Efrapeptin J, a New Down-regulator of the Molecular Chaperone GRP78 from a Marine *Tolypocladium* sp.

Yoichi Hayakawa, Yuki Hattori, Takashi Kawasaki, Kaneo Kanoh, Kyoko Adachi, Yoshikazu Shizuri, Kazuo Shin-ya

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Abstract A new down-regulator of the molecular chaperone GRP78, efrapeptin J, was isolated from a marine fungus, *Tolypocladium* sp. AMB18. The molecular formula of efrapeptin J was established as $C_{81}H_{139}N_{18}O_{16}^{+}$ by high-resolution FAB-MS. The structure was elucidated to be a linear pentadecapeptide containing a hexahydropyrrolo[1,2-*a*]pyrimidinium moiety by NMR and MS analyses. Efrapeptins F, G and J dose-dependently inhibited 2-deoxyglucose-induced luciferase expression in HT1080 human fibrosarcoma cells transfected with a luciferase reporter plasmid containing the GRP78 promoter. Efrapeptin J also inhibited the protein expression of GRP78 in HT1080 cells and MKN-74 human gastric cancer cells. Efrapeptin J induced cell death in HT1080 cells under endoplasmic reticulum stress.

Keywords efrapeptin, GRP78, *Tolypocladium*, transcriptional down-regulator

Introduction

The endoplasmic reticulum (ER) is a cellular organelle involved in protein synthesis and quality control of protein folding. ER stress induces the expression of several molecular chaperones that prevent unfolded protein

accumulation [1]. The molecular chaperone GRP78 can protect cells from ER stress-induced death and is regulated by the promoter ERSE (ER-stress element) [2, 3]. Increased expression of GRP78 causes the resistance of solid tumor cells to low-glucose stress due to poor blood vessel formation [4]. Decreased expression of GRP78 is known to be involved in neuronal cell death in Alzheimer's disease [5]. Thus, down-regulators of GRP78 expression under ER stress are expected to show antitumor activity against solid tumors and up-regulators of GRP78 may prevent ER stress-induced neuronal cell death. In the course of screening for transcriptional regulators of GRP78 using luciferase reporter assay, a marine fungus *Tolypocladium* sp. AMB18 was found to produce a new GRP78 down-regulator designated efrapeptin J (**1**, Fig. 1). This paper describes the fermentation, isolation, structure elucidation and biological activity of **1**.

Results

Fermentation and Isolation

The producing organism was cultured in Potato Dextrose Broth (Difco) containing 50% seawater on a rotary shaker at 25°C for 7 days. One-ml portions of the seed culture were inoculated into stainless steel plates (320×190 mm)

Y. Hayakawa (Corresponding author), **Y. Hattori**, **T. Kawasaki**: Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, E-mail: hykw@rs.noda.tus.ac.jp

K. Kanoh, **K. Adachi**, **Y. Shizuri**: Marine Biotechnology Institute Co. Ltd., 3-75-1 Heita, Kamaishi, Iwate 026-0001, Japan

K. Shin-ya: Biological Information Research Center, National Institute of Advanced Industrial Science and Technology, 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan

Fig. 1 Structures of efrapeptins J (**1**), G and F.

containing 400 ml of an agar medium consisting of glucose 0.5%, glycerol 2.0%, yeast extract 0.2%, Pharmamedia (Traders Protein) 2.0%, sodium chloride 0.25% and agar 1.5% (pH 6.5 before autoclaving). The fermentation was carried out at 25°C for 14 days under static condition.

The whole culture (4.0 liters) was extracted with Me_2CO . After evaporation, the aqueous concentrate was extracted with EtOAc. The extract was applied to a silica gel column, which was washed with $CHCl_3$ -MeOH (10:1) and eluted with $CHCl_3$ -MeOH (5:1). The active eluate was subjected to HPLC (Senshu Pak PEGASIL ODS 7 μ , 20 i.d. $\times$ 250 mm). Development of the column with 50% CH_3CN -0.2% trifluoroacetic acid gave three active fractions, two of which were identified as efrapeptins F and G [6] on the basis of NMR and MS data. The remaining active fraction was concentrated to dryness to yield a colorless powder of **1** (50 mg).

Structure Elucidation

The molecular formula of **1** was determined to be $C_{81}H_{139}N_{18}O_{16}^+$ (ion) by high-resolution FAB-MS. The IR spectrum showed strong absorption peaks at 3300, 1670 and 1540 cm^{-1} due to amide groups. The ^{13}C -NMR spectrum exhibited 16 amide carbonyl carbons. These data indicated that **1** belongs to the peptide antibiotics. The NMR data are summarized in Table 1. One-bond 1H - ^{13}C connectivities were established by a heteronuclear multiple-quantum coherence (HMQC) [7] experiment.

COSY and heteronuclear multiple-bond correlation (HMBC) [8] experiments revealed the presence of 15 amino acid residues (Fig. 2). A couple of isolated *N*-methylene protons (δ 3.73, 3.67) displayed long-range correlations to a carbonyl carbon (δ 171.5) to construct a glycine residue (Gly). Four methylene protons including an *N*-methylene group (δ 3.74, 3.32) were coupled to each other and to a carbonyl carbon (δ 174.9), indicating the presence of a β -alanine residue (β -Ala). An alanine residue

Fig. 2 Partial structures of efrapeptin J (**1**).

Bold lines show 1H - 1H spin networks and arrows indicate 1H - ^{13}C long-range correlations.

(Ala) was established by 1H - ^{13}C long-range correlations to a carbonyl carbon (δ 177.6) from doublet methyl (δ 1.44) and *N*-methine (δ 4.02) protons, which were coupled to each other. Each of three *N*-methine protons (δ 5.14, 5.03, 4.95) displayed $CH-CH_2-CH_2$ spin networks and exhibited long-range correlations to each couple of carbonyl (δ 173.3, 173.6, 173.5) and *N*-methylene (δ 45.8, 45.3, 44.7) carbons. The three *N*-methylene groups were part of three CH_2-CH_2 sequences in the COSY spectrum. Comparison of their 1H and ^{13}C chemical shifts with those of pipecolic acid [9, 10] identified three pipecolic acid residues (Pip¹, Pip², Pip³). Pip¹ was required to be *N*-acetylated by 1H - ^{13}C

Table 1 ^{13}C - and ^1H -NMR data for efrapeptin J (**1**).

No.	δ_{C}	δ_{H}	No.	δ_{C}	δ_{H}
Ac-Pip ¹	1	173.3	Aib ⁵	1	175.8
	2	54.1		2	58.4
	3	27.4		3	28.0
	4	21.5		4	25.3
	5	26.0	Pip ³	1	173.5
	6	45.8		2	57.0
	MeCO	173.8		3	26.5
	MeCO	21.7		4	21.2
Aib ¹	1	176.2		5	25.8
	2	58.1		6	44.7
	3	27.6	Aib ^{6*}	1	177.3
	4	25.4		2	58.3
Pip ²	1	173.6		3	28.3
	2	56.8		4	23.9
	3	26.3	Ala	1	177.6
	4	21.4		2	53.3
	5	25.8		3	17.0
	6	45.3	Leu ²	1	174.7
Aib ^{2*}	1	178.2		2	55.7
	2	58.1		3	40.8
	3	27.1		4	26.1
	4	24.2		5	23.5
Aib ³	1	177.7		6	21.4
	2	58.0	Aib ^{7*}	1	177.1
	3	27.9		2	58.1
	4	23.3		3	26.7
Leu ¹	1	175.4		4	25.0
	2	54.3	HPP	2	45.3
	3	40.9		3	20.0
	4	26.3		4	43.5
	5	23.9		6	55.6
	6	21.6		7	19.1
β -Ala	1	174.9		8	31.9
	2	37.0		8a	166.8
	3	37.2		9	57.7
Gly	1	171.5		10	46.0
	2	45.4		11	42.0
Aib ^{4*}	1	177.4		12	25.8
	2	58.1		13	23.7
	3	26.7		14	21.5
	4	24.2			0.88

* Interchangeable.

long-range couplings from the α -methine of Pip¹ and a singlet methyl (δ 2.17) to a carbonyl carbon (δ 173.8). An *N*-methine proton (δ 4.19) showed long-range correlations to carbonyl (δ 175.4), methylene (δ 40.9) and methine (δ 26.3) carbons, the latter two of which were coupled to two doublet methyl groups (δ_{H} 0.96, 0.86) to form a leucine

residue (Leu¹). A similar spin system from an *N*-methine proton (δ 3.95) identified another leucine residue (Leu²). Seven couples of singlet methyls (δ_{H} 1.40~1.57) revealed ^1H - ^{13}C long-range couplings to the relevant seven quaternary carbons bearing nitrogen (δ 58.0~58.4) and seven carbonyl carbons (δ 176.2, 178.2, 177.4, 177.7,

Fig. 3 Mass fragmentation analysis for efrapeptin J (**1**).

Bold numbers show significant fragment peaks.

175.8, 177.3, 177.1), respectively. These data indicated the presence of seven α -aminoisobutyric acid residues (Aib¹~Aib⁷).

In the remaining part, C₁₃H₂₅N₃⁺, the COSY spectrum displayed three spin systems (shown by bold lines in Fig. 2), one of which was expanded to include geminal methyls (δ 0.92, 0.88) attached at C-12 due to long-range couplings from both 13-H₃ and 14-H₃ to C-11 and C-12 (Fig. 2). The ¹H and ¹³C chemical shifts required C-2, C-4, C-6, C-9 and C-10 to be bound to nitrogen atoms. Long-range couplings from 9-H₂ to C-2 and from 6-H₂ to C-4 indicated the connections *via* nitrogen atoms between C-2 and C-9 and between C-4 and C-6. The remaining carbon (δ 166.8) coupled with the four methylenes (2-H₂, 6-H₂, 8-H₂, 9-H₂) was attributed to an amidinium carbon in a hexahydropyrrolo[1,2-*a*]pyrimidinium skeleton (HPP), and this assignment completed all the units of **1**.

In the HMBC spectrum, the α -protons of Pip², Leu¹, Gly, Pip³ and Leu² and the β -methylene protons of β -Ala revealed long-range correlations to carbonyl carbons in adjacent amino acid residues to establish Aib¹-Pip², Aib³-Leu¹- β -Ala-Gly, Aib⁵-Pip³ and Ala-Leu² sequences (Fig. 2).

In the FAB mass spectrum, most fragment peaks included the C-terminal amidinium ion according to

charge-remote fragmentation [11] (Fig. 3). Distinct fragment peaks with 43 or 83 mass unit differences were assigned to the cleavage at both sides of the quaternary carbon of Aib and both sides of the piperidine ring of Pip. The amino acid sequence was established by assignments of sequential cleavages from the N-terminal of **1** as shown in Fig. 3. Efrapeptin J (**1**), a new member of the efrapeptin family, is a linear pentadecapeptide containing a hexahydropyrrolo[1,2-*a*]pyrimidinium moiety as shown in Fig. 1.

Biological activity

Luciferase reporter assay was carried out using human fibrosarcoma HT1080 cells transformed with the luciferase gene under the control of the GRP78 promoter (HT1080 G-L). Treatment of HT1080 G-L cells with 10 mM of 2-deoxyglucose (2DG) for 18 hours increased luciferase activity about 5-fold compared with non-treated control. Efrapeptins J, F and G dose-dependently inhibited the luciferase expression by 2DG with IC₅₀s of 18, 8.5 and 3.3 nM, respectively (Fig. 4). Under the same condition, the efrapeptins did not release lactate dehydrogenase (LDH) from the cells (Fig. 4), indicating that these inhibitory effects were not caused by cytotoxicity. Western blot analysis showed that **1** at 30 or 100 nM remarkably reduced

Fig. 4 Effects of efrapeptins on the luciferase expression and LDH release in HT1080 G-L cells.

HT1080 G-L cells were treated with efrapeptin J (●, ○), F (■, □) or G (▲, △) in the presence of 2-deoxyglucose (10 mM) for 18 hours at 37°C. The relative luciferase activity compared with non-treated control (●, ■, ▲) was measured with a luminometer. The LDH release (○, □, △) was calculated as free LDH/total LDH using LDH-Cytotoxic Test Wako.

Fig. 5 Western blot analysis of GRP78 expression.

HT1080 cells or MKN-74 cells were treated with efrapeptin J (1) in the presence or absence of an ER-stress inducer for 18 hours. The cell lysate was analyzed by SDS-PAGE followed by Western blotting. TM, A23187 and 2DG indicate 6.25 µg/ml of tunicamycin, 4.0 µM of A23187 and 10 mM of 2-deoxyglucose, respectively.

the expression of GRP78 protein in 2DG-treated HT1080 cells (Fig. 5). Efrapeptin J also inhibited GRP78 expression in MKN74 human gastric cancer cells (Fig. 5). Various ER-stress inducers were tested for their effect on the activity of 1. GRP78 induction by tunicamycin or glucose deprivation was inhibited by 1, although 1 did not affect the induction of GRP78 by the calcium ionophore A23187 (Fig. 5). The effect of 1 on cell viability under ER-stress condition was

Fig. 6 Selective cytotoxicity of efrapeptin J (1) in the presence of an ER-stress inducer.

HT1080 cells were treated with efrapeptin J for 18 hours in the presence or absence of an ER-stress inducer. The cells were plated and incubated for 7 days to form colonies. The cell viability was calculated as percent of colony number in control culture. 2DG: 2-deoxyglucose (10 mM).

evaluated using colony formation assay. Efrapeptin J induced cell death in HT1080 cells in the presence of 2DG or under a glucose-deprived condition, whereas no cytotoxicity was observed in HT1080 cells treated with only 1 (Fig. 6).

Discussion

A series of fungal peptides are known as the peptaibols [12], which contain a high proportion of α,α -dialkylated amino acids such as Aib and isovaline, an acetylated *N*-terminus and a *C*-terminal amino alcohol. The structure of 1 appears to be related to those of the peptaibols, but is distinguishable due to the presence of a *C*-terminal amidinium. Structurally related pyrrolo[1,2-*a*]pyrimidines are rarely found in natural products, which include efrapeptins and adenopeptin [9], an apoptosis inducer from *Chrysosporium* sp.

Efrapeptins have been reported to inhibit mitochondrial F_1F_0 -ATPase [13]. Among down-regulators of GRP78 reported, valinomycin [14] and piericidin A [15] are known as respiratory inhibitors and prunastatin A [16], JBIR-04 and JBIR-05 [17] are derivatives of antimycin A, a respiratory inhibitor targeting complex III. Thus respiratory inhibitors might inhibit transcriptional expression of GRP78 induced by ER stress. Further studies on the biological activities of efrapeptins are in progress.

Experimental

Microorganism

The efrapeptin J producing fungus, AMB18, was isolated from sea mud collected in Aomori prefecture (N 41°02'29.9", E 140°19'37.6"), Japan, and identified as *Tolypocladium* sp. from its 28S rDNA (D1-D2 region) sequence [18].

Efrapeptin J (1)

Colorless powder; melting point 132~137°C; FAB-MS (*m/z*) found: 1620.0610 (M^+), calcd. for $C_{81}H_{139}N_{18}O_{16}^+$: 1620.0616; $[\alpha]_D^{23} + 14^\circ$ (*c* 0.24, MeOH); UV (MeOH) end absorption; IR ν_{max} (KBr) 3300, 2940, 1670, 1540, 1440 cm^{-1} ; ^{13}C - and 1H -NMR (CD_3OD) see Table 1.

Spectroscopic Measurements

UV and IR spectra were measured on Shimadzu UV-1700 and JASCO FT/IR-410 spectrometers, respectively. Mass spectra were obtained on a JEOL SX-102A spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. NMR spectra were obtained on a JEOL JNM-LA400 spectrometer with 1H -NMR at 400 MHz and with ^{13}C -NMR at 100 MHz. Chemical shifts are given in ppm relative to CHD_2OD at 3.30 ppm for 1H -NMR and CD_3OD at 49.0 ppm for ^{13}C -NMR.

Cells and Cell Culture

HT1080 G-L cells were established by transfection of HT1080 human fibrosarcoma cells with the plasmid pGRP70pro160-Luc containing a firefly luciferase gene under the control of the GRP78 promoter [19]. MKN-74 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB). All the cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose at 37°C.

Reporter Assay

HT1080 G-L cells were plated into 96-well plates at a density of 2×10^3 cells/well. After incubation for 6 hours at 37°C, the cells were treated with samples in the presence or absence of 2-deoxyglucose (10 mM) for 18 hours at 37°C. After the medium was removed, 100 μ l of Cell Culture Lysis Reagent (Promega) were added to each well. Each 50 μ l portion of the cell lysate was transferred into a white 96-well plate. Luciferase activity was measured with an ABI TR717 microplate luminometer (Applied Biosystems) using Luciferase Assay Reagent (Promega).

LDH Release Assay

HT1080 G-L cells were plated into 96-well plates at a density of 2×10^3 cells/well. After incubation for 6 hours at 37°C, the cells were cultured with various concentrations of samples for 18 hours at 37°C. The conditioned medium was transferred into another 96-well plate, and the residual cells were dissolved in 100 μ l of 0.5% Triton X-100. LDH-Cytotoxic Test Wako (Wako Pure Chemical Industries) was used to measure lactate dehydrogenase (LDH) concentrations in the conditioned medium and cell lysate. The rate of free LDH was calculated according to the equation below by using the LDH concentration in the conditioned medium as the amount of free LDH and the LDH concentration in the cell lysate as the amount of intracellular LDH. Rate of free LDH (%) = (amount of free LDH)/(amount of free LDH + amount of intracellular LDH) $\times 100$.

Western Blotting

HT1080 cells or MLN-74 cells were plated in 9-cm Petri dishes at a density of 2×10^5 cells/dish. After incubation with or without samples under ER stress for 18 hours at 37°C, the cells were collected using a cell scraper and lysed in a SDS sample buffer consisting of 10% glycerol, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS) and 62.5 mM Tris-HCl (pH 6.8). Protein concentrations of the lysates were measured with a Bio-Rad protein assay kit. Aliquots containing 45 μ g of protein were resolved on 5~20% SDS-polyacrylamide gel and transferred by electroblotting to a polyvinylidene difluoride (PVDF) membrane. The membrane was pretreated with a blocking buffer (0.1% Tween 20, 80 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , 100 mM NaCl) containing 10% skim milk for 20 minutes and incubated with 2.0 μ g/ml of mouse monoclonal anti-KDEL (Stressgen) in a blocking buffer containing 4.0% skim milk for 1 hour. The membrane was washed with a blocking buffer containing 4.0% skim milk and incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin antibody (1:2000) (Amersham Pharmacia Biotech). After washing with a blocking buffer, the protein expression of GRP78 was detected with an ECL detection system (Amersham Pharmacia Biotech).

Colony Formation Assay

HT1080 cells were plated in 9-cm Petri dishes at a density of 2×10^5 cells/dish. The cells were treated with various concentrations of samples in the presence or absence of 2DG (10 mM) or under a glucose-deprived condition for 18 hours. Trypsinized cells were plated at 200 cells/dish and incubated for 7 days to form colonies. After the colonies

were stained with 0.01% crystal violet, the cell viability was calculated as percent of colony number in control culture.

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