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Michellamine B, a Novel Plant Alkaloid, Inhibits Human Immunodeficiency Virus-Induced Cell Killing by at Least Two Distinct Mechanisms†

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Studies of the mechanism of action of michellamine B, a novel anti-human immunodeficiency virus (HIV) alkaloid from the tropical plant *Ancistrocladus korupensis*, have revealed that the compound acts at two distinct stages of the HIV life cycle. The compound had no direct effect on HIV virions and did not block the initial binding of HIV to target cells. Postinfection time course studies revealed that the agent partially inhibited HIV-induced cell killing and syncytium formation when added up to 48 h following acute infection; however, viral reproduction was fully inhibited only when the compound was added immediately after infection. Time-limited treatments of HIV-infected cells revealed that michellamine B had to be present continuously to provide maximum antiviral protection. HIV replication in cells in which infection was already fully established or in chronically infected cells was unaffected by michellamine B. Biochemical studies showed that michellamine B inhibited the enzymatic activities of reverse transcriptases (RTs) from both HIV type 1 and HIV type 2 as well as two different nonnucleoside drug-resistant RTs with specific amino acid substitutions. In addition, human DNA polymerases α and β were inhibited by the alkaloid. Michellamine B exerted a potent dose-dependent inhibition of cell fusion in two independent cell-based fusion assays. Thus, michellamine B acts both at an early stage of the HIV life cycle by inhibiting RT as well as at later stages by inhibiting cellular fusion and syncytium formation.

The escalating worldwide pandemic of AIDS continues to present an urgent requirement for new drug development candidates with antiviral activity against the causative human immunodeficiency viruses (HIV). The U.S. National Cancer Institute (NCI) has undertaken a major initiative to discover novel anti-HIV agents from natural sources (3). Stemming from these efforts, several new classes of HIV-inhibitory compounds from diverse families of plants have been isolated and characterized (e.g., see references 2, 11, 13–15, and 18). Bioassay-guided fractionation of the aerial parts of the tropical liana *Ancistrocladus korupensis* provided a chemically novel, atropisomeric trio of anti-HIV alkaloids, michellamines A, B, and C (4, 21). Despite relatively modest potency, all three compounds were capable of complete inhibition of the cytopathic effects of HIV type 1 (HIV-1) and HIV type 2 (HIV-2) on human lymphoblastoid target cells in vitro. At the effective anticytopathic concentrations, the alkaloids also inhibited the production of reverse transcriptase (RT), p24, and infectious virions in HIV-infected cells, indicating a diminution of viral replication. Of the three compounds, michellamine B was the most potent against HIV and was also the most prevalent in the extracts of *A. korupensis* (4). Because of its unusual structure, broad range of anti-HIV activity, chemical stability, and solubility, michellamine B has been committed to preclinical drug development by the NCI. To help further ascertain the biological novelty and potential importance of this lead, we have investigated the mechanisms underlying the anti-HIV effects of michellamine B.

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MATERIALS AND METHODS

Reagents. Michellamine B was obtained as previously described (4, 21) from extracts of the leaves of the tropical liana *A. korupensis*. XTT (2, 3-*bis*[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt), AZT (3′-azido-3′-deoxythymidine, NSC 602670) and ddC (2′3′-dideoxycytidine, NSC 606170) were provided by the NCI Drug Synthesis and Chemistry Branch. XTT was prepared at a concentration of 1 mg/ml in serum-free RPMI 1640. Phenazine methosulfate (Sigma) was prepared at 0.153 mg/ml in phosphate-buffered saline and stored at −20°C. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was also purchased from Sigma. Polymerase assays utilized the reagents poly(rA-dT)$_{12–18}$, poly(rC-dG)$_{12–18}$, and poly(dT-dA)$_{12–18}$ (Pharmacia LKB, Piscataway, N.J.) and 16S-23S rRNA (Boehringer Mannheim, Indianapolis, Ind.) and random nonamer primers (Synthetic Genetics, San Diego, Calif.).

Cells and viruses. The CEM-SS lymphocytic cell line was obtained from Peter Nara (NCI). H9 cells chronically infected with HIV-1 IIIB were obtained from Makoto Matuskura (NCI). The cell-cell fusion experiments utilized the HeLa-CD4-LTR-β-gal cells (19) and the HL 2/3 cells (9). Both cell lines were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health. All cells were maintained in RPMI 1640 medium without phenol red, and the medium was supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 50 μg of gentamicin per ml (complete medium). All cell-washing steps were carried out with complete medium, and all incubations were routinely performed at 37°C in an atmosphere containing 5% CO_2.

Anti-HIV assays. Cells were seeded into each well of a 96-well microtiter plate at a density of 5×10^3 cells per well. The cells were infected with virus at a multiplicity of infection (MOI) previously determined to give complete cell killing. Following 6 days of incubation at 37°C, the viability of the cells in each well was determined spectrophotometrically by the metabolic reduction of XTT to a soluble colored formazan (12). The effect of michellamine B on chronically infected cells was assessed by the determination of supernatant RT, core p24, and CEM-SS infectivity as previously described (1, 12).

Effect of pretreatment of target cells or virus with michellamine B. CEM-SS cells were incubated with 50 μM michellamine B or complete medium for 1 to 4 h with gentle mixing and then washed free of michellamine B in two centrifugation steps. The pretreated cells were suspended in complete medium and then plated at a density of 5×10^3 cells per 50 μl into the individual wells of a 96-well microtiter plate (each well contained 100 μl of 50 μM michellamine B or medium alone). The cells were infected at an MOI of 0.6 with 50 μl of diluted HIV stock, incubated for 6 days, and then evaluated by the XTT assay. In a similar fashion, concentrated HIV-1 was pretreated for 1 h with 10 μM michellamine B or complete medium. After the incubation, the pretreated virus preparations were diluted to provide an MOI of 0.6 in 50 μl and also to cause the michellamine B level to fall below an effective antiviral concentration. CEM-SS cells at a density of 5×10^3 cells per 50 μl were plated into the individual wells of a 96-well microtiter plate, with each well containing 100 μl of michellamine B or medium. Fifty microliters of the pretreated virus was added to the appropriate wells, incubated for 6 days, and then evaluated by XTT formazan production.

Time course studies. To determine the effects of a late addition of michellamine B on freshly infected cells, CEM-SS cells (5,000 per well) were incubated with virus at an MOI of 1.0; 50 μM michellamine B was then added to both the infected and uninfected cells after 0, 2, 4, 8, 12, 24, and 48 h during the 6-day incubation. After the 6-day incubation, cellular viability was assessed by the XTT assay. In addition, aliquots were removed from each test well so that supernatant RT determinations to monitor viral replication could be made. AZT (final concentration, 1 μM) was used as a control.

To determine the ability of michellamine B to abort a new infection, limited-treatment experiments were performed. CEM-SS cells were pelleted and infected in bulk with HIV-1 at an MOI of 1.0. The infection was carried out at 22°C for 45 min with constant agitation. After the infection, the cells were suspended in complete medium with or without michellamine B (final concentration, 50 μM). At intervals of 0, 4, 8, 24, 48, and 72 h, aliquots of cells were removed, washed free of michellamine B, and plated into the individual wells of a 96-well microtiter plate on which each well contained medium with or without michellamine B (50 μM). The plates were then incubated for 6 days from the time of the initial infection. At the end of the incubation, cellular viability was assessed by the XTT assay. ddC (final concentration, 1 μM) was used as a control.

Enzyme inhibition assays. All RTs used in this study were recombinant enzymes expressed in *Escherichia coli*. The HIV-1 and HIV-2 RT expression plasmids have been described elsewhere (16, 17). The two variant HIV RTs used in this study were a Tyr-181 → Ile RT (Δ181) and a Tyr-188 → Leu RT (Δ188). The preparation, enzymatic activities, and drug sensitivities of these two variant RTs have been previously described (5, 6). The three template-primer systems used to evaluate the inhibition of the various RT preparations by michellamine B consisted of poly(rA-dT)$_{12-18}$, poly(rC-dG)$_{12-18}$, and rRNA-primer. Recombinant-derived DNA polymerases α and β were the kind gift of Sam Wilson (University of Texas Medical Branch, Center for Molecular Sciences). The assay conditions have been described previously in detail (8, 25).

Multinuclear activation of a galactosidase indicator assay (MAGI assay). The assay utilized CD4$^+$ HeLa cells which employ a Tat protein-induced transactivation of a β-galactosidase gene driven by the HIV-1 long terminal repeat promoter (β-gal cells) (19). The MAGI assay can be used to quantitate both the binding of infectious virions to cells as well as cell-cell fusion events. Individual infected cells, or syncytia, can be easily counted microscopically after they have been incubated with X-Gal. β-Gal cells were used to assess the effect of michellamine B on HIV-1 infectivity by cell-free virions and in a cell-cell fusion assay with HL 2/3 cells (9). The assays were performed as previously described (19), with several modifications. The HIV infectivity assay involved the plating of 10^4 β-gal cells in 100 μl in 96-well, flat-bottom microtiter plates. After an overnight incubation, the medium was then removed and replaced with 100 μl of various concentrations of michellamine B. One hour later, 100 μl containing the desired amount of virus was added to each well. The cells were incubated for an additional 48 h, fixed, and stained with X-Gal as described previously (19). Blue multinuclear cells were then counted with a microscope.

The cell-cell fusion assay was performed in 96-well, flat-bottom microtiter plates. First, 5×10^3 β-gal cells in complete medium were plated in each well and incubated with test compounds for 1 h before the addition of 5×10^3 HL 2/3 cells. The cells were then incubated for an additional 48 h, fixed, and stained with X-Gal as described previously (19). The blue syncytia were counted with a microscope.

Fusion inhibition assays were also performed with a cocultivation assay system comprising uninfected CEM-SS cells and CEM-SS cells chronically infected with HIV, as previously described (8). Syncytia were quantitated at 24 and 48 h. The effects were expressed as the percent reduction from the number of syncytia obtained in the absence of the compound (virus control). All tests were performed in triplicate.

RESULTS

Direct-effects studies. Direct incubation of HIV-1 with michellamine B did not affect viral infectivity (data not shown). Similarly, pretreatment of CEM-SS cells with michellamine B followed by extensive washing prior to infection failed to protect cells from HIV-1-induced killing (data not shown).

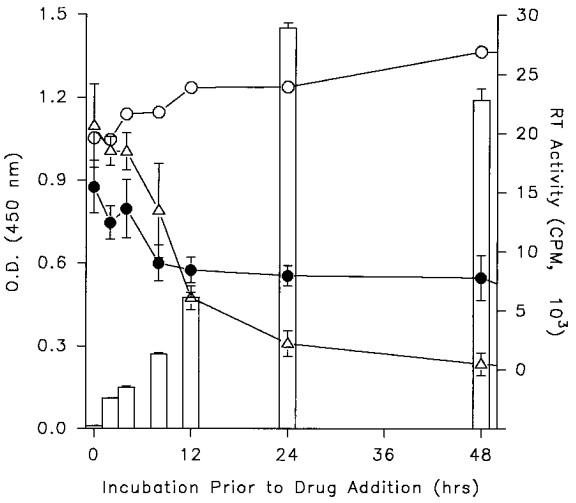

FIG. 1. Postinfection time course. At 0, 2, 8, 12, 24, and 48 h after the initial infection of CEM-SS cells with HIV-1, 50 μM michellamine B was added to infected (●) and uninfected (○) cells and 1 μM AZT was added to infected cells (△) as a control. All cells were then incubated for 6 days and assayed for cellular viability by the XTT assay. In addition, supernatant RT levels were obtained from michellamine B-infected cells at each time point (bars). All values are graphically represented as the mean values ±1 standard deviation for quadruplicate determinations, except for the drug-treated, uninfected cells, which are represented as the mean of duplicate determinations. O.D., optical density.

Time course and limited-treatment studies. A series of time course experiments was performed to estimate the stage(s) in the HIV life cycle at which michellamine B acted. As shown in Fig. 1, the viability of HIV-infected CEM-SS cells (as assessed by the XTT assay) dropped rapidly if the addition of either michellamine B or AZT was delayed for more than 2 to 4 h. Delayed addition of both compounds for 12 h resulted in a 50% loss of viability. However, at 24 and 48 h postinfection, the addition of michellamine B resulted in a level of cytoprotection that was approximately 30 to 40% of that of the uninfected michellamine B-treated cultures. Microscopic examination of the infected michellamine B-treated cultures at the end of the 6-day assay revealed a much lower level of syncytium formation than that seen with the untreated infected cells, even when the addition of michellamine B was delayed for 48 h. In contrast, the delayed addition of AZT for greater than 12 h resulted in a continued loss of cytoprotection. Microscopic examination of infected cultures in which the addition of AZT was delayed for 24 or 48 h revealed an abundance of syncytia comparable to that seen with the untreated infected cells. Despite the cytoprotection observed at the 24- and 48-h delayed-addition time points for michellamine B, it can be seen in Fig. 1 that viral reproduction (supernatant RT levels) was clearly not inhibited. These results indicate that michellamine B must be present early after acute HIV infection to produce complete cytoprotection and cessation of viral reproduction but that it can at least partially inhibit HIV cytopathicity when introduced well after the establishment of a productive infection.

Limited-treatment experiments were performed to further delineate the mechanism(s) of action of michellamine B. As shown in Fig. 2, the removal of michellamine B as late as 72 h after the time of infection resulted in failure to protect CEM-SS cells. Since residual extracellular virus was separated from the cultures along with michellamine B, the association of HIV-1 with its cellular receptors occurred in the presence of

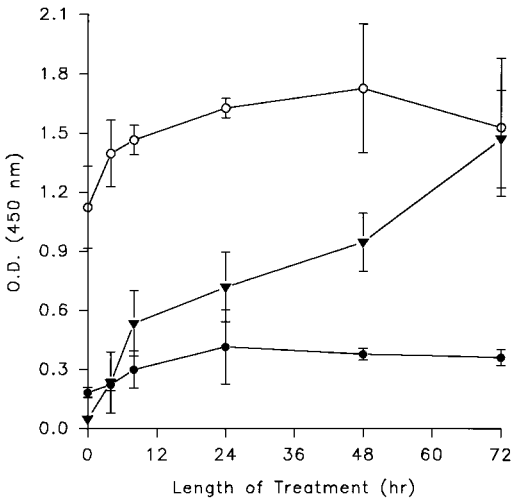

FIG. 2. Limited-treatment time course. CEM-SS cells infected with HIV-1 (●) or sham-infected CEM-SS cells (○) were suspended in culture medium containing 50 μM michellamine B and incubated at 37°C. At 0, 4, 8, 24, 48, and 72 h, aliquots of cells were removed, washed free of michellamine B, and seeded into the individual wells of a 96-well microtiter plate (each well contained drug-free, complete medium). Control, ddC (1 μM)-treated infected cells (▼) were treated in an identical fashion. Cellular viability was assessed by the XTT assay. All values are graphically represented as the mean values ±1 standard deviation for quadruplicate determinations. O.D., optical density.

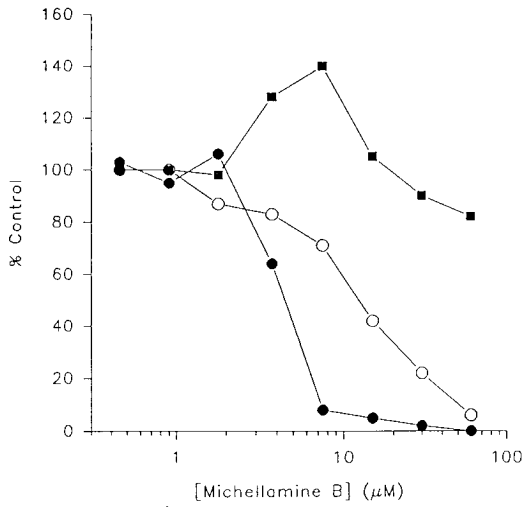

FIG. 3. Effect of michellamine B on HIV-1 infectivity and cell-cell fusion. The MAGI assay was used to assess the HIV-1 infectivity of β-gal cells (■) and the fusion of β-gal cells with HL 2/3 cells (○) in the presence of michellamine B. Also depicted graphically is the effect of michellamine B on syncytium formation of uninfected and chronically infected CEM-SS cells (●). Points are the mean values of triplicate samples expressed as the percentages of non-michellamine-treated cell controls. Standard errors of the means, ≤15%.

michellamine B. Under identical conditions, ddC began to exert cytoprotective activity after 8 h of treatment (Fig. 2).

Enzyme inhibition studies. The effects of michellamine B against HIV-1 and HIV-2 RT and two nonnucleoside-resistant variant RTs are shown in Table 1. The 50% inhibitory concentrations (IC_{50}s) obtained were dependent on the template and primer used for each of the four RT preparations. There was about a fivefold difference in the IC_{50}s of the poly(rA-dT) and rRNA template-primer systems. When rRNA was used as the template, michellamine B inhibited all RT preparations with IC_{50}s within the range of 50% effective concentrations (EC_{50}s) obtained in the cytoprotection assays. Michellamine B showed no inhibition of poly(rC-dG)-directed activity at concentrations up to 200 μM; however, it did inhibit both DNA polymerase α and β.

Fusion inhibition studies. The MAGI assay was utilized to determine whether michellamine B interfered with viral infectivity. After β-gal cells were infected with HIV_{IIIB} (MOI = 1.0) in the presence of various concentrations of michellamine B, the infection was not inhibited. The resulting blue-stained syncytia were of the same size and number as those of the virus-infected control β-gal cells (Fig. 3). The MAGI assay was then used to monitor the effect of michellamine B on the fusion of β-gal cells with HL 2/3 cells. Figure 3 shows the results of these experiments. Michellamine B inhibited syncytium formation in a dose-dependent fashion, with an IC_{50} of approximately 20 μM. In fusion inhibition experiments performed with cocultivation of uninfected and chronically infected CEM-SS cells, inhibition of syncytium formation was observed at an IC_{50} of approximately 10 μM (Fig. 3).

TABLE 1. Inhibition of RT by michellamine B

Enzyme	IC_{50} (μM)[a]		
	poly(rA-dT)$_{12-18}$	poly(rC-dG)$_{12-18}$	rRNA
HIV-1 RT	121.7	>200	22.2
HIV-2 RT	167.2	>200	18.7
HIV-1 Δ181 RT	196.8	>200	31.6
HIV-1 Δ188 RT	105.5	>200	21.7
DNA polymerase α	39.2		
DNA polymerase β	187.6		

[a] Values are averages of triplicate determinations. Standard errors averaged 10% of the respective means.

DISCUSSION

The rapid appearance of resistance to available and investigational drugs that has been observed both in vitro as well as in the clinic has underscored the need for the discovery of new antiviral compounds with novel mechanisms of action.

Michellamine B is a structurally novel naphthalene tetrahydroisoquinoline alkaloid (4, 21) having an unprecedented C-5 to C-8′ linkage between the naphthalene and isoquinoline ring systems. Furthermore, none of the previously known alkaloids of this class have the dimeric structure of michellamine B. It also has the greatest number of free phenolic hydroxy groups of this class and therefore is the most polar compound in this class.

Michellamine B has been reported elsewhere (4) to be capable of completely inhibiting the cytopathic effects of diverse laboratory and clinical strains of HIV-1 and HIV-2 on human lymphoblastoid target cells in vitro. The alkaloids also inhibited the production of RT, p24, and infectious virions in HIV-infected CEM-SS cells at antiviral effective concentrations (EC_{50}s were typically 1 to 20 μM). The replication of HIV in freshly isolated peripheral blood cells and macrophages was also inhibited by michellamine B (4).

These inhibitory effects were realized at michellamine concentrations that had no obvious adverse morphological or physiological effects on the target cells. These results suggested that michellamine B acts on some specific feature of virus replication rather than on general or nonspecific aspects of cellular metabolism. Virion-receptor interactions are unlikely candidates for the michellamine B inhibitory mechanism, since viral binding occurs in the presence of michellamine B. In

addition, inhibition of virus binding was not observed in the β-gal binding inhibition assay.

Postinfection time course studies revealed that michellamine B was able to provide a significant degree of protection against HIV-induced cell killing and syncytium formation even when it was added as late as 48 h after the time of infection. However, viral replication, as assessed by supernatant RT production, was not inhibited by michellamine B when it was added 24 to 48 h after the time of infection. These results suggested that in this in vitro system, michellamine B inhibited cell killing by blocking cell-cell fusion and the formation of syncytia without necessarily inhibiting viral replication.

The inhibition of cell fusion by michellamine B was further established with the MAGI assay system. Michellamine B effectively blocked fusion in two separate cell systems. Because of the amphoteric nature of the compound, this activity may result from reversible interactions with membrane components that play a role in cell-cell fusion. The importance of this cytoprotective activity of michellamine B remains to be determined, especially in light of the fact that michellamine B effectively inhibited viral replication in normal human peripheral blood cells (4). HIV-induced cell-cell fusion does not readily occur with these cells.

Several lines of experimental evidence point also to a mechanism of action of michellamine B involving the early stages of HIV replication. Michellamine B failed to inhibit virus production from chronically infected H9 cells, indicating that michellamine B acted at some step prior to provirus transcription. However, michellamine B substantially differed from AZT and ddC in that its cytoprotective activity was reversible and the compound had to be present continuously for maximal antiviral protection.

These observations focused attention on the events subsequent to virus attachment but prior to the integration of the viral DNA into the host DNA, thus warranting a specific examination of the effects of michellamine B on viral RT. Preliminary experiments first seemed to rule out the RT as the target of michellamine B action because the IC_{50}s first measured with homopolymeric templates were substantially higher than the EC_{50}s for antiviral activity (cytoprotection) found in cell cultures. However, subsequent studies revealed that when heteropolymeric rRNA was employed as the template in the assay, the IC_{50}s for RT and the antiviral EC_{50}s of michellamine B were about equivalent.

The importance of the choice of the template used to assess the ability of a compound to inhibit RT has also been demonstrated for diverse, nonnucleoside, HIV-1-specific RT inhibitors (7, 22, 24, 25). As with michellamine B, compounds of that general class showed the greatest inhibition of RT activity when a heteropolymer RNA template was used. However, unlike the nonnucleoside RT inhibitors, michellamine B afforded complete cytoprotection against several strains of HIV-2 and was equally potent against the pyridinone-resistant virus strain A17 (23).

Condra et al. (10) recently reported on molecular interactions between the nonnucleoside RT inhibitors and a region of the RT molecule defined by amino acid residues 176 to 190, with specific contributions by the tyrosines at positions 181 and 188. In order to determine whether the amino acid residues at positions 181 and 188 were involved in the inhibitory activity of michellamine B, we utilized two HIV RT mutants with single amino acid substitutions at these positions (5). Previous studies have shown these variants to be resistant to several nonnucleoside RT inhibitors (5). Michellamine B inhibited both of the variant RTs with approximately the same potency as that seen with the wild-type HIV-1 and HIV-2 RT recombinant preparations (Table 1). These results indicate that michellamine B acted at a site(s) on the RT enzyme distinct from the hydrophobic pocket recently described for nevirapine (20). Preliminary studies of the mechanism of RT inhibition by michellamine B revealed that the compound acted as a noncompetitive inhibitor with respect to deoxynucleoside triphosphates and functioned through a mixed inhibition mechanism with respect to the template-primer (10a). Our findings that michellamine B was effective in inhibiting the cellular DNA polymerases α and β may perhaps explain why, in some cell systems, michellamine B had a relatively narrow in vitro therapeutic index.

In summary, the michellamines represent a novel anti-HIV chemotype distinct from any previously known pharmacologic class. Their broad range of anti-HIV activity, including the ability to inhibit drug-resistant strains of HIV, merits careful consideration for drug development from this class.

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