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Inhibition of human immunodeficiency virus type 1 infectivity by a new amine bellenamine

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Abstract

Bellenamine, (R)-3,6-diamino-N-(aminomethyl)hexanamide (molecular weight 174), produced by *Streptomyces nashvillensis*, which has been reported to have weak antibacterial activity and to slightly enhance the immune response, showed potent activity against human immunodeficiency virus type 1 (HIV-1). Its mode of action was investigated. Bellenamine inhibited de novo infection of human T cells with HIV-1, at a 50% effective concentration (EC_{50}) of 0.62 μg/ml (3.6 μM). Its 50% cytotoxic concentration (CC_{50}) was over 2000 μg/ml (11.5 mM) and thus its cytotoxicity was quite low. When HIV-1-infected cells were treated with bellenamine or glycosylation inhibitors, they produced virus with reduced infectivity, and thus bellenamine inhibited the secondary spread of HIV-1 in vitro similarly to glycosylation inhibitors. However, bellenamine did not change the apparent molecular weights of env or gag proteins, unlike glycosylation inhibitors. Bellenamine showed no significant activity against virus adsorption, reverse transcriptase, viral protease or the glycosylation process. The antiviral mechanism of bellenamine remains to be examined further.

Keywords: Antiviral agent; Infectivity; Immunofluorescence assay; Plaque assay; Bellenamine

1. Introduction

Acquired immune deficiency syndrome (AIDS) is caused by human immunodeficiency virus (HIV) (Barré-Sinoussi et al., 1983; Gallo et al., 1984). As there is an urgent need for effective chemotherapeutic agents against AIDS, many compounds have been evaluated for anti-HIV-1 activy in vitro. Some of the compounds that have been reported to be effective against HIV-1 replication in vitro are currently being investigated clinically for their potential therapeutic use in

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patients with AIDS (Mitsuya et al., 1990). 3′-Azido-3′-deoxythymidine (AZT) (Mitsuya et al., 1985), 2′,3′-dideoxyinosine (DDI), 2′,3′-dideoxycytidine (DDC) (Mitsuya and Broder, 1986) and 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) (Baba et al., 1987) are currently commercially available and have been prescribed to patients suffering from AIDS or AIDS-related complex (ARC) (Fischl et al., 1987; Merigan et al., 1989; Yarchoan et al., 1989). Treatment with these drugs has often resulted in the improvement of immunological functions and other clinical symptoms in patients. However, the appearance of severe dose-dependent toxicities or the emergence of drug-resistant mutant viruses has been reported after long-term administration of these drugs (Larder and Kemp, 1989; St. Clair et al., 1991; Fitzgibbon et al., 1992). AIDS is a life-long disease unless HIV-1 can be eliminated from patients. The long-term treatment of patients with AIDS necessitates the development of various types of anti-AIDS agents that have little toxicity and different modes of action. The emergence of drug-resistant virus strains should be carefully monitored during clinical treatment. Potential new anti-HIV agents may have novel chemical structures or inhibit HIV replication by mechanisms different from those currently in use.

In our screening program, a number of compounds from microbial origin were examined for anti-HIV activity. Bellenamine (Ikeda et al., 1986) markedly inhibited HIV-1 infection in vitro. Bellenamine (formerly named D-β-lysylmethanediamine), (R)-3,6-diamino-N-(aminomethyl)hexanamide, produced by *Streptomyces nashvillensis* has a weak antibacterial activity against Gram-positive bacteria, enhances delayed-type hypersensitivity to sheep red blood cells and increases the number of antibody-forming cells in the spleen of immunized mice (Ikeda et al., 1986). We also investigated its mode of action as a potential anti-HIV-1 agent.

2. Materials and methods

2.1. Compounds

Bellenamine and related compounds were isolated or synthesized (Ikeda et al., 1992a; Ikeda et al., 1992b; Ikeda et al., 1992c) in the Institute of Microbial Chemistry (Tokyo, Japan). Castanospermine and deoxynojirimycin were purchased from Takara Syuzo (Kyoto, Japan), and AZT was from Sigma Chemical (St. Louis, MO, USA). These compounds were dissolved at 20 mg/ml in phosphate-buffered saline (PBS) for antiviral evaluation and stored at −20°C as stock solutions.

2.2. Cells and HIV-1

The human T cell lines MT-4 (Miyoshi et al., 1981) and MOLT-4 (Minowada et al., 1972) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). MOLT-4/III_B cells were MOLT-4 cells persistently infected with the HTLV-III_B (or LAI) strain of HIV-1 (Popovic et al., 1984). MOLT-4/GUN-1 cells were MOLT-4 cells persistently infected with the GUN-1 strain of HIV-1 (Takeuchi et al., 1987), which was a clinical isolate of the syncytium-inducing (SI) type. Peripheral blood lymphocytes (PBL) were isolated from the blood of healthy subjects by Ficoll-paque gradient centrifugation. PBL were stimulated with phytohemagglutinin (PHA) prior to HIV-1 infection and cultured in RPMI 1640 medium containing 10% FCS and 100 units/ml of recombinant interleukin-2. HIV-1 was prepared from the culture supernatant of MOLT-4/III_B and MOLT-4/GUN-1 cells and stored in small aliquots at −80°C.

2.3. Anti-HIV-1 assay

The measurement of activities of compounds against HIV-1 replication was based on the inhibition of viral protein expression in MT-4 cells infected with HIV-1 (Takeuchi et al., 1987). Briefly, MT-4 cells (0.5 ml) suspended in culture medium at a density of 1×10^5 cells/ml were seeded into wells of 48-well plates containing various amounts of test compounds in an aqueous solution (0.05 ml). After 2 h, the cells were infected with HIV-1 at a multiplicity of infection (m.o.i.) of 0.05. After a 4-day incubation at 37°C,

percentages of HIV-1 antigen-positive MT-4 cells were determined by the indirect immunofluorescence assay (IFA). In the case of mock infection or infection with HIV-1 in the presence of AZT at 1 μg/ml, fluorescence-positive cells were not observed, suggesting that there was no cross reactivity of HIV-1-seropositive serum used to other antigens, such as HTLV-I antigens, in MT-4 cells. The 50% effective concentration (EC_{50}) was defined as the concentration of a compound that was estimated to reduce the percentage of HIV-1 antigen-positive MT-4 cells to half that in untreated control cultures.

Anti-HIV-1 activities of compounds using PBL were evaluated by the quantitative detection of HIV-1 p24 antigen in culture supernatants of PBL. PHA-stimulated PBL (5×10^6 cells/ml) were infected with HIV-I at an m.o.i. of 0.1. After virus adsorption for 1 h, the cells were washed twice to remove unadsorbed virus particles and suspended in culture medium at 2.5×10^5 cells/ml. The cell suspension (0.2 ml) was dispensed into wells of 96-well microplates containing test compounds. After incubation for 6 days at 37°C, the amounts of HIV-1 p24 antigen in culture supernatants were determined by ELISA. The 50% effective concentrations (EC_{50}) were calculated as the concentrations of each compound capable of reducing the amount of HIV-1 p24 antigen in culture supernatants of drug-treated cultures to 50% of that in untreated controls.

The same experiments were repeated two or three times for each assay system and similar results were obtained. Representative results were shown.

2.4. Cytotoxicity assay

Cytotoxicities of compounds to MT-4 cells were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Pauwels et al., 1988). Briefly, MT-4 cells (0.1 ml) suspended in culture medium at 1×10^5 cells/ml were seeded into wells of 96-well microplates containing various amounts of test compounds. After incubation for 4 days at 37°C, 10 μl of MTT solution (7.5 mg/ml) was added to cultures to stain viable cells. After 4 h, 100 μl of solvent (10% Triton X-100 and 0.4% HCl in isopropanol) was added to cultures to solubilize formazan. After an overnight incubation, the absorbance of each well was measured using a microplate reader (Bio-Rad, model 450) at 560 nm. All data represent average values for at least three wells. Variations in values between each of the triplicate wells were less than 10% of the mean. The 50% cytotoxic concentration (CC_{50}) was calculated as the concentration of a compound capable of reducing absorbance (OD_{560}) to 50% of that in untreated controls.

2.5. Reverse transcriptase (RT) assay

RT activities in culture supernatants were determined after precipitation of virions with polyethylene glycol (PEG). HIV-1 lysates containing RT were prepared by disrupting precipitates with lysis buffer. Ten-μl aliquots of HIV-1 lysates were mixed with 50 μl of the reaction mixture consisting of 40 mM Tris-HCl (pH 7.8), 0.25 mg/ml BSA, 4 mM dithiothreitol, 45 mM KCl, 10 mM $MgCl_2$, 0.2 mM poly(rA), 0.4 unit/ml oligo$(dT)_{12-18}$, 13.5 μM TTP and 45 μCi/ml [^{3}H]TTP (40–70 Ci/mmol), (ICN, Costa Mesa, CA, USA) and incubated at 37°C for 1 h. The reaction mixtures were spotted onto DE-81 filters, which were then air-dried, washed three times with 0.5 M disodium hydrogenphosphate, twice with distilled water and once with ethanol. The radioactivity bound to filters was then counted in a liquid scintillation counter (Hoshino et al., 1983).

2.6. Plaque assay

The infectivity of HIV-1 was determined by plaque assay as described by Harada et al. (1985) with a slight modification. MT-4 cells (2×10^6 cells/100 μl) were infected with 10-fold diluted virus samples for 1 h at 37°C. The cells were washed twice with PBS to remove unadsorbed virus particles and resuspended in 0.5 ml of RPMI

1640 medium containing 10% FCS and 0.28% Sea Plaque agarose (FMC Corp., Rockland, ME, USA). The cell suspension was transferred to 6-well plates (9.4 cm^2/well) containing 0.5 ml of 1.2% agarose medium. After the cell suspension had solidified, it was overlaid with 0.5 ml of 0.28% agarose medium and 2 ml of liquid medium was further added over the agarose medium. The plates were incubated at 37°C, and on days 2 and 4 the liquid medium was replaced by fresh medium. On day 5, the liquid medium was removed and the medium containing 0.01% neutral red was added. Plates were then incubated at 37°C for 1 day and plaques were counted.

2.7. Immunoprecipitation

To determine the effects of bellenamine on the synthesis and processing of gag proteins and envelope glycoproteins, immunoprecipitation was performed. MOLT4/III_B cells (1×10^5 cells/ml) were incubated in 2 ml of RPMI 1640 medium containing various concentrations of test compounds at 37°C for 3 days. The cells were then extensively washed with PBS and resuspended in 2 ml of methionine-free RPMI 1640 medium supplemented with 10% dialyzed FCS and 100 μCi/ml of Tran ^{35}S-label (> 1000 Ci/mmol) (ICN, Costa Mesa, CA, USA). After incubation for 1 day at 37°C, the cells and culture supernatants were harvested. The cells were washed with PBS and lysed in RIPA buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 1 mM phenylmethlysulfonyl fluoride (PMSF). HIV-1 in the culture supernatants was concentrated with PEG and lysed with RIPA buffer. Both samples were treated with serum from healthy subjects and protein A-Sepharose before immunoprecipitation. Pre-cleared samples were incubated with serum from an HIV-1-infected subject for 1 h. The protein A-Sepharose suspension was then added and placed on a rotator for 16 h at 4°C. The Sepharose was washed and resuspended in loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue). Samples were electrophoresed under reducing conditions in 7.5% polyacrylamide gels, and labeled proteins were detected by fluorography.

3. Results

3.1. Antiviral activity and cytotoxicity of bellenamine

The effect of bellenamine on acute infection with HIV-1 was evaluated using MT-4 human T-cells (Fig. 1). In the absence of bellenamine, about 95% of the cells were positive for HIV-1 antigens after incubation for 4 days. At concentrations as low as 3 μg/ml, bellenamine markedly inhibited the expression of HIV-1 antigens in MT-4 cells, and its EC_{50} was 0.62 μg/ml (3.6 μM). However, even high concentrations of bellenamine, e.g. 100 μg/ml, did not completely inhibit HIV-1 infection. The cytotoxicity of bellenamine to MT-4 cells was determined by the MTT method. Even at a concentration of 2000 μg/ml (11.5 mM), bellenamine was not markedly cytotoxic to MT-4 cells (Fig. 1).

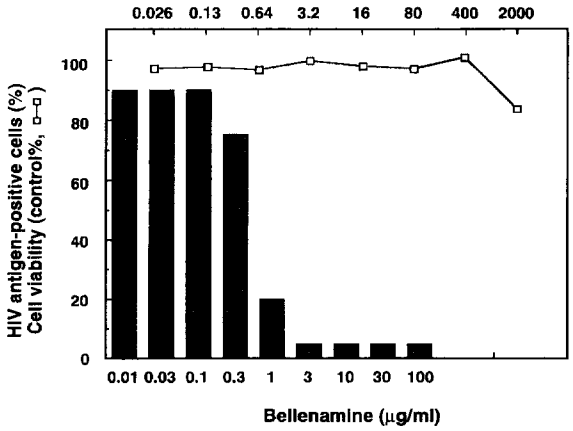

Fig. 1. Anti-HIV-1 activity and cytotoxicity of bellenamine. MT-4 cells were infected with HIV-1 and cultured in the presence of bellenamine. After incubation for 4 days, the percentage of HIV-1 antigen-positive MT-4 cells was determined by IFA. The viability of MT-4 cells was determined by the MTT assay after incubation with bellenamine for 4 days. The same experiments were repeated three times for two assay systems and similar results were obtained.

Table 1
Anti-HIV activities of bellenamine and its related compounds

Compound	Structure	EC_{50}^{a}	IC_{50}^{b}
Bellenamine	(*R*) $H_2NCH_2CH_2CH_2CHCH_2CONHCH_2NH_2$ (NH$_2$)	0.62 (3.6)	>2000 (>11 500)
L-Bellenamine	(*S*) $H_2NCH_2CH_2CH_2CHCH_2CONHCH_2NH_2$ (NH$_2$)	>100	ND
SMD	$HOOCCH_2CH_2CONHCH_2NH_2$	>100	ND
D-β-LysNH_2	$H_2NCH_2CH_2CH_2CHCH_2CONH_2$ (NH$_2$)	>100	ND
AcLMD	$H_2NCH_2CH_2CH_2CHCH_2CONHCH_2NHCOCH_3$ (NH$_2$)	>100	ND
CyLMD	$H_2NCH_2CH_2CH_2CHCH_2CO$ (NHCH$_2$ NH)	>100	ND

ND, not determined.[a]Inhibitory activities of compounds against HIV-1 replication were evaluated by the inhibition of viral protein expression in acutely infected MT-4 cells. They are shown as μg/ml (μM).[b]Cytotoxicities of compounds to MT-4 cells were evaluated by the MTT method. They were shown as μg/ml (μM).

The effect of bellenamine on acute infection of PBL with HIV-1 was also evaluated by ELISA. Inhibition of HIV-1 p24 antigen production after infection was used to estimate antiviral activities of samples. Bellenamine was highly inhibitory to HIV-1 infection as detected by reduced production of HIV-1 p24 antigen in culture media, its EC_{50} being about 1.3 μg/ml (7.5 μM) (data not shown).

The effect of bellenamine on acute infection was also examined using an SI-type clinical isolate, i.e. GUN-1 strain: infection of MT-4 cells with this strain was also inhibited by bellenamine (data not shown).

3.2. Antiviral activities of bellenamine-related compounds

Anti-HIV-1 activities of bellenamine-related compounds were determined using MT-4 cells (Table 1). L-Bellenamine, N-(aminomethyl)succinamic acid (SMD) (Ikeda et al., 1992a), D-β-lysinamide (D-β-LysNH_2) (Ikeda et al., 1992c), 1′-N-acetylbellenamine (AcLMD) (Ikeda et al., 1992a) and cyclized bellenamine (CyLMD) (Ikeda et al., 1992c) in parallel with bellenamine were examined for anti-HIV-1 activities. All compounds other than bellenamine had little anti-HIV-1 activities.

3.3. Effect of bellenamine on HIV-1 replication in chronically infected cells

We next examined whether bellenamine affected HIV-1 production in chronically infected cells. Production of HIV-1 by chronically infected MOLT-4/III_B cells which had been treated with bellenamine was determined by measuring RT activity in their culture supernatants. No apparent inhibition of HIV-1 production was detected by treatment with bellenamine at concentrations up to 100 μg/ml (data not shown).

3.4. Inhibition of the spread of HIV-1 infection by bellenamine

As shown in Fig. 1, the expression of HIV-1 antigens in MT-4 cells was not completely inhib-

ited even in the presence of high concentrations of bellenamine: about 5% of the cells were still positive for HIV-1 antigens. There are two possible explanations for this result. Firstly, although infection of MT-4 cells with HIV-1 was inhibited almost completely by bellenamine during the first cycle of infection, HIV-1 gradually spread to 5% of the cells during cultivation for 4 days. Secondly, bellenamine permitted the first cycle of infection with HIV-1, but inhibited the secondary spread of HIV-1. As MT-4 cells were infected with HIV-1 at an m.o.i. of 0.05, about 5% of MT-4 cells would become positive for HIV-1 antigens after the first cycle of HIV-1 infection. To determine which was the case, ratios of HIV-1 antigen-positive MT-4 cells in cultures treated with bellenamine were determined at days 1, 2, 3 and 4 of infection (Fig. 2). About 5% of MT-4 cells were positive for HIV-1 antigens after cultivation for 2 days regardless of whether they had been treated with bellenamine. In the absence of bellenamine, the percentage of infected cells increased rapidly to 95% during the next 2 days. In the presence of high concentrations of bellenamine, the percentages of HIV-1-positive cells, however, did not further increase. This result suggested that bellenamine did not inhibit de novo infection with HIV-1, but instead inhibited the spread of HIV-1 infection.

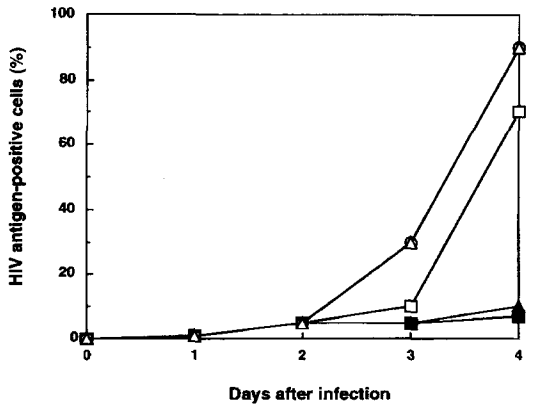

Fig. 2. Inhibition of the secondary spread of HIV-1 infection in MT-4 cells by bellenamine. MT-4 cells were infected with HIV-1 at a multiplicity of infection of 0.05 and cultured in the presence of bellenamine: 0 (△), 0.1 (○), 0.3 (□), 1 (▲), 3 (●) and 10 (■) μg/ml. Percentages of HIV-1 antigen-positive MT-4 cells were determined at day 0, 1, 2, 3 and 4 of infection. Experiments were performed three times under the same assay conditions and similar results were obtained.

To confirm the latter possibility diluted virus suspension (1/10, 1/20, 1/50 and 1/100, which corresponded to m.o.i. of 0.8, 0.4, 0.16 and 0.08, respectively) were inoculated into MT-4 cells in the presence or absence of 10 μg/ml of bellenamine. The HIV-1-antigen-positive cells at 4 days after infection were proportional to virus dilutions. In the presence of bellenamine (10 μg/ml), expression of HIV-1 in MT-4 cells was not markedly inhibited when infected with large amounts of virus (data not shown). This result also suggested that bellenamine did not inhibit primary infection.

Incubation of HIV-1 with bellenamine for 1 h before infection did not reduce the infectivity of virus as measured by the plaque assay using MT-4 cells (data not shown), suggesting that bellenamine did not react with the HIV-1 virions directly.

3.5. *Effect of bellenamine on HIV-1 infectivity*

Our findings that bellenamine might inhibit the secondary infection of MT-4 cells by HIV-1 indicated two possible explanations. (1) HIV-1 produced by MT-4 cells that have been treated with bellenamine may lack infectivity, or (2) MT-4 cells treated with bellenamine may become less susceptible to HIV-1. Therefore, we examined the infectivity of HIV-1 produced by bellenamine-treated cells and also the susceptibility to HIV-1 of MT-4 cells treated with bellenamine.

To compare the infectivities of HIV-1 samples prepared from cultures incubated in the presence or absence of bellenamine, MT-4 cells were infected with HIV-1 under conditions where about 80% MT-4 cells, either untreated or treated with bellenamine (10 μg/ml), became positive for HIV-1 antigens. MT-4 cells were inoculated with diluted HIV-1 in the presence or absence of bellenamine at 10 μg/ml. When MT-4 cells were infected with 1/50-diluted HIV-1 (m.o.i. of 0.16) and incubated in the absence of bellenamine, about 80% of the cells became positive for HIV-1 antigens. Similar percentages of MT-4 cells expressed HIV-1 antigens when infected with 1/10-

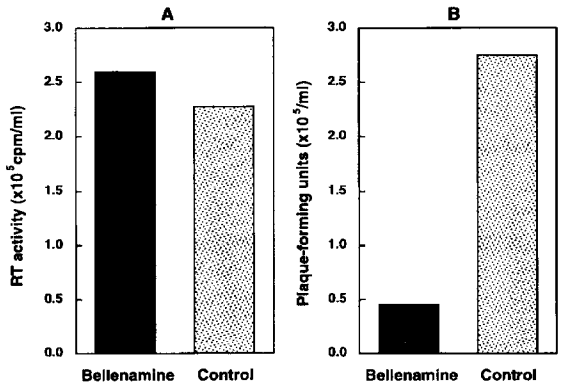

Fig. 3. Effect of bellenamine on production of HIV-1 by acutely infected cells and its infectivity. Viral infectivity of the culture supernatants was determined comparatively by (A) virus production by RT assay and (B) virus infectivity by plaque assay. Experiments were repeated three times under the same assay conditions and results were similar.

diluted HIV-1 (m.o.i. of 0.8) and cultured in the presence of 10 μg/ml of bellenamine. The production of virus by cultures incubated with or without bellenamine were similar as shown by assaying their culture supernatants for RT activities (Fig. 3A). The infectivity of the virus produced by cultures treated with bellenamine was reduced to about 10% of the control (Fig. 3B). As shown in Fig. 4, a marked reduction of HIV-1 infectivity by treatment with bellenamine was observed in the culture of chronically HIV-1 infected MOLT-4/III$_B$ cells. Virus treated with 1, 0.1 and 0.01 mM of bellenamine showed a dose-dependent reduction in infectivity. The infectivity of virus treated with glycosylation inhibitors, castanospermine or deoxynojirimycin at 1 mM were also lower than the controls, as reported by Gruters et al. (1987). Thus, bellenamine did not inhibit the production of HIV-1, but reduced the infectivity of virus particles produced by bellenamine-treated cells.

RT assays of the culture supernatants suggested that similar amounts of HIV-1 particles were released from infected cells regardless of whether the cells had been treated with bellenamine or not, indicating at least that the expression of pol genes, synthesis and processing of gag-pol protein were not inhibited by bellenamine. Stimulation of the HIV-1 long terminal repeat by HIV-1 Tat determined by LTR-CAT assay (Okamoto et al., 1990), the synthesis of viral mRNA and proteins, and the activity of HIV-1 proteinase were not inhibited by bellenamine (data not shown).

3.6. Effect of bellenamine on the sensitivity of indicator cells to HIV-1 infection

It is known that the CD4 antigen is the principal receptor for HIV (Dalgleish et al., 1984; Klatzmann et al., 1984). Therefore, we examined whether bellenamine affected expression of CD4 antigen on target cells. MT-4 cells were cultured for 4 days in the presence of bellenamine, and then the cells were examined by flow cytometry for expression of CD4 antigen. Expression of the CD4 antigen on the cell surface was not reduced by treatment with bellenamine at concentrations up to 100 μg/ml (data not shown). Reverse transcription of viral RNA was not inhibited by bellenamine, as the amount of proviral DNA formed in bellenamine-treated cells 1 day after infection was similar to that in controls (data not shown).

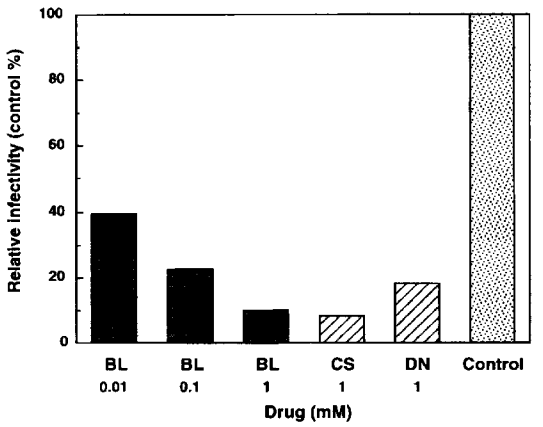

Fig. 4. Inhibition by bellenamine of the infectivity of HIV-1 produced by chronically infected MOLT-4/III$_B$ cells. Relative infectivities of samples were determined by dividing plaque-forming units of the samples by RT activity (pfu/cpm), and then these were compared with the control. Bellenamine (BL) was used at 0.01, 0.1 and 1 mM, castanospermine (CS) at 1 mM, and deoxynojirimycin (DN) at 1 mM (1 mM bellenamine was 174 μg/ml). No apparent inhibition of HIV-1 production was detected by treatment with bellenamine, castanospermine and deoxynojirimycin at concentrations up to 1 mM (data not shown). Three independent experiments gave similar results.

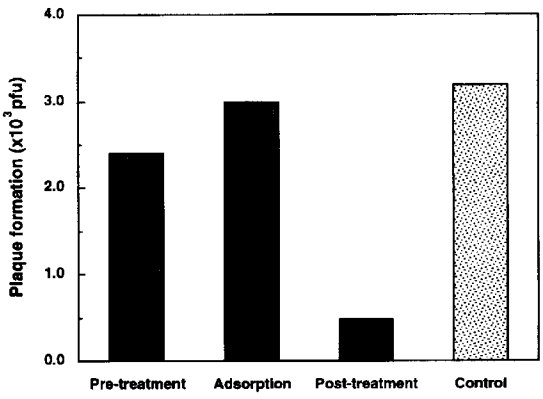

Fig. 5. Susceptibilities of cells treated with bellenamine to HIV-1 infection. The effect of bellenamine on the susceptibility of MT-4 cells to HIV-1 infection was determined by plaque-forming assay. The cells were treated with 10 μg/ml of bellenamine either for 1 day before infection (pre-treatment), during the adsorption period of 1 hour (adsorption) or for 5 days after infection (post-treatment). The number of plaque-forming units in inocula determined using bellenamine-untreated MT-4 cells was 3.2×10^3 (Ccntrol). Experiments were repeated three times to confirm results.

To further determine whether bellenamine may affect the sensitivity of indicator cells to viral infection, MT-4 cells were treated with bellenamine before or after infection with HIV-1 and applied to the plaque assay (Fig. 5). That is, the cells were treated with bellenamine (10 μg/ml) for 1 day before infection (pre-treatment), during adsorption of HIV-1, i.e. 1 hour, or for 5 days after infection (post-treatment). In the absence of bellenamine, the virus stock contained 3.2×10^3 plaque-forming units/ml. Similar numbers of plaques were formed by MT-4 cells treated with bellenamine before infection or during adsorption. However, treatment of MT-4 cells with bellenamine after adsorption resulted in a reduction in the number of plaques. These findings indicated that bellenamine neither reduced the sensitivity of indicator cells to viral infection nor inhibited the adsorption of HIV-1 to the cells.

3.7. Effect of bellenamine on synthesis and maturation of HIV-1 proteins

The infectivities of HIV-1 samples produced by cells treated with bellenamine were low (Fig. 3 and 4). The infectivity of HIV-1 produced by cells treated with glycosylation inhibitors has been reported to be reduced, and the apparent molecular weights of HIV-1 envelope glycoproteins produced by these cells are different from those produced by untreated cells (Gruters et al., 1987). Therefore, we examined the possibility that bellenamine inhibited the glycosylation of HIV-1 envelope glycoproteins by monitoring the effect of bellenamine in comparison with that of castanospermine on apparent molecular weights of HIV-1 proteins by SDS-PAGE (Fig. 6). MOLT-4/III_B cells were treated with bellenamine or castanospermine at 10 μg/ml for 3 days. Then the cells were labeled for 1 day, and viral proteins were immunoprecipitated with HIV-1-specific antiserum. Apparent molecular weights of viral proteins were determined by SDS-PAGE and fluorography. In the presence of castanospermine, a band corresponding to envelope glycoprotein gp120 was detected, but it migrated to a higher position on the gel than that in the control, indicating inhibition of glycosylation of the env protein (Montefiori et al. (1988). No alteration of the mobility of envelope glycoproteins was, however, detected in the samples treated with bellenamine. Furthermore, the mobilities of other viral proteins, i.e. gag proteins or gag precursor protein, were not changed as compared with those of the control. Thus, unlike virus protease inhibitors, bellenamine did not affect processing of the gag precursor protein.

4. Discussion

The inhibitory effect of bellenamine on HIV-1 infection was clearly observed in an acute infection system. However, even high concentrations of bellenamine did not completely inhibit HIV-1 infection in MT-4 cells. Several lines of evidence indicated that bellenamine did not inhibit single-cycle infection with HIV-1, but instead inhibited the secondary spread of infection. This was shown to be due to the reduced infectivity of newly produced virus (Fig. 3 and 4). It will be important to examine anti-HIV-1 activities of bellenamine against various HIV-1 strains including clinical isolates and we are planning to do so.

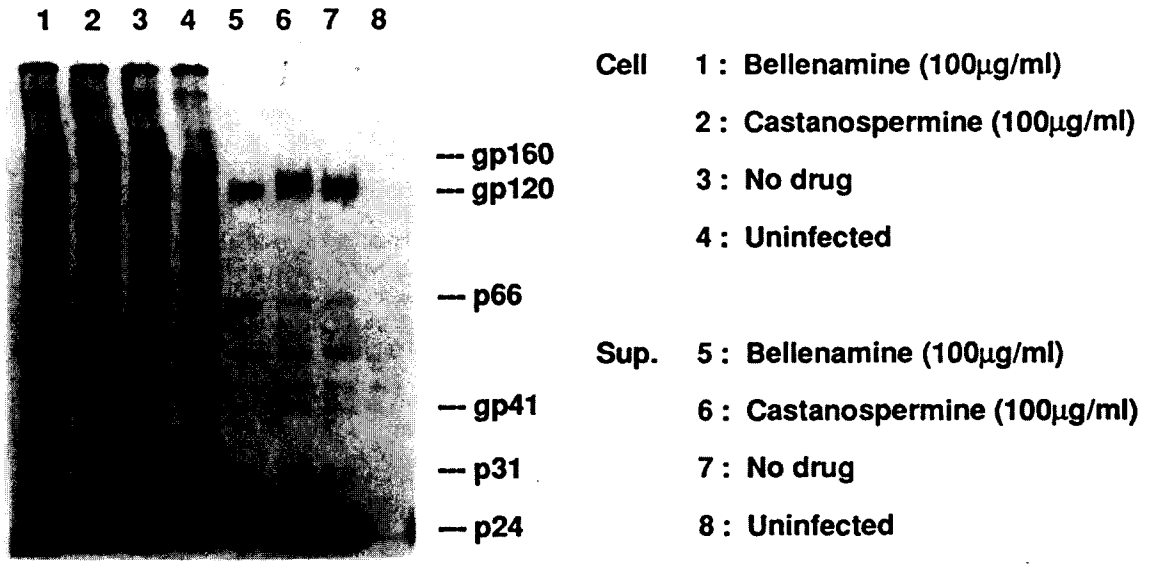

Fig. 6. Effect of bellenamine on the processing of viral proteins detected by SDS-PAGE. MOLT-4/III_B (lanes 1–3, 5–7) and MOLT-4 (lanes 4 and 8) cells were incubated with bellenamine or castanospermine (100 μg/ml) for 3 days, and the cells were then labeled with Tran ^{35}S-label for 1 day in the presence of the drugs. Cell lysates (lanes 1–4) and concentrated culture supernatants (lanes 5–8) were immunoprecipitated with HIV-1-seropositive human serum. Lanes: 1 and 5, bellenamine; 2 and 6, castanospermine; 3 and 7, no drug; 4 and 8, uninfected control. Similar results were obtained in the repeated experiment.

It has been reported that the infectivity of HIV-1 produced by cells treated with viral protease inhibitors or glycosylation inhibitors is reduced (Gruters et al., 1987). Therefore, we examined the possibility that bellenamine may act in a similar manner. Apparent molecular weights of viral proteins, however, were not altered in samples treated with bellenamine relative to those in controls. Another possibility is that the inhibitory mechanism of bellenamine may be related to a glycosylation step of the viral envelope protein. It is known that glycosylation of the viral envelope is essential for the infectivity of HIV-1. There are many steps in glycosylation of cellular and viral proteins, and several glycosylation inhibitors which act on different steps have been reported to have anti-HIV-1 activities with infectivity-reduced viruses being produced by cells treated with these inhibitors (Gruters et al., 1987). Castanospermine, an inhibitor of glucosidase I, and other inhibitors of glycosylation such as deoxynojirimycin, deoxymannojirimycin, swainsonine or tunicamycin have been reported to clearly affect the apparent molecular weights of HIV-1 glycoproteins as detected by SDS-PAGE of immunoprecipitates (Montefiori et al., 1988). It is still possible that only a small change in the molecular weights of glycoproteins might have been induced by treatment with bellenamine if it acts at a late step of the glycosylation pathway. The infectivity of HIV-1 may also be reduced by inhibition of a step other than glycosylation: i.e. HIV-1 *vif* gene has been reported to enhance viral infectivity (Strebel et al., 1987), and bellenamine might affect the function and/or expression of this or other regulatory genes. So far, we could not identify the precise step of HIV-1 infection at which bellenamine exerts its antiviral effect.

Anti-HIV-1 activities of bellenamine-related compounds were determined using MT-4 cells (Table 1). All of these compounds, except for bellenamine, showed little anti-HIV-1 activity. Thus, the anti-HIV-1 activity of bellenamine depends on a stringent structure–function relationship because small structural modifications are not tolerated.

It is well documented that drug-resistant HIV-1 mutants appear during treatment of patients with

a single drug. Combination therapy with compounds having different modes of action, therefore, will be necessary to circumvent the appearance of drug-resistant HIV-1 strains (Chow et al., 1993). Recently, a number of anti-HIV-1 agents have been studied and their modes of action often differ from one another (Mitsuya et al., 1990; De Clercq, 1995). Our study suggests that bellenamine might have a unique mode of action which is different from those of known anti-HIV-1 agents, and, in addition, bellenamine has very low cytotoxicity. Thus, bellenamine may represent a new class of anti-HIV-1 agents and be a good candidate for therapeutic use against HIV-1 infection.

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