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6-[1-(4-Fluorophenyl)methyl-1*H*-pyrrol-2-yl)]-2,4-dioxo-5-hexenoic acid ethyl ester a novel diketo acid derivative which selectively inhibits the HIV-1 viral replication in cell culture and the ribonuclease H activity in vitro

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Abstract

The human immunodeficiency virus-type 1 (HIV-1) reverse transcriptase (RT) is a multifunctional enzyme which displays DNA polymerase activity, which recognizes RNA and DNA templates, and a degradative ribonuclease H (RNase H) activity. While both RT functions are required for retroviral replication, until now only the polymerase function has been widely explored as drug target. We have identified a novel diketo acid derivative, 6-[1-(4-fluorophenyl)methyl-1*H*-pyrrol-2-yl)]-2,4-dioxo-5-hexenoic acid ethyl ester (RDS 1643), which inhibits in enzyme assays the HIV-1 RT-associated polymerase-independent RNase H activity but has no effect on the HIV-1 RT-associated RNA-dependent DNA polymerase (RDDP) activity and on the RNase H activities displayed by the Avian Myeloblastosis Virus and *E. coli*. Time-dependence studies revealed that the compound is active independently on the order of its addition to the reaction mixture, and inhibition kinetics studies demonstrated that RDS 1643 inhibits the RNase H activity noncompetitively, with a K_I value of 17 μM. When RDS 1643 was combined with non-nucleoside RT inhibitors (NNRTI), such as efavirenz and nevirapine, results indicated that RDS 1643 does not affect the NNRTIs anti-RDDP activity and that, vice versa, the NNRTIs do not alter the RNase H inhibition by RDS 1643. When assayed on the viral replication in cell-based assays, RDS 1643 inhibited the HIV-1_{IIIB} strain with an EC_{50} of 14 μM. Similar results were obtained against the Y181C and Y181C/K103N HIV-1 NNRTI resistant mutant strains. RDS 1643 may be the first HIV-1 inhibitor selectively targeted to the viral RT-associated RNase-H function.
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Keywords: HIV; AIDS; Reverse transcriptase; Ribonuclease H; Antiviral agents; Diketoacid

1. Introduction

Conversion of the single-stranded RNA retroviral genome into double-stranded proviral DNA is an obligatory event in the human immunodeficiency virus (HIV) replication cycle which is accomplished, in the host cell cytoplasm, by the virus-coded enzyme reverse transcriptase (RT). This process requires a combination of an RNA- and DNA-dependent DNA polymerase activity together with a degradative function which hydrolyzes the RNA component of the RNA-DNA replication hybrid intermediate and is termed, therefore, ribonuclease H (RNase H). The two RT-associated enzymatic functions are carried out by two distinct catalytic sites which, albeit the HIV-1 RT is composed by two polypeptide subunits (p66 and p51), reside in the p66 subunit at a distance of approximately 18 base pairs from each other (Arts and Le Grice, 1998; Hughes et al., 1998; Klarmann et al., 2002). The N terminus of p66 accommodates the active site which catalyzes the RNA- and DNA-dependent DNA polymerase

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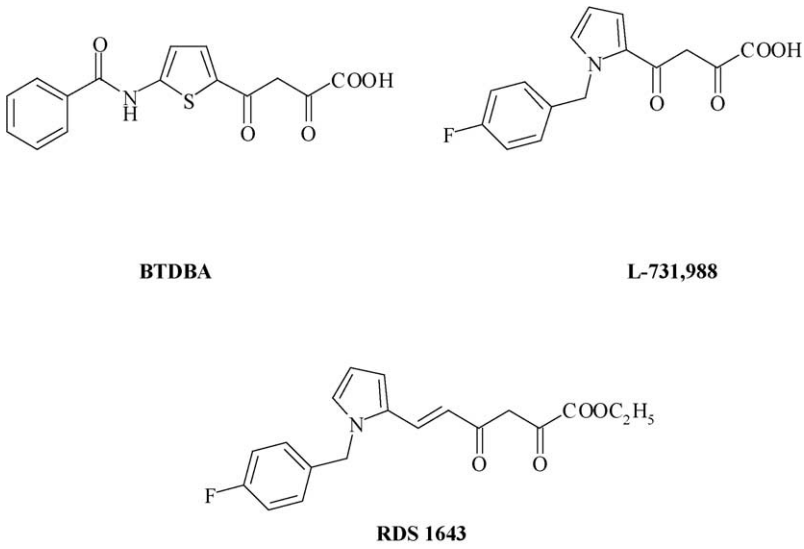

Fig. 1. Chemical structures of RNase H and IN inhibitors.

activity, while the C terminus accommodates the active site which catalyzes the RNase H activity. Since both activities are absolutely required for DNA provirus formation (Hughes et al., 1998; Klarmann et al., 2002), the RT-associated RNase H function is an attractive target for the development of new antiretroviral agents (Klarmann et al., 2002; Tarrago-Litvak et al., 2002).

In spite of these considerations, all RT inhibitors currently approved for the treatment of HIV infection, or presently under investigation in clinical trials, inhibit its polymerase activity, while none of them blocks its RNase H activity (Parniak and Sluis-Cremer, 2000; Gulick, 2003). Until now in fact, only a few compounds have been described to inhibit the HIV-1 RNase H function in vitro (Tarrago-Litvak et al., 2002). Among them are sulfated polyanions (Moelling et al., 1989), the natural marine product illimaquinone (Loya et al., 1990; Loya and Hizi, 1993), the azidothymidylate (AZTMP) (Tan et al., 1991), the *N*-(4-*tert*-butylbenzoyl)-2-hydroxyl-1-naphthaldehydfe hydrazone (BBNH) (Borkow et al., 1997), the 4-chlorophenylhydrazone of mesoxalic acid (CPHM) (Davis et al., 2000), the *N*-hydroxyimides (Klumpp et al., 2003) and few others (Klarmann et al., 2002; Tarrago-Litvak et al., 2002). In reality, with the exception of the *N*-hydroxyimides which do not inhibit the *E. coli* RNase H and block the HIV-1 RT-associated polymerase activity at a concentration 40-fold higher than the concentration required to inhibit the RNase H function (Klumpp et al., 2003), none of them is truly selective in vitro for the HIV-1 RNase H either because they inhibit also other viral or cellular recombinant enzymes (Moelling et al., 1989; Loya et al., 1990; Loya and Hizi, 1993; Davis et al., 2000), or because they are active on both HIV-1 RT-associated polymerase and RNase H functions (Borkow et al., 1997). Moreover, with the exception of BBNH (Borkow et al., 1997), none of them inhibits the HIV-1 replication in cell-based assays (Klarmann et al., 2002; Tarrago-Litvak et al., 2002; Moelling et al., 1989; Loya et al., 1990; Loya and Hizi, 1993; Tan et al., 1991; Davis et al., 2000; Klumpp et al., 2003). Recently, the diketo acid (DKA) derivative 4-[5-(benzo-ylamino)thien-2-yl]-2,4-dioxobutanoic acid (BTDBA, Fig. 1) has been reported to inhibit the HIV-1 RT RNase H function without affecting its polymerase activity (Shaw-Reid et al., 2003). Even though this DKA derivative was not highly selective for RNase H since (i) it inhibited in the same concentration range also the HIV-1 integrase (IN) in enzyme assays and (ii) it did not block the viral replication in cell-based assays, it provided the proof of concept for direct inhibition of the HIV-1 RT RNase H associated activity by DKAs (Shaw-Reid et al., 2003).

In this report we identified a novel DKA derivative, the 6-[1-(4-fluorophenyl)methyl-1*H*-pyrrol-2-yl)]-2,4-dioxo-5-hexenoic acid ethyl ester (RDS 1643, Fig. 1), which selectively inhibits the RNase H function of the HIV-1 RT, without affecting neither its DNA polymerase associated activity, nor the RNase H activity associated to the Avian Myeloblastosis Virus (AMV) RT or the *E. coli* RNase H, while it barely affected the HIV-1 IN activity. We also performed a partial characterization of the interaction between RDS 1643 and the HIV-1 RT by time of addition, kinetic and drug association studies. Finally, we demonstrated that RDS 1643 inhibits the replication of wild type (wt) and drug-resistant HIV-1 strains in cell-based assays.

2. Materials and methods

2.1. Materials

P6HRT-prot plasmid was kindly provided by Dr. S. Le Grice (NCI at Frederick). All buffer components were obtained from Sigma–Aldrich unless specified; [γ^{32}P]-ATP, [^{3}H]-dGTP, poly(rC)-oligo(dG)$_{12–18}$ and Hi-Trap desalting column were obtained from Amersham Biosciences; G-25

Sephadex quick spin column, T4 polynucleotide kinase, AMV rRT and *E. coli* RNase H were from Roche; His-binding resin was from Novagen. The tC5U RNA oligonucleotide (5′-CCCCCUCUCAAAAACAGGAGCAGAAAGACAAG-3′) and the p12 DNA oligonucleotide (5′-GTCTTTCTGCTC-3′) were purchased by MWG Biotech, Italy.

2.2. HIV-1 rRT purification

Heterodimeric rRT was expressed essentially as described (Le Grice et al., 1995). Briefly, *E. coli* strain M15 containing the P6HRT-prot vector were grown up to an OD_{600} of 0.8 and induced with IPTG 1.7 mM for 5 h. Protein purification was carried out with an AKTA-prime FPLC (Amersham Biosciences) with a Ni^{2+}-sepharose chromatography. Briefly, cell pellets were resuspended in Lyses buffer (20 mM Hepes pH 7.5, 0.5 M NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole, 0.4 mg/mL lysozime), incubated on ice for 20 min, sonicated and centrifuged at 30,000 × *g* for 1 h. The supernatant was applied to a His-binding resin column and washed thoroughly with Wash buffer (20 mM Hepes pH 7.5, 0.3 M NaCl, 5 mM β-mercaptoethanol, 60 mM imidazole, 10% glycerol). rRT was gradient-eluted with Elute buffer (Wash buffer with 0.5 M imidazole), fractions were collected and enzyme activity was assessed; protein purity was checked by SDS-PAGE and found to be higher than 90%. Enzyme containing fractions were pooled and loaded onto a Hi-Trap desalting column equilibrated with Storage buffer (50 mM Tris–Hcl pH 7.0, 25 mM NaCl, 1 mM EDTA, 10% glycerol). Fractions were collected, catalytic activities and protein concentration were determined. Enzyme-containing fractions were pooled and aliquots were stored at −80 °C.

2.3. RNase H polymerase-independent cleavage assay

When the tC5U/p12 hybrid was used as reaction substrate the RNase H activity was measured as described (Shaw-Reid et al., 2003). Briefly, the tC5U RNA oligonucleotide was labeled at its 5′-end with [γ^{32}P]-ATP and T4 polynucleotide kinase. The unincorporated [γ^{32}P]-ATP was separated from the labeled RNA by running the sample through a G-25 Sephadex quick spin column. The labeled tC5U oligonucleotide was annealed to the p12 DNA oligonucleotide in 0.1 M NaCl by heating at 80 °C and slowly cooling to room temperature overnight. RNase H activity was measured in 15 μL reaction volume containing 50 mM Tris–HCl pH 7.8, 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 80 mM KCl, 50 nM tC5U/p12 labeled hybrid, 2 nM rRT and different drug concentrations. The reaction mixture was incubated for 30 min at 37 °C and stopped by the addition of 7.5 μL of sample buffer (96% formamide, 20 mM EDTA, 0.08% Bromophenol blue and 0.08% Xylene cyanol). Samples were heated at 100 °C for 3 min, and 10 μL of them were layered onto a denaturing 20% polyacrylamide gel (7 M urea, 0.09 M Tris–borate (pH 8.3), 2 mM EDTA, 20% acrylamide) and run for 1.5 h at 80 W. The gel was dried and reaction products were visualized and quantified by a Bio-Rad FX Phosphoimager.

When the poly(dC)-[^{3}H]poly(rG) hybrid was used as reaction substrate the RNaseH activity was measured as described (Starnes and Cheng, 1989). Briefly, RNaseH activity was measured in 50 μL reaction volume containing the same reaction mixture as above with the exception of 4 nM poly(dC)-[^{3}H]poly(rG) and 1 nM rRT. The reaction mixture was incubated for 30 min at 37 °C and 40 μL aliquots were spotted on glass fiber filters (Whatman GF/A) and processed for determination of trichloroacetic acid-insoluble radioactivity (Starnes and Cheng, 1989). When AMV and *E. coli* RNase H activities were measured, the poly(dC)-[^{3}H]poly(rG) hybrid was used as reaction substrate with the buffer conditions indicated by the manufacturer, and the trichloroacetic acid-insoluble radioactivity was determined as above.

2.4. Kinetic studies

The analysis of the kinetic of inhibition was performed according to Lineaweaver–Burke plots; ν was expressed as fmoles/min, K_I was calculated by replotting the intercept values versus the inhibitor concentration. Reversibility studies were conducted according to Segel by plotting V_{max}, obtained in the presence or absence of the inhibitor, versus $[E]_t$ (Segel, 1975). In fact, for a reversible non competitive inhibitor, the "plus inhibitor" curve should have a smaller slope than the control curve and should go through the origin. For an irreversible inhibitor, the "plus inhibitor" curve will have the same slope as the control curve, but should intersect the horizontal axis at a position different from the origin (Segel, 1975).

2.5. RNA-dependent DNA polymerase assay

The RDDP activity of HIV-1 rRT was measured as described (Tramontano and Cheng, 1992) in 50 μL volume containing 50 mM Tris–HCl pH 7.8, 80 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL bovine serum albumine, 0.3 O.D./mL poly(rC)-oligo$(dG)_{12–18}$, 10 μM [^{3}H]-dGTP (1 Ci mmol^{-1}). The reaction mixture was incubated for 30 min at 37 °C, 40 μL aliquots were spotted on glass fiber filters (Whatman GF/A) and processed for determination of trichloroacetic acid-insoluble radioactivity (Tramontano and Cheng, 1992).

2.6. HIV-1 replication assay

Wt and resistant HIV-1 strains stock solutions had titers of (2–4.5) × 10^6 50% cell culture infectious dose ($CCID_{50}$)/mL. Cytotoxicity evaluation was based on the viability of mock-infected MT-4 cells, as monitored by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels et al., 1988). Activity of the compounds against the HIV-1 multiplication in acutely infected cells was based on inhibition of virus-induced cytopathicity in MT-4 cells and was determined by the MTT method (Pauwels et al., 1988).

3. Results

3.1. Inhibition of HIV-1 rRT polymerase-independent RNase H activity

The HIV-1 RNase H belongs to the superfamily of polynucleotidyl transferases which includes also the core domain of the HIV-1 IN, another essential retroviral enzyme (Rice et al., 1996; Rice and Baker, 2001). The structural analysis of these proteins revealed that they share similar active sites that have been recruited to serve different biological functions (Rice et al., 1996; Rice and Baker, 2001). These observations have led to hypothesize that compounds which have been shown to inhibit the HIV-1 IN could be able to inhibit also the viral RNase H function. In fact, the DKA derivative BTDBA, which belongs to a class of IN inhibitors (Hazuda et al., 2000), has recently been shown to inhibit both HIV-1 IN and RNase H functions without blocking the rRT-associated RDDP activity (Shaw-Reid et al., 2003). Given the structural similarities (Fig. 1) between the DKA derivative BTDBA and RDS 1643, an analogue that we had previously synthesized in an IN inhibition screening program, we tested the latter against the HIV-1 rRT-associated polymerase-independent RNase H activity in the same assay system previously described (Shaw-Reid et al., 2003). Fig. 2 shows that RDS 1643 inhibited the RNase H reaction at concentrations in the low micromolar range, with an IC_{50} value of 13 μM (Table 1). To further verify this observation, we also prepared a longer hybrid poly(dC)-[^{3}H]poly(rG) and used it as reaction substrate as described (Starnes and Cheng, 1989). Also in this system, RDS 1643 inhibited the RNase H activity with an IC_{50} value of 15 μM.

Subsequently, in order to explore the selectivity of action of RDS 1643, we assayed the HIV-1 rRT-associated RDDP activity, the AMV rRT-associated RNase H activity and the *E. coli* RNase H activity in the presence of compound concentrations up to 100 μM. RDS 1643 did not inhibit any of these enzymes (Table 1). We also tested the RDS 1643 effect on both HIV-1 IN 3′-processing and strand transfer reactions finding that it affected both of them with an IC_{50} value of 92–98 μM (Table 1). Nevirapine, efavirenz and L-731,988 (Hazuda et al., 2000) were used as reference compounds for HIV-1 RDDP and IN reactions, respectively.

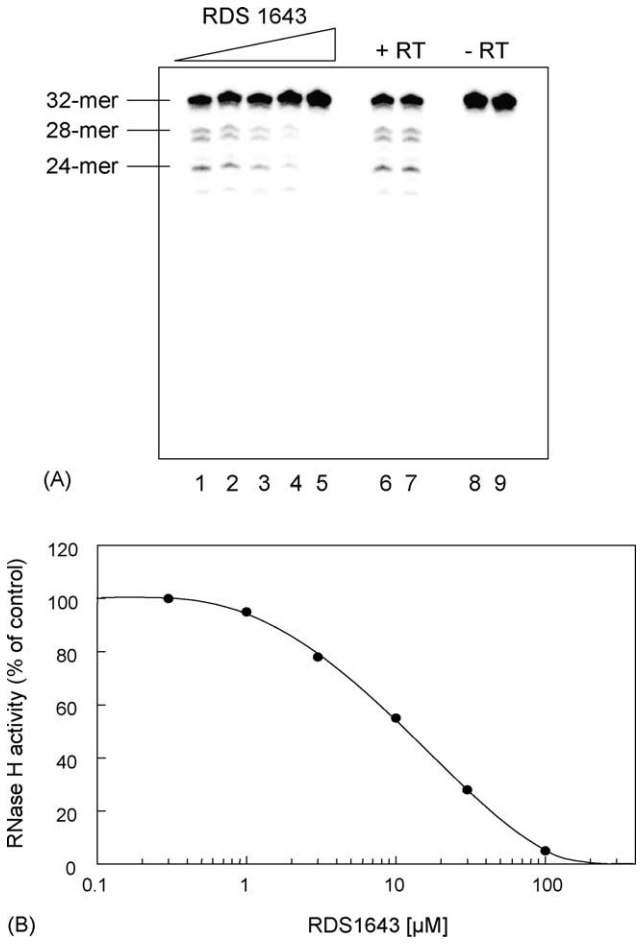

Fig. 2. Inhibition of polymerase-independent RNase H by RDS 1643. Reactions were carried out as described under Section 2. Panel A, PAGE analysis of the RNase H reaction. Three major bands were resolved as reaction products, each of them coming from a single cleavage event of the 32^{mer} substrate. Lane 1–5 plus rRT and RDS 1643 (1, 3, 11, 33, 100 μM); lane 6–7 plus rRT without drug; lane 8–9 without rRT. Panel B, plot of percent activity of the 24^{mer} band production measured in the presence of different RDS 1643 concentrations (similar data were obtained for the other bands). Data represent mean values from three independent determinations.

3.2. Time-dependence study

In order to gain some insights into the interaction between the HIV-1 rRT and RDS 1643, we first evaluated whether this interaction could be affected by the time of addition of the

Table 1
Inhibition of viral and cellular enzymes by RDS 1643

Compound	[a]IC_{50}					
	HIV-1				AMV	*E. coli*
	RT RNase H	RT RDDP	IN 3′-processing	IN strand-transfer	RT RNase H	RNase H
RDS 1643	13 ± 4	>100	92 ± 5	98 ± 8	>100	>100
Nevirapine	>100	0.6 ± 0.1	>100	>100	>100	>100
Efavirenz	>10	0.0013 ± 0.0004	>10	>10	>10	>10
L-731,988	>100	>100	2 ± 0.5	0.3 ± 0.2	>100	>100

Enzyme reactions were performed as described under Section 2. Values represent the mean value and S.D. of at least three independent experiments.
[a] Compound concentration (μM) required to reduce enzyme activity by 50%.

reaction mixture components. Results showed that the RNase H inhibition by RDS 1643 was identical when (i) the compound was preincubated with the enzyme in the absence of the substrate; (ii) the compound and all the components of the reaction mixture were incubated at the same time (the standard experimental condition); (iii) the compound was added to the reaction mixture after the enzyme had been preincubated with the substrate to allow the formation of the rRT/substrate complex (data not shown).

Next, we wanted to investigate the reversibility of the rRT/RDS 1643 interaction. A standard reaction mixture was incubated with either 100 or 10 μM RDS 1643 at 37 °C for 5 min, then it was 10-fold diluted into a solution containing only the salt components of the reaction mixture (i.e. a mixture without enzyme, substrate and drug) and further incubated for 30 min. In these conditions, the RNase H activity in the diluted samples was inhibited to the same extent to which it was inhibited in the respective undiluted control samples (i.e. totally inhibited in the presence of 100 μM drug or partially inhibited in the presence of 10 μM drug) (data not shown). In these conditions therefore, the inhibition of the HIV-1 RNase H activity by RDS 1643 appears to be either slowly reversible or irreversible.

3.3. Kinetics of HIV-1 rRT polymerase-independent RNase H inhibition by RDS 1643

The hydrolysis of the poly(dC)-[^{3}H]poly(rG) hybrid substrate by RNase H is a processive reaction which can be monitored according to the Michaelis–Menten kinetic assumptions. In this system, RDS 1643 resulted a non competitive inhibitor of the polymerase-independent RNase H activity, with a K_I value of 17 μM (Fig. 3).

However, given the results obtained in the time-dependence study, it could be possible that RDS 1643 could behave as an irreversible rRT inhibitor rather than as a reversible non competitive inhibitor. Therefore, to distinguish between irreversible and reversible non competitive inhibition we plotted V_{max} versus $[E]_t$ in the presence or absence of the inhibitor as described by Segel (Segel, 1975). Results showed that RDS 1643 is a reversible non competitive inhibitor of the RNase H function (Fig. 4).

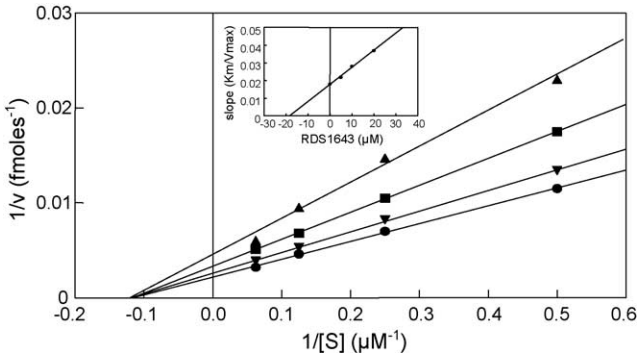

Fig. 3. Lineweaver–Burk plot of the inhibition of the HIV-1 rRT-associated RNase H activity by RDS 1643. HIV-1 rRT was incubated in the absence (●) or in the presence of 5 μM (▼), 10 μM (■) or 20 μM (▲) RDS 1643. Reactions were performed as described under Section 2. Inset, replot of the line slopes obtained in the Lineweaver–Burk plot against the RDS 1643 concentration to calculate the K_I value.

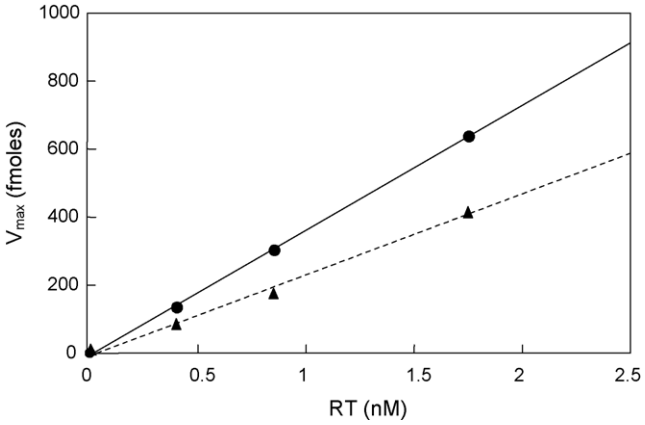

Fig. 4. Reversibility of the inhibition of the HIV-1 RNase H rRT activity by RDS 1643. HIV-1 rRT was incubated in the absence (●) or in the presence of 15 μM RDS 1643 (▲). Reactions were performed as described under Section 2.

3.4. Binding of Mg^{2+} to RDS 1643

It has been shown that the *N*-hydroxyimides inhibit the HIV-1 RNase H activity by binding to the metal ions (Klumpp et al., 2003). Furthermore, it has been proposed that the structurally similar DKA derivative BT-DBA might interact with the metal cofactor (Shaw-Reid et al., 2003). Therefore, to verify whether also RDS 1643 might interact with the metal ions, we measured its UV–vis spectrum in the absence or in the presence of 6 mM $MgCl_2$. Results showed that the cation addition shifted the RDS 1643 maximum absorbance from 393 nm to 408 nm (Fig. 5).

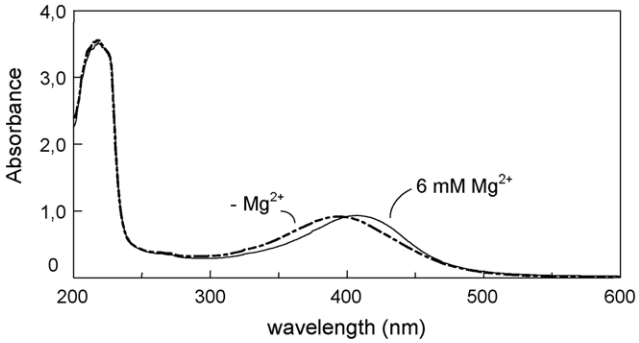

Fig. 5. Chelation of Mg^{2+} by RDS 1643. UV–vis spectrum of RDS 1643 measured alone (broken line) or in the presence of 6 mM $MgCl_2$ (unbroken line).

3.5. Combination of RNase H and RDDP inhibitors

It has been shown that there is a significant interdomain communication between the NNRTI binding site domain and the RNase H domain. In fact, in enzymatic assays, TIBO was reported to activate the HIV-1 RNase H activity (Gopalakrishnan and Benkovic, 1994) and nevirapine was reported to alter its cleavage specificity (Palaniappan et al., 1995). Therefore, it was of interest to investigate whether the combination of compounds that selectively inhibit either the RDDP or the RNase H activity could affect in any way their respective interaction with the rRT. For this purpose we assayed RDDP and RNAse H HIV-1 rRT-associated activities in the presence of a combination of increasing concentrations of RDS 1643 and either nevirapine or efavirenz. Results showed that RDS 1643 did not affect the RDDP inhibition by the two NNRTIs and that, on the other hand, the two NNRTIs did not vary the inhibition of the polymerase-independent RNase H activity by RDS 1643 (Table 2).

Table 2
Effect of combination of RDS 1643 and NNRTIs on the HIV-1 rRT-associated polymerase-independent RNase H and RDDP activities

Compounds	IC_{50} (μM)[a]	
	RNase H	RDDP
RDS 1643	13	
RDS 1643 + EFV 30 μM	18	
RDS 1643 + EFV 10 μM	14	
RDS 1643 + EFV 3 μM	11	
RDS 1643 + EFV 1 μM	13	
RDS 1643	13	
RDS 1643 + NVP 30 μM	20	
RDS 1643 + NVP 10 μM	19	
RDS 1643 + NVP 3 μM	12	
RDS 1643 + NVP 3 μM	16	
NVP		0.60
NVP + RDS 1643 100 μM		0.50
NVP + RDS 1643 30 μM		0.65
NVP + RDS 1643 10 μM		0.81
NVP + RDS 1643 3 μM		0.68
EFV		0.0013
EFV + RDS 1643 100 μM		0.0008
EFV + RDS 1643 30 μM		0.0021
EFV + RDS 1643 10 μM		0.0015
EFV + RDS 1643 3 μM		0.0026

Enzyme reactions were performed as described under Section 2. Values represent average of two independent determinations.
[a] Compound concentration required to reduce enzyme activity by 50%.

3.6. Inhibition of wt and NNRTI-resistant HIV-1 replication by RDS 1643

Recent studies have shown that the vast majority of the DKA derivatives which were synthesized as HIV-1 IN inhibitors are unable to block the viral replication in cell based assays (Tarrago-Litvak et al., 2002). Consistently, the BT-DBA analogue did not inhibit the HIV-1 proliferation in cell culture (Shaw-Reid et al., 2003). Therefore, in order to explore whether RDS 1643 was able to stop the retroviral multiplication, we assayed the replication of wt HIV-1 in MT-4 cells. Results showed that RDS 1643 inhibited the HIV-1 proliferation in cell-based assays with an EC_{50} value of 14 μM and with a selectivity index of 4 (Table 3).

Furthermore, since it is well known that the presence of the single amino acid substitutions K103N and Y181C, or the triple substitution K103R, V179D and P225H, in the HIV-1 RT result in a more than 100-fold decrease in sensitivity to NNRTI such as nevirapine and efavirenz (Corbett and Rodgers, 2002), we wanted to verify the impact of these mutations on the HIV-1 inhibition by RDS 1643. Therefore, we tested the replication of these mutant viruses in the presence of the compound and found that, interestingly, RDS 1643 possesses the same potency of inhibition for wt and resistant strains (Table 3). Nevirapine, efavirenz and L-731,988 were used as reference compounds.

4. Discussion

In our search for HIV-1 IN inhibitors we have synthesized a new DKA derivative which is able to selectively inhibit the polymerase-independent HIV-1 rRT associated RNase H function in in vitro assays and to stop the viral replication in cell-based assays.

In vitro assays have demonstrated that the inhibition pattern of retroviral and bacterial recombinant enzymes by RDS 1643 is unique with respect to all the other molecules which have been identified to inhibit the HIV-1 rRT-associated RNase H function so far. In fact, RDS 1643 inhibited selectively the HIV-1 rRT RNase H activity without affecting

Table 3
Inhibition of wt and NNRTI-resistant HIV-1 by different compounds

Compound	CC_{50} (μM)[a]	EC_{50} (μM)[b]			
	MT-4	wt	Y181C	K103N/Y181C	K103R/V179D/P225H
RDS 1643	63 ± 4	14.3 ± 2.0	12.5 ± 3.1	7.3 ± 0.9	18.9 ± 1.5
Nevirapine	100 ± 8	0.4 ± 0.1	>30	>30	>30
Efavirenz	35 ± 3	0.004 ± 0.001	0.02 ± 0.004	0.15 ± 0.02	2.0 ± 0.2
L-731,988	54 ± 4	1.5 ± 0.2	5.0 ± 0.8	8.2 ± 1.1	2.4 ± 0.3

Assays were performed as described under Section 2. Values represent average ± range of two independent determinations.
[a] Compound concentration (μM) required to reduce the viability of mock-infected MT-4 cells by 50%.
[b] Compound concentration (μM) required to achieve 50% protection of MT-4 cells from the HIV-1 induced cytopathogenicity.

its RDDP action while RNase H inhibitors such as BBNH (Borkow et al., 1997), quinones (Min et al., 2002), naphthalenesulfonic acid derivatives (Mohan et al., 1994) and others (Tarrago-Litvak et al., 2002) inhibit both HIV-1 rRT functions. In addition, RDS 1643 was inactive on the *E. coli* RNase H and was 7-fold more potent on the HIV-1 RNase H than on the HIV-1 IN activity, while other RNase H inhibitors which do not affect the RDDP function such as illimaquinone, CPHM and BTDBA inhibit in the same concentration range also either the *E. coli* RNase H hydrolysis, the formers, or the HIV-1 IN, the latter (Loya et al., 1990; Loya and Hizi, 1993; Shaw-Reid et al., 2003).

To investigate the mode of interaction between RDS 1643 and the HIV-1 rRT we performed time-dependence and kinetics studies. Results indicated that RDS 1643 is a reversible non competitive inhibitor of the RNase H activity and that both its binding to RT and its effect on it are independent from nucleic acid binding. This result is somehow consistent with the observations that CPHM, which does not inhibit the HIV-1 polymerase function, has no effect on the binding of the hybrid substrate to the enzyme (Davis et al., 2000), meanwhile BBNH, which inhibits both HIV-1 RT activities, competes with the DNA:RNA substrate (Borkow et al., 1997). Furthermore, it is consistent with the observation that also the structurally similar DKA derivative BTDBA does not require the nucleic acid hybrid for its binding to the rRT and with the proposal that BTDBA might have one binding site in the RNase H domain, in which it probably interacts with the metal cofactor (Shaw-Reid et al., 2003). The shift in the RDS 1643 maximum of absorbance observed in the presence of 6 mM $MgCl_2$ suggests that also this DKA may interact with the Mg^{2+} ions. It is worth to note that, as a consequence of the cations chelation by the CPHM dicarboxylic acid moiety, also the CPHM maximum of absorbance was reported to shift from 305 nm to 335 nm in the presence of 9 mM $MgCl_2$ (Davis et al., 2000). In addition, also the *N*-hydroxyimide derivatives, that were shown to inhibit the HIV-1 RNase H activity (which has been proposed to be provided by two-metal ion active site structure) by binding to the metal ions, were inactive on the *E. coli* RNase H (which probably possesses a one-metal ion mechanism of RNA cleavage) (Klumpp et al., 2003).

Taken together these results seem to indicate that, similarly to what has been suggested for the IN inhibition by DKA compounds (Grobler et al., 2002) also the RNase H inhibition by DKAs may involve the sequestration of the divalent cofactor and that it may exist a specific pocket in the HIV-1 RNase H active site domain to which DKAs could bind. In fact, despite their structural homology, bacterial and retroviral RNase H share a low amino acid residues homology (Hughes et al., 1998; Klarmann et al., 2002) and, therefore, the lack of RDS 1643 activity on both AMV and bacterial RNase H may suggest that the RDS 1643/HIV-1 rRT interaction may take place through the contact with some HIV-1 specific amino acid residue. However, it is worth to note that significant differences between the interactions DKA/HIV-1 RT and DKA/HIV-1 IN are likely to exist since it has been shown that DKAs compete with the IN substrate DNA (Espeseth et al., 2000).

To examine the possible interdependence of the NNRTIs/rRT and RDS 1643/rRT interactions, we assayed the effect of the simultaneous presence of one NNRTI and RDS 1643 on both polymerase and RNase H functions. The absence of any kind of effect on the inhibition of the rRT functions by either drug clearly indicates the complete lack of interference between these two interactions. It is worth to note that the polymerase and RNase H domains of the HIV-1 RT present a high degree of interdependence (Hughes et al., 1998), and that the NNRTIs nevirapine and TIBO, which bind to a hydrophobic pocket near the contact between RT and the nucleic acid, cause a distortion of the enzyme conformation which is sufficient to affect the cleavage specificity of the RNase H and to increase the RNase H activity (Gopalakrishnan and Benkovic, 1994; Palaniappan et al., 1995). Therefore, our results suggest that this conformational change does not affect the RDS 1643 binding to the rRT and that, vice versa, the RDS 1643 interaction with the rRT either (i) does not induce a significant distortion of the RNase H domain, or (ii) determines a conformational change in the RNase H domain which does not impact on the polymerase domain.

The difference in the mode of interaction with the HIV-1 RT between the NNRTIs and RDS 1643 is also confirmed by the fact that RDS 1643 is able to effectively block the viral replication of mutant strains resistant to NNRTIs.

In summary, RDS 1643 is the first compound which, at the best of our knowledge, has been reported to inhibit selectively, in the same concentration range, both the HIV-1 RNase H function in vitro and the viral replication in cell culture. Even though further studies would be needed to definitively prove that the inhibition of the viral proliferation is due indeed to the inhibition of the genomic RNA hydrolysis by RT, our findings further substantiate the hypothesis that the development of selective RNase H inhibitors may lead to the improvement of the chemotherapeutic armamentarium for HIV-1 infection.

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