Anti-HIV-1 Activity of the *Iboga* Alkaloid Congener 18-Methoxycoronaridine

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Original Paper

Abstract

The *Iboga* alkaloid congener 18-methoxycoronaridine (18-MC) exhibits *in vitro* leishmanicidal and *in vivo* anti-addiction properties. In this paper, we describe that 18-MC inhibits HIV-1 infection in human peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages. We found that 18-MC inhibits the replication of primary isolates of HIV-1 in a dose-dependent manner, regardless of the preferential chemokine receptor usage of the isolates, at non-cell-toxic concentrations. The antiretroviral activity of 18-MC resulted in EC_{50} values of 22.5 ± 4.7 μM and 23 ± 4.5 μM for R5 and X4 isolates, respectively, in PBMCs, and a therapeutic index (TI) of 14.5. Similar findings were observed for inhibition of HIV-1 replication in macrophages: EC_{50} equal to 12.8 ± 5 μM and 9.5 ± 3 μM for an R5 virus after 14 and 21 days of infection, respectively, with TI equal to 25.6 and 34.5. 18-MC moderately inhibits the HIV-1 enzyme reverse transcriptase (IC_{50} = 69.4 μM), which at least partially explains its antiretroviral activity.

Key words
HIV-1 · AIDS · 18-methoxycoronaridine · *Iboga* alkaloid · reverse transcriptase

Introduction

Infection by the human immunodeficiency virus type 1 (HIV-1), the etiological agent of the acquired immunodeficiency syndrome (AIDS), is a global health problem affecting more than 42 million people worldwide [1]. It has been estimated that 5 million new infections occurred in 2003, and about 3 million individuals died from AIDS in the same period. HIV-1 infects and replicates in $CD4^+$ T lymphocytes and monocyte/macrophages, using the CD4 molecule and the chemokine receptors CCR5 or CXCR4 to enter the target cell [2]. HIV-1 persistently replicates in the lymphoid tissues [3], leading to a progressive deterioration of the immune system, and to a severe clinical outcome of immunosuppression, the foremost characteristic of AIDS. An effective vaccine against HIV-1 infection has not been developed yet.

Two decades after the discovery of the first cases of AIDS, the clinical use of the abundant antiretroviral repertoire has resulted in an unequivocally favorable effect, decreasing the morbidity and mortality of HIV-1 infection [4]. Treatment with highly ac-

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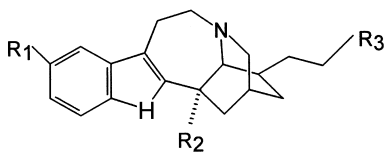
tive antiretroviral therapy (HAART), a combination of drugs that inhibit the HIV-1 enzymes reverse transcriptase (RT) and protease, promotes a sustained decrease in the viral load and a restoration of the immune response, even in patients who have developed a severe immunosuppression [4]. However, this treatment does not completely eradicate HIV-1 from the infected tissues [5], and its long-term use is restricted by metabolic disorders and toxicities, emergence of drug-resistant viruses and complex administration [6]. Thus, the search for other antiretroviral compounds is critical, and numerous new anti-HIV-1 agents that target different phases of viral replication cycle are under development or in clinical trials [7].

The *Iboga*-type indole alkaloid coronaridine (COR) is found in many species of the plant kingdom and has been studied for its potential anti-addictive properties [8], [9]. Because of the side effects such as tremor, cerebellar neurotoxicity and bradycardia associated with COR, chemical structure modifications were made to reduce its side effects, which was attained with a methoxylation at carbon-18 [8], [9], resulting in the analogue 18-methoxycoronaridine (Fig. **1**). In preclinical studies, 18-methoxycoronaridine (18-MC) exerted few to none of the non-specific or neurotoxic side effects associated with COR administration [8], [9]. We recently reported that the natural alkaloid COR presents an antiparasite activity against *Leishmania amazonensis*[10], a property showed by 18-MC as well [11]. Since many alkaloids have been described as capable of inhibiting HIV-1 infection i*n vitro* [12], we investigated whether 18-MC is also endowed with antiretroviral properties. We found that 18-MC inhibits HIV-1 replication in human peripheral blood mononuclear cells and in monocyte-derived macrophages, and that this activity is at least partially mediated by reducing the activity of the HIV-1 enzyme reverse transcriptase.

Materials and Methods

Cells

Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained by density gradient centrifugation (Hystopaque, Sigma Chem. Co., St Louis, MO) from buffy coat preparations. Cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), penicillin (100 U/mL), streptomycin (100 ug/mL), 2 mM glutamine and 10 mM HEPES. PBMCs were stimulated with 5 μg/mL of phytohemagglutinin (PHA, Sigma) during two to three days, and further maintained in culture medium containing 5 U/mL of recombinant human interleukin-2 (Sigma). Monocyte-derived human macrophages were isolated from PBMCs by plastic adherence. Briefly, 3×10^5 PBMCs were plated in 24-well plates in RPMI medium without serum for 1 h in 5% CO_2 at 37 °C. Non-adherent cells were washed out, and adherent cells were maintained with Dulbecco's modified Eagle's medium (DMEM) with 10% human serum (Sigma) for 7 days, for differentiation in macrophages. Macrophage purity was higher than 90%, as determined by flow cytometry analysis (FACScan, Becton Dickinson) using anti-CD3 (PharMingen, San Diego, CA) and anti-CD16 (Southern Biotech, Birmingham, AL) monoclonal antibodies.



Compounds	R_1	R_2	R_3
COR	H	CO_2CH_3	H
18-MC (racemic)	H	CO_2CH_3	OCH_3

Fig. **1** Chemical structures of coronaridine (COR) and 18-methoxycoronaridine (18-MC).

Viruses

The following HIV-1 primary isolates were used in this study: the R5-tropic isolate Ba-L (donated by Michael A. Norcross, CBER/FDA, Bethesda, MD, USA), the X4-tropic virus 95BRRJ010, and the R5X4 dual-tropic isolate 95BRBA07. The latter two isolates were obtained from the Brazilian Network for HIV Isolation and Characterization [13], and their chemokine receptor usage was reported elsewhere [14]. Virus stocks were prepared in PHA-activated PBMCs from normal donors.

Reagents

The compound (±) 18-MC (Fig. **1**) was synthesized as described [15], and its water-soluble hydrochloride salt was used for antiretroviral investigation. 18-MC is racemic, and the compound was pure according to chemical analysis as reported elsewhere [15]. To evaluate the safety of 18-MC to human cells, PBMCs and macrophages were treated with different concentrations of the compound, and cell viability was examined using the trypan blue dye exclusion assay. The antiretroviral agent 3′-azido-3′-deoxythymidine (AZT; Sigma) was used as a positive control for infection and enzymatic assays.

Anti-HIV-1 inhibitory activity

To study the antiretroviral activity of 18-MC, PBMCs were exposed to viral suspensions containing 5 to 10 ng/mL of HIV-1 p24 Ag, during 2 to 3 h. Cells were washed, resuspended in complete medium, plated in 96-well culture plates (2×10^5 cells/well) in triplicates, and treated with the indicated concentrations of 18-MC. After 7 days at 37 °C in 5% CO_2, viral replication was assessed by measuring the HIV-1 p24 Ag in culture supernatants by an ELISA capture assay (ZeptoMetrix Co., Buffalo, NY). In some experiments, HIV-1-infected PBMCs were treated with 18-MC (50 μM) during three days only; the compound was washed out and infected cells were cultured during a further 7 days only with medium. In parallel, other HIV-1-infected PBMCs were exposed to 18-MC during the entire culture period of 10 days. The antiretroviral activity of 18-MC was also evaluated in macrophages, which were infected by the R5 isolate Ba-L with 10 to 20 ng/mL of HIV-1 p24 Ag. After incubation overnight at 37 °C, 5% CO_2, excess virus was washed out, cell monolayers were replenished with fresh medium, and infected macrophages were treated with 18-MC. Cultures were maintained during three weeks, and half of the culture medium was renewed each 7 days, adding back the compound in order to keep the original concentration. Viral replication was measured as for infected PBMCs.

Anti-HIV-1 reverse transcriptase (RT) inhibitory activity

The inhibitory effect of 18-MC on the RT polymerase activity was evaluated using recombinant HIV-1 RT, obtained as previously described [16]. The polymerization reactions contained 50 mM Tris HCl (pH 7.8), 6 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 20 μM dTTP, 10 μM of [^3H]dTTP (47 Ci/mmol), and 150 μg/mL poly(rA) oligo(dT) template primer (Pharmacia) and 1 U of enzyme (defined as the amount of enzyme that incorporates 1 pmol of dTTP in 30 min at 37 °C under standard assay conditions). The reaction mixture was incubated at 37 °C for 30 min, and stopped by adding ice-cold 5% trichloroacetic acid containing 20 mM of sodium pyrophosphate. The precipitates were collected on Whatman DAE 81 filters, washed with sodium phosphate 0.1 M, and the incorporated triphosphate was measured (counts-per-minute – CPM) in a liquid scintillation counter. The RT inhibitor AZT was used as an indicator, and the results are shown as the percentage of inhibition of RT activity relative to polymerase activity found in the absence of enzyme inhibitors.

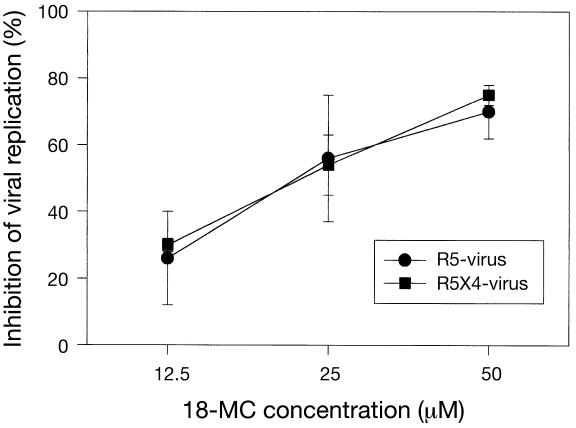


Fig. **2** Effect of 18-MC on HIV-1 replication in PBMCs. Cells were infected with HIV-1, exposed to 18-MC, and viral replication was measured in the culture supernatants after seven days. Data represent means ± SEM of three independent experiments for each virus (R5-tropic virus: Ba-L; R5X4-tropic virus: 95BRBA07). Virus production in the positive controls (HIV-1-infected cells cultured only with medium): 47.5 ± 4 and 36.5 ± 9.5 ng/mL p24 Ag for R5 and R5X4 isolates, respectively.

Results and Discussion

In this paper, we report that the indole alkaloid congener 18-MC inhibits HIV-1 infection, independently of the preferential coreceptor usage of the viral isolates. The infection assays were performed with primary cells (acutely infected PBMCs and macrophages) to avoid the genotypic and phenotypic changes that might occur during viral passages in tumor cell lines. We also used primary isolates, which are phenotypically closer to the viral population present in HIV-1-infected patients.

In preliminary experiments, we found that the naturally occurring COR decreased the infection mediated by R5- and R5X4-tropic primary isolates of HIV-1 in PBMCs, in a dose dependent manner (data not shown). Since 18-MC is an improved molecule of the natural compound COR, presenting little to none of the adverse effects associated with the original molecule [8], [9], we continued our antiretroviral studies using the new alkaloid congener 18-MC.

We initially examined the safety of 18-MC in human cells by exposing macrophages and PBMCs to this compound, and cell viability was evaluated by trypan blue dye exclusion assay. Cells were treated with a large concentration range of 18-MC during 7 days (PBMCs, n = 6) or up to 21 days (macrophages, n = 4), and we detected a CC_{50} = 328 μM.

18-MC mediated a substantial anti-HIV-1 effect in infected PBMCs, regardless of the preferential coreceptor usage of the viral isolates (Fig. **2**). A dose-dependent inhibition was observed for the R5 (Ba-L) and R5X4 (95BRBA07) variants, which were mildly to strongly neutralized by 25 μM and 50 μM of 18-MC, respectively. The antiretroviral activity of 18-MC resulted in EC_{50} values of 22.5 ± 4.7 μM for the R5 virus, and 23 ± 4.5 μM for the X4 isolate, and a therapeutic index (TI) equal to 14.5. Treatment of PBMCs infected with the X4 isolate (95BRRJ010) with 50 μM of 18-MC consistently inhibited viral replication, with inhibitory levels similar to those reached for the other two phenotypes (Fig. **3**, left side). AZT, a nucleoside analogue inhibitor of the HIV-1 enzyme reverse transcriptase, blocked viral replication as

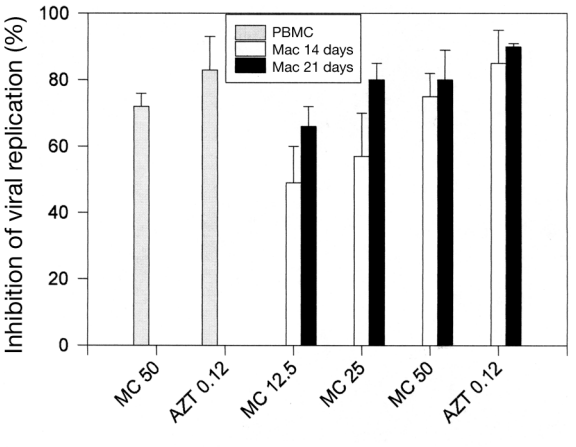


Fig. **3** Effect of 18-MC on HIV-1 replication in PBMCs and macrophages. PBMCs (two bars on the left) were infected with the X4-tropic HIV-1 isolate 95BRRJ010 and exposed to 18-MC or AZT, and viral replication was measured in the culture supernatants after seven days. Macrophages (eight bars on the right) were infected with the R5-tropic isolate Ba-L, treated with 18-MC or AZT, and viral replication was measured after 14 or 21 days. Data represent means ± SEM of three independent experiments for each virus (MC = 18-MC). Virus production in the positive controls (HIV-1-infected cells cultured only with medium): 18 ± 1 for X4 virus in PBMCs; 11.2 ± 5 (14 days) and 24.7 ± 9 (21 days) ng/mL p24 Ag for R5 isolate in macrophages.

expected (Fig. **3**, left side). In some experiments with the X4 isolate, HIV-1-infected PBMCs were cultured for an extended period, and exposed to 18-MC (50 μM) during either three days only, or 10 days. Using this approach, we found that the inhibition of HIV-1 replication was 50% higher when infected cells were treated with 18-MC during the whole period of the assay (10 days), in opposition to a shorter exposure to the compound (3 days): mean ± SEM of viral inhibition = 65 ± 5% vs. 42 ± 8%, respectively. This finding suggests that the optimal antiretroviral activity may be dependent of the permanent exposure of the infected cells to 18-MC.

We further evaluated the antiretroviral activity of 18-MC in HIV-1-infected macrophages, and we detected that 18-MC mediated a dose-dependent inhibition of HIV-1 replication (Fig. **3**, right side). This effect is more evident two to three weeks after infection, when viral production by macrophages maintained only with culture medium usually reaches higher levels in our experimental conditions. Thus, we observed intensities of inhibition ranging from 49% to 75% 14 days after infection, and from 66% to 80% after 21 days, induced by the three 18-MC concentrations tested. At this final time-point, the inhibitory efficiency of 25 μM of 18-MC was similar to that exhibited by 50 μM. The EC_{50} values for 14 and 21 days of infection were, respectively, 12.8 ± 5 μM and 9.5 ± 3 μM, resulting in TI of 25.6 and 34.5. Because macrophages function as an HIV-1 reservoir through their ability to resist HIV-1-mediated cytopathicity and continuously replicate the virus [5], these results are particularly relevant. AZT vigorously controlled HIV-1 growth in macrophages, as predicted.

Since some alkaloids limit HIV-1 replication *in vitro* through inhibition of the enzyme reverse transcriptase [17], [18], we investigated whether 18-MC is endowed with this property. We found that 12.5 μM to 50 μM of 18-MC moderately reduced the polymerase activity of the recombinant HIV-1 RT (Fig. **4**). A peak of inhibition of RT relative activity (54%) was reached with 100 μM, plateauing thereafter. These values resulted in an IC_{50} equal to 69.4 ± 3 μM (Fig. **4**).

The antiretroviral effect of the alkaloids may be due to their action on different steps of viral replication, such as inhibition of syncytium formation and of the RT activity [17], [18], [19]. Since 18-MC mediates only a moderate dose-dependent RT inhibition, it is possible that other concurrent mechanisms contribute to reduce HIV-1 replication. For example, 18-MC exhibits affinity for κ-opioid receptors [9], and it is known that the κ-opioid agonist U50488 inhibits HIV-1 replication in macrophages [20] and lymphocytes [21]. Thus, the anti-HIV activity of 18-MC may also, to some extent, result from its binding to and stimulation of κ-opioid receptors. A potential additional mechanism is the reduction of lymphoproliferation, since preliminary experiments performed in our laboratory have shown that 25 μM and 50 μM of 18-MC decreased by 30% and 50% the blastogenesis of PHA-activated PBMCs cultured with IL-2, respectively. This property has been suggested to contribute to the strong antiretroviral activity of mycophenolic acid [22].

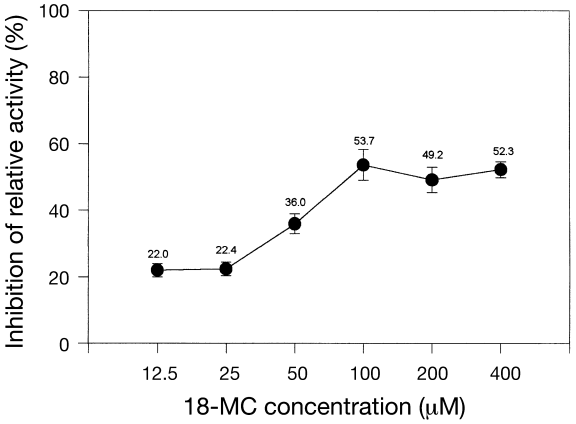


Fig. **4** Effect of 18-MC on HIV-1 RT activity. Recombinant HIV-1 RT was incubated with the indicated concentrations of 18-MC, and the enzyme polymerase activity was evaluated as described in Material and Methods. Data represent means ± SEM of three experiments done in triplicates. AZT was used as a positive control for RT inhibition at 1 μM, inhibiting 78% of the RT activity. For the sake of clarity, inhibition values are shown over the circles. Positive control (RT activity in the absence of enzyme inhibitors): 10.747 ± 845 CPM.

Our present results warrant further investigation on the mechanisms by which 18-MC decreases HIV-1 replication *in vitro,* in addition to inhibition of HIV-1 reverse transcriptase. Finally, considering that 18-MC demonstrates a vigorous leishmanicidal activity *in vitro* [11], its potential therapeutic properties may be uniquely useful for the treatment of HIV-1-infected individuals as well as patients coinfected with *Leishmania* and HIV-1.

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