

Prunustatin A, a Novel GRP78 Molecular Chaperone Down-regulator Isolated from *Streptomyces violaceoniger*

Yukiko Umeda, Shuhei Chijiwa, Kazuo Furihata, Keiko Furihata, Shohei Sakuda, Hiromichi Nagasawa, Hidenori Watanabe, Kazuo Shin-ya

Received: October 18, 2004 / Accepted: February 14, 2005

© Japan Antibiotics Research Association

Abstract In the course of our screening program for regulators of a molecular chaperone GRP78 expression, we isolated a novel inhibitor of GRP78 expression, designated as prunustatin A, from *Streptomyces violaceoniger* 4521-SVS3. The structure of prunustatin A was determined by a series of NMR analyses to be an oxidized type of the neoantimycin family. Prunustatin A inhibited the expression of GRP78 induced by 2-deoxyglucose in human fibrosarcoma HT1080 cells accompanied by global cell death without showing cytotoxicity under normal nutrient condition.

Keywords molecular chaperone, GRP78, glucose deprivation, prunustatin

GRP78 acts as a molecular chaperone in the endoplasmic reticulum (ER) to promote protein folding. The enhancement of ER stress response has been proven to play a role in mechanisms of resistance to chemotherapy and hypoglycemic stress in solid tumor [1], while the reduction of ER stress response is involved in the pathology of central nervous diseases such as Alzheimer's and Parkinson's diseases [2]. The ER stress response causes an increase in gene expression of a number of ER chaperones such as GRP78/Bip and GRP94 [3]. Thus, substances that directly, either down- or up-regulate *grp78* transcription

are expected to be promising drugs for the treatment of cancer and Alzheimer's disease, respectively. In the course of our screening program for regulators of GRP78/Bip molecular chaperone expression, we isolated versipelostatatin [4] by employing a reporter gene assay system under the control of the GRP78 promoter. Further screening resulted in the isolation of a novel inhibitor of GRP78 expression, designated as prunustatin A (**1**, Figure 1), a new member of neoantimycin [5] family from an actinomycete identified as *Streptomyces violaceoniger* 4521-SVS3. In this paper, we report the fermentation of the producing organism and the isolation, physicochemical properties, structure elucidation and brief biological activities of prunustatin A.

The prunustatin producing strain was isolated from a soil sample collected on Kumejima island in Okinawa

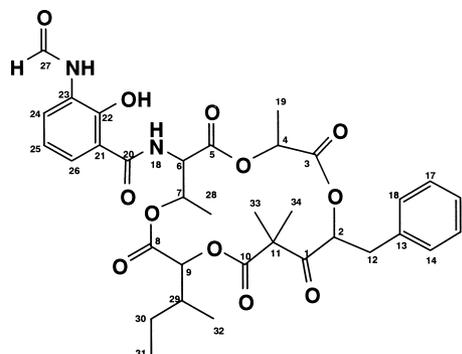


Fig. 1 Structure of prunustatin A (**1**).

K. Shin-ya (Corresponding author), **Y. Umeda**, **S. Chijiwa**, **Keiko Furihata**: Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan, E-mail: kshin@iam.u-tokyo.ac.jp

Kazuo Furihata, **S. Sakuda**, **H. Nagasawa**, **H. Watanabe**: Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Table 1 Physico-chemical properties of prunustatin A (**1**)

Appearance	Pale yellow powder
Melting point	103~106°C
$[\alpha]_D^{27}$ (c 0.01, CHCl ₃)	+21.2°
Molecular formula	C ₃₄ H ₄₀ N ₂ O ₁₂
HR-FABMS (<i>m/z</i>)	found: 669.2668 [M+H] ⁺ calcd: 669.2660
UV λ_{\max} (MeOH) nm (ϵ)	320 (6480)
IR ν_{\max} (KBr) cm ⁻¹	3400, 1750, 1720, 1690

prefecture, and was subcultured on a Benett agar slant. It was cultivated in a seed medium consisting of starch 1.0%, polypepton 1.0%, molasses 1.0% and meat extract 1.0% (pH 7.2) for 2 days at 27°C on a rotary shaker. The seed culture was inoculated into 500-ml Erlenmeyer flasks each containing 100 ml of the production medium composed of starch 2.5%, soybean meal 1.5%, dry yeast 0.2% and CaCO₃ 0.4% (pH 6.4 before sterilization), and these were cultivated on a rotary shaker (200 rpm) for 5 days at 27°C.

The fermented whole broth (2 liters) was centrifuged to obtain a mycelial cake, which was extracted with acetone (1 liter ×2). The extract was concentrated *in vacuo*, and the residual aqueous solution was extracted twice with ethyl acetate. The separated organic layer was dried over Na₂SO₄, filtered and the filtrate was concentrated *in vacuo*. The active material was applied to a silica gel column packed with the solvent mixture of *n*-hexane and ethyl acetate (2:1). The column was developed with the same solvent system and active fractions were pooled and concentrated to dryness. The dry residue was rechromatographed on Sephadex LH-20 in CHCl₃-MeOH (1:1). The active fraction was evaporated *in vacuo* to dryness and the residue was further purified by preparative HPLC using PEGASIL-ODS (Senshu Pak, 20 i.d. ×250 mm) developed with 80% aqueous MeOH to give a pure sample of prunustatin A (**1**) as pale yellow powder.

The physico-chemical properties of **1** are summarized in Table 1. The molecular formula of **1** was established as C₃₄H₄₀N₂O₁₂ on the basis of HRFAB-MS analysis. The direct connectivity of protons and carbons were established by the HMQC spectrum and the tabulated ¹³C and ¹H NMR spectral data for **1** are shown in Table 2. The DQF-COSY and HMBC spectra established four partial structures (Fig. 2). The proton-proton correlations observed in the DQF-COSY include 14, 18-H (δ_{H} 7.03), 15, 17-H (δ_{H} 7.23) and 16-H (δ_{H} 7.22) revealed the presence of a benzene ring moiety. The ¹H-¹H couplings between an oxymethine proton 2-H (δ_{H} 5.66, δ_{C} 78.6) and methylene

Table 2 ¹H and ¹³C NMR data for prunustatin A (**1**) in CDCl₃

No.	δ_{C}	δ_{H}
1	202.9	
2	78.6	5.66 (t, 6.0 Hz)
3	168.3	
4	69.9	5.40 (q, 7.0 Hz)
5	167.7	
6	55.5	5.13 (dd, 9.0, 2.0 Hz)
7	71.4	5.85 (dq, 7.0, 2.0 Hz)
8	168.4	
9	76.2	4.78 (d, 8.0 Hz)
10	172.1	
11	54.4	
12	36.9	3.32 (dd, 14.0, 6.0 Hz) 3.24 (dd, 14.0, 6.0 Hz)
13	135.0	
14,18	129.9	7.03 (2H, dd, 8.0, 2.0 Hz)
15,17	128.5	7.23 (2H, m)
16	127.3	7.22 (m)
19	17.5	1.43 (3H, d, 7.0 Hz)
20	170.3	
21	112.8	
22	150.6	
23	127.3	
24	124.8	8.55 (dd, 8.0, 1.0 Hz)
25	119.0	6.93 (t, 8.0 Hz)
26	120.5	7.37 (d, 8.0 Hz)
27	158.9	8.49 (s)
28	16.7	1.29 (3H, d, 7.0 Hz)
29	36.2	1.92 (m)
30	24.6	1.46 (m) 1.16 (m)
31	10.5	0.84 (3H, d, 7.0 Hz)
32	14.4	0.86 (3H, d, 7.0 Hz)
33	22.2	1.28 (3H, s)
34	20.7	1.02 (3H, s)
23-NH		7.90 (s)
6-NH		7.16 (d, 9.0 Hz)

¹H and ¹³C NMR were observed at 500 and 125 MHz, respectively.

protons 12-H (δ_{H} 3.32, 3.24), together with ¹H-¹³C long-range couplings in HMBC spectrum from 2-H, 12-H and 15, 17-H to C-13 (δ_{C} 135.0), 14-H and 18-H to C-12 (δ_{C} 36.9) established the phenethyl residue. The protons of two singlet methyl groups 33-H (δ_{H} 1.28) and 34-H (δ_{H} 1.02) were each long-range coupled to an ester carbonyl carbon C-10 (δ_{C} 172.1), a quaternary carbon C-11 (δ_{C} 54.4) and a ketone carbonyl carbon C-1 (δ_{C} 202.9), which in turn long-range coupled to 2-H. These HMBC

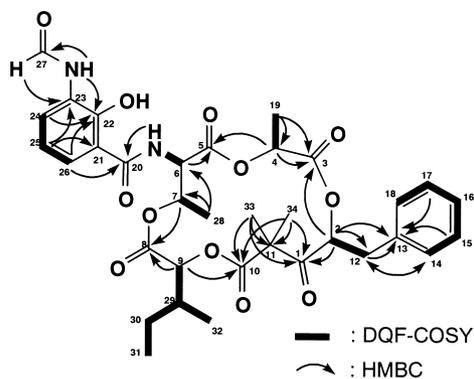


Fig. 2 DQF-COSY and HMBC analyses of **1**.

correlations indicated the successive connectivity of C-2, C-1 and C-11 as shown in Figure 2. Thus, a 4-hydroxy-2,2-dimethyl-3-oxo-5-phenylpentanoic acid moiety was elucidated as a partial structure of **1** as shown in Figure 2. A 2-hydroxypropionic acid moiety was established by ^1H - ^1H correlation between an oxymethine proton 4-H (δ_{H} 5.40, δ_{C} 69.9) and a methyl proton 19-H (δ_{H} 1.43), and ^1H - ^{13}C long-range correlations between 19-H and C-3 (δ_{C} 168.3).

The proton sequence between the aromatic protons 24-H (δ_{H} 8.55), 25-H (δ_{H} 6.93) and 26-H (δ_{H} 7.37) indicated a 1,2,3-trisubstituted benzene ring moiety. An amide proton 23-NH (δ_{H} 7.90) was long-range coupled to an aldehyde carbon C-27 (δ_{C} 158.9), which was considered to connect directly with this amide nitrogen atom from its ^{13}C chemical shift. The aldehyde proton 27-H (δ_{H} 8.49) and the aromatic proton 25-H were long-range coupled to an aromatic carbon C-23 (δ_{C} 127.3). These results suggested that the formylamino group was substituted at the C-23 position. The aromatic proton 26-H was long-range coupled to a carbonyl carbon C-20 (δ_{C} 170.3) at the peri position, which indicated that this carbonyl carbon was substituted at C-21. The aromatic protons 24-H and 26-H in addition to an amide proton 23-NH (δ_{H} 7.90) were long-range coupled to C-22 (δ_{C} 150.6). By taking into consideration these ^{13}C chemical shifts of aromatic carbons, an oxygen atom should be substituted at the C-22 position. The UV spectrum of **1** also suggested the existence of the 2-hydroxy-3-formylamino-benzoic acid moiety, which is the same chromophore as that of the antimycins [6, 7]. An amide proton 6-NH (δ_{H} 7.16) was coupled with 6-H (δ_{H} 5.13) which is considered to be an α -methine proton of an amino acid residue from its typical ^{13}C chemical shift (δ_{C} 55.5). A methyl proton 28-H (δ_{H} 1.29), which was coupled with an oxymethine proton 7-H (δ_{H} 5.85), was long-range coupled to C-6 and C-7 (δ_{C} 71.4). In addition to these correlations, the long-range

couplings from the methine proton 6-H to a carbonyl carbon C-5 (δ_{C} 167.7), and from the amide proton 6-NH to C-20 were observed. These results indicated the threonine residue and the connectivity with the chromophore moiety.

The remaining substructure was also determined by the interpretation of ^1H - ^1H spin couplings and ^1H - ^{13}C long-range couplings as follows. The long-range coupling between 9-H (δ_{H} 4.78) and a carbonyl carbon C-8 (δ_{C} 168.4), along with the sequence from 9-H to 31-H (δ_{H} 0.84) through 29-H (δ_{H} 1.92), which was additionally coupled to a methyl proton 32-H (δ_{H} 0.86), and 30-H (δ_{H} 1.16, 1.46) observed in the DQF-COSY spectrum of **1** established a 2-hydroxy-3-methylpentanoic acid moiety as shown in Figure 2. The connectivity of these partial structures were elucidated by the long-range couplings between 2-H and C-3, 4-H and C-5, 7-H and C-8, and 9-H and C-10. In this manner, the structure of **1** was determined as shown in Figure 1. As an analog of prunustatin A with a 15-membered macrocyclic, SW-163A, which can be considered as a reduced type of prunustatin A at the C-1 position, has been reported as an immunosuppressant [8].

The human fibrosarcoma cell line HT1080 cells which were transformed with the luciferase reporter gene under the regulation of the GRP78 promoter (designated as HT1080 G-L), produced luciferase in four times greater quantity than did the control when treated with 10 mM of 2-deoxyglucose (2-DG). In this evaluation system employed as a screen, prunustatin A reduced the expression of the reporter gene with an IC_{50} value of 11.5 nM. In the same manner, the structurally related compound SW-163 A [8], which was also produced by the prunustatin producing strain, exhibited inhibitory activity in this assay with an IC_{50} value of 22.9 nM. Prunustatin A completely inhibited the expression of GRP78 at the protein level at the concentrations of more than 80 nM in HT1080 cells without affecting actin level. Under normal condition, prunustatin A showed cytostatic effect but not exhibited cytotoxicity even at the concentration of 500 nM. On the other hand, prunustatin A treatment became highly toxic in 2-DG containing medium resulting in approximately six times greater cell death ratio at the concentration of 100 nM than that in normal conditioned medium.

Since SW-163A showed the similar activity to **1**, the redox state at the C-1 position is not essential for this biological activity. Although the chromophore of **1** is same as that of antimycins [6, 7], which has a 9-membered macrocyclic substructure, it is interesting that prunustatin A is stable to oxidative insult, contrary to the vulnerability of antimycins to oxidative and acidic condition. As observed with versipelostatin [9], prunustatin A induced global cell death under glucose deprived condition such as

the treatment with 2-DG but not under normal nutrient conditions. Thus, it is expected that prunostatin A would be a useful cancer chemotherapeutic agent against solid tumors. Studies on the elucidation of the absolute structure and detailed biological activities of **1** are now underway.

Acknowledgments This work was supported in part by Industrial Technology Research Grant Program in 01A04006b from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

Reference

1. Katschinski DM, Jacobson EL, Wiedemann GJ, Robins HI. Modulation of VP-16 cytotoxicity by carboplatin and 41.8°C hyperthermia. *Cancer Res Clin Oncol* 127: 425–435 (2001)
2. Imaizumi K, Miyoshi K, Katayama T, Yoneda T, Taniguchi M, Kubo T, Tohyama M. The unfolded protein response and Alzheimer's disease. *Biochim Biophys Acta* 1536: 85–96 (2001)
3. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 13: 1211–1233 (1999)
4. Park HR, Furihata K, Hayakawa Y, Shin-ya K. Versipelostatin, a novel GRP78/Bip molecular chaperone down-regulator of microbial origin. *Tetrahedron Lett* 43: 6941–6945 (2002)
5. Caglioti L, Misiti D, Mondelli R, Selva A, Arcamone F, Cassinelli G. The structure of neoantimycin. *Tetrahedron* 25: 2193–2221 (1969)
6. Watanabe K, Tanaka T, Fukuhara K, Miyairi N, Yonehara H, Umezawa H. Blastmycin: a new antibiotic from *Streptomyces* sp. *J Antibiot* 10A: 39–45 (1957)
7. Kinoshita M, Wada M, Umezawa S. The total syntheses of a diastereomeric mixture of antimycin A3 (blastmycin). *J Antibiot* 22: 580–582 (1969)
8. Takahashi K, Tsuda E, Kurosawa K. SW-163A and B, novel immunosuppressants produced by *Streptomyces* sp. *J Antibiot* 54: 867–873 (2001)
9. Park HR, Tomida A, Sato S, Tsukumo Y, Yun J, Yamori T, Hayakawa Y, Tsuruo T, Shin-ya K. Blockade of survival response to glucose deprivation for selective killing of tumor cells. *J Natl Can Inst* 96: 1300–1330 (2004)