

ORIGINAL ARTICLE

Beauvericin and enniatins H, I and MK1688 are new potent inhibitors of human immunodeficiency virus type-1 integrase

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Some enniatins (ENs) reportedly exhibit antiretroviral activities *in vivo*. The potential inhibitory activities of cyclic hexadepsipeptides such as beauvericin (BEA) and ENs H, I and MK1688 were investigated *in vitro* against human immunodeficiency virus type-1 (HIV-1) integrase and Moloney murine leukemia virus reverse transcriptase. BEA, EN I and EN MK1688 exhibited strong inhibitory activities against HIV-1 integrase, whereas EN H showed relatively weak activity. None of the examined compounds showed anti-reverse transcriptase activity. BEA was the most effective inhibitor of the tested cyclic hexadepsipeptides in inhibiting HIV-1 integrase. These results indicate the potential of cyclic hexadepsipeptides as a new class of potent inhibitors of HIV-1 integrase.

The Journal of Antibiotics (2009) 62, 687–690; doi:10.1038/ja.2009.102; published online 6 November 2009

Keywords: beauvericin; enniatins; HIV-1; inhibitor; integrase

INTRODUCTION

Human immunodeficiency virus type-1 (HIV-1) is the causative agent of AIDS (acquired immunodeficiency syndrome).¹ An essential process in the retroviral life cycle is integration of the viral DNA into the host genome, which is mediated by the viral enzyme integrase² via two consecutive steps.³ The first step, which is called the 3'-processing reaction, proceeds as integrase removes the terminal dinucleotide from the 3'-end of each strand of the viral cDNA in the cytoplasm of the infected cell. In the second step, known as the strand transfer reaction, the processed 3'-end of the viral DNA attaches to the host-cell DNA in the cell nuclei. Extensive efforts over the past few years have focused on frustrating these reactions, which have resulted in the discovery of a large number of HIV-1 integrase inhibitors.⁴ Various classes of the compounds, including natural products and synthetic compounds, have been suggested. Enniatins (ENs) B, B₁ and A₁ produced by *Fusarium* species reportedly exhibit anti-HIV-1 activity,⁵ but the mechanism for EN inhibition of viral replication remains ambiguous. Our group has recently reported the successful copurification of beauvericin (BEA) and ENs H, I, and MK1688 from culture broth of *Fusarium oxysporum* isolated from soil samples collected in Korea.^{6–8} As ENs B, B₁ and A₁ have been shown to protect human lymphoblastoid cells in HIV-1-induced cell death,⁵ we decided to test the antiviral activity of BEA and these ENs, H, I and MK1688, by studying whether or not they inhibit viral enzymes such as reverse transcriptase and integrase *in vitro*. In our assay, BEA, EN I and EN MK1688 were found to strongly inhibit the 3'-processing activity of HIV-1 integrase.

MATERIALS AND METHODS

Preparation of cyclic hexadepsipeptides

BEA and ENs were produced from the liquid culture of *F. oxysporum* strain FB1501 (KFCC 11363P) in *Fusarium* defined medium (FDM; the medium contained sucrose 25 g, NaNO₃ 4.25 g, NaCl 5 g, MgSO₄·7H₂O 2.5 g, KH₂PO₄ 1.36 g, FeSO₄·7H₂O 0.01 g and ZnSO₄·7H₂O 0.0029 g in 1.0 l tap water) as described previously.⁶ For submerged cultures, 100 ml of medium in a 250 ml Erlenmeyer flask was inoculated with approximately 1×10⁵ spores and the culture was incubated at 25 °C with shaking at 120 r.p.m. BEA and ENs H, I and MK1688 were purified from chloroform in two steps on HPLC. A GROM-Sil pack ODS preparative column (1.0×25 cm²; Herrensberg, Germany) was used for the first purification, in which the mobile phase was CH₃CN–H₂O, 65:35 (in volume), at a flow rate of 4 ml min⁻¹. The compounds were further purified with a Shiseido pack C18 column (0.46×25 cm², Shiseido, Tokyo, Japan). The second HPLC purification was performed over 45 min at a constant flow rate (1 ml min⁻¹) with a CH₃CN–H₂O, 70:30 (in volume).

Analysis and identification of BEA and ENs H, I and MK1688 by HPLC, mass spectrometry and liquid chromatography/tandem mass spectrometry

BEA and ENs were analyzed as described previously⁶ with a reverse-phase column (0.46×25 cm, 5 μm, Shiseido) using HPLC with an LC-305 device, 306 pumps and a 151 UV-visible detector from Gilson (Middleton, WI, USA). They were eluted with CH₃CN:H₂O:phosphoric acid, 70:30:0.01 (in volume), at a constant flow rate (1 ml min⁻¹) for 40 min, and the elution was monitored at 210 nm. The BEA and ENs were identified using an electrospray ionization mass spectrometer (LC-MSD Trap VL, Agilent, Santa Clara, CA, USA). An aliquot of 10 μl of the sample was injected in the analytical column

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Received 31 August 2009; revised 5 October 2009; accepted 6 October 2009; published online 6 November 2009

(0.5×30 cm², C₁₈, 5 μm, Shiseido) for liquid chromatography analysis, in which the composition of elute was CH₃CN–H₂O, 70:30 (in volume), at a flow rate of the mobile phase of 0.5 ml min⁻¹. During liquid chromatography/mass spectrometry analysis, the liquid chromatography effluent entered the mass spectrometer with a source voltage of 4.5 kV. The heated capillary was maintained at 325 °C, the sheath gas and auxiliary gas were adjusted to 89.7 and 14.6 arbitrary units, respectively, and the capillary voltage was 71.6 V. The mass spectrometer was initially programmed to perform full scans at *m/z*=100–900 for BEA and ENs H, I and MK1688 in order to observe the protonated molecular ion signals of these compounds, as well as possible fragment ions and adducts.

Evaluation of anti-HIV-1 integrase activity

The 3'-processing assays of HIV-1 integrase were performed as described previously.⁹ For the *in vitro* 3'-processing reaction, two 20-mer oligonucleotides the sequences of which resemble those of the end of HIV-1 U5-LTR were synthesized at Takara: K16 (U5-LTR, + strand), 5'-TGTGGAAATCTCTAG CAGT-3'; and K17 (U5-LTR, - strand), 5'-ACTGCTAGAGATTTCCACA-3'. Oligonucleotide K16 (15 pmol) was labeled at the 5'-end using 250 μCi [γ-³²P]-ATP (Amersham, Richmond, VA, USA) and 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) in a 40 μl reaction buffer (70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂ and 5 mM dithiothreitol) for 60 min at 37 °C. After adding 18 pmol of complementary oligonucleotide K17, the mixture was boiled for 3 min and then cooled slowly. The labeled substrates were separated from unincorporated nucleotide by passage through a Biospin 6 column (Biorad, Hercules, Canada). The standard reaction of the 3'-processing activity contained 0.1 pmol of radiolabeled substrate, 3 pmol of HIV-1 integrase and inhibitors at the stated concentrations, 15 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM MnCl₂, 2 mM 2-mercaptoethanol, 2.5 mM CHAPS, 0.1 mM EDTA, 0.1 mM PMSE, 1% glycerol and 10 mM imidazole in a total volume of 10 μl. The reaction solutions were incubated at 37 °C for 60 min and stopped by the addition of 4 μl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. The reaction products were analyzed on a 15% denaturing polyacrylamide gel. Reactions were visualized and quantified by autoradiography of the dried gel (GS525 Molecular Dynamic Phosphoimager (Biorad)). The percentage inhibition was calculated according to the equation $100 \times [1 - (D - C) / (N - C)]$, where C, N and D are the fractions of 20-mer substrate converted to 18-mer (3'-processing product) for DNA alone, DNA plus integrase and integrase plus drug, respectively. The IC₅₀ value was determined by plotting the drug concentration versus the percentage inhibition and determining the concentration that produced 50% inhibition.¹⁰

Evaluation of antireverse transcriptase activity

Reverse transcriptase assays were carried out using Moloney murine leukemia virus (MMLV) reverse transcriptase (New England Biolabs). The standard reaction contained 24 pmol poly A(dT), 48 pmol of [³H]-dTTP, 0.6 units of MMLV reverse transcriptase and inhibitors in 50 μl of 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl₂ and 10 mM dithiothreitol. The reaction mixtures were incubated at 37 °C for 1 h and then spotted onto a DE81 filter (Whatman, Maidstone, UK), which was washed three times with 2× SSC, twice with 95% ice-cold ethanol, and then dried. Radioactivity was measured in a liquid scintillation counter.

RESULTS AND DISCUSSION

Production of cyclic hexadepsipeptides and their identification

Cyclic peptides have been successfully used as enzyme inhibitors, antifungal and antibacterial agents, immunomodulating substances, and anticancer drugs.^{11–15} BEA and ENs share a common cyclohexadepsipeptide structure as a basic skeleton (Figure 1). They are composed of an alternating sequence of three *N*-methyl-*L*-amino acids and three *D*- α -hydroxyisovaleric acids.^{6,16,17} Mixtures of ENs B, EN B₁ and A previously showed anti-HIV-1 activity by protecting human lymphoblastoid cells against HIV-1-induced cell death with an *in vitro* therapeutic index of approximately 200.⁵ Our group has copurified BEA, and ENs H, I and MK1688 from culture broth of *F. oxysporum*, and the production of these cyclic hexadepsipeptides were further characterized during fermentation (Figure 2). The growth

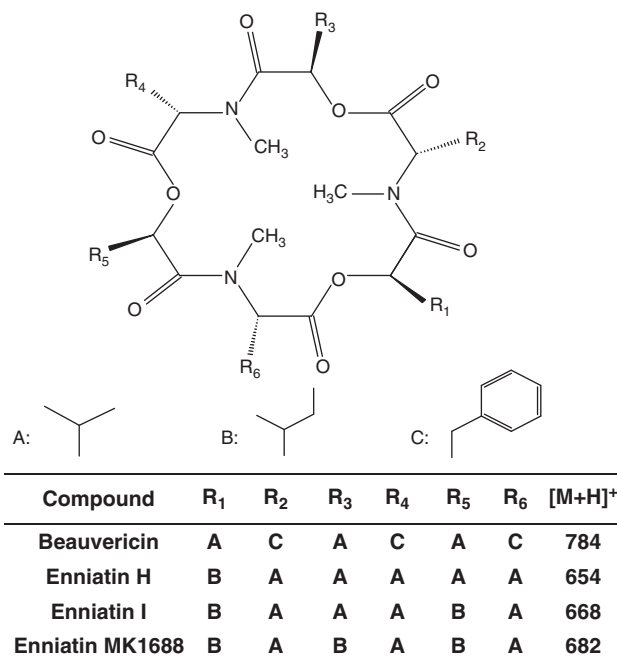


Figure 1 Chemical structures of (A) beauvericin (BEA) and (B) new enniatins (ENs) H (R₁=CH₂CH₃, R₂ and R₃=CH₃), I (R₁ and R₂=CH₂CH₃, R₃=CH₃) and MK1688 (R₁, R₂, and R₃=CH₂CH₃).

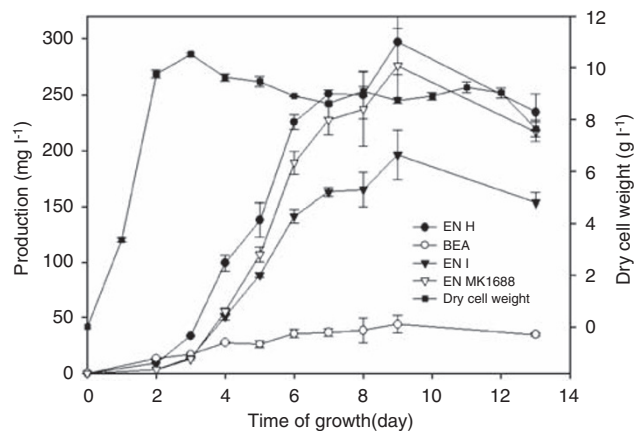


Figure 2 Growth of *Fusarium oxysporum* KFCC11363P and production of BEA and ENs. The levels of BEA and ENs were analyzed from a liquid culture of *F. oxysporum* FB1501 (KFCC 11363P) in FDM.

and production of BEA and ENs H, I and MK1688 by *F. oxysporum* strain KFCC 11363P in FDM are shown in Figure 2. The mycelium weight was maximal at day 3 of cultivation (10 g l⁻¹), and the amounts of BEA and ENs H, I and MK1688 in FDM were maximal at day 9 of cultivation (0.044, 0.29, 0.19 and 0.28 g l⁻¹, respectively). The productions of ENs H, I and MK1688 were first reported from the insect pathogenic fungus *Verticillium hemipterigenum* strain BCC 1449, and their amounts were highest in YES media (yeast extract sucrose; the medium consisted of yeast extract 20 g and sucrose 150 g in 1.0 l tap distilled water): 0.065, 0.004 and 0.0003 g l⁻¹, respectively.¹⁸ These data show that markedly higher amounts of ENs H, I and MK1688 were produced by *F. oxysporum* strain KFCC 11363P than by *V. hemipterigenum*. Moreover, the molecular weights of BEA and

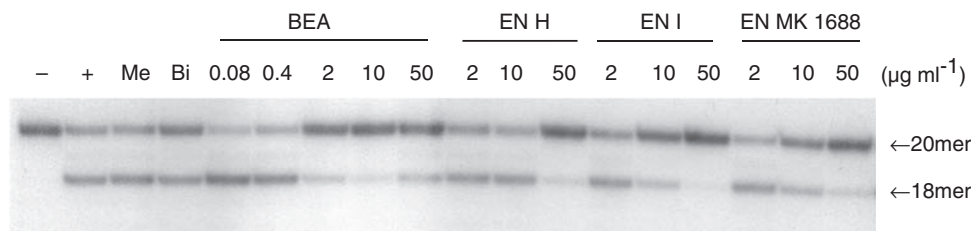


Figure 3 Inhibitory effects of cyclic hexadepsipeptides isolated from *Fusarium oxysporum* KFCC11363p on human immunodeficiency virus type-1 (HIV-1) integrase. Products from the *in vitro* 3'-processing reactions were analyzed on a 15% denaturing polyacrylamide gel, and visualized and quantified by autoradiography of the dried gel. -, no integrase added; +, integrase added; Me, 5% methanol as a final concentration (solvent control); Bi, 5 µg ml⁻¹ baicalein (with a known HIV-1 integrase inhibitor used as inhibitor control).

Table 1 Inhibitory effects of beauvericin and enniatins on human immunodeficiency virus type-1 (HIV-1) IN and Moloney murine leukemia virus (MMLV) RT^a

Compound	IC ₅₀ (µM) ^b	
	IN	RT
Beauvericin	1.9 ± 0.4	95.6 ± 18.2
Enniatin I	10.6 ± 1.8	> 100
Enniatin H	37.9 ± 2.8	> 100
Enniatin MK 1688	11.8 ± 3.1	89.8 ± 15.5

Abbreviations: IN, integrase; RT, reverse transcriptase.

^aData are mean ± s.e. values from three independent experiments.

^bInhibitor concentration required to reduce the 3'-processing activity of HIV-1 IN or MMLV RT activity by 50%.

ENs H, I, and MK1688 are 784.5, 654.7, 669.0 and 683.6, respectively, on the basis of molecular ion peaks in *m/z* plots. EN H, BEA, EN I and EN MK1688 could be eluted with retention times of 16.0, 17.6, 20.8 and 27.2 min, respectively, in HPLC chromatograms (data not shown). The following ion products [M+H]⁺ were found in liquid chromatography/tandem mass spectrometry analysis (*m/z* values): BEA: 756.4, 623.4, 523.4, 495.4, 362.4, 280.2 and 262.2; EN H: 626.6, 541.5, 513.6, 441.4, 328.3 and 228.1; EN I: 640.6, 555.5, 527.4, 441.3, 328.4 and 228.2; EN MK1688: 654.4, 569.5, 455.4, 427.2, 342.4 and 228.0.

Evaluation of anti-reverse transcriptase activity

After identifying the cyclic hexadepsipeptides, their inhibitory activities on retroviral enzymes were investigated. Their potentials to inhibit *in vitro* reactions using purified HIV-1 integrase were examined first, with baicalein, a known HIV-1 integrase inhibitor, used as a positive control. BEA and ENs inhibited HIV-1 integrase activity dose-dependently, as shown in Figure 3. BEA strongly inhibited the 3'-processing activity of HIV-1 integrase (IC₅₀, 1.9 ± 0.4 µM, mean ± s.e.) (Table 1), followed by ENs I and MK1688 (IC₅₀, 10.6 ± 1.8 and 11.8 ± 3.1 µM, respectively), and then by EN H (IC₅₀, 37.9 ± 2.8 µM). The inhibitory activities of these cyclic hexadepsipeptides against the viral enzyme were tested in MMLV reverse transcriptase. In contrast to their strong inhibition of HIV-1 integrase, BEA and ENs did not exert any inhibitory effects on reverse transcriptase, as indicated by their IC₅₀ values being around 100 µM or more (Table 1). The relative inhibitory potencies of BEA, EN I and EN MK1688 on HIV-1 integrase were comparable to those of baicalein (IC₅₀, 1.2 µM) and robinetin (IC₅₀, 5.9 ± 1.9 µM), which are strong HIV-1 integrase inhibitors.¹⁹ Lutzke *et al.*²⁰ reported a hexapeptide inhibitor of HIV-1 integrase, the sequence of which is HCKFWW, and suggested that it

acts at or near the catalytic site of integrase. Owing to their structural characteristics, cyclic hexadepsipeptides could inhibit the oligomerization of the enzyme that is essential for catalytic activity.²¹

As there is no known human counterpart of HIV-1 integrase, it is an attractive target for antiviral drug design. To date, several classes of compounds have been identified as being active against HIV-1 integrase. Even though raltegravir and elvitegravir have been shown to be promising in clinical trials, and the first has been recently made available for clinical practice,²² more extensive screening of various biological sources is necessary to identify a clinically effective inhibitor. To our knowledge, this is the first report of cyclic peptides that can inhibit HIV-1 integrase. We surmise that cyclic hexadepsipeptides represent a new class of potent inhibitors of HIV-1 integrase.

ACKNOWLEDGEMENTS

We are grateful to Se-Young Choi for performing the phosphoimage analyses of the 3'-processing assays of HIV-1 integrase. This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2009-0059098).

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