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Flazinamide, a novel β-carboline compound with anti-HIV actions

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Abstract

A β-carboline compound, flazin isolated from *Suillus granulatus* has been shown weak anti-HIV-1 activity. Based on the structure of flazin, flazinamide [1-(5′- hydromethyl-2′-furyl)-β-carboline-3-carboxamide] was synthesized and its anti-HIV activities were evaluated in the present study. The cytotoxicity of flazinamide was about 4.1-fold lower than that of flazin. Flazinamide potently reduced syncytium formation induced by HIV-1IIIB with EC50 value of 0.38 μM, the EC50 of flazinamide was about 6.2-fold lower than that of flazin. Flazinamide also inhibited HIV-2ROD and HIV-2CBL-20 infection with EC50 values of 0.57 and 0.89 μM, respectively. Flazinamide reduced p24 antigen expression in HIV-1IIIB acute infected C8166 and in clinical isolated strain HIV-1KM018 infected PBMC, with EC50 values of 1.45 and 0.77 μM, respectively. Flazinamide did not suppress HIV-1 replication in chronically infected H9 cells. Flazinamide blocked the fusion between normal cells and HIV-1 or HIV-2 chronically infected cells. It weakly inhibited activities of recombinant HIV-1 reverse transcriptase, protease or integrase at higher concentrations. In conclusion, the conversion of the carboxyl group in 3 position of flazin markedly enhanced the anti-viral activity (TI value increased from 12.1 to 312.2) and flazinamide might interfere in the early stage of HIV life cycle.

Keywords: β-Carboline; Flazinamide; Synthesis; Anti-HIV agents; HIV-1; HIV-2

In the past decades, the highly active anti-retroviral therapy (HAART) decreased the number of AIDS cases dramatically [1]. However, HAART is not able to eradicate HIV-1 from patients completely. The long-term clinical effectiveness of approved anti-HIV drugs has been hampered by the ascendance of drug-resistant mutants in response to anti-retroviral therapies [2]. The rates of success of HAART are predicated to decrease gradually with the increase in the emergence of drug-resistant strains. Therefore, continuous development of new anti-viral agents is necessary.

β-Carboline alkaloids were found in several medicinal plants and displayed a variety of actions on the central nervous, muscular, and cardiovascular systems. It was discovered that β-carboline derivatives might function their anti-tumor activity through multiple mechanisms, such as targeting DNA [3], suppression the activity of topoisomerase [4], CDK [5] and IκK [6]. Lee et al. reported this class of compounds possesses the anti-HIV activity [7,8]. Recently, Yu et al. found that some β-carboline derivates inhibit HIV replication by interfering with TAT–TAR interaction [9]. A highly fluorescent carboline compound, flazin, was primarily found in sake (Japanese rice wine) and its structure [1-(5′-hydroxymethyl-2′-furyl)-β-carboline-3-carboxylic acid] was determined by chemical transformation

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[10]. Recently, we reported that flazin purified from the fruiting bodies of *Suillus granulatus* possesses weak anti-HIV activity [11]. In this study, we converted the carboxyl group in 3 position of flazin to the formamido group, and synthesized 1-(5′-hydroxymethyl-2′-furyl)-β-carboline-3-carboxamide and named as flazinamide. Anti-HIV activities of flazinamide and its possible action mechanisms were investigated in present study.

Materials and methods

Reagents. AZT was purchased from Sigma. Horseradish peroxidase (HRP)-labeled goat anti-human IgG was purchased from Sino-America Biotechnology Company (China). p5F1, monoclonal antibody (McAb) against HIV-1 p24, was prepared by our laboratory. Human polyclonal anti-HIV-1 serum was kindly donated by Dr. Hiroo Hoshino (Gunma University School of Medicine, Japan). Structures of flazin and flazinamide are presented in Fig. 1.

Cells and virus. Cell lines (C8166, H9, and H9/HIV-1_{IIIB}) were maintained in RPMI-1640 supplemented with 10% heat-inactivated newborn calf serum (Gibco). The cells used in all experiments were in log-phase growth. PBMC from healthy donors were isolated by Ficoll–Hypaque centrifugation and incubated in complete medium containing 5 μg/ml phytohemagglutinin (PHA) (Sigma) and 50 U/ml human recombinant IL-2 for 72 h prior to use for anti-viral assays. The laboratory-derived viruses (HIV-1_{IIIB}, HIV-2_{ROD}, and HIV-$2_{CBL\text{-}20}$) were obtained from MRC, AIDS Reagent Project, UK. The clinically isolated HIV-1_{KM018} was obtained from a HIV-1 infected individual in Yunnan Province of China as described [12]. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) was determined and calculated by the Reed and Muench method. Virus stocks were stored in aliquots at −70 °C.

MTT-based cytotoxicity assay. Cellular toxicity of compounds was assessed by MTT method as described previously [13]. Briefly, cells were seeded on a microtiter plate in the absence or presence of various concentrations of compounds in triplicate and incubated at 37 °C in a humid atmosphere of 5% CO_2 for 3 days. Twenty microliters of MTT reagent (5 mg/ml in PBS) was added to each well, then incubated at 37 °C for 4 h, 100 μl of 50% DMF–20% SDS was added. After the formazan was dissolved completely, the plates were read on a Bio-Tek ELx 800 ELISA reader at 595 nm/630 nm ($A_{595/630}$). The cytotoxic concentration that caused the reduction of viable cells by 50% (CC_{50}) was calculated from dose–response curve.

Syncytia assay. In the presence of 100 μl various concentrations of compounds, C8166 cells (4×10^5/ml) were infected with virus (HIV-1_{IIIB}, HIV-$2_{CBL\text{-}20}$ or HIV-2_{ROD}) at a multiplicity of infection (M.O.I) of 0.06. The final volume per well was 200 μl. Control assays were performed without the testing compounds in HIV-1_{IIIB} infected and uninfected cultures. AZT was included as positive control. After 3 days of culture, the cytopathic effect (CPE) was measured by counting the number of syncytia

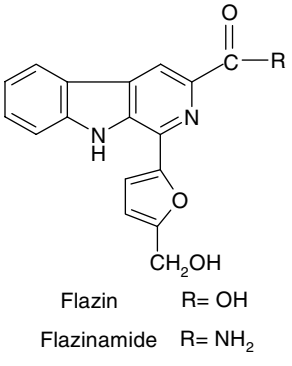

Fig. 1. The structures of flazinamide and flazin.

(multinucleated giant cell). Percentage inhibition of syncytia formation was calculated and 50% effective concentration (EC_{50}) was calculated [14].

Inhibition of HIV-1 p24 antigen production in acute infection. The inhibitory effect of compound on HIV-1 replication in vitro was further examined by quantification of p24 expression using capture ELISA as previously described. Briefly, C8166 cells were inoculated with HIV-1_{IIIB} (M.O.I. = 0.03) in the absence or presence of various concentrations of compound at 37 °C for 2 h to allow for viral absorption. It was then washed three times with PBS. The cells were plated at 3×10^4 cells/well with or without various concentrations of compound and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 4 days. HIV-1 p24 expression in cell-free supernatants was assayed by ELISA.

Inhibition of HIV-1 p24 antigen production in PBMC. Adequate numbers of PHA-activated normal PBMC were incubated with HIV-1_{KM018} (M.O.I. = 0.03) in presence of various concentration of compound. After 3 h of virus adsorption, the cells were washed twice with PBS and incubated with or without various concentration of compound in culture medium supplemented with 50 U/ml human recombinant IL-2 at 1.2×10^6 cells/ml for 7 days. Half of the medium was changed with corresponding compound concentrations on the third day. At 7 days post-infection, HIV-1 p24 antigen in the culture supernatants was analyzed by ELSIA. The inhibition of HIV-1 p24 antigen production in PBMC was calculated.

Inhibition of HIV-1 p24 antigen production in chronically infected H9 cells. H9 cells chronically infected with HIV-1_{IIIB} were washed three times with PBS to remove free virus particle. Two hundred microliters per well (3×10^5 cell/ml) of the cell suspension was cultured for 3 days in a 96-well culture plate with different concentrations of compound and then p24 antigen in the culture supernatants was tested by ELISA.

Co-cultivation assay. C8166 cells (3×10^4) co-cultured with 1×10^4 virus (HIV-1_{IIIB}, HIV-2_{ROD} or HIV-$2_{CBL\text{-}20}$) infected H9 cells in the presence or absence of the compound with various concentrations at 37 °C in a humidified atmosphere of 5% CO_2. Dextran sulfate (DS) was used as positive control. After 6 h incubation, the number of syncytia was scored under an inverted microscope [14].

Inhibition assay for recombinant HIV-1 RT activity. HIV-1 reverse transcriptase (RT) activity was measured by ELISA RT kit using a commercially available kit (Roche) according to the instructions of the manufacturer. The compounds were incubated with DIG-labeled reaction mixture at 37 °C for 2 h, then anti-DIG-POD solution was added, followed by substrate ABTS. Foscarnet was used as a positive control. The absorbency at 405 nm/490 nm ($A_{405/490}$) was read on Bio-Tek ELx 800 ELISA reader [14].

Inhibition assay of recombinant HIV-1 protease activity. The recombinant HIV-1 protease (PR) was expressed and purified as previously described [15]. HIV-1 PR was diluted in reaction buffer. The compounds were added and incubated for 30 min at room temperature. Fluorescent substrate DABCYL-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS (Anaspec, San jose) was added to initiate the reaction. The mixture was allowed to react for 90 min and the change of the fluorescent signal was monitored. Negative (double distilled water) and positive (1 μM indinavir) control was included. The percentage of inhibition was calculated.

Binding potency of compound to HIV-1 integrase. The interaction between compound and HIV-1 integrase (IN) was determined by SPR using a BIAcore 3000™ biosensor system (Biacore Inc., Piscataway, NJ). Integrase were immobilized on the surface of the chip. To allow association, various concentration of the compound diluted in HBS-EP was applied to a chip containing immobilized integrase. Dissociation of compound from the integrase was monitored in real-time after application of buffer to wash the chip and the kinetic rate constants for dissociation (K_d) was obtained by fitting the real-time data using BIA evaluation software.

Results

Anti-HIV-1 activities

The structures of flazinamide and flazin are shown in Fig. 1. The CC_{50s} of flazinamide and flazin against

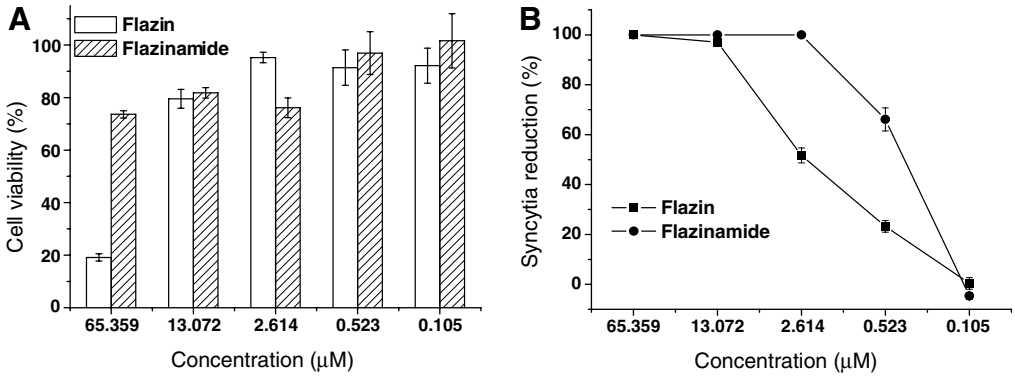

Fig. 2. Comparison of flazinamide and flazin on cytotoxicities (A) and anti-HIV-1 activities (B). Cytotoxicity on C8166 cells was measured by MTT assay. Anti-HIV-1 activity was determined by syncytia reduction assay. Data are expressed as means ± SD.

C8166 cells were 118.64 and 28.71 μM, respectively (Fig. 2A). The cytotoxicity of flazinamide was about 4.1-fold lower than that of flazin. The flazinamide and flazin inhibited HIV-1_{IIIB} replication in C8166 cells with EC_{50} values of 0.38 and 2.37 μM, respectively (Fig. 2B). The EC_{50} of flazinamide was about 6.2-fold lower than that of flazin. Thus the conversion of the carboxyl group in 3 position of flazin to the formamido group (Fig. 1) markedly enhanced the anti-viral activity, therapy index (TI) value increased from 12.1 to 312.2. The cytotoxicities and anti-HIV activities of flazinamide and flazin are summarized in Table 1.

Suppression of flazinamide on viral replication was also assessed by measuring expression of HIV-1 p24 antigen. Flazinamide dramatically reduced p24 production in HIV-1_{IIIB} acute infected C8166 with EC_{50} value of 1.45 μM (Fig. 3A). It also inhibited low-passage clinical isolated virus HIV-1_{KM018} replication in PBMC with EC_{50} value of 0.77 μM (Fig. 3B). Flazinamide potently inhibited syncytia formation induced by HIV-2_{ROD} or HIV-2_{CBL-20} in a dose-dependent manner with EC_{50} value of 0.57 and 0.89 μM, respectively (Fig. 3C).

Table 1
The summary of anti-HIV activities of flazinamide and flazin

Compounds	Cells	HIV strains or enzymes	Assays	EC_{50} (μM)
Flazinamide	C8166	HIV-1_{IIIB}	Syncytia	0.38
			p24	1.45
		HIV-2_{ROD}	Syncytia	0.57
		HIV-2_{CBL-20}	Syncytia	0.89
	PBMC	HIV-1_{KM018}	p24	0.77
	C8166	HIV-1_{IIIB}/H9	Co-cultivation	1.52
		HIV-2_{ROD}/H9	Co-cultivation	0.40
		HIV2_{CBL-20}/H9	Co-cultivation	0.63
	H9/HIV-1_{IIIB}	HIV-1_{IIIB}	p24	>65.36
	—	Protease	Fluorescent	35.74
	—	Integrase	BIAcore	5.99 (K_d)
	—	Reverse transcriptase	ELISA	>65.36
Flazin	C8166	HIV-1_{IIIB}	Syncytia	2.37

EC_{50} is the effective concentration that inhibits 50% of viral production. K_d is the kinetic rate constants for dissociation. The data shown in the table are a representative of three independent experiments.

Mechanisms of action

To address the action mechanisms, uninfected C8166 cells were co-cultured with infected H9 cells in presence of compound. Flazinamide significantly inhibited cell-to-cell transmission of HIV-1_{IIIB}, HIV-2_{ROD}, and HIV-2_{CBL-20} with EC_{50} values of 1.52, 0.40, and 0.63 μM, respectively (Fig. 3D). Moreover, the enzymatic assays were carried out. In vitro, flazinamide bound the HIV-1 integrase potently, with a K_d value of 5.99 μM (Fig. 4 and Table 1). It also inhibited recombinant HIV-1 protease with EC_{50} value of 35.74 μM. Flazinamide at a concentration of 65.359 μM weakly inhibited recombinant HIV-1 reverse transcriptase activity by 26.57% (Table 1) and p24 production in chronically infected H9 cells by 32.64%. In time-of-analysis, when C8166 cells were infected with HIV-1_{IIIB} for 3 h before flazinamide treatment, the EC_{50} value that flazinamide inhibits the formation of syncytia increased to higher than 65.36 μM (data not shown).

Discussion

Although clinically effective when used in combination, none of the currently available anti-HIV drugs represent ideal therapies, due to drug-related side effects, inconvenient dosing requirements, and/or the emergence of drug-resistant virus [16]. This issue of drug resistance is particularly problematic given that viral variants resistant to one drug often exhibit some level of cross-resistance to other drugs within the same class. Thus, development of new anti-viral agents with novel action mechanisms is necessary. Botanical compounds provide a wide spectrum of biological and pharmacological properties [17]. They may offer more opportunities to find anti-HIV drugs or lead compounds.

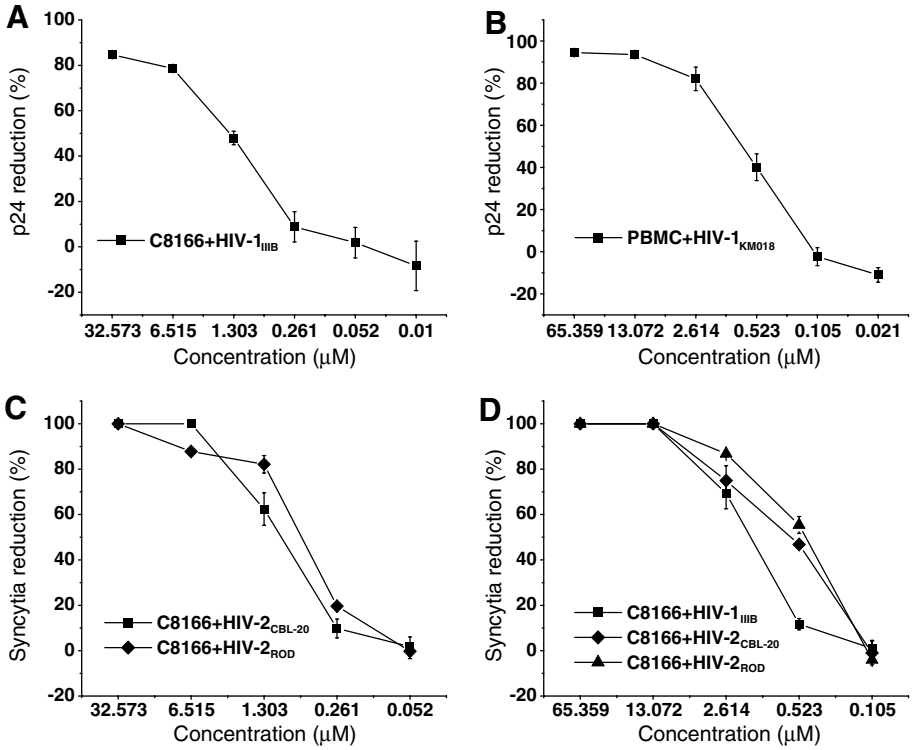

Fig. 3. Inhibitory activities of flazinamide on HIV replication. Replication of HIV-1_{IIIB} in C8166 (A) and HIV-1_{KM018} in PBMC (B) were measured by quantification of p24 antigen production; inhibiton of HIV-2_{CBL-20} (■) and HIV-2_{ROD} (♦) induced CPE in C8166 cells (C), as well as inhibitory effect on cell-to-cell fusion between normal C8166 cells and HIV-1_{IIIB} (■), HIV-2_{CBL-20} (♦) or HIV-2_{ROD} (▲) infected H9 cells (D) were measured by counting the syncytia formation under inverted microscope. Data are expressed as means ± SD.

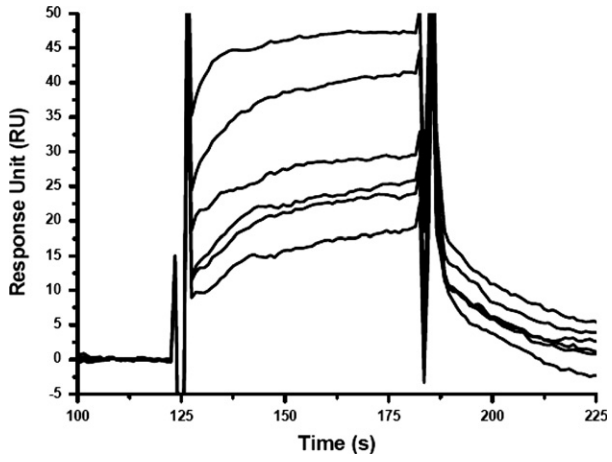

Fig. 4. Interaction between flazinamide and recombinant HIV-1 intergrase. The interaction was analyzed by Biacore 3000. The concentrations of flazinamide from top to bottom were 8.143, 1.629, 0.326, 0.065, 0.013, and 0.003 μM, respectively.

We found that flazin isolated from fruiting bodies of *S. granulatus* possesses weak anti-HIV-1 activities previously [11]. In the present paper, the structure of flazin was modified and got a derivative, flazinamide. Flazinamide showed more effective anti-HIV-1 activity and lower cytotoxicity than flazin, TI value increased from 12.07 to 312.21. The conversion of the carboxyl group in 3 position of flazin to the formamido group improved the anti-viral activity.

The anti-HIV activities of the flazinamide were further evaluated. The results showed that this compound inhibited HIV-1_{IIIB} and clinical isolate HIV-1_{KM018} potently. Similar results were obtained when anti-viral activities on HIV-2_{ROD} and HIV-2_{CBL-20} strains were tested.

Multiple bioactivities of β-carboline were previously reported [18–20]. It was also reported that β-carboline inhibited HIV-1 replication by interfering with TAR–TAT interaction [9]. The anti-HIV mechanisms of flazinamide were investigated in this research, too. Flazinamide inhibited cell-to-cell transmission of HIV-1 and HIV-2 potently indicated that it interfered in the early stage of HIV life cycle. In the enzymatic assay, flazinamide bound recombinant HIV-1 integrase potently, with *K*d value of 5.99 μM. It also inhibited recombinant HIV-1 protease activity with EC50 value of 35.74 μM and weakly suppressed HIV-1 RT by 26.57% at concentration of 65.359 μM (Table 1). To identify major target of flazinamide, more tests were carried out. Flazinamide did not show anti-viral activity in HIV-1_{IIIB} chronically infected H9. When the flazinamide was added at 3 h post-infection, the ability of flazinamide to inhibit HIV-1_{IIIB} induced CPE in C8166 cells decreased dramatically. This result also suggested that flazinamide may mainly interfered in early steps of HIV-1 life cycle. The molecular mechanisms of this compound deserve further investigation.

In conclusion, flazinamide, a flazin analogue, inhibited HIV-1 and HIV-2 replication potently. Flazinamide showed more effective anti-HIV-1 activity and lower cytotoxicity than flazin. The conversion of the carboxyl group in 3 position of flazin to the formamido group markedly enhanced the anti-viral activity, TI value increased from 12.1 to 312.2. This type of compound might regulate early stage of HIV-1 life cycle. More studies on anti-HIV mechanisms of this compound are in progress.

Acknowledgments

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