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# Antiviral activity of diterpenes isolated from the Brazilian marine alga *Dictyota menstrualis* against human immunodeficiency virus type 1 (HIV-1)

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**Abstract**

The antiviral effect of the $CH_2Cl_2$/MeOH-soluble fraction from the alga *Dictyota menstrualis* on HIV-1 replication was evaluated in vitro. The antiretroviral activity was attributed to two diterpenes: (6R)-6-hydroxydichotoma-3,14-diene-1,17-dial, named Da-1, and (6R)-6-acetoxi-dichotoma-3,14-diene-1,17-dial, named AcDa-1. Da-1 or AcDa-1 were added to the culture medium of HIV-1-infected PM-1 cells at different times post-infection or during virus adsorption/penetration. The results indicated that the compounds affected an early step of the virus replicative cycle. Virus binding and entry into the host cells were evaluated in the presence of each diterpene, but no inhibitory effect was observed. To evaluate provirus DNA synthesis/integration into the host genome, the viral protease coding sequence was amplified from total cellular DNA. Proviral DNA was not detected in infected cells incubated with the diterpenes. To investigate the effect of the diterpenes on the reverse transcription of the viral genomic RNA, the recombinant HIV-1 reverse transcriptase (RT) was assayed in vitro in the presence of each diterpene. Da-1 and AcDa-1 inhibited the RNA-dependent DNA-polymerase activity of HIV-1 RT in a dose-dependent manner. Taken together, our results demonstrate that both diterpenes inhibit HIV-1 RT and consequently virus replication.


*Keywords:* *Dictyota menstrualis*; HIV-1; Diterpenes; Antivirals; HIV-1 reverse transcriptase; HIV-1 RT

## 1. Introduction

The acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV), has become a serious threat to global public health in the last decades. The first generation drugs, such as AZT, ddC, ddI and D4T (Huang et al., 1992; Sergheraert et al., 1993) have been extensively used in the clinic, but the rapid development of virus resistance to these nucleoside analogs represents a significant obstacle to anti-HIV therapy (Biesert et al., 1991; St. Clair et al., 1991; Najera et al., 1994; Menendez-Arias, 2002). Furthermore, these drugs have limited or transient benefits due to their adverse side effects (Tozser, 2001). In the light of these considerations, new classes of drugs that can supplement, or partially replace, existing drugs are definitely needed for a suitable long-term use.

The discovery and characterization of new anti-HIV agents with novel structures or mechanism(s) of action and low toxicity to the host remain priority (Barreca et al., 2003). Specific inhibitors of several steps of the viral replicative cycle, including viral attachment and entry, reverse transcription, provirus DNA integration and RNA packaging,

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have been subjected to preclinical investigation or have already entered clinical trials. A considerable number of these inhibitors have been isolated from natural sources, such as marine algae and plants (De Clercq, 2000; Jung et al., 2000).

In this work, the inhibitory activity of the Brazilian marine alga *Dictyota menstrualis* on HIV-1 replication was investigated. Two diterpenes were isolated from the alga extract as the biologically active compounds and the viral molecular target for both compounds was determined.

## 2. Materials and methods

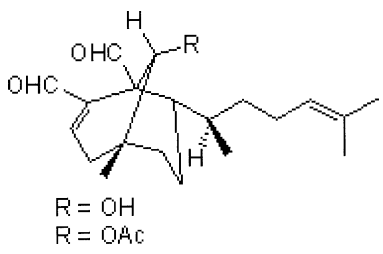
### 2.1. Samples

Fractionation of the alga *D. menstrualis*, purification and chemical characterization of the (6R)-6-hydroxydichotoma-3,14-diene-1,17-dial and its natural acetate-derivative (6R)-6-acetoxi-dichotoma-3,14-diene-1,17-dial, named here as Da-1 and AcDa-1 (Scheme 1) were previously described (Teixeira et al., 2001). Briefly, the brownish residue ($CH_2Cl_2$/MeOH fraction) obtained after extraction from the fresh alga was partitioned between *n*-hexane (*n*-hexane fraction 1) and MeOH. The *n*-hexane fraction 1 was further separated into four fractions on silica gel. One of the fractions ($CH_2Cl_2$-fraction) was further separated into seven fractions through the silica gel-column chromatography. One of the fractions (*n*-hexane fraction 2) contained the crude "pachydictyol A" while the other ($CHCl_3$/EtOAc-fraction) contained the crude 1. The last fraction was further purified by preparative TLC giving rise to the pure 1. Treatment of pure 1 with acetic anhydride in pyridine gave rise to the acetate derivative pure 2. Pure 1 and 2 were compared by physical and $^1$H NMR spectroscopic data (reported elsewhere) and identified as Da-1 and AcDa-1, respectively.

The samples described above, obtained in the sequential steps of the purification process of the diterpenes, were dissolved in sterile DMSO 100%, immediately before each experiment, and diluted at least 1:100 in complete culture medium, unless otherwise stated.

### 2.2. Cell line, virus isolate and virus quantification

PM-1 cells and viral isolates were provided by Dr. Amilcar Tanuri (Universidade Federal do Rio de Janeiro). PM-1 cells




Scheme 1.

are derived from the neoplastic T-cell line Hut78 and show phenotypic characteristics similar to those of the parental cell line (i.e., $CD3^+$ $CD4^+$ $CD8^-$ $CD26^+$ HLA-$DR^+$). Cells were grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum, L-glutamine and antibiotics (Lusso et al., 1995). The HIV-1 subtype B isolate BR48 was previously characterized (Ramos et al., 1999). Quantification of virion production in the cell-free supernatants obtained from infected peripheral blood lymphocytes (PBLs) was performed by $p24^{Gag}$ (CA) ELISA capture assays (Vironostika-bioMerieux-Bostel, The Netherlands) according to the manufacturer's instructions. The cell-free supernatants containing titered virus were stored at −70 °C.

### 2.3. Cytotoxicity assay

PM-1 cells were grown in 24-well plates ($2 \times 10^6$ cells/well) and incubated for 7 days in the presence of each isolated-fractions or isolated-diterpenes. Thereafter, cell viability was evaluated by trypan blue exclusion assay (Phillips, 1973) and cell proliferation was evaluated by [$^3$H]thymidine incorporation and liquid scintillation count. Cytotoxicity of each sample was determined along with their antiviral activity and was based on the viability of mock-infected PM-1 cells and expressed as the 50% cytotoxic concentration ($CC_{50}$).

### 2.4. Antiviral assay

Analysis of the antiviral activity of samples of the *D. menstrualis* isolated-fractions and purified diterpenes on HIV-1 replication was based on the inhibition of the p24 viral antigen production. Briefly, PM-1 cells were grown in 24-well plates and infected with $2 \times 10^5$ viral particles of the BR48 isolate (100 ng of p24 viral antigen) for 2 h in the absence of samples. After this period, virus inoculum was replaced by fresh culture medium containing or not different concentrations of some samples obtained in the process of purification of the diterpenes. At the seventh day post-infection, virion production in cell-free supernatants was quantified by $p24^{Gag}$ (CA) ELISA capture assays. The anti-HIV-1 activities of some samples were expressed as the 50% effective concentration ($EC_{50}$).

### 2.5. Virus attachment/internalization assay

Virus entry assays were performed as previously described (Aiken and Trono, 1995). PM-1 cells, grown in 24-well plates, were washed twice and infected with $2 \times 10^6$ viral particles (1 μg of p24 viral antigen), in the absence or in the presence of each diterpene at either 4 °C, a permissive temperature for virus binding but not internalization, or 37 °C, a permissive temperature for both processes. After 2 h of incubation with virus inoculum, the cells infected at 4 °C were washed and either lysed (with 0.5 mL of PBS containing 0.5% Triton X-100) or resuspended (with 0.5 mL PBS con-

taining 0.5 M EDTA–0.25% trypsin) and further incubated for 30 min at 37 °C. Trypsin-treated cells were washed twice with PBS to remove virus particles attached to the cell surface but not internalized and lysed. Cells infected at 37 °C were also trypsinized prior to lysis. Attached and internalized $p24^{Gag}$ (CA) in the cell lysates were quantified by $p24^{Gag}$ (CA) ELISA capture assays. For comparative purposes virus infection was alternatively carried out in the presence of the monoclonal anti-CD4 Leu3a antibody (Becton Dickinson, San Jose, CA).

### 2.6. PCR analysis of the proviral DNA synthesis/integration

PM-1 cells were grown in 24-well plates and infected with $2 \times 10^5$ viral particles (100 ng of p24 viral antigen) for 2 h in the absence of samples. After this period, the virus inoculum was replaced by fresh medium containing or not 100 μM Da-1 or AcDa-1 and infected-cells were incubated for 2 days. Alternatively, infected-cells were incubated with the non-nucleoside RT inhibitor nevirapine (3.2 and 5.0 μM), for comparison purpose. After this period of time, genomic DNA was isolated by GFX™ genomic blood purification kit (Pharmacia Biotech) and used for nested-PCR amplification of the viral protease region on HIV-1 genome. Primers and amplification conditions were described elsewhere (Stuyver et al., 1997). The final 290 pb product was visualized on the UV transilluminator in 1% agarose gels stained with ethidium bromide.

### 2.7. Expression and purification of the HIV-1 reverse transcriptase

The HIV-1 RT recombinant plasmid pUC12N was kindly provided by Dr. Stephen H. Hughes (NCI-Frederick Cancer Research). The expression and purification of the 66 kDa HIV-1 RT recombinant enzyme were previously described (Hizi et al., 1988; Fletcher et al., 1996). Briefly, *E. coli* cells (DH5α), transformed with the HIV-1 RT recombinant plasmid, were grown in 100 μg/mL ampicilin-supplemented NZYM liquid broth, for 12–16 h with shaking at 37 °C. The bacterial cells were collected by centrifugation in a Microfuge for 2 min at 10,000 rpm. The pellet was washed once with cold 100 mM NaCl, 20 mM Tris chloride, 1 mM EDTA, pH 7.4. Bacterial cells were then disrupted in 0.2 mM NaCl, 20% (v/v) glycerol, 1% Triton X-100, 1 mM EDTA, 2 mM dithiothreitol, 25 mM Tris chloride, pH 8.0. The lysates were kept at 4 °C for 15 min. The insoluble material was removed by centrifugation at 10,000 rpm in Microfuge. The supernatant was applied to the commercial Econo-Pac™ Q- and S-matrix cartridge columns (BioRad) connected in series. The columns were washed with low-salt buffer and the enzyme was eluted from the S column using a salt-gradient buffer, with medium-pressure chromatographic system (Shimadzu liquid chromatography system). The purified enzyme was collected and stored at −70 °C.

The purified-recombinant enzyme was shown to be active in RT assays and, in its sensitivity towards the inhibitory properties of several known retroviral agents (e.g. azidothymidine 5′-triphosphate and suramin), could not be distinguished from the viral enzyme (Hizi et al., 1988). The protein concentration of the purified recombinant HIV-1 RT used in all experiments was 0.75 mg/mL and the specific activity was 750 U/mg protein.

### 2.8. HIV-1 RT assays

The purified-HIV-1 RT recombinant enzyme was assayed as previously described (Hizi et al., 1988; Fletcher et al., 1996). The compounds were diluted in milli-Q water immediately before use. Each assay was performed in a 0.1 mL reaction mixture containing 25 mM Tris chloride, pH 7.8, 75 mM KCl, 8 mM $MgCl_2$, 2 mM dithiotreitol, 50 μM of each nucleotide (dATP, dCTP, dGTP, dTTP), [$^3$H]dTTP (49 Ci/mmol), poly(rA).oligo$(dT)_{12–18}$. Two microliters of the HIV-1 RT purified-enzyme was assayed for 30 min at 37 °C, unless otherwise stated. The reactions were stopped by addition of 10 μL EDTA 0.5 M, collected on Whatman DE81 fiber and washed three times with 100 mM sodium pyrophosphate. [$^3$H]dTTP-incorporated nucleotides were measured by liquid scintillation.

## 3. Results

### 3.1. Inhibition of HIV-1 replication in PM-1 cells by isolated fractions and purified diterpenes obtained from the extract of the alga D. menstrualis

To evaluate the antiviral potential of the marine alga *D. menstrualis*, PM-1 cells were infected with HIV-1 subtype B isolate BR48 in the presence of increasing concentrations of the $CH_2Cl_2$/MeOH fraction of the alga extract and at 7 days post-infection virion production was quantified. A dose-dependent inhibition of virus replication was observed (Fig. 1). To find out the substance that could account for the antiviral activity of the $CH_2Cl_2$/MeOH fraction of the alga extract, it was further fractionated through several steps and some different fractions were tested. A similar dose-dependent inhibitory effect was also observed in infected-cells incubated with the *n*-hexane fraction 1. More pronounced effects were observed for the $CH_2Cl_2$-fraction and $CHCl_3$/EtOAc-fraction, reaching 95% of inhibition at 200 μg/mL. No inhibitory effect was detected for fraction *n*-hexane fraction 2 (Fig. 1).

The $CHCl_3$/EtOAc-fraction was further fractionated and two diterpenes were isolated and characterized; (6R)-6-hydroxydichotoma-3,14-diene-1,17-dial (named here as Da-1) and its derivative acetate: (6R)-6-acetoxi-dichotoma-3,14-diene-1,17-dial (named here as AcDa-1) (Teixeira et al., 2001). Incubation of PM-1 infected-cells with Da-1 or AcDa-1 inhibited virus replication in a dose-dependent man-

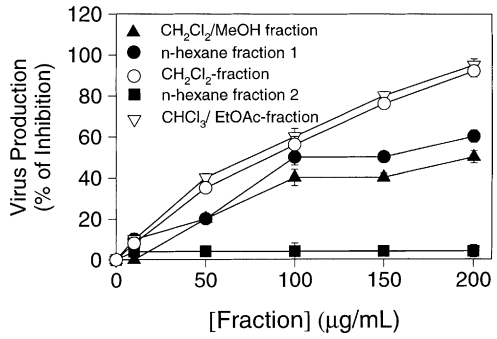



Fig. 1. Antiviral activity of the isolated-fractions from *D. menstrualis* extract: PM-1 cells were infected with HIV-1 for 2 h in the absence of isolated fractions. At the end of this period of time, virus inoculum was replaced by fresh medium supplemented with isolated-fractions. Infected cells were incubated for 7 days in the supplemented medium. After this period, cell-free supernatants were monitored for virus production by $p24^{Gag}$ (CA) ELISA capture assays. The data, expressed in terms of percentage of inhibition using the values obtained for non-treated infected-cells as reference, are means and vertical error bars show ±S.E. of assays performed in triplicate and are representative of at least two experiments.

ner (Fig. 2). The $EC_{50}$ values obtained for Da-1 and AcDa-1 were 40 and 70 μM, respectively. At 100 μM, Da-1 inhibited 97% of the virus production while AcDa-1 inhibited nearly to 70%. These results demonstrate that at least a significant part of the anti-HIV-1 effect of the $CH_2Cl_2$/MeOH fraction of the extract obtained from the alga *D. menstrualis* could be attributed to both diterpenes. The cytotoxicity of each fraction or isolated compound was determined in parallel with the antiviral activity assays. The isolated fractions and purified diterpenes showed no significant effects on the host cell viability or proliferation within the concentration range at which the antiviral activity was detected (Table 1).

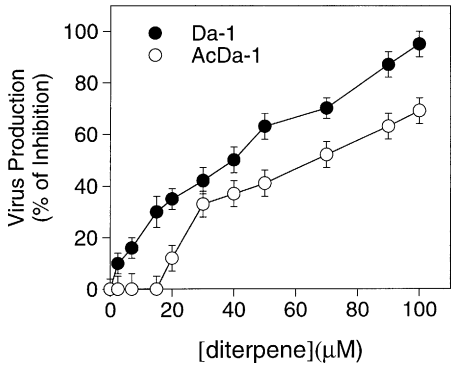



Fig. 2. Antiviral activity of diterpenes isolated from *D. menstrualis*: PM-1 cells were infected with HIV-1 for 2 h in the absence of the diterpenes. At the end of this period of time, virus inoculum was replaced by fresh medium supplemented with Da-1 or AcDa-1. Infected-cells were incubated for 7 days in the supplemented medium. Cell-free supernatants were monitored for virus production by $p24^{Gag}$ (CA) ELISA capture assays. The data, expressed in terms of percentage of inhibition using the values obtained for non-treated infected-cells as reference, are means and vertical error bars show ±S.E. of assays performed in triplicate and are representative of three experiments.

### 3.2. Effects of Da-1 and AcDa-1 on early steps of the HIV-1 replication cycle

To investigate the antiviral mechanisms of the diterpenes on HIV-1 replication, Da-1 or AcDa-1 were either present since the beginning of infection or added at different periods post-infection. In the former condition, PM-1 cells were incubated with virus inoculum in the presence of either Da-1 or AcDa-1 for 2 h (from −2 to 0 h) and, after this period, virus inoculum was replaced by fresh culture medium supplemented with Da-1 or AcDa-1. In both conditions, virus production was measured at 7 days post-infection. Infection of PM-1 cells in the presence of either Da-1 or AcDa-1 reduced virus production as compared to non-treated cells (Fig. 3). Inhibition of virus production was also observed if Da-1 or AcDa were added to the culture medium from 0 to 4 h post-infection, but no significant inhibitory effect was observed if the diterpenes were added at 10 h post-infection or later. These results demonstrate that both diterpenes affected virus production in a time-dependent manner, and that they may affect an early stage of virus replicative cycle.

### 3.3. Effects of Da-1 and AcDa-1 on virus adsorption and internalization

In order to further characterize the early viral process affected by the diterpenes, a p24 antigen uptake assay was used to evaluate the efficiency of virus adsorption and internalization (Aiken and Trono, 1995). As expected, treatment with trypsin prior to cell lysis removed more than 90% of the cell-associated $p24^{Gag}$ (CA) when the virus infection was carried out at 4 °C, demonstrating that

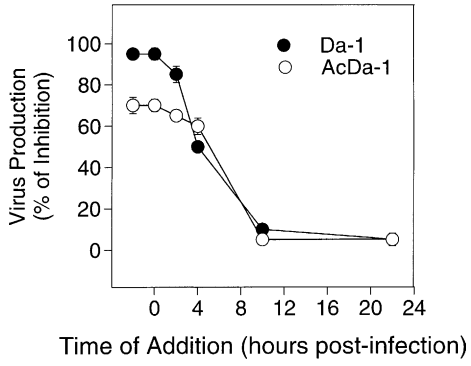



Fig. 3. Effects of Da-1 or AcDa-1 on the early phase of virus replication cycle: PM-1 cells were infected with HIV-1 for 2 h. The 0 h post-infection was considered as the time where virus inoculum was replaced by fresh culture medium. Da-1 or AcDa-1 (100 μM) were added to the culture medium together with virus inoculum or at different times post-infection. In the former case, virus inoculum was replaced by fresh culture medium containing Da-1 or AcDa-1. In all cases, infected-cells were incubated with the compounds for 7 days. After this period, cell-free supernatants were used to measure virus production. The data, expressed in terms of percentage of inhibition using the values obtained for non-treated infected-cells as reference, are means and vertical error bars show ±S.E. of assays performed in triplicate and are representative of two experiments.

Table 1
Determination of $CC_{50}$ and $EC_{50}$, for isolated-fractions and diterpenes obtained from *D. menstrualis* organic extract

| | Cells | | [3H]Thymidine incorporation | Cytotoxicity ($CC_{50}$[a]) | Antiviral activity ($EC_{50}$[b]) |
|---|---|---|---|---|---|
| | Total ($\times 10^6$) | Dead (%) | CPM ($\times 10^3$) | | |
| Control | 3.2 ± 0.2 | 1.0 ± 0.3 | 20 ± 2.2 | – | – |
| $CH_2Cl_2$/MeOH fraction | 3.7 ± 0.4 | 1.9 ± 0.3 | 22 ± 2.4 | >300 μg/mL | 200 μg/mL |
| *n*-Hexane fraction 1 | 3.0 ± 0.3 | 2.0 ± 0.5 | 20 ± 1.0 | >300 μg/mL | 100 μg/mL |
| $CH_2Cl_2$-fraction | 3.2 ± 0.3 | 1.5 ± 0.3 | 21 ± 1.0 | >300 μg/mL | 70 μg/mL |
| $CHCl_3$/EtOAc-fraction | 3.6 ± 0.2 | 1.0 ± 0.2 | 19 ± 1.8 | >300 μg/mL | 70 μg/mL |
| Da-1 | 3.3 ± 0.4 | 2.3 ± 0.6 | 20 ± 1.2 | >200 μM | 40 μM |
| AcDa-1 | 3.0 ± 0.3 | 1.0 ± 0.3 | 17 ± 2.0 | >200 μM | 70 μM |
| Nevirapine[c] | | | | >100 μM | 40 nM |

[a] 50% cytotoxic concentration ($CC_{50}$), based on cell viability and proliferation at 300 μg/mL of isolated fractions or 200 μM of isolated diterpenes.
[b] 50% effective concentration ($EC_{50}$), based on virus production measured by ELISA-p24 assay.
[c] In CEM T-lymphoblastoid cells and primary human macrophages (Richman et al., 1991).

virion internalization but not adsorption was prevented (Fig. 4). In contrast, trypsinization of cells infected at 37 °C before lysis eliminated adsorbed-virus and therefore only internalized virus particles could be detected. Based on these data our results demonstrate that Da-1 and AcDa-1 did not affect virus attachment to the cell surface nor internalization into PM-1 cells. For comparative purposes virus infection was alternatively carried out in the presence of the monoclonal anti-CD4 Leu3a antibody (Becton Dickinson, San Jose, CA), and we observed that the specific inhibitor of virus attachment reduced cell-associated $p24^{Gag}$ (CA) at both 37 and 4 °C. Similarly to Da-1 and AcDa-1, nevirapine, a specific inhibitor of the reverse transcriptase, did not affect either adsorption or penetration.

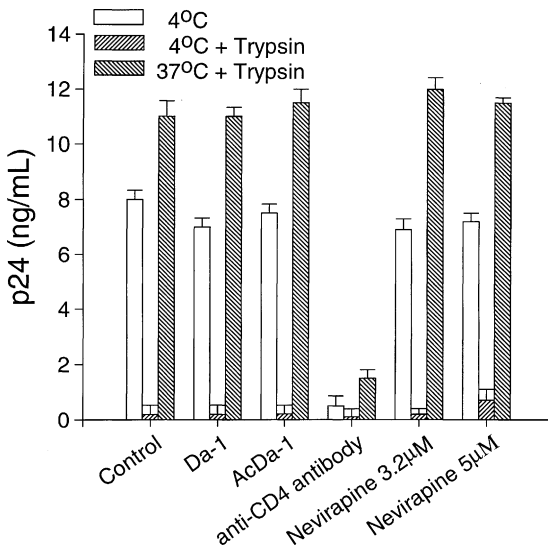



Fig. 4. Effects of Da-1 and AcDa-1 on virus attachment/internalization. PM-1 cells were infected with HIV-1 (1 μg of $p24^{Gag}$ (CA), for 2 h in the absence or in the presence of 100 μM Da-1 or AcDa-1, or nevirapine (3.2 or 5.0 μM), or 1 μg/mL monoclonal anti-CD4 Leu3a antibody, and maintained at 4 or 37 °C. After this period, cells were either washed and lysed or trypsinized and washed prior to lysis. Lysates were processed for virus titer. The data are representative of three independent experiments.

### 3.4. Effects of Da-1 and AcDa-1 on proviral DNA synthesis/integration

Synthesis and integration of proviral DNA are the following steps of the HIV-1 replicative cycle after virus particle internalization. To investigate the effect of both diterpenes on these processes, HIV-1 infected-cells were treated for 2 days in the absence or in the presence of 100 μM Da-1 or AcDa-1 and genomic DNA was isolated for amplification of a specific viral coding sequence only present within the fully synthesized double-stranded proviral DNA. A fragment of 290 bp encompassing the viral *protease* gene could only be detected in non-treated infected cells, indicating that incubation of infected cells with Da-1 or AcDa-1 inhibited the synthesis of the double-stranded proviral DNA (Fig. 5, compare lanes 3 and 4 with lane 2). Similar inhibition was observed in infected cells incubated with nevirapine, a classical non-nucleoside inhibitor of the reverse transcriptase (Fig. 5, compare lanes 5 and 6 with lane 2) or with the anti-CD4 antibody used as inhibitor of virus adsorption (Fig. 5, compare lane 7 with lane 2). These results suggest that both diterpenes Da-1 and AcDa-1 could be acting after virus adsorption and penetration, or, more precisely, during the synthesis of the proviral DNA.

### 3.5. Effects Da-1 and AcDa-1 on HIV-1 reverse transcriptase (RT) activity

To investigate the effect of both diterpenes on the synthesis of the double-stranded proviral DNA an in vitro assay was performed with the purified recombinant HIV-1 RT. The RNA-dependent DNA polymerase activity of the viral enzyme was evaluated in the absence and in the presence of increasing concentrations of Da-1 or AcDa-1 (Fig. 6). The enzyme was inhibited in a dose-dependent manner by both compounds with $IC_{50}$ values of 10 and 35 μM for Da-1 and AcDa-1, respectively. The maximal inhibitory effect of Da-1 (95%) was observed at the concentration of 80 μM. At the same concentration, AcDa-1 also reached a maximal inhibitory effect, that is near to 70%.

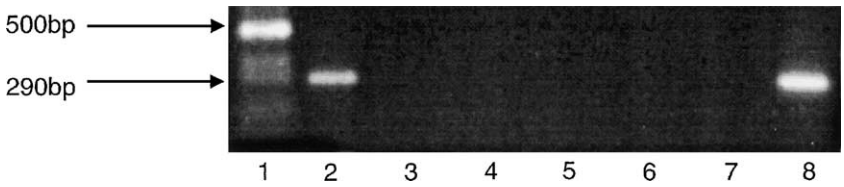



Fig. 5. Detection of proviral DNA in HIV-1-infected PM-1 cell incubated with Da-1 or AcDa-1: PM-1 cells were infected with HIV-1 for 2 h in the absence of the diterpenes. At the end of this period, virus inoculum was replaced by fresh medium supplemented with 100 μM Da-1 or AcDa-1 or 3.2 or 5.0 μM of nevirapine. Infected cells were incubated for 2 days in the supplemented medium. After this period, genomic DNA was isolated and amplification of the viral *protease* gene was performed by nested-PCR. The final 290 pb product was separated in 1% agarose gels, stained with ethidium bromide and visualized on UV transilluminator: 100 pb ladder (1); infected-cells in the absence (2) or in the presence of 100 μM Da-1 (3); AcDa-1 (4); nevirapine 3.2 μM (5); nevirapine 5.0 μM (6); 1 μg/mL monoclonal anti-CD4 Leu3a antibody (7); positive control (HIV-1 subtype B American-HXB2) (8).

## 4. Discussion

The search for new classes of antiretroviral drugs has become imperative if we consider the numerous reports on the long-term toxicity to the host and the acquired resistance to the currently available drugs for anti-HIV therapy (Lucas et al., 1999; Menendez-Arias, 2002). The number of natural products with antiviral activity derived from microorganisms, plants, marine invertebrates and particularly algae has increased in the last decade (Cardellina et al., 1993; Loya et al., 1994, 2002; De Clercq, 2000).

In this work the anti-HIV potential of the Brazilian marine brown alga *D. menstrualis* was evaluated in vitro. The anti-HIV-1 activity of the organic extract was determined in PM-1 cells in vitro. The inhibitory effect observed led us to isolate compounds that could account for this biological activity. Considering that two isolated-diterpenes denominated hydroxidictiodial and dictiodial showing inhibitory effect against HIV-RT activity have been previously isolated from the related brown alga *D. dichotoma* (Ninomya et al., 1995), a procedure for isolation of diterpenes from *D. menstrualis* was developed (Teixeira et al., 2001). Isolated fractions obtained in some steps of the procedure were evaluated for anti-HIV activity in PM-1 cells and our results showed that only one of them (*n*-hexane 2) do not affect virus production at a significant level. Two diterpenes were isolated and characterized by the procedure used: 6(R)-6-hydroxydichotoma-3,14-dieno-1,17-dial (denominated Da-1) and the natural acetate 6(R)-6-acetoxi-dichotoma-3,14-dieno-1,17-dial (denominated AcDa-1) (Teixeira et al., 2001). Da-1 and AcDa-1 inhibited virus replication with $EC_{50}$ values of 40 μM (12.7 μg/mL) and 70 μM (24.1 μg/mL), respectively. Furthermore, Da-1 and AcDa-1 reduced HIV-1 RT activity with $IC_{50}$ values of 10 μM and 35 μM, respectively. Considering that neither Da-1 nor AcDa-1 affected cell viability or proliferation, the synthesis of proviral DNA is a specific target for the anti-HIV-1 activity of the diterpenes.

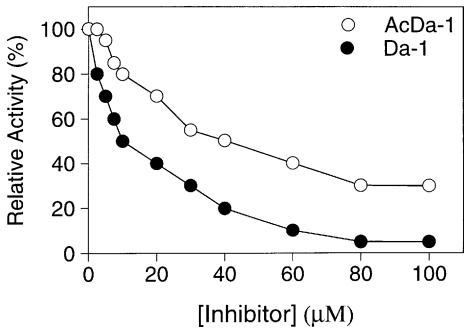



Fig. 6. Effects of Da-1 and AcDa-1 on the RNA-dependent DNA polymerase activity of the recombinant HIV-1 RT: the RNA-dependent DNA-polymerase activity of the HIV-1 RT was assayed by incorporation of [$^3$H]dTTP in the reaction product with poly(rA).oligo(dT) as the template primer, as described in Section 2. The assays were carried out at the concentration near to the $K_m$ value for [$^3$H]dTTP (10 μM) and saturating concentration for the template primer (150 μg/mL) (Wu et al., 1988). The data are means and vertical error bars show ±S.E. of assays performed in triplicate and are representative of two experiments.

The antiviral activities described here for Da-1 and AcDa-1 are as effective as those described for previously isolated diterpenes (Ninomya et al., 1995), hydroxidictiodial and dictiodial, that showed $EC_{50}$ values of 4.3 and 9.2 μg/mL, respectively. The well-known non-nucleoside inhibitor of the HIV-1 RT nevirapine is more effective as inhibitor of virus production and enzyme activity than the diterpenes described here. HIV p24 antigen production by both HIV-$1_{BRU}$-infected CEM T-lymphoblastoid cells and HIV-$1_{Bal-85}$-infected primary human monocytes/macrophages was inhibited by nevirapine at an $EC_{50}$ of 40 nM, while the recombinant HIV-1 RT was inhibited at an $IC_{50}$ of 84 nM (Merluzzi et al., 1990; Richman et al., 1991). There are few reports on the anti-HIV-1 properties of diterpenes and we believe that they may represent an interesting class of anti-HIV-1 agents.

Inhibition of HIV-1 replication by terpenes such as betulinic acid and derivatives has been previously demonstrated (Fujioka et al., 1994; Kashiwada et al., 1996). The inhibitory mechanism of the betulinic acid derivative RPR 103611, which is structurally different from Da-1 and AcDa-1, involves blockade of the fusion mechanism of HIV-1 envelope with cell receptor by targeting the gp41 viral glycoprotein (Mayaux et al., 1994). Our results demonstrate that both diterpenes affected an early step of the virus replicative cycle. However, we could not observe any inhibitory effect of Da-1 and AcDa-1 on virus attachment or entry. These results suggest that a post-internalization event on the replicative cycle could be targeted by the diterpenes.

Synthesis and integration of the proviral DNA into the host genome are the following events in the virus replicative cycle after virus internalization (Turner and Summers, 1999). The lack of amplification of a fully synthesized proviral DNA from infected-cells incubated with the diterpenes suggested that viral DNA synthesis and/or integration could be the targets for these compounds. Synthesis of the proviral DNA is carried out by the HIV-1 RT (Turner and Summers, 1999). A cell-free in vitro assay of the RNA-dependent DNA polymerase activity of the recombinant HIV-1 RT in the presence of Da-1 and AcDa-1 demonstrated a dose-dependent inhibition of the viral RT activity with $IC_{50}$ values of 10 and 35 μM, respectively. Inhibition of virus reverse transcription and consequently inhibition of synthesis of the proviral DNA by the diterpenes is likely the mechanism involved in the inhibition of virus replication in PM-1 cells.

Nevertheless, our data do not rule out a possible inhibitory effect of the diterpenes on the viral integrase. If HIV-1 integrase would be a second target, the inhibitory effect of the diterpenes on HIV-1 replication should be the outcome of their inhibitory effects on both HIV-1 RT and integrase. However, since the $IC_{50}$ values obtained for Da-1 and AcDa-1 in the HIV-1 RT assay are of the same order of magnitude as the $EC_{50}$ values obtained by the analysis of HIV-1 production in infected cells, HIV-1 RT is likely the only viral target for both diterpenes.

Further studies on the kinetic mechanism of HIV-1 RT inhibition by Da-1 and AcDa-1 could improve our knowledge on the interactions between this important viral target for antiretroviral therapy and non-nucleoside inhibitors.

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