3′-hydroxyls obtained in the previous reaction are used in a direct transesterification in which the 3′-viral ends are joined to 5′-phosphates in the host DNA to produce the integration intermediate. In the third, the provirus formation is completed by the further removal of the unpaired nucleotides at the 5′-ends of the viral DNA and the repair of the single-strand gaps between viral and host DNA: both these steps are probably accomplished by cellular enzymes [3].

Since integration is an essential requirement for the HIV-1 replication, IN is considered an attractive target for the development of new antiviral therapies [4]. To date, the efficacy of potential IN inhibitors is typically assessed in cell-free assays which use recombinant enzyme (rIN) and a variety of synthetic oligonucleotides as DNA substrates, since its assessment in partially purified PIC assays is rather cumbersome [4,5]. In recent years, a good number of rIN inhibitors have been described. However, with few exceptions, the rIN inhibitors identified in high-throughput cell-free screenings rarely prove to be able to block both the virus replication in cell-based assays and the DNA integration in PIC-based assays [4,6–9]. The striking lack of correlation between the in vitro and in vivo activity of the large majority of anti-IN compounds, irrespectively of the chemical class to which they belong to [4] most likely reflects the inadequacy of the cell-free assays to reproduce the cellular integration process.

The common substrate of the most diffuse in vitro assay used for drug screenings is a short dsDNA (18–24^{mer}), usually a 21^{mer} DNA, which reproduces the 3′-end of the U5 region of the proviral DNA and which can be labeled at either its 5′-end or its 3′-end [4,5,10]. rIN cleaves the last two nucleotides (GT) at the 3′-end of the 21^{mer} dsDNA oligonucleotide producing a 19^{mer} and a dimer as reaction products (the 5′-end labeling allows to track the 19^{mer} production, whereas the 3′-end labeling evidences the dimer production). Any dsDNA can be used by rIN as target DNA and, in the absence of excess of target DNA, integration occurs into an unprocessed molecule of the 21^{mer} dsDNA giving rise to a 19–38^{mer} oligonucleotide as reaction product, since the 3′-hydroxyl of the 19^{mer} can attack any base of the 21^{mer} dsDNA. This assay allows to asses both 3′-processing and strand-transfer reactions and is used to identify compounds that are targeted to both reactions or to only one of them [4,5]. A nonradioactive microtiter-plate assay which uses the same principles has also been described for high-throughput screening of strand-transfer inhibitors [11]. This assay uses an immobilized 30^{mer} dsDNA oligonucleotide, which also reproduces the U5 LTR end, as 3′-processing reaction substrate and an heterologous biotinylated oligonucleotide as target DNA for the subsequent strand-transfer reaction.

All these systems uses only one viral termini as DNA substrate, however, even though in vitro an individual 3′-end serves as rIN substrate, in vivo the mechanism and efficiency of integration is determined by the interaction between IN and both 3′-ends. Indeed, Wei et al., reported that formation of the Moloney murine leukemia virus preintegration complex requires the presence of two functional viral DNA 3′-ends [12]. More recently, Brin and Leis reported, in an elegant system, but too complex to be used for screening purpose, that both U3 and U5 HIV-1 3′-ends are required to reconstitute an in vitro complete integration process [13,14].

With the aim of better reproducing in vitro the in vivo integration reaction, we designed a 40^{mer} double-stranded oligonucleotide containing the 3′-end of both HIV-1 U3 and U5 regions. Using this 40^{mer} dsDNA as reaction substrate, in comparison with the standard 21^{mer} dsDNA, we first performed a biochemical characterization of the HIV-1 rIN activities. Subsequently, we verified whether the use of the new substrate could improve the correlation between the effects of the HIV-1 IN inhibitors observed in cell-free assays and the ones observed in cell-based assays, therefore, allowing to elicit from the in vitro results the ‘‘false positive’’ rIN inhibitors. For this reason, we evaluated the rIN-catalyzed reactions in the presence of different classes of rIN inhibitors, either active or inactive on the HIV-1 replication, using both 21^{mer} and 40^{mer} oligonucleotides as DNA substrates. Furthermore, the experimental design compared the efficacy of the rIN inhibitors both pre and post of the assembly between the rIN and the DNA substrate.

2. Materials and methods

2.1. Materials

The pINSD.His vector was kindly provided by Dr. R. Craigie (NIH). *Escherichia coli* BL21(DE3), His-bind resin was purchased from Novagen. Isopropylthio-β-D-galactoside (IPTG), CHAPS, aurintricarboxylic acid (ATA), baicalein and myricetin were purchased from Sigma. The Klenow polymerase, G-25 Sephadex quick spin columns and dTTP were purchased from Roche. [α-^{32}P]dGTP and HiTrap desalting columns were purchased from Amersham Pharmacia Biotech.

2.2. Synthetic oligonucleotides

Gel-purified oligonucleotides were purchased from the DNA Synthesis Laboratory, Department of Pathology of Yale University. The following oligonucleotides were used in this study: 19U5A, 5′-GTGTGGAAAATCTCTAGCA-3′; 21U5B, 5′-ACTGCTAGAGATTTTCCACAC-3′; 38U-3U5A, 5′-ACTGGAAGGGCTAATTCACTTGTGGAAA-ATCTCTAGCA-3′; 38U3U5B, 5′-ACTGCTAGAGATTT-TCCACAAGTGAATTAGCCCTTCCA-3′; 40U3U5A, 5′-ACTGGAAGGGCTAATTCACTTGTGGAAAATCTCT-AGCAGT-3′; 40U3U5B, 5′-ACTGCTAGAGATTTTCCA-CAAGTGAATTAGCCCTTCCAGT-3′; 40U30A, 5′-AC-

TGGAAGGGCTAATTCACTGGATTAATGGTTACGCC-TAA-3′; 38U30B, 5′-TTAGGCGTAACCATTAATCCAG-TGAATTAGCCCTTCCA-3′; 380U5A, 5′-TTAGGCGT-AACCATTAATCCTGTGGAAAATCTCTAGCA-3′; 400-U5B, 5′-ACTGCTAGAGATTTTCCACAGGATTAATG-GTTACGCCTAA-3′; 38U3U3, 5′-ACTGGAAGGGCTA-ATTCACTAGTGAATTAGCCCTTCCAGT-3′; 38U5U5, 5′-ACTGCTAGAGATTTTCCACATGTGGAAAATCTC-TAGCAGT-3′.

Couples of oligonucleotides were annealed in 0.1 M NaCl by heating at 80 °C and slowly cooling to room temperature overnight to prepare the following dsDNA reaction substrates (between parentheses): 19U5A and 21U5B (21^{mer} U5 dsDNA), 38U3U5A and 38U3U5B (40^{mer} U3U5 dsDNA), 40U3U5A and 38U3U5B (40^{mer} U3U5A dsDNA), 38U3U5A and 40U3U5B (40^{mer} U3U5B dsDNA), 40U30A and 38U30B (40^{mer} U30 dsDNA), 380U5A and 400U5B (40^{mer} 0U5 dsDNA). Oligonucleotides 38U3U3 and 38U5U5 were self-annealed to form dsDNA 40^{mer} U3U3 and 40^{mer} U5U5 dsDNAs, respectively. dsDNA substrates were labeled by introducing at the 3′-ends of the 38^{mer} oligonucleotides the two missing nucleotides using [α-^{32}P]dGTP, cold TTP and Klenow polymerase. Unincorporated [α-^{32}P]dGTP was separated from the duplex substrate with a G-25 Sephadex quick spin column.

2.3. HIV-1 rIN purification

Expression of the HIV-1 rIN protein with an amino-terminal polyhistidine tag was obtained by IPTG induction of the *E. coli* strain BL21(DE3) containing the pINSD.His vector. Protein purification was carried out with an AKTA-prime FPLC (Amersham Pharmacia Biotech) as described [10]. Briefly, cell pellet was resuspended in Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9, 5 mM CHAPS), sonicated and centrifuged at 30,000 × *g* for 30 min at 4 °C. The supernatant was applied to a His-bind resin column and washed thoroughly with binding buffer and with washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9, 5 mM CHAPS). rIN was eluted with Elute Buffer (1 M imidazole, 0.5 M NaCl, 50 mM Tris–HCl, pH 7.9, 5 mM β-mercaptoethanol, 5 mM CHAPS), fractions were collected and enzyme activity was determined. Protein purity was checked by SDS–PAGE and found to be higher than 90%. Enzyme-containing fractions were pooled and loaded onto an HiTrap desalting column equilibrated in Elute Buffer without imidazole. Fractions were collected and catalytic activity and protein concentration were determined. Enzyme-containing fractions were pooled and aliquots were frozen at −80 °C.

2.4. HIV-1 rIN assay

Standard 3′-processing and strand-transfer reaction conditions were: 10 mM HEPES, pH 7.5, 10 mM $MnCl_2$, 1 mM dithiothreitol, 60 mM NaCl, 2.5 nM of 40^{mer} dsDNA and 250 nM rIN (considered as monomer). When the dsDNA substrate was the 21^{mer} U5 oligonucleotide (1.25 nM concentration), the experimental conditions were the same with the exception of the presence of 40 mM NaCl and the addition of 2% glycerol. Samples of 15 μl volume were incubated at 37 °C for 1 h, reactions were stopped by adding 7.5 μl of Sample Buffer (96% formamide, 20 mM EDTA, 0.08% bromophenol blue and 0.08% xylene cyanol) [5] and analyzed by denaturing PAGE. Samples were layered onto a denaturing 15% polyacrylamide gel (7 M urea, 0.09 M Tris borate, pH 8.3, 2 mM EDTA, 15% acrylamide) and run for 1 h at 80 W. Reaction products were monitored, quantified with a Bio-Rad Personal FX Phosphoimager and fmol of product calculated. When a preincubation period was performed, a 10-min incubation was carried out at 37 °C in a volume of 15 μl. Then, 3 μl of buffer A (10 mM HEPES, pH 7.5, 10 mM $MnCl_2$, 60 mM NaCl) or 3 μl of serial dilutions of test compounds (dissolved in buffer A) were added to the mixture and reactions were allowed to proceed at 37 °C for 1 h. Reactions were stopped by adding 9 μl of Sample Buffer and analyzed as above. In order to correctly compare samples with different volumes, quantifications were normalized according to the sample volume.

3. Results

3.1. Cleavage of the two-ended 40^{mer} U3U5 oligonucleotide by the HIV-1 rIN

In the present study we designed and used in the HIV-1 rIN in vitro assay a 40^{mer} duplex DNA oligonucleotide reaction substrate (U3U5 40^{mer} dsDNA) consisting of sequences from the two viral DNA termini: the first 20 nt residues reproduce the sequence of the HIV-1 U3 end while the last 20 nt residues reproduce the sequence of the HIV-1 U5 end (Table 1). The two-ended U3U5 40^{mer} dsDNA oligonucleotide was ^{32}P-labeled at both 3′-ends as described in Section 2. Hence, we could examine (i) the rIN 3′-processing reaction by monitoring the formation of a ^{32}P-labeled GT dimer product coming from both U3 and U5 dsDNA 3′-ends, and (ii) the rIN strand-transfer reaction by monitoring the formation of a ^{32}P-labeled $40–78^{mer}$ oligonucleotide produced by the joining of the 38^{mer} dsDNA, obtained from the 3′-processing reaction, to another unprocessed ^{32}P-labeled U3U5 40^{mer} dsDNA, acting as acceptor substrate surrogate of the cellular DNA. Therefore, similarly to the assay system which uses the 21^{mer} dsDNA as substrate [5], this assay allows to evaluate compounds which target both enzymatic reactions or only one of them.

Initially, in order to verify that the enzyme could efficiently process both U3 and U5 3′-ends of the two-ended 40^{mer} U3U5 dsDNA, we prepared 40^{mer} U3U5 reaction

Table 1
Sequences of 21^{mer} and 40^{mer} dsDNA oligonucleotides used as HIV-1 rIN reaction substrate

21^{mer} U5	5′-GTGTGGAAAATCTCTAGCAGT-3′ (U end) 3′-CACACCTTTTAGAGATCGTCA-5′
40^{mer} U3U5	5′-*ACTGGAAGGGCTAATTCACT*TGTGGAAAATCTCTAGCAGT-3′ (U3 end; U5 end) 3′-*TGACCTTCCCGATTAAGTGA*ACACCTTTTAGAGATCGTCA-5′
40^{mer} U30	5′-*ACTGGAAGGGCTAATTCACT*GGATTAATGGTTACGCCTAA-3′ (U3 end; 0 end) 3′-*TGACCTTCCCGATTAAGTGA*CCTAATTACCAATGCGGATT-5′
40^{mer} 0U5	5′-*TTAGGCGTAACCATTAAT*CCTGTGGAAAATCTCTAGCAGT-3′ (0 end; U5 end) 3′-*AATCCGCATTGGTAATTAGG*ACACCTTTTAGAGATCGTCA-5′
40^{mer} U3U3	5′-*ACTGGAAGGGCTAATTCACT*AGTGAA TT AGCCCTTCCAGT-3′ (U3 end; U3 end) 3′-*TGACCTTCCCGATTAAGTGA*TCACTTAATCGGGAAGGTCA-5′
40^{mer} U5U5	5′-*ACTGCTAGAGATTTTCCACA*TGTGGAAAATCTCTAGCAGT-3′ (U5 end; U5 end) 3′-TGACGATCTCTAAAAGGTGTACACCTTTTAGAGATCGTCA-5′

21^{mer} substrate reproduces the U5 3′-end of the HIV-1 LTR. 40^{mer} dsDNA substrates were designed to reproduce: (i) 20 nt from U3 (in italics) and 20 nt from U5 (underlined) 3′-end, two-ended 40^{mer} dsDNA substrate (U3U5); (ii) 20 nt from either U3 or U5 3′-end and 20 nt from a random sequence, one-ended 40^{mer} dsDNA substrates (U30 and 0U5); (iii) 20 nt from either U3 or U5 3′-end repeated twice, two-repetitive-ended 40^{mer} dsDNA substrates (U3U3 and U5U5).

substrates (i) doubly ^{32}P-labeled at both 3′-ends, (ii) singly ^{32}P-labeled at the U3 3′-end (U3U5A dsDNA), (iii) singly ^{32}P-labeled at the U5 3′-end (U3U5B dsDNA). The standard singly ^{32}P-labeled U5 21^{mer} was used as control substrate. Fig. 1 shows that the HIV-1 rIN proved to be able to perform its 3′-processing activity on both U3 and U5 ends of the 40^{mer} dsDNA oligonucleotide and that the 38^{mer} product of the above reactions was efficiently used as reaction substrate for the subsequent rIN strand-transfer catalysis. As expected [10], similar results were obtained labeling the 5′-ends of the 40^{mer} oligonucleotides (data not shown). Furthermore, since Mg^{2+} is the divalent cation which is believed to be relevant for viral replication in vivo [1–3], we compared the enzyme activity in the presence of Mg^{2+} and Mn^{2+}, the cation which is frequently used in rIN in vitro assays instead of Mg^{2+} [5]. Using the two-ended 40^{mer} U3U5 dsDNA as reaction substrate, the presence of Mg^{2+} instead of Mn^{2+} reduced the 3′-processing activity by sixfold and no strand-transfer reaction was observed even after long time exposures (data not shown). Therefore, the subsequent study was conducted with Mn^{2+}, the standard cation used in in vitro assays [5].

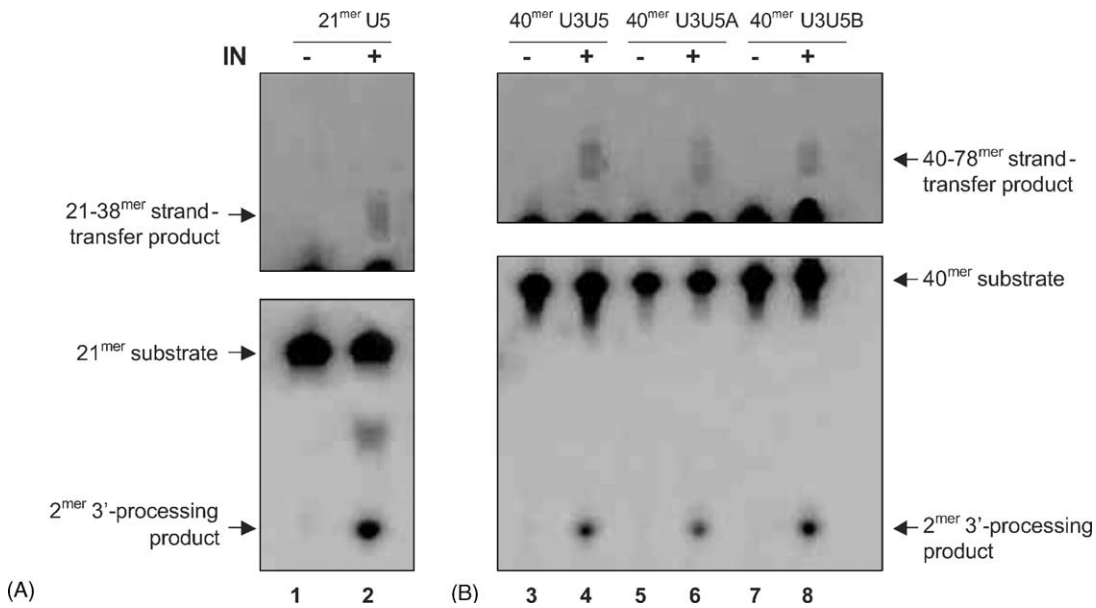

Fig. 1. HIV-1 rIN catalytic activities on U3 and U5 3′-end of the two-ended 40^{mer} U3U5 dsDNA substrate. 10 nM of differently labeled dsDNA oligonucleotides were incubated in a standard reaction mixture at 37 °C for 1 h with or without rIN and processed as described in Section 2. The dsDNA substrates were: (A) Lanes 1 and 2, 21^{mer} U5 dsDNA labeled at the U5 3′-end; (B) lanes 3 and 4, 40^{mer} U3U5 dsDNA labeled at both U3 and U5 3′-ends; lanes 5 and 6, 40^{mer} U3U5 dsDNA labeled only at the U3 3′-ends (U3U5A); lanes 7 and 8, 40^{mer} U3U5 dsDNA labeled only at the U5 3′-ends (U3U5B). Strand-transfer products were visualized with longer screen exposures.

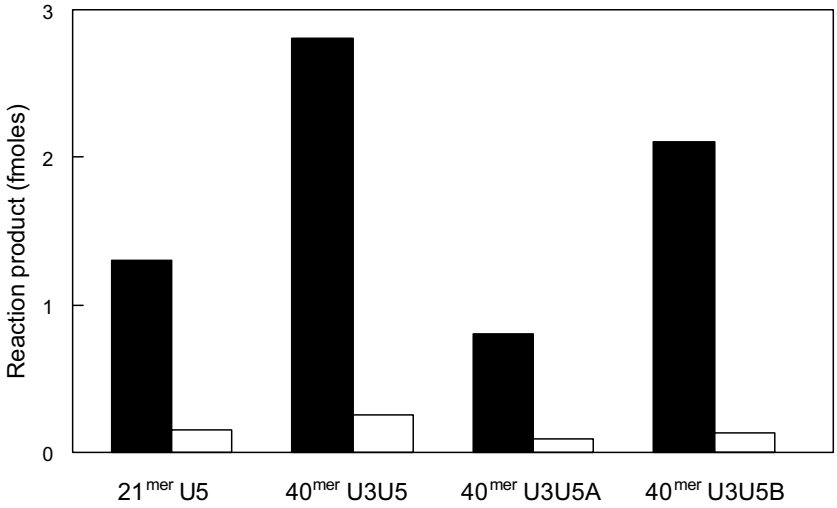

Fig. 2. Comparison of the HIV-1 rIN reaction efficiency on the U3 and U5 3′-end of the two-ended 40^{mer} U3U5 dsDNA. Differently labeled dsDNA oligonucleotides [21^{mer} U5 dsDNA labeled at the U5 3′-end (21^{mer} U5); 40^{mer} U3U5 dsDNA labeled at both U3 and U5 3′-ends (40^{mer} U3U5); 40^{mer} U3U5 dsDNA labeled at the U3 3′-end (40^{mer} U3U5A); 40^{mer} U3U5 dsDNA labeled at the U5 3′-end (40^{mer} U3U5B)] were incubated with HIV-1 rIN in a standard reaction mixture for 1 h at 37 °C and processed as described in Section 2. Close bars represent the rate of the 3′-processing reaction and open bars the rate of the strand-transfer reaction.

It has been reported that oligonucleotides representing the HIV-1 U5 3′-end are more efficient substrates for rIN reactions than the U3 counterparts [13,15–17]. Therefore, using the same ^{32}P-labeled 3′-end dsDNA substrates as above we confirmed that the HIV-1 rIN processed the U5 3′-end of the two-ended 40^{mer} dsDNA oligonucleotide twofold more efficiently than its U3 3′-end and with the same efficiency as the U5 3′-end of the 21^{mer} (Fig. 2). Differently, the rIN strand-transfer efficiency was obviously identical independently by which 3′-end was labeled, since the reaction products produced by the 3′-processing reaction, and used thereafter for the strand-transfer activity, were the same regardless the 3′-end labeling position.

3.2. Determination of kinetic constants for the HIV-1 rIN 3′-processing activity

In order to further characterize the differential activity of the HIV-1 rIN 3′-processing on the U3 and U5 3′-end of the U3U5 40^{mer} dsDNA we determined the steady-state kinetic parameters using the same ^{32}P-labeled dsDNA substrates as described above.

Kinetic constants were calculated according to Dixon [10,18,19] since in our reaction mixture the rIN and substrate concentrations result in the same order of magnitude, bringing a significant fraction of the dsDNA substrate to be likely bound to the rIN. Therefore, in these experimental conditions, the Henry–Michaelis–Menten equations are inadequate to determine steady-state kinetic parameters and the more general method described by Dixon should be used [10,18,19].

Table 2 shows that the rIN K_m values obtained with all dsDNA substrates were similar, confirming that the interaction rIN–dsDNA substrate is not influenced by the length of the substrate. Differently, the enzyme showed a catalytic constant twofold higher when processing the U5 end than when processing the U3 end of the U3U5 40^{mer} dsDNA, confirming the HIV-1 rIN preference for the U5 end of the viral LTRs.

3.3. Inhibition of the HIV-1 rIN by selected compounds using two-ended 40^{mer} U3U5 as dsDNA reaction substrate

To examine whether the use of the two-ended 40^{mer} U3U5 as DNA substrate in the rIN in vitro assay could allow a more reliable correlation between the effects of putative IN inhibitors observed in cell-free assays and their effects in cell-culture assays, we tested three molecules which are able to block the rIN catalysis by different means (Fig. 3): (i) ATA, a mixture of polymeric substances which

Table 2
Steady-state parameters of the HIV-1 rIN 3′-processing reaction on the U3 and U5 3′-ends of the two-ended 40^{mer} U3U5 oligonucleotide

dsDNA 3′-end labeled	K_m (nM)	k_p (h^{-1})[a]
21^{mer} U5	1.1 ± 0.3	0.09 ± 0.04
40^{mer} U3U5	2.1 ± 0.5	0.25 ± 0.06
40^{mer} U3U5A	1.9 ± 0.2	0.07 ± 0.02
40^{mer} U3U5B	1.5 ± 0.4	0.17 ± 0.03

Differently labeled dsDNA oligonucleotides [21^{mer} U5 dsDNA labeled at the U5 3′-end (21^{mer} U5); 40^{mer} U3U5 dsDNA labeled at both U3 and U5 3′-ends (40^{mer} U3U5); 40^{mer} U3U5 dsDNA labeled at the U3 3′-end (40^{mer} U3U5A); 40^{mer} U3U5 dsDNA labeled at the U5 3′-end (40^{mer} U3U5B)] were incubated in a standard reaction mixture at 37 °C for 1 h with HIV-1 rIN and processed as described in Section 2. Kinetics values were calculated as described [10,18,19].

[a] k_p (turnover number) values were calculated from the ratio V_{max}/[IN], where [IN] was obtained from kinetic analysis.

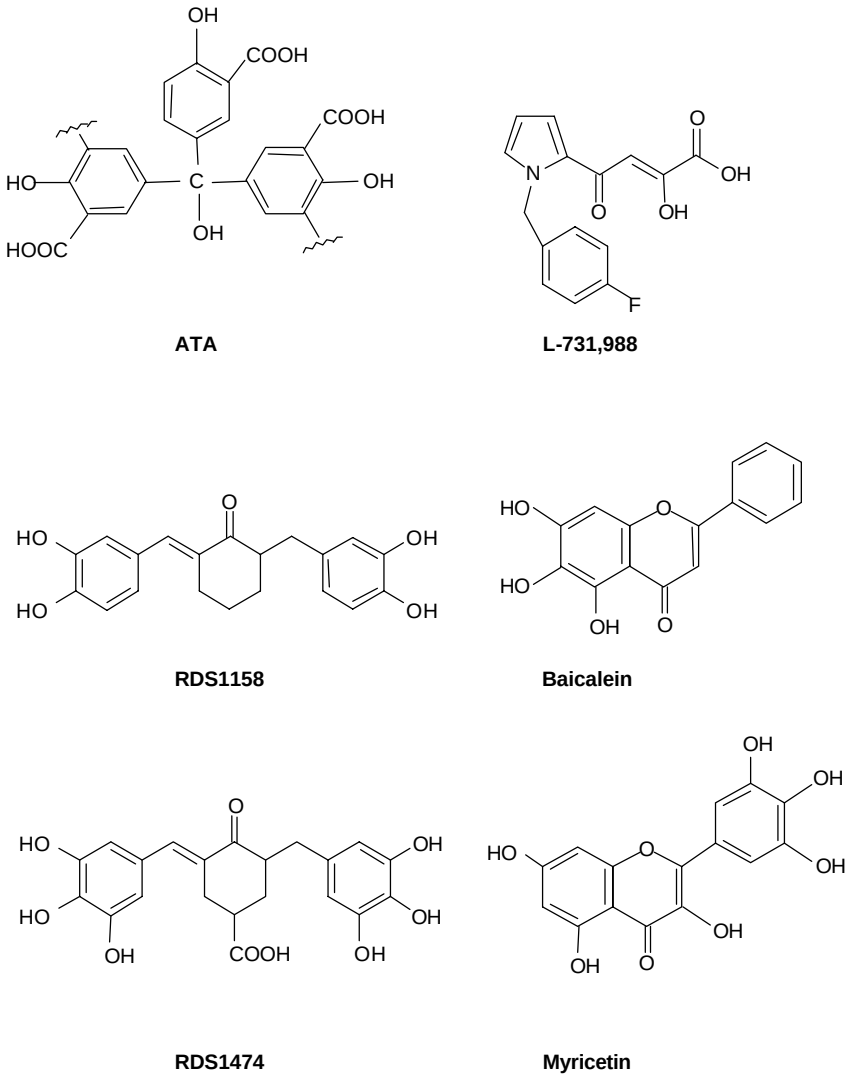

Fig. 3. Chemical structures of selected molecules that inhibit the HIV-1 rIN.

inhibits the binding to DNA of a variety of different proteins (i.e. RNA and DNA polymerases, nucleases, HIV-1 reverse transcriptase and rIN) [20]. ATA also inhibits the HIV-1 replication in cell culture by blocking the virus entry [20]. Therefore, it can be considered a non-specific rIN inhibitor which probably could inhibit the rIN activity regardless the in vitro system used; (ii) an hydroxylated aromatic derivative, 2-(3,4-dihydroxybenzyl)-6-(3,4-dihydroxybenzylidene)cyclohexanone (RDS1158), which we reported to inhibit the rIN activity in vitro, but which is ineffective on the HIV-1 reverse transcriptase, the cellular topoisomerases, DNA and RNA polymerases as well as on the HIV-1 replication in cell-based assays [21]. RDS1158, therefore, can be considered the kind of selective "false positive" rIN inhibitor which a more predictive in vitro assay should be able to eliminate; (iii) a diketo acid derivative, 4-[1-(4-fluorobenzyl)-4-iodopyrrole-2-yl]-2,4-diketobutanoic acid (L-731,988), which has been reported to inhibit both the rIN activity in vitro and the viral replication in cell culture and is considered a selective IN inhibitor [6]. Noteworthy, the activity of L-731,988 is remarkable in that it discriminates between the two IN catalytic activities, inhibiting the 3′-processing with an IC50 of 5 μM and the strand-transfer with an IC_{50} of 0.1 μM [6].

It has been proposed that many in vitro rIN inhibitors, among which the majority of the compounds which are inactive in cell-based assays, may act chelating the metal ion Mn^{2+} which is required for rIN activity and which induces conformational changes in the enzyme [22]. It has been also proposed that such changes take place in the reaction mixture within few minutes, and that the subsequent binding of the rIN to the substrate DNA is fast and brings to a tightly associated and stable protein–DNA complex [23]. Therefore, we decided to assay the inhibitory activity of the three compounds adding the molecules to the reaction mixture either before that the enzyme could bind to the DNA substrate (−preincubation), or after a 10-min incubation at 37 °C that allows the rIN–dsDNA complex formation (+preincubation).

Table 3
Effect of selected inhibitors on the HIV-1 rIN activities using the 21^{mer} U5 and the 40^{mer} U3U5 dsDNA as reaction substrate

Compound	IC_{50}[a]			
	3′-processing		Strand-transfer	
	−Preincubation	+Preincubation	−Preincubation	+Preincubation
21^{mer} U5				
ATA	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.8 ± 0.2
RDS1158	0.7 ± 0.2	0.7 ± 0.1	0.5 ± 0.2	0.6 ± 0.3
L-731,988	2.4 ± 1.6	3.0 ± 0.9	0.2 ± 0.1	0.3 ± 0.2
40^{mer} U3U5				
ATA	0.3 ± 0.2	0.5 ± 0.1	0.7 ± 0.2	0.4 ± 0.1
RDS1158	0.6 ± 0.1	>100	0.3 ± 0.1	>100
L-731,988	3.4 ± 0.8	2.9 ± 1.0	0.3 ± 0.1	0.2 ± 0.1

Tested compounds were added either before the reaction mixture was incubated at 37 °C (−preincubation), or after a 10-min incubation at 37 °C (+preincubation). Reaction mixture were prepared and processed as described in Section 2.

[a] Compound concentration required to reduce the HIV-1 rIN catalysis of 3′-end-labeled 21^{mer} or 40^{mer} dsDNA substrate by 50%. Values were expressed in μM for RDS1158 and L-731,988 and in μg/ml for ATA.

Table 3 shows that ATA and L-731,988 inhibited the rIN catalysis with the same potency regardless the substrate used and the preincubation performed. Similarly, when the reaction substrate was the 21^{mer} U5 dsDNA, RDS1158 blocked the enzyme activities with the same IC_{50} values regardless the preincubation step. Differently, even at the highest tested concentration (100 μM), RDS1158 did not inhibit the HIV-1 rIN activities when it was added, after the 10-min preincubation, to a reaction mixture containing the 40^{mer} U3U5 oligonucleotide as DNA substrate (Table 3 and Fig. 4). These results imply that the rIN–U3U5 40^{mer} dsDNA complex may be more stable, and hence more resistant to the RDS1158 inhibition, than the rIN–U5 21^{mer} dsDNA complex which maintains its activity only partially when higher compound concentrations are added after the preincubation period (Fig. 4B).

3.4. Inhibition of the HIV-1 rIN by selected compounds using one-ended or two-repetitive-ended 40^{mer} oligonucleotides as dsDNA reaction substrate

In order to discern whether the different rIN susceptibilities to RDS1158 obtained using the one-ended 21^{mer} U5 dsDNA or the two-ended 40^{mer} U3U5 dsDNA as reaction substrate might be due to the diverse DNA length or to the presence of only one or both viral 3′-ends, we designed four new 40^{mer} oligonucleotides (Table 1). In two of them, a random sequence replaced either the U5 3′-end (40^{mer} U30) or the U3 3′-end (40^{mer} 0U5). In the third, a second U3 3′-end sequence substituted the U5 3′-end (40^{mer} U3U3), in the fourth—vice versa—a second U5 3′-end sequence substituted the U3 3′-end (40^{mer} U5U5). Table 4 shows that when the HIV-1 rIN reaction substrate was either the U30 or the 0U5 one-ended 40^{mer} oligonucleotide, all compounds inhibited the enzyme activities regardless the preincubation step, as they did when the one-ended 21^{mer} U5 was the rIN DNA substrate.

Similarly, when the rIN reaction substrate was either the U3U3 or the U5U5 two-repetitive-ended 40^{mer} oligonucleotide, ATA and L-731,988 inhibited the rIN activities independently from the preincubation step. On the contrary, using in the reaction these two DNA substrates RDS1158 inhibited the rIN catalysis with IC_{50} values which were 10-fold higher in the presence of the preincubation step (3.5–8 μM) than in its absence (0.2–0.6 μM). This demonstrates that the presence of the U3 and/or U5 IN recognition sequences in the dsDNA substrate is correlated to the rIN susceptibility to the RDS1158 hydroxylated aromatic derivative.

3.5. Inhibition of the HIV-1 rIN activities by other selected compounds using one-ended or two-ended 40^{mer} oligonucleotides as dsDNA reaction substrate

Subsequently, we wanted to verify whether the above experimental conditions lead to a rIN cell-free assay which is able to discriminate among other in vitro rIN inhibitors which are either inactive or active in cell-based assays. Therefore, we evaluated the effect of baicalein, myricetin and 4-oxo-3-(3,4,5-trihydroxybenzyl)-5-(3,4,5-trihydroxybenzylidene)cyclohexanecarboxylic acid (RDS1473) (Fig. 3) on the enzyme activities. Baicalein and myricetin are hydroxylated aromatic derivatives which were reported to inhibit several cellular functions (i.e. topoisomerase II, DNA polymerases α, β and γ) [24,25], HIV-1 rIN and reverse transcriptase in vitro, but were not able to block the IN activity in PIC assays [8,26,27]. RDS1473 is an RDS1158 analogue which is able to inhibit both rIN in cell-free assays and viral replication in cell-based assay with an EC_{50} and CC_{50} values of 4 and 70 μM, respectively, and which is ineffective on the HIV-1 reverse transcriptase, cellular topoisomerases, DNA and RNA polymerases (E.T., personal communication). Table 5 shows that, consistently with other reports [8], baicalein

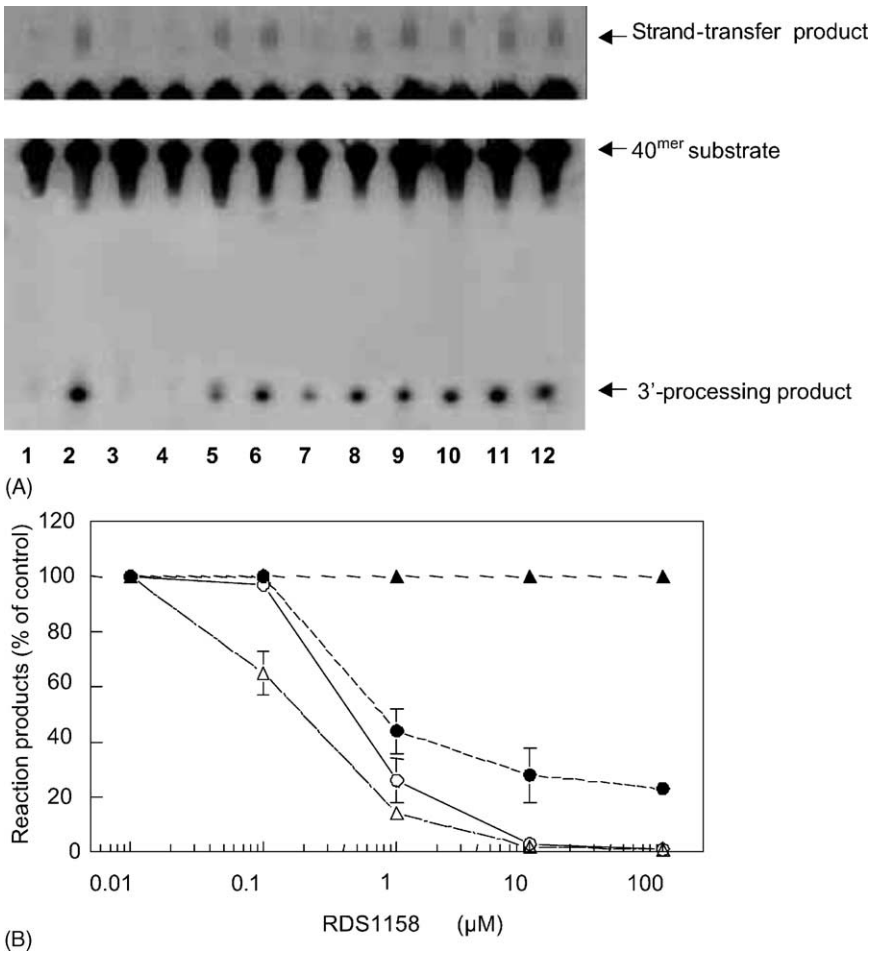

Fig. 4. Inhibition of the HIV-1 rIN by RDS1158. (A) PAGE analysis of the rIN reactions assessed using the 40^{mer} U3U5 dsDNA as substrate. The 3′-processing products are shown in the bottom panel (lighter exposure) and the strand-transfer products in the upper panel (darker exposure). Lane 1, without rIN; lane 2, plus rIN without drugs; lanes 3–5, plus rIN and RDS1158 (100, 33, 11, 3.3 μM) added before incubation at 37 °C (−preincubation); lane 7, plus rIN only 10-min incubation at 37 °C (zero time for +preincubation); lane 8, plus rIN (control for +preincubation); lanes 9–12, plus rIN and RDS1158 (100, 33, 11, 3.3 μM) added after 10-min incubation at 37 °C (+preincubation). (B) Graphical representation of the effect of the RDS1158 on the rIN 3′-processing reaction assessed using either 3′-end-labeled 21^{mer} U5 dsDNA (circles) or 3′-end-labeled two-ended 40^{mer} U3U5 dsDNA (triangles) as substrate. The compound was added either before the reaction mixture was incubated at 37 °C (−preincubation, empty circles and triangles), or after a 10-min incubation at 37 °C (+preincubation, full circles and triangles). Values represent data and standard deviation from three independent determinations.

and myricetin inhibited the HIV-1 rIN activities when added to the reaction mixture before the enzyme (−preincubation), whereas they were inactive when added after the preincubation step regardless the length of the dsDNA used as reaction substrate. Differently, when the reaction substrate was the 21^{mer} U5 dsDNA, RDS1473 inhibited the rIN catalysis in the submicromolar range, independently of the preincubation step, whereas when the reaction substrate was the 40^{mer} U3U5 dsDNA, the performance of preincubation step increased its IC_{50} values by roughly 30-fold. Noteworthy, the latter IC_{50} values (11–19 μM) obtained in vitro resulted in the same range of activity of the EC_{50} value (4 μM) observed in cell-based assay.

Finally, to test the possibility that the stability of the rIN–dsDNA complex in vitro could be influenced by the oligonucleotide length or recognition sequence we used cold 21^{mer} U5 and 40^{mer} U3U5 oligonucleotides as DNA substrate competitors. Table 5 shows that both 21^{mer} U5 and 40^{mer}U3U5 competitor dsDNAs resulted inactive when added after the preincubation step in spite of which oligonucleotide substrate was used as reaction substrate.

4. Discussion

We have designed a new 40^{mer} duplex DNA oligonucleotide reaction substrate for the HIV-1 rIN in vitro reactions consisting of recognition sequences from both U3 and U5 viral LTR termini. In this system, kinetic studies showed that the turnover number observed for the 3′-processing reaction on the U5 recognition sequence is twofold higher than the turnover number on the U3 counterpart. These data confirm that the HIV-1 U5 IN recognition sequence is the ‘‘dominant’’ LTR end [14–17,26].

Table 4
Effect of selected inhibitors on the HIV-1 rIN activities using different 40[mer] dsDNAs as reaction substrate

Compound	IC_{50}[a]			
	3′-processing		Strand-transfer	
	−Preincubation	+Preincubation	−Preincubation	+Preincubation
40[mer] U30				
ATA	1.0 ± 0.1	0.6 ± 0.2	0.8 ± 0.3	1.1 ± 0.4
RDS1158	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.2
L-731,988	2.0 ± 1.6	1.5 ± 0.9	0.3 ± 0.1	0.1 ± 0.1
40[mer] 0U5				
ATA	0.7 ± 0.2	1.1 ± 0.3	0.7 ± 0.2	0.9 ± 0.3
RDS1158	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.7 ± 0.2
L-731,988	3.0 ± 0.8	1.2 ± 1.0	0.3 ± 0.1	0.2 ± 0.1
40[mer] U3U3				
ATA	0.5 ± 0.2	0.2 ± 0.1	0.8 ± 0.3	0.4 ± 0.1
RDS1158	0.6 ± 0.1	6.0 ± 1.2	0.7 ± 0.2	8.2 ± 1.8
L-731,988	4.0 ± 1.2	2.0 ± 0.6	0.5 ± 0.3	0.3 ± 0.2
40[mer] U5U5				
ATA	0.9 ± 0.2	1.6 ± 0.3	0.3 ± 0.1	0.7 ± 0.2
RDS1158	0.2 ± 0.2	3.5 ± 1.1	0.5 ± 0.2	4.5 ± 1.3
L-731,988	2.4 ± 0.2	1.0 ± 0.2	0.4 ± 0.2	0.2 ± 0.1

Tested compounds were added either before the reaction mixture was incubated at 37 °C (−preincubation), or after a 10-min incubation at 37 °C (+preincubation). Reaction mixture were prepared and processed as described in Section 2.

[a] Compound concentration required to reduce the HIV-1 rIN catalysis of 3′-end-labeled 21[mer] or 40[mer] dsDNA substrate by 50%. Values were expressed in μM for RDS1158 and L-731,988 and in μg/ml for ATA.

However, while the difference in substrate efficiency of the two LTR 3′-ends observed for the HIV-1 rIN 3′-processing activity was relatively modest, twofold in this work and not precisely quantified by others [15,16,26], the difference observed for integration efficiency (that comprises both reactions) is somehow more prominent, sixfold [17] to ninefold [14]. Even though it is not clear whether this difference can be (i) considered to be significant and (ii) simply due to diverse experimental conditions (particularly the length of the substrate used for integration studies was at least 300 nt whereas the substrates used for 3′-processing studies were 21–40[mer] oligonucleotides), it may indicate that both enzymatic activities present an higher efficiency when the U5 recognition sequence is used and that, therefore, the observed effects in integration are cumulative of 3′-processing and strand-transfer preference.

Table 5
Effect of selected inhibitors on the HIV-1 rIN activities using the 21[mer] U5 and 40[mer] U3U5 dsDNA as reaction substrate

Compound	IC_{50}[a]			
	3′-processing		Strand-transfer	
	−Preincubation	+Preincubation	−Preincubation	+Preincubation
21[mer] U5				
Baicalein	0.7 ± 0.2	84 ± 12	0.6 ± 0.3	95 ± 8
Myricetin	7.0 ± 0.6	>100	8.3 ± 1.2	>100
RDS1473	0.3 ± 0.1	0.3 ± 0.2	0.7 ± 0.3	0.5 ± 0.2
21[mer] U5	1.8 ± 0.4	>250	2.5 ± 1.0	>250
40[mer] U3U5	2.5 ± 0.8	>250	1.9 ± 0.7	>250
40[mer] U3U5				
Baicalein	1.2 ± 0.5	>100	0.4 ± 0.1	98 ± 5
Myricetin	13 ± 1.6	>100	15.0 ± 2.4	>100
RDS1473	0.5 ± 0.2	11.0 ± 3.2	0.6 ± 0.2	19.0 ± 2.9
21[mer] U5	2.1 ± 0.6	>250	1.4 ± 0.8	>250
40[mer] U3U5	3.5 ± 1.1	>250	2.9 ± 0.7	>250

Tested compounds were added either before the reaction mixture was incubated at 37 °C (−preincubation), or after a 10-min incubation at 37 °C (+preincubation). Reaction mixture were prepared and processed as described in Section 2.

[a] Compound concentration required to reduce the HIV-1 rIN catalysis of 3′-end-labeled 21[mer] or 40[mer] dsDNA substrate by 50%. Values were expressed in μM for baicalein, myricetin and RDS1473 and nM for 21[mer] U5 and 40[mer] U3U5.

To verify whether the use of the two-ended 40^{mer} U3U5 duplex DNA oligonucleotide as rIN DNA substrate could improve the correlation between the effects of putative IN inhibitors observed in cell-free assays with their effects observed in cell-culture assays, we examined the rIN inhibition curve of compounds that exert different impacts on viral replication. The first tested compound was the polyanion ATA which is inactive in PIC assays that use unpurified complexes, but it inhibits IN catalysis in PIC assays which use partially purified complexes, as well as rIN activities and rIN binding to DNA in vitro [8]. Probably, its strong anionic charge destroys the IN–DNA interaction, and only the unpurified PIC posses IN–DNA interactions sufficiently stable to maintain enzymatic activity in its presence. Consistently, we found that ATA inhibits rIN activities regardless the dsDNA recognition sequences of the oligonucleotide substrate and the rIN preincubation with the dsDNA substrate before ATA addition.

The second tested compound was the cinnamoyl compound RDS1158, a typical example of polyhydroxylated aromatic molecule active in in vitro assays but inactive in cell-culture assays [21]. The finding that RDS1158 was not able to inhibit rIN activities when the enzyme was preincubated with the two-ended 40^{mer} U3U5 dsDNA before the addition of the compound, seems to suggest that the latter assay conditions may be more selective than the standard assay conditions. Noteworthy, both the presence of two-ended 40^{mer} U3U5 dsDNA and the performance of its preincubation with rIN were required to avoid the RDS1158 inhibition. These results support the concept that the preincubation of the enzyme with the two-ended 40^{mer} U3U5 dsDNA allows a binding rIN–dsDNA which is more resistant than the rIN 21^{mer} dsDNA complex.

The third tested compound was the diketo acid derivative L-731,988, which has been reported to inhibit both the rIN activity in vitro and the viral replication in cell culture [6]. Consistently, L-731,988 inhibits rIN activities regardless the dsDNA recognition sequences of the oligonucleotide substrate and the preincubation conditions.

To examine the role of the U3 and U5 IN recognition sequence and the role of the dsDNA substrate length in the increased selectivity of the rIN assay versus the RDS1158 molecule, one-ended and two-repetitive-ended 40^{mer} oligonucleotides substrates dsDNA were prepared. Results clearly indicate that the length of the dsDNA has no impact on the assay selectivity, whereas the presence of two-repetitive ends in the DNA substrate (either U3U3 or U5U5) leads to an intermediate assay sensitivity to RDS1158.

Taken together these results suggest that the architecture of the rIN–dsDNA complex is influenced by the sequence of the DNA substrate. This is consistent with the previous observations that HIV-1 IN requires both U3 and U5 recognition sequences to catalyze a concerted DNA integration [13] and that the formation of the preintegration complex also requires two functional viral DNA ends [12]. Actually, it has been proposed that this requirement may be due either to the fact that the two LTR ends bind to different regions of a multimer IN complex or that, alternatively, IN assumes different conformations upon the binding to one or two 3′-ends [13]. Our data suggest that the presence in the DNA substrate of either only one viral end, or two repetitive viral ends or the natural two different viral ends, leads to different rIN–dsDNA architectures that may result in diverse enzyme sensitivities to some compounds. Such sensitivities may reflect different complex stability.

To further investigate the reliability of the new assay conditions we examined the effects on rIN activity of other polyhydroxylated aromatic molecules, such as baicalein, myricetin and RDS1473. The first two are flavons which were not able to block enzyme activities when added after the formation of the rIN–dsDNA complex, confirming that their interaction with the HIV-1 rIN is weak and non-specific [8,24–29]. Differently, RDS1473 is a cinnamoyl analogue which blocks the HIV-1 replication in cell-based assays and the rIN in in vitro assays. The fact that RDS1473 is able to inhibit the enzyme catalysis when added after the formation of the rIN 40^{mer} U3U5 dsDNA complex at a concentration ($IC_{50} = 14\ \mu M$) comparable to the concentration ($EC_{50} = 4\ \mu M$) needed to stop viral replication confirms the good correlation between the rIN inhibition results obtained in cell-free and in cell-based assays when the 40^{mer} U3U5 dsDNA is the in vitro substrate. In addition, it may suggest that the interaction between this molecule and the enzyme could be specific.

In summary, we propose that the use of the two-ended 40^{mer} U3U5 oligonucleotide as DNA substrate of the HIV-1 rIN in vitro assay may lead to the formation of a more stable rIN–dsDNA complex which, coupled with an experimental design which allows the formation of this complex before the addition of the putative IN inhibitor, may lead to an assay which is more reliable and predictive of the IN in vivo sensitivity to new compounds.

References

[1] Hindmarsh P, Lei J. Retroviral DNA integration. Microbiol Mol Biol Rev 1999;63:836–43.
[2] Sherman MP, Greene WC. Slipping through the door: HIV entry into the nucleus. Microbes Infect 2002;4:67–73.
[3] Andrake MD, Skalka AM. Retroviral integrase, putting the pieces together. J Biol Chem 1996;271:19633–6.
[4] Pani A, Marongiu ME. Anti-HIV-1 integrase drugs: how far from the shelf? Curr Pharm Des 2000;6:569–84.
[5] Debyser Z, Cherepanov P, Pluymers W, De Clercq E. Assays for the evaluation of HIV-1 integrase inhibitors. Methods Mol Biol 2001;160:139–55.
[6] Hazuda DJ, Felock P, Witmer M, Wolfe A, Stillmock K, Grobler JA, et al. Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. Science 2000;28:646–50.
[7] Goldgur Y, Craigie R, Cohen GH, Fujiwara T, Yoshinaga T, Fujishita T, et al. Structure of the HIV-1 integrase catalytic domain complexed with an inhibitor: a platform for antiviral drug design. Proc Natl Acad Sci USA 1999;96:13040–3.

[8] Farnet CM, Wang B, Lipford JR, Bushman FD. Differential inhibition of HIV-1 preintegration complexes and purified integrase protein by small molecules. Proc Natl Acad Sci USA 1996;93: 9742–7.
[9] Hansen MS, Smith GJ, Kafri T, Molteni V, Siegel JS, Bushman FD. Integration complexes derived from HIV vectors for rapid assays in vitro. Nat Biotechnol 1999;17:578–82.
[10] Tramontano E, Colla PL, Cheng YC. Biochemical characterization of the HIV-1 integrase 3′-processing activity and its inhibition by phosphorothioate oligonucleotides. Biochemistry 1998;37:7237–43.
[11] Hazuda DJ, Hastings JC, Wolfe AL, Emini EA. A novel assay for the DNA strand-transfer reaction of HIV-1 integrase. Nucleic Acids Res 1994;22:1121–2.
[12] Wei SQ, Mizuuchi K, Craigie R. Footprints on the viral DNA ends in Moloney murine leukemia virus preintegration complexes reflect a specific association with integrase. Proc Natl Acad Sci USA 1998;95: 10535–40.
[13] Brin E, Leis J. Changes in the mechanism of DNA integration in vitro induced by base substitutions in the HIV-1 U5 and U3 terminal sequences. J Biol Chem 2002;277:10938–48.
[14] Brin E, Leis J. HIV-1 integrase interaction with U3 and U5 terminal sequences in vitro defined using substrates with random sequences. J Biol Chem 2002;277:18357–64.
[15] Bushman FD, Craigie R. Activities of human immunodeficiency virus (HIV) integration protein in vitro: specific cleavage and integration of HIV DNA. Proc Natl Acad Sci USA 1991;88:1339–43.
[16] Sherman PA, Dickson ML, Fyfe JA. Human immunodeficiency virus type 1 integration protein: DNA sequence requirements for cleaving and joining reactions. J Virol 1992;66:3593–601.
[17] Cherepanov P, Surratt D, Toelen J, Pluymers W, Griffith J, De Clercq E, et al. Activity of recombinant HIV-1 integrase on mini-HIV DNA. Nucleic Acids Res 1999;27:2202–10.
[18] Segel IH. Enzyme kinetics. New York: Wiley; 1975. p. 18–80.
[19] Dixon M. The graphical determination of K_m and K_i. Biochem J 1972; 129:197–215.
[20] Cushman M, Sherman P. Inhibition of HIV-1 integration protein by aurintricarboxylic acid monomers, monomer analogs, and polymer fractions. Biochem Biophys Res Commun 1992;185:85–90.
[21] Artico M, Di Santo R, Costi R, Novellino E, Greco G, Massa S, et al. Geometrically and conformationally restrained cinnamoyl compounds as inhibitors of HIV-1 integrase: synthesis, biological evaluation and molecular modeling. J Med Chem 1998;41:3948–60.
[22] Neamati N. Structure-based HIV-1 integrase inhibitor design: a future perspective. Expert Opin Invest Drugs 2001;10:281–96.
[23] Pemberton IK, Buc H, Buckle M. Displacement of viral DNA termini from stable HIV-1 integrase nucleoprotein complexes induced by secondary DNA-binding interactions. Biochemistry 1998;37:2682–90.
[24] Ono K, Nakane H, Fukushima M, Chermann JC, Barre-Sinoussi F. Differential inhibitory effects of various flavonoids on the activities of reverse transcriptase and cellular DNA and RNA polymerases. Eur J Biochem 1990;190:469–76.
[25] Austin CA, Patel S, Ono K, Nakane H, Fisher LM. Site-specific DNA cleavage by mammalian DNA topoisomerase II induced by novel flavone and catechin derivatives. Biochem J 1992;282:883–9.
[26] LaFemina RL, Callahan PL, Cordingley MG. Substrate specificity of recombinant human immunodeficiency virus integrase protein. J Virol 1991;65:5624–30.
[27] Fesen MR, Pommier Y, Leteurtre F, Hiroguchi S, Yung J, Kohn KW. Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. Biochem Pharmacol 1994;48:595–608.
[28] Inouye Y, Yamaguchi K, Take Y, Nakamura S. Inhibition of avian myeloblastosis virus reverse transcriptase by flavones and isoflavones. J Antibiot 1989;42:1523–5.
[29] Mahmood N, Pizza C, Aquino R, De Tommasi N, Piacente S, Colman S, et al. Inhibition of HIV infection by flavanoids. Antiviral Res 1993; 22:189–99.