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The dolabellane diterpene Dolabelladienetriol is a typical noncompetitive inhibitor of HIV-1 reverse transcriptase enzyme

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Abstract

We recently described that a dollabelane diterpene isolated from the marine algae *Dictyota pfaffii* (Dolabelladienetriol) inhibits the human immunodeficiency virus type 1 (HIV-1) enzyme reverse transcriptase (RT), and HIV-1 replication in primary cells. Based on these findings, we investigated additional antiretroviral properties of Dolabelladienetriol. Here, we describe that Dolabelladienetriol blocked the synthesis and integration of HIV-1 provirus and completely abrogated viral replication in primary cells. Also, studies of kinetic mode of action revealed that the Dolabelladienetriol is a nonnucleoside RT inhibitor (NNRTI), acting as a noncompetitive inhibitor, with a K_i value equal to 7.2 μM. To assess whether Dolabelladienetriol could potentiate the anti-HIV-1 effects of other HIV-1 inhibitors, HIV-1-infected cells were treated with Dolabelladienetriol at its EC_{50} dose plus sub-optimal concentrations of classical antiretrovirals. Dolabelladienetriol provided an additive effect with the nucleoside RT inhibitor AZT, and a synergistic effect with the protease inhibitor atazanavir sulphate. There was no increment of the anti-HIV-1 effect resulting from the combination between Dolabelladienetriol and the NNRTI nevirapine. Using a large panel of HIV-1 isolates harboring NNRTI resistance mutations, we found no cross-resistance between Dolabelladienetriol and clinical available NNRTIs. Thus, Dolabelladienetriol is an NNRTI, with potent activity against HIV-1 isolates carrying common NNRTI-associated resistance mutations. Dolabelladienetriol may be considered as a potential new agent for anti-HIV-1 therapy.

Keywords: AIDS; HIV-1; Reverse transcriptase; Diterpene; Dolabelladienetriol

1. Introduction

Since the discovery that human immunodeficiency virus type 1 (HIV-1) is the etiological agent of the acquired immunodeficiency syndrome (AIDS), a large panel of efficient antiretrovirals has been developed and is available for clinical use. The introduction of the highly active anti-retroviral therapy (HAART) effectively reduced the mortality rate and prolonged the life expectancy of HIV-1-infected individuals in developing and developed countries (Snyder et al., 2000; Stebbing et al., 2006). However, the current antiretroviral treatment does not eradicate HIV-1 from the infected tissues, and viral replication remains active in cellular reservoirs (Rodríguez-Arenas et al., 2006; Sabin et al., 2006). Moreover, the long-term use of the

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combined therapy is restricted by the increased prevalence of HIV-1 resistant strains, and, in some instances, by metabolic disorders (Zhang et al., 2006). Thus, current search for new antiretroviral agents is crucial, and compounds that inhibit different steps of HIV-1 replication cycle are under development or in clinical investigation.

The HIV-1 genome encodes three essential enzymes for its replicative cycle, reverse transcriptase (RT), integrase and protease. RT is a heterodimer consisting of a p66 (66 kDa) and a p51 (51 kDa) subunits (Arts et al., 1994; Hottiger and Hübscher, 1996), and is a multifunctional enzyme showing a RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and an RNase H activities, all of which required to convert the viral RNA genome into a double-stranded cDNA. Antiretroviral agents that inhibit the HIV-1 RT enzyme can be classified into two main groups, the nucleoside/nucleotide RT inhibitors (N(t)RTIs), which, following two or three phosphorylation steps, act as chain terminators, and the nonnucleoside RT inhibitors (NNRTIs), that inhibit the enzyme in a noncompetitive manner by interacting with the allosteric binding site at the palm region of the RT (Pomerantz and Horn, 2003).

A number of marine natural products have been demonstrated to present pharmacological activities against a wide range of pathogens, including HIV-1 (Alakurtti et al., 2006; Balunas and Kinghorn, 2005). Terpenes are natural compounds largely distributed in the plant kingdom and also found in marine algae, and diterpenes exhibit significant antiviral effects, particularly on HIV-1 replication (de Souza Pereira et al., 2005). Recently, we described that the dollabelane diterpene (1R*,2E,4R*,6E,8S*,10S*,11S*,12R*)-8,10,18-trihydroxy-2,6-dolabelladiene (henceforth denominated Dolabelladienetriol), extracted from the marine alga *Dictyota pfaffii*, is a potent inhibitor of the HIV-1 replication in primary cells in vitro through the inhibition of the enzyme RT. However, the mechanism of action by which Dolabelladienetriol inhibits the RT enzyme has remained unclear. Thus, we measured the RT enzyme kinetics in the presence of Dolabelladienetriol, and in this paper we report that this compound inhibits RT through a typical noncompetitive mode. Corroborating these findings, we were unable to observe proviral integration in the HIV-1-infected cells treated with Dolabelladienetriol.

2. Materials and methods

2.1. Reagents

The compound (1R*,2E,4R*,6E,8S*,10S*,11S*,12R*)-8,10,18-trihydroxy-2,6-dolabelladiene (Dolabelladienetriol; Fig. 1) was obtained through reduction of the rare dolabellane diterpene 10,18-diacetoxy-8-hydroxy-2, 6-dolabelladiene, as already described (Barbosa et al., 2004). The NRTIs 3′-azido-3′-deoxythymidine (AZT) and 3′-azido-3′-deoxythymidine 5′-triphosphate (AZT-TP) were from Sigma Chemical Co. St. Louis, MO, and Sierra Bioresearch (Tuscon, AZ), respectively, and the NNRTI nevirapine was kindly donated by Dr. Nubia Boechat (Department of Organic Synthesis, Farmanguinhos, Fiocruz, Rio de Janeiro, RJ, Brazil). The protease inhibitor

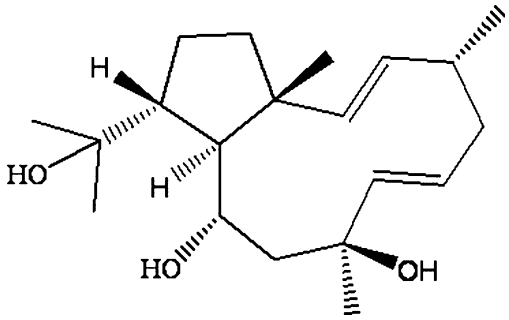

Fig. 1. Chemical structure of Dolabelladienetriol.

(PI) atazanavir sulphate and the recombinant HIV-1 enzyme RT were obtained from NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIH, Bethesda, MD). The template/primer poly(rA)–oligo $(dT)_{12–18}$, was purchased from Amershan Pharmacia, Biotech, Inc., Piscataway, NJ.

2.2. Cells and virus

Peripheral blood mononuclear cells (PBMCs) from healthy human donors were obtained by density gradient centrifugation (Hystopaque, Sigma) from buffy coat preparations. PBMCs were resuspended in RPMI 1640 (LGC Bio, São Paulo, SP, Brazil) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM glutamine and 10 mM HEPES, stimulated with 5 μg/mL of phytohemagglutinin (PHA, Sigma) during 2–3 days, and further maintained in culture medium containing 5 U/mL of recombinant human interleukin-2 (Sigma). The virus isolate Ba-L (R5-tropic, subtype B (Lima et al., 2002) was used for cell infections, and virus stocks were prepared in PHA-activated PBMCs from normal donors.

2.3. Evaluation of HIV-1 proviral DNA integration

We analyzed the effect of Dolabelladienetriol on HIV-1 proviral integration in the host cell genome by polymerase chain reaction (PCR). PBMCs were initially exposed to viral suspensions containing 5–10 ng/mL of HIV-1 p24 antigen during 2–3 h. Cells were washed, resuspended in complete medium, plated in 24-well culture plates (1.5×10^6 cells/well), treated with the indicated concentrations of Dolabelladienetriol and, after 7 days at 37 °C in 5% CO_2, infected PBMCs were collected for nested PCR analyses. DNA was extracted using the UltraClean GelSpin DNA purification kit (MO Bio Laboratories, Inc., Carlsbad, CA), and the integrated viral DNA sequences were amplified by using specific primers for the protease region of HIV-1 genome. The reaction mixtures and PCR conditions were adapted from Stuyver et al. (1997). The final 290 bp product was visualized following electrophoresis through a 1% agarose gel stained with ethidium bromide. The NRTI AZT (1 μM) and the protease inhibitor atazanavir sulphate (10 μM) were used as controls. HIV-1 replication was assessed by measuring the HIV-1 p24 antigen in culture supernatants by an ELISA capture assay (ZeptoMetrix Co., Buffalo, NY).

2.4. Effect of Dolabelladienetriol on RNA-dependent DNA polymerase (RDDP) activity of HIV-1 RT

The inhibitory effect of Dolabelladienetriol on RDDP activity of RT was evaluated using a solution containing 25 mM Tris–HCl pH 7.8, KCl 75 mM, $MgCl_2$ 8 mM and DTT 2 mM at final volume of 25 μL (buffer A). To analyze the nucleotide uptake, saturating concentrations of poly(rA)–oligo $(dT)_{12–18}$ (80 μg/mL) and various concentrations of dTTP (ranging from 0 to 30 μM) were added to buffer A. To analyze the template-primer uptake, saturating, concentrations of dTTP (60 μM) and various concentrations of poly(rA)–oligo $(dT)_{12–18}$ (ranging from 0 to 10 μg/mL) were added to buffer A. The isotopic dilutions were performed with the ratio of 2 μCi [^{3}H]dTTP (49 Ci/mmol)/2.7 μM dTTP, and all reactions were performed according steady-state kinetics by using 3 U of recombinant HIV-1 RT (1 U defined as the amount of enzyme necessary to catalyze 1 pmol of radiolabeled dTTP per min per mg of enzyme) at 37 °C for 30 min. Reactions were stopped by the addition of 10 μL of EDTA at 0.5 M and the entire volume was filtered through Whatman DE81 filters to collect the newly synthesized strand of DNA. Then, filters were washed three times with sodium phosphate (100 mM), and incorporated nucleotides were measured by liquid scintillation counting (Packard, model Tri-carb 2100). The K_i values were determined by Dixon's plot with 5 μM dTTP, the K_m and V_{max} values by Lineweaver–Burk plot, and the K_{cat} by dividing the V_{max} by the amount of total RT used.

2.5. Combination of Dolabelladienetriol with other antiretrovirals

To evaluate whether the combination of Dolabelladienetriol with other antiretroviral agents could result in either additive or synergistic effects on HIV-1 replication, PBMCs infected with HIV-1 Ba-L were treated with Dolabelladienetriol at its EC_{50} (8.4 μM) (Cirne-Santos et al., 2006) in combination with sub-optimal concentrations of a NRTI (AZT), an NNRTI (nevirapine), or a protease inhibitor (atazanavir sulphate), as described elsewhere (Marquez et al., 2005). After 7 days, viral replication was evaluated by measuring HIV-1 p24 antigen in culture supernatant, as described above. We considered synergistic, additive, or antagonistic effect, respectively, when the combination effect is greater, equal or less than the sum of the effects of all individual drugs (Ingarro et al., 2003).

2.6. Phenotypic analysis of isolates carrying NNRTI-related drug mutations

A recombinant virus assay technology was used to generate viruses carrying NNRTI mutations located at the RT palm-finger region (RT codons 37–225). Eleven mutant viral isolates were utilized carrying different pattern of NNRTI mutations (see Table 2). Donor sequence was obtained from PCR targeting a 1009 kb fragment originated from primers RT19F and RT20R; pNL43 infectious clone was used as a subtype B wild type (wt) prototype. These fragments were co-transfected into $CD4^+$ T cell line (MT4) with the pGEMT3/HXB2/ΔRT Sma I-linearized plasmid carrying the defective (ΔRT) HIV HXB2 genomic DNA generating a chimeric virus. Before starting the phenotypic assay, sequencing was performed to confirm the absence of drug resistance mutations (DRM) and integrity of the RT gene in all chimeric viruses. The susceptibility of chimeric viruses to the NNRTI compounds nevirapine and efavirenz, as well as to Dolabelladienetriol, was determined in three independent assays in MT4 cells using the Rizasurin-based cell viability assay, as previously described (Hirsch et al., 2000). Statistical analysis to calculate the IC_{50} was performed using the Analyse-it® v. 1.62 for Microsoft Excel statistics package and Sigmaplot® software. Briefly, the percentage of viable cells developed by Rizasurin after 5 days post-infection was plotted in a semi-log graphic against the concentration of drugs tested. A Hill's 3-parameter non-linear regression was performed to obtain the sigmoid curve of viable cells, and the IC_{50} value for each drug and virus tested was estimated. The IC_{50} values means are always shown together with the standard deviation of the replicates (mean ± S.D.). Fold resistance was calculated dividing the IC_{50} from the isolates carrying NNRTIs mutations by the IC_{50} obtained from pNL43 (wt).

3. Results

3.1. Dolabelladienetriol inhibits DNA proviral synthesis and integration

Reverse transcription is an essential early step of the HIV-1 replicative cycle, and this event is responsible for producing virus-specific double-stranded proviral DNA, which integrates into the genome of the host cells. We previously described that Dolabelladienetriol inhibits HIV-1 replication targeting the enzyme RT (Cirne-Santos et al., 2006). Now, we show that Dolabelladienetriol inhibits the synthesis/integration of HIV-1 proviral DNA in infected cells (Fig. 2). HIV-1-infected PBMCs were treated with Dolabelladienetriol, or with other antiretro-

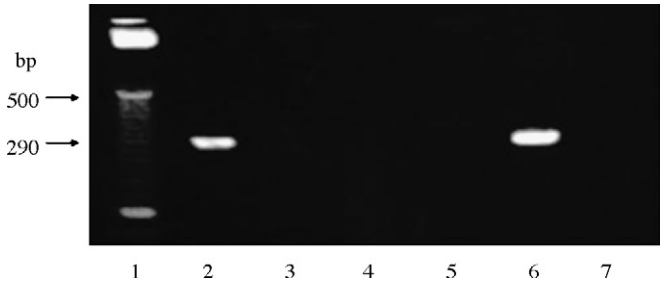

Fig. 2. Effect of Dolabelladienetriol on HIV-1 proviral DNA integration. PBMCs were infected with HIV-1 and exposed or not to Dolabelladienetriol and other antiretrovirals. Viral integration was detected by nested-PCR of the *protease* gene, and the gel shown is representative of three assays with similar results. 100 bp (1), infected cells plus culture medium (2), uninfected cells plus culture medium (3), infected cells plus Dolabelladienetriol (50 or 25 μM; 4 and 5, respectively), atazanavir sulphate (10 (M; 6), or AZT (1 (M; 7). HIV-1 replication was measured in the supernatants of the same cell cultures 7 days after infection. Means ± S.E.M. of p24 antigen (ng/mL): medium = 85 ± 2.8; Dolabelladienetriol 50 μM = 4.7 ± 0.6; Dolabelladienetriol 25 μM = 10.8 ± 0.8; atazanavir sulphate = 3.2 ± 0.2; AZT = 9.8 ± 0.3.

virals, and integration of proviral DNA was evaluated by PCR methods. Addition of Dolabelladienetriol at 25 or 50 μM prevented HIV-1 integration (lanes 4 and 5) when compared to non-treated infected cells, which display the fragment of 290 bp (lane 2). Our results show that Dolabelladienetriol inhibited the reverse transcription step of HIV-1 life cycle, and the proviral DNA formation. As expected, AZT completely blocked HIV-1 integration (lane 7), and atazanavir did not (lane 6). Very low amounts of HIV-1 p24 antigen were found in the supernatants of the same cell cultures, showing that Dolabelladienetriol, as well as AZT and atazanavir, markedly inhibited viral replication (see legend to Fig. 2).

3.2. Kinetic analysis of the nature of inhibition of HIV-1 RT by Dolabelladienetriol

We used a cell-free based assay to obtain insight into the mechanism of action of Dolabelladienetriol against HIV-1 RT activity. Initially, we determined that the K_i values of Dolabelladienetriol and AZT were 7.2 μM and 0.01 μM, respectively (Fig. 3). These results are in agreement with the Dolabelladienetriol EC_{50} values previously found (Cirne-Santos et al., 2006), and confirm that RT is the main target of Dolabelladienetriol in the HIV-1 replication cycle.

Next, we investigated the kinetic mode of action with respect to dTTP as a variable substrate, or poly(rA)–oligo $(dT)_{12–18}$ as a variable template-primer. A noncompetitive mode of inhibition was observed in both cases (Fig. 4 and Table 1), indicating that interaction of Dolabelladienetriol with HIV-1 RT enzyme is independent of the prior binding of the substrate or of the template-primer to the HIV-1 RT. Thus, Dolabelladienetriol might interact with the NNRTIs binding site at the palm region of HIV-1 RT (Maga et al., 2000; Sluis-Cremer et al., 2004).

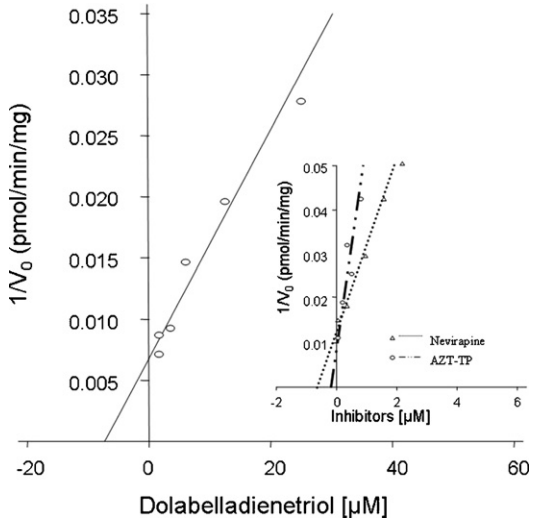

Fig. 3. Effect of Dolabelladienetriol on HIV-1 RT activity. Recombinant RT was incubated with various concentration of either Dolabelladienetriol or nevirapine, or AZT-TP (inset) for 30 min, and RNA-dependent DNA polymerase activity was assayed as described in Section 2. Data represent means of five independent experiments, and the inhibition of RT activity (K_i values) was calculated by Dixon's plot, using SigmaPlot 8.0.

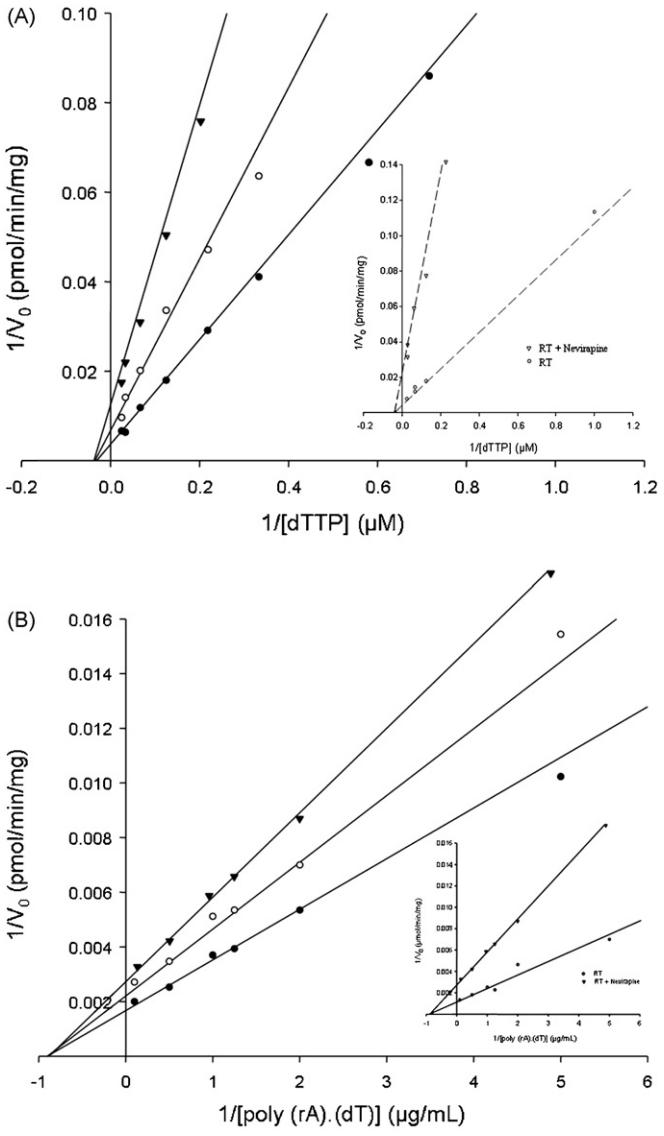

Fig. 4. Effect of Dolabelladienetriol on kinetic parameters of HIV-1 enzyme RT. The enzyme RT was incubated with no compounds (●), with one-fold the K_i (7.2 μM; ○) or with three-fold the K_i (21 μM; ▼) of Dolabelladienetriol with respect to dTTP (A) or template-primer uptake (B), as described in Section 2. Data represent means of five independent experiments, and the Lineweaver–Burk plots were generated by SigmaPlot 8.0. The insets (A and B) show the effect of nevirapine on the same kinetic parameters.

Since this mechanism of action is typical of NNRTIs, but not of NRTIs, these results predict that Dolabelladienetriol could have an additive effect with NRTIs, but not with NNRTIs.

3.3. Effects of combination of Dolabelladienetriol with other antiretroviral agents

Our kinetic experiments demonstrated that Dolabelladienetriol inhibits the HIV-1 RT in a noncompetitive fashion, with respect to dTTP template-primer uptake, similar to nevirapine (Fig. 4). Since these findings show that Dolabelladienetriol acts as a classical NNRTI, we addressed whether this compound could present an either additive or synergistic effect with other HIV-1 inhibitors. Thus, we treated HIV-1-infected PBMCs

Table 1
Kinetic parameters of RT with respect to dTTP or template-primer uptake

Conditions	K_m (μM)	V_{max} (pmol/min/mg)	K_{cat} (1/s)
Variable dTTP concentration			
RT	5.8 ± 0.3	260 ± 1.1	86.83 ± 0.4
RT + Dolabelladienetriol	4.5 ± 0.18	45.74 ± 4.09	15.24 ± 1.36
Variable template/primer concentration			
RT	1.3 ± 0.27	567.8 ± 1.0	189.09 ± 0.31
RT + Dolabelladienetriol	1.6 ± 0.23	442.2 ± 3.7	147.4 ± 1.22

with Dolabelladienetriol at its EC_{50} (8.4 μM) combined with sub-optimal concentrations of AZT, nevirapine or atazanavir sulphate, as described elsewhere (Marquez et al., 2005). The combination of Dolabelladienetriol and AZT resulted in an additive inhibitory effect, from 30 to 60% inhibition with AZT only to ~90% inhibition with both together (Fig. 5). The combination between Dolabelladienetriol and atazanavir produced an evident synergistic effect, from 25 to 50% inhibition with atazanavir alone to almost complete blockage of HIV-1 replication after combination of both compounds (Fig. 5). As expected, based on the similar mode of action, there was no increment of anti-HIV-1 effect resulting from the combination between Dolabelladienetriol and nevirapine (Fig. 5).

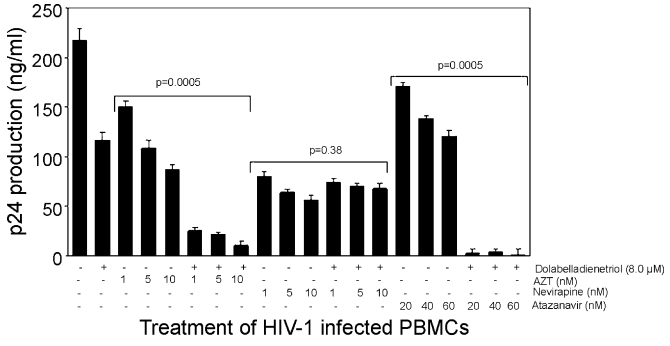

Fig. 5. Combination of Dolabelladienetriol with other antiretrovirals. PBMCs were infected with HIV-1 (Ba-L strain), exposed or not to Dolabelladienetriol at its EC_{50} (8 μM) combined or not with sub-optimal concentrations of AZT, nevirapine, or atazanavir. Viral replication was measured in culture supernatant after 7 days, and data represent mean ± S.E.M. of three independent experiments. Data were statistically analyzed using Students' *t*-test, and the *p*-values are shown above the bars.

3.4. Dolabelladienetriol antiretroviral effect on HIV-1 isolates carrying mutations related to NNRTI resistance

Twelve samples were selected among patients failing antiretroviral regimens using NNRTI (nevirapine and efavirenz), and one sample from the same cohort with no NNRTI mutations (BR321) was used as a negative control. The mutation profile found in these 12 isolates is shown in Table 2. Several different patterns of known NNRTI mutations (L100I, K103N, V106M/A, Y181C, Y188L, G190E/A, and P225H; see Hirsch et al., 2000 for details) were found in the palm-finger RT domain. The RT palm-finger region was amplified and a series of chimeric viruses was generated carrying the NNRTI mutations in their backbone. These isolates were then used to generate the IC_{50} of nevirapine, efavirenz and Dolabelladienetriol. The fold resistance was calculated as described in Section 2. As depicted in Table 2, we found samples showing high phenotypic resistance to nevirapine and efavirenz, however there was no cross-resistance to Dolabelladienetriol. In fact, the IC_{50} values for Dolabelladienetriol in mutant strains were similar to the one found in the wild type prototypic HIV-1 isolate (NL43). Our results clearly demonstrate that Dolabelladienetriol is endowed with the abil-

Table 2
Antiviral activity of Dolabelladienetriol and current NNRTIs against NNRTI-resistant HIV-1 strains

Samples	Subtypes[a]	NNRTI mutations	Nevirapine	Efavirenz	Dolabelladienetriol
BR-321	B	WT	3[b]	1.5	1.1
NNRTI-322	B	K103N; V106M; Y188L	45	15	1
NNRTI-350	C	V106M; P225H	80	120	1.1
NNRTI-393	F	V106A; Y181I	3	1.4	1
NNRTI-421	B	V106A; Y181C; G190S	>200	2.2	1.2
NNRTI-465	B	K103N; Y188L	8	6	1
NNRTI-470	F	K103N; Y188L; G190E	40	20	1.2
NNRTI-528	B	K103N; Y188L	>200	>200	1.1
NNRTI-551	C	Y188L	5	3	1
NNRTI-600	B	K103N; G190A	>200	40	1
NNRTI-605	B	K103N; Y181C; G190A	>200	20	1
NNRTI-607	B	L100I; K103N; Y181C; G190A	120	45	1
NNRTI-609	B	K103N; Y181C	>200	>200	1.1

HIV-1 strains harboring one or more NNRTI resistance-associated mutations, and wild type control isolate (BR321) are described in Section 2.

[a] The subtype assignments of the isolates were generated using the HIV subtyping tool located at Stanford HIV Drug Resistance Database (http://hivdb.Stanford.edu).

[b] Results are presented in fold resistance using IC 50% prototypic NL43 wild type isolate as reference (IC 50% NNRTI isolates/IC 50% NL43; see details in Section 2).

ity to inhibit the replication of HIV-1 isolates carrying common NNRTI-associated resistance mutations.

4. Discussion

We previously described that the dolabellane diterpene 8,10,18-trihydroxy-2,6-dolabelladien (Dolabelladienetriol, Fig. 1) inhibits the replication of HIV-1 in human primary cells and in tumor cell lines (Cirne-Santos et al., 2006). The suppression of HIV-1 infection resulted from the ability of Dolabelladienetriol to inhibit the HIV-1 enzyme RT. Here, we further report that Dolabelladienetriol prevents the formation/integration of HIV-1 proviral DNA by inhibiting the HIV-1 RT activity in a noncompetitive manner, exhibits an additive effect with AZT, and potentiates the antiretroviral activity of atazanavir.

Initially, we detected that there was no HIV-1 proviral DNA synthesis and integration in HIV-1-infected PBMCs treated with Dolabelladienetriol, likewise to what happened when infected cells were exposed to AZT (Fig. 2), a classical inhibitor of the HIV-1 proviral DNA synthesis (Sobanski et al., 1996). Reverse transcription is an essential early step of the HIV-1 replicative cycle, and this event is responsible for producing virus-specific double-stranded proviral DNA, which integrates into the host cell genome. Thus, the inhibition of this step abolishes the production of virus particles by infected cells (Arts and Wainberg, 1996; Dukes et al., 1996). Accordingly, no production of HIV-1 p24 antigen was detected in the culture supernatants of Dolabelladienetriol-treated HIV-1-infected cells during the viral integration experiments. These results are in agreement with our previous findings regarding the potent antiretroviral activity of Dolabelladienetriol.

Because Dolabelladienetriol is a nonucleoside RT inhibitor (Barbosa et al., 2004), we performed kinetic assays in order to determine its mechanism of action concerning substrate uptake. For this purpose, we started by Dixon's plot analysis to calculate K_i values, and we found that Dolabelladienetriol K_i (7.2 μM) showed a good correlation with its previously described EC_{50} (8.4 μM) (Cirne-Santos et al., 2006). Although we cannot rule out that Dolabelladienetriol presents an additional mechanism of action, the similarity between its K_i and EC_{50} values suggests that RT is the main target of Dolabelladienetriol. Whereas the Dolabelladienetriol K_i was 14.4 times higher than that of the reference compound nevirapine (0.5 μM in our assays), Dolabelladienetriol was much more efficient than other diterpenes that inhibit the RT enzyme, such as Da-1 and AcDa-1, that showed K_i values equal to 10 and 35 μM, respectively (Pereira et al., 2004). As already described for other natural products, minor chemical modifications at the dollabelane ring, such as hydroxylation, acetylation, or addition of hallogenic moieties (De Lucca et al., 1997; Maga et al., 2000), could reduce Dolabelladienetriol K_i value, thus improving its anti-RT activity and antiretroviral efficiency.

To understand at which catalytic form of HIV-1 RT Dolabelladienetriol might act, we analyzed the Lineweaver–Burk plots of the tested compound towards this enzyme with respect to dTTP or template/primer as variable substrates. We observed that this compound is a noncompetitive inhibitor of RT regarding the uptake of both substrates, indicating that Dolabelladienetriol and nevirapine share the same mechanism of action (Fig. 4A). Therefore, Dolabelladienetriol acts as a classical NNRTI, since it inhibits RT catalysis independently of the prior binding of the dTTP or template/primer to the enzyme. In general, NNRTIs bind to the allosteric site at the palm region of RT, and keep the "thumb" of the enzyme at open conformation, thus preventing the DNA polymerization (Matthée et al., 1999; Sluis-Cremer et al., 2004). Comparison of the structure of Dolabelladienetriol with other classical NNRTIs, such as nevirapine and efavirenz (Maga et al., 2000), may suggest that the dolabellane ring of the Dolabelladienetriol shares some homology with the rings of these reference molecules. However, because Dolabelladienetriol possesses different substituting radicals, it might interact with amino acid residues not sorted out in nevirapine or efavirenz-resistant RT molecules. Thus, the study of Dolabelladienetriol, and possibly chemically modified Dolabelladienetriol molecules (as above suggested), may result in the development of new compounds that may inhibit the replication of NNRTIs-resistant HIV-1 strains (Clotet, 1999; Deeks, 2001). Indeed, our results using NNRTI-resistant isolates (Table 2), suggest that Dolabelladienetriol interacts in a manner that is different from that of nevirapine and efavirenz. Dolabelladienetriol is an NNRTI with high activity against a large panel of clinical isolates showing elevated drug resistance phenotype to clinical available NNRTIs. The effects of Dolabelladienetriol on other properties of HIV-1 RT, such as DNA-dependent DNA polymerase and ribonuclease H, as well as ATP-dependent pyrophosphorolysis of the RT, should be further investigated. The latter activity is considerably important, since some NNRTIs only synergize in the presence of ATP (Meyer et al., 2004; Odriozola et al., 2003).

Our studies on combination of Dolabelladienetriol with other antiretrovirals revealed that this compound presents an additive effect with a classical NRTI (AZT), but not with an NNRTI (nevirapine) (Fig. 5). Importantly, these results harmonize with our kinetic data, since Dolabelladienetriol interacts with a distinct region of the RT molecular structure in comparison to AZT. Moreover, it is possible that Dolabelladienetriol and other NNRTIs do not target the same amino acid residues on RT palm region, which would be consistent with the noncompetitive mechanism of action of both molecules. Thus, results of inhibition of HIV-1 replication in infected cells treated with Dolabelladienetriol plus AZT or with nevirapine consolidate that Dolabelladienetriol acts according to an NNRTI mechanism of action. Furthermore, Dolabelladienetriol synergizes with a protease inhibitor (atazanavir sulphate; Fig. 5), meaning that Dolabelladienetriol could be associated with drugs that interfere with different steps of the HIV-1 replicative cycle, or with distinct region of the same enzyme.

In this work we corroborated our previous findings concerning the strong anti-HIV-1 activity of Dolabelladienetriol (Cirne-Santos et al., 2006), confirmed that RT enzyme is the main target of this natural product, and demonstrated its kinetic nature of RT inhibition. Studies regarding new antiretroviral agents are expected to reveal either new mechanisms of action,

or the ability of the new compounds to synergize with the current antiretroviral agents (Hazen et al., 2005; Robinson et al., 2000). Since drug combinations for AIDS treatment are designed to prevent the emergence or replication of multi-drug resistant HIV-1 strains, we propose that Dolabelladienetriol could be considered as a potential component of drug associations for antiretroviral therapy.

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