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MK800-62F1, a New Inhibitor of Apoptotic Cell Death, from *Streptomyces diastatochromogenes* MK800-62F1

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activity

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A new compound, MK800-62F1, was isolated from a cultured broth of *Streptomyces diastatochromogenes* MK800-62F1. It inhibited H_2O_2-induced apoptosis in human small cell lung carcinoma Ms-1 cells as well as in human T-cell leukemia Jurkat cells. In addition, MK800-62F1 also inhibited camptothecin-induced apoptosis in Jurkat cells, which was mediated by intracellular H_2O_2 generation. MK800-62F1 did not exhibit antioxidative activity *in vitro*, suggesting that inhibition of apoptosis by MK800-62F1 was not due to the scavenging of H_2O_2, rather it was due to the modulation of the downstream event of H_2O_2 generation.

Apoptosis is of central importance for development and homeostasis in tissues of multicellular organisms[1]. In addition, it plays a major role in many diseases including cancer[2], acquired immunodeficiency syndrome[3] and neurodegenerative disorders[4]. Thus, the elucidation of the molecular mechanisms of apoptosis is important for the development of new drugs for these various diseases.

Caspase-3 protease plays important roles in the signaling pathway controlling mammalian apoptosis. Recent studies have suggested that proteolytic cleavage and activation of caspase-3 may be functionally important in the induction of apoptosis. The immature 32-kDa caspase-3 is cleaved into a 12-kDa fragment and 17-kDa biologically active caspase-3[5]. Previously, we reported that an anticancer drug-induced apoptosis was commonly mediated by intracellular H_2O_2 generated through activation of caspase-3 protease[6]. However, the signaling pathway from the H_2O_2 generation to cell death is still unclear. Thus, the inhibitor of this signaling pathway may be useful for the elucidation of the mechanism of apoptosis.

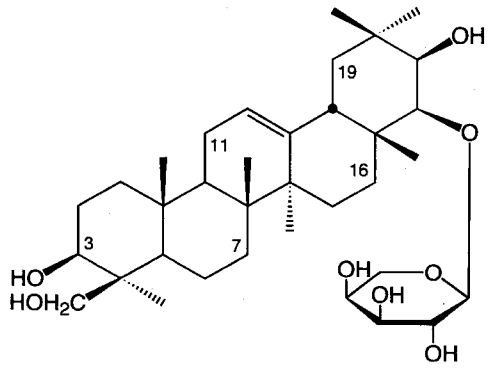
In the course of screening for inhibitors of H_2O_2-induced apoptosis from the cultured broth of microorganisms, we found that a strain of *Streptomyces diastatochromogenes* MK800-62F1 produced a novel compound named MK800-62F1 (Fig. 1). In this paper, the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties and biological activities of MK800-62F1 are described. The structure elucidation of MK800-62F1 will be described in the following paper[7].

Materials and Methods

Microorganism

The MK800-62F1 producing organism, the strain MK800-62F1, was isolated from a soil sample collected in Syasui-gun, Toyama Prefecture, Japan. *Streptomyces diastatochromogenes* IMC S-0712 (ISP 5449) and *Streptomyces neyagawaensis* IMC S-0815 (ISP 5588) were compared taxonomically with strain MK800-62F1.

Fig. 1. Structure of MK800-62F1.



Taxonomic Studies

Morphological observations were made with a scanning electron microscope (Hitachi S-570). Cultural and physiological characteristics of the strain MK800-62F1 were determined by the methods of SHIRLING and GOTTLIEB[8] and by the methods of WAKSMAN[9]. Permissive temperatures for growth were determined on glucose-asparagine agar (glucose 1.0%, asparagine 0.05%, K_2HPO_4 0.05%, agar 2.0%, pH adjusted to 7.0 before sterilization). Color codes were assigned to the substrate, and aerial mass color including soluble pigments according to the Color Harmony Manual, 1958 (Container Corporation of America, Chicago, Illinois). Carbohydrate utilization was investigated by using the procedure of PRINDHAM and GOTTLIEB[10]. Diaminopimelic acid isomers in the grown culture were investigated according to the method of BECKER *et al.*[11]. Menaquinones were extracted and analyzed using atmospheric pressure chemical ionization mass spectrometry with a Hitachi M-1200H mass spectrometer.

Fermentation

A slant culture of the strain MK800-62F1 was inoculated into 125 ml of a seed culture medium containing sucrose 4%, soybean meal 2.5%, NaCl 0.25%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $MnCl_2 \cdot 4H_2O$ 0.0005%, $ZnSO_4 \cdot 7H_2O$ 0.0005% (adjusted to pH 7.4 before sterilization) in a 500-ml Sakaguchi flask and cultured by incubating at 27°C for 2 days on a reciprocal shaker (110 rpm). For production of MK800-62F1, the seed culture (4 ml) was inoculated into 125 ml of the same medium in a 500-ml Sakaguchi flask, and incubated at 27°C for 5 days on a reciprocal shaker.

Cell Culture

Human small cell lung carcinoma Ms-1 cells[12] and human T-cell leukemia Jurkat cells were cultured in RPMI 1640 medium (Nissui) supplemented with 5% FBS at 37°C in a 5% CO_2-95% air atmosphere. The inhibitory activities of MK800-62F1 for apoptotic cell death were determined by trypan blue dye exclusion assay.

Measurement of *In Vitro* Antioxidative Activity

2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA, Molecular Probes) was added to the RPMI 1640 medium containing 5% FBS at the final concentration of 5 μM. After addition of H_2O_2 to 0.3 mM in the presence or absence of various concentrations of MK800-62F1, the solution was incubated for 30 minutes at room temperature. Levels of DCFH-DA oxidation by H_2O_2 were measured by using a spectrofluorometer (Hitachi F-1300) with excitation at 488 nm and emission at 530 nm.

Results and Discussion

Taxonomic Studies

Strain MK800-62F1 formed branched substrate mycelia and aerial hyphae which bore spirals (5~10 turns). The mature spore chain consisted of 10 to 50 or more spores. The spore was 0.5~0.6×1.0~1.1 μm in size with a smooth surface. No synnemata, sclerotia, sporangia or motile spores were observed. The cultural characteristics of the strain MK800-62F1 on various agar media are summarized in Table 1. On various agar media, the substrate mycelia of this strain were pale yellow to pale yellowish-brown. The aerial mycelia were light-gray. The soluble pigments, without melanoid pigment, were not formed. Physiological characteristics and carbohydrate utilization of this strain are summarized in Table 2. The permissive temperature range for growth was 20°C to 37°C, with the optimal temperature for growth at 30°C. Formation of melanoid pigment was positive on ISP No. 6 medium, probably positive on ISP No. 1 and No. 7 media. This strain hydrolyzed starch. Whole-cell hydrolysates of strain MK800-62F1 contained L,L-diaminopimelic acid. The strain has MK-9 (H_8) and MK-9 (H_6) as the major components of menaquinones.

The taxonomic properties given above suggested that this strain belonged to the genus *Streptomyces*, and we searched the data of the known *Streptomyces* species. As a result, it was considered that *S. diastatochromogenes* and *S. neyagawaensis* were closely related to strain MK800-62F1. Accordingly, strain MK800-62F1 was compared with *S. diastatochromogenes* IMC S-0712 (ISP 5449) and *S.*

Table 1. Cultural characteristics of strain MK800-62F1.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Pale yellowish orange [3ca, Pearl Pink]~Pale yellow [2ea, Lt Wheat]	Thin, white	None
Glycerol-asparagine agar (ISP No.5)	Pale yellow [2ca, Lt Ivory~ 2ea, Lt Wheat]	Light gray [2fe, Covert Gray~ 3fe, Silver Gray]	None
Inorganic salts-starch agar (ISP No.4)	Colorless~ Pale yellow [2gc, Bamboo]	Light gray [2fe, Covert Gray~ 3fe, Silver Gray]	None
Tyrosine agar (ISP No.7)	Pale yellow [2gc, Bamboo]~Grayish yellow brown [3lg, Adobe Brown]	Light gray [2dc, Natural~ 2fe, Covert Gray]	Brown
Yeast extract-malt extract agar (ISP No.2)	Pale yellowish orange [3gc, Lt Tan]~ Pale yellowish brown [3ne, Topaz]	Light gray [2dc, Natural~ 2fe, Covert Gray]	None
Oatmeal agar (ISP No.3)	Pale yellow [2ea, Lt Wheat]	Light gray [2dc, Natural~ 2fe, Covert Gray]	None

Observation after incubation at 27℃ for 21 days.

neyagawaensis IMC S-0815 (ISP 5588) in more detail. As shown in Table 2, strain MK800-62F1 was considered to correspond to *S. diastatochromogenes* on the basis of the results. Therefore, the strain MK800-62F1 was identified as *S. diastatochromogenes* MK800-62F1.

A subculture of this strain has been deposited in the National Institute of Bioscience and Human-Technology, The Agency of Industrial Science and Technology, Tsukuba-shi, Ibaragi Prefecture, Japan, under the accession No. FERM P-17068.

Isolation and Purification

The time course study indicated that production of inhibitor of H_2O_2-induced apoptosis began at 3 days and reached a maximum after 5 days' incubation. The cultured broth (6.0 liters) at 5 days was separated into a mycelial cake and cultured filtrate (5.2 liters) by filtration. The mycelial cake was extracted with acetone (2.0 liters) and concentrated *in vacuo* to an aqueous solution (1.0 liter). The solution was combined with the broth filtrate, and extracted with *n*-hexane (5.0 liters, twice). The aqueous layer was applied to Diaion HP-20 column (600 ml), and the column was washed with water (2.0 liters) and then with 50% aqueous MeOH (2.0 liters). The active substance was eluted with MeOH (2.0 liters) and concentrated *in vacuo* to remove methanol. The concentrate was extracted with *n*-BuOH (1.0 liter, twice). The organic layer was concentrated to give a brown oily material (858.3 mg). The oily material was applied to silica gel column and the active substance was eluted with $CHCl_3$-MeOH (100 : 10 and 100 : 20). The active fractions were concentrated to give a yellow oil (25.1 mg). The yellow oil was applied to Toyopearl HW-40 column and eluted with $CHCl_3$-MeOH (1 : 1). The active fractions were pooled and concentrated to give a yellow powder (12.9 mg). The powder, thus obtained, was applied on preparative TLC, developed with $CHCl_3$-MeOH (100 : 30), and the active band was scraped off, and then eluted with $CHCl_3$-MeOH (100 : 30). The active material was applied to Toyopearl HW-40 column, eluted with MeOH and concentrated to give a pure MK800-62F1 as a white powder (3.5 mg).

Physico-chemical Properties

The physico-chemical properties of MK800-62F1 are summarized in Table 3. The compound was obtained as a white powder and is soluble in DMSO, pyridine, slightly

Table 2. Comparison of strain MK800-62F1, *Streptomyces diastatochromogenes* and *S. neyagawaensis*.

	Strain MK800-62F1	*S. diastatochromogenes* IMC S-0712 (ISP 5449)	*S. neyagawaensis* IMC S-0815 (ISP 5588)
Spore chain morphology	spiral	wave~spiral	spiral
Spore surface	smooth	smooth	smooth
Aerial mass color	Light gray	Light gray	Grayish white~Light gray
Reverse side color	Pale yellow~ Pale yellowish brown	Pale yellow~ Pale yellowish brown	Pale yellow~ Gray yellowish brown
Soluble pigment	−	−	−
Formation of melanoid pigment			
ISP No.1	(+)	+	(+)
ISP No.6	+	+	+
ISP No.7	(+)	+	+
Reduction of nitrate	−	−	−
Hydrolysis of starch	+	+	+
Carbon utilization:			
L-Arabinose	+	+	+
D-Xylose	±	±	±
D-Glucose	+	+	+
D-Fructose	+	+	+
Sucrose	+	+	+
Inositol	±	+	(+)
Rhamnose	+	+	+
Raffinose	+	+	+
D-Mannitol	+	+	+

+ : utilization, (+) : probably utilization, ± : doubtful

soluble in MeOH, chloroform, but insoluble in *n*-hexane and water. The molecular formula for MK800-62F1 was determined to be $C_{35}H_{58}O_8$ on the basis of FAB-MS, HRFAB-MS and NMR spectra. MK800-62F1 gave a positive color reaction with molybdophosphoric acid-sulfuric acid and anisaldehyde-sulfuric acid reagents, but a negative color reaction with ninhydrin and Rydon-Smith reagents.

Biological Activity

When Ms-1 cells were treated with 0.3 mM H_2O_2, cell viability was dramatically decreased as estimated by trypan blue dye exclusion assay (Fig. 2). This H_2O_2-induced cell death was apoptosis as previously reported[6]. The MK800-62F1 (0.3~10 μg/ml) showed a weak cytotoxic effect in Ms-1 cells; however, it inhibited H_2O_2-induced apoptosis in a dose-dependent manner up to 10 μg/ml (Fig. 2, A). Similar effects were obtained when Jurkat cells were used instead of Ms-1 cells (Fig. 2, B).

Next, to rule out the possibility that inhibition of H_2O_2-induced apoptosis by MK800-62F1 was due to the scavenging of H_2O_2, we measured the antioxidative activity of MK800-62F1 by using 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) *in vitro*. Fluorescin is reactive with H_2O_2 to give a fluorescent compound, fluorescein. As shown in Table 4, H_2O_2 scavengers, such as *N*-acetyl-L-cysteine (NAC), inhibited DCFH-DA oxidation by H_2O_2. However, MK800-62F1 did not affect DCFH-DA oxidation by H_2O_2 up to 30 μg/ml, suggesting that inhibition of H_2O_2-

Table 3. Physico-chemical properties of MK800-62F1.

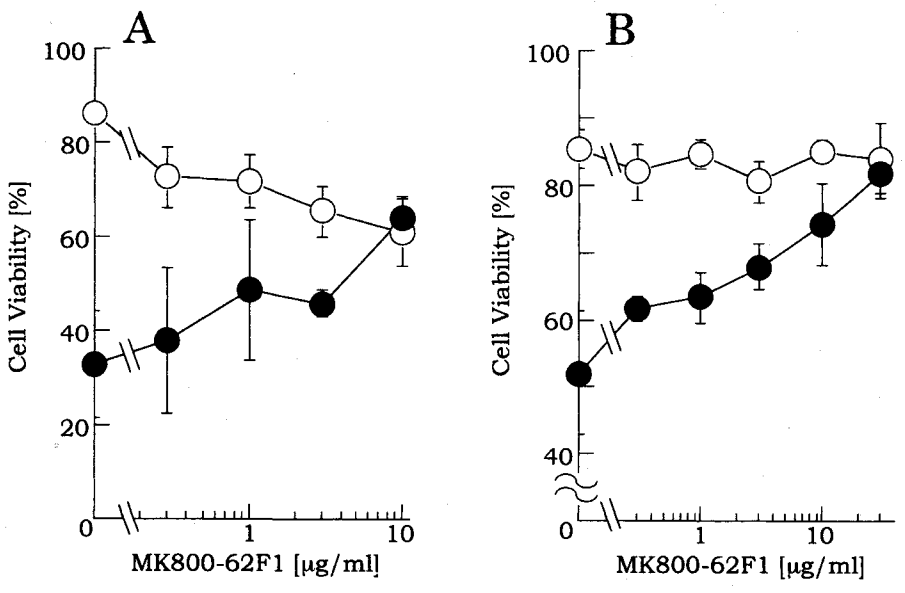
Appearance	White powder
MP (℃)	>200
$[\alpha]_D^{22}$ (c=1, pyridine)	+65°
Molecular formula	$C_{35}H_{58}O_8$
FAB-MS (*m/z*)	
Positive	607 $(M+H)^+$, 629 $(M+Na)^+$
Negative	605 $(M-H)^-$
HRFAB-MS (*m/z*, positive)	
Found	629.4029 $(M+Na)^+$
Calcd. for $C_{35}H_{58}O_8Na$	629.4058 $(M+Na)^+$
UV λ_{max}^{MeOH} (nm)	End absorption
IR ν_{max} (cm^{-1})	3413, 2929, 1643, 1384, 1080
Rf value on TLC [a]	0.42

[a] Silica gel 60 F_{254} (Art.5715, Merck) with $CHCl_3$-MeOH (5:1)

Table 4. Effect of MK800-62F1 on DCFH-DA oxidation by H_2O_2.

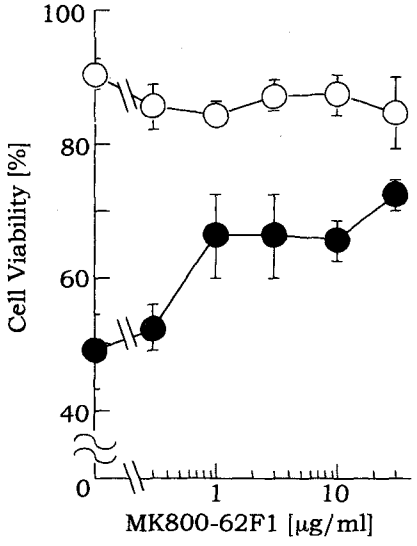
H_2O_2 [0.3 mM]	1577-33C [μg/ml]	Relative Fluorescence Intensity [%]
-	0	13.8
+	0	100.0
+	0.3	105.0
+	1	112.1
+	3	115.3
+	10	116.6
+	30	115.2
+	NAC 2 mM	15.8

Fig. 2. Effect of MK800-62F1 on H_2O_2-induced cell death.



Human small cell lung carcinoma, Ms-1 cells (A), or human T-cell leukemia, Jurkat cells (B), were treated with various concentrations of MK800-62F1 in the presence (●) or absence (○) of 0.2 mM H_2O_2 for 6 hours, 0.3 mM H_2O_2 for 8 hours, respectively. Cell viability was assessed by trypan blue dye exclusion assay. Values are means of four independent determinations; bars, SD.

Fig. 3. Effect of MK800-62F1 on camptothecin-induced cell death.



Jurkat cells were treated with various concentrations of MK800-62F1 in the presence (●) or absence (○) of 100 ng/ml of camptothecin for 16 hours. Cell viability was assessed by trypan blue dye exclusion assay. Values are means of four independent determinations; bars, SD.

induced apoptosis by MK800-62F1 was not due to the scavenging of H_2O_2.

Generation of H_2O_2 mediated by active caspase-3 is required for apoptosis induced by anticancer drugs such as camptothecin, vinblastine, inostamycin and adriamycin in Ms-1 cells and Jurkat cells[6]. Therefore, we examined whether MK800-62F1 inhibited anticancer drug-induced apoptosis. As shown in Fig. 3, camptothecin-induced apoptosis was inhibited by MK800-62F1 in Jurkat cells. These results indicate that MK800-62F1 inhibits apoptosis by modulating downstream events of H_2O_2 generation.

It is reported that soybean saponins inhibit H_2O_2 damage to mouse fibroblast cells[13,14]. These saponins might also inhibit anticancer drug-induced apoptosis because they have similar chemical structure to MK800-62F1. Thus, MK800-62F1 and its related compounds would be useful tools to investigate the biological significance of intracellular H_2O_2 generation in the signaling pathway of apoptosis. Furthermore, because neurodegenerative and inflammatory disorders are considered to be caused by H_2O_2-induced apoptosis, MK800-62F1 may provide a novel therapeutic drug for these diseases. Detailed studies on the biological activity of MK800-62F1 are in progress.

Acknowledgement

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References

1) STELLER, H.: Mechanisms and genes of cellular suicide. Science 267: 1445~1449, 1995
2) BERGMANN, A.; J. AGAPITE & H. STELLER: Mechanisms and control of programmed cell death in invertebrates. Oncogene 17: 3215~3223, 1998
3) THOMPSON, C. B.: Apoptosis in the pathogenesis and treatment of disease. Science 267: 1456~1462, 1995
4) MIZUNO, Y.; H. MOCHIZUKI, Y. SUGITA & K. GOTO: Apoptosis in neurodegenerative disorders. Intern. Med. 37: 192~193, 1998
5) HANNUN, Y. A.: Apoptosis and the dilemma of cancer chemotherapy. Blood 89: 1845~1853, 1997
6) SIMIZU, S.; M. TAKADA, K. UMEZAWA & M. IMOTO: Requirement of caspase-3(-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. J. Biol. Chem. 273: 26900~26907, 1998
7) YOSHIMOTO, Y.; T. SAWA, H. NAGANAWA, T. SUGAI, T. TAKEUCHI & M. IMOTO: MK800-62F1, a new inhibitor of apoptotic cell death, from *Streptomyces diastatochromogenes* MK800-62F1. II. Structural elucidation. J. Antibiotics 53: 575~578, 2000
8) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriology 16: 313~340, 1966
9) WAKSMAN, S. A.: Classification, identification and descriptions of genera and species. *In* The Actinomycetes, Vol. II. pp. 1~363, The Williams & Wilkins Co., Baltimore, 1961
10) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriology 56: 107~114, 1948
11) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
12) TAMURA, K.; M. TAKADA, I. KAWASE, T. TADA, S. KUDOH, K. OKISHIO, M. FUKUOKA, N. YAMAOKA, Y. FUJIWARA & M. YAMAKIDO: Enhancement of tumor radio-response by irinotecan in human lung tumor xenografts. Jpn. J. Cancer Res. 88: 218~223, 1997
13) YOSHIKOSHI, M.; Y. YOSHIKI, K. OKUBO, J. SETO & Y. SASAKI: Prevention of hydrogen peroxide damage by soybean saponins to mouse fibroblasts. Planta Med. 62: 252~255, 1996
14) MACFARLANE, N. G.; D. J. MILLER, G. L. SMITH & D. S. STEELE: Effects of oxidants on the sarcoplasmic reticulum of saponin-treated rat ventricular trabecular trabeculae. Cardiovasc. Res. 28: 1647~1652, 1994