MS-347a, A NEW INHIBITOR OF MYOSIN LIGHT CHAIN KINASE FROM *Aspergillus* sp. KY52178

SATOSHI NAKANISHI, KATSUHIKO ANDO, ISAO KAWAMOTO[†] and YUZURU MATSUDA*

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machida-shi, Tokyo 194, Japan

(Received for publication August 3, 1993)

MS-347a was isolated from the culture broths of *Aspergillus* sp. KY52178 as an inhibitor of smooth muscle myosin light chain kinase (MLCK). MS-347a inhibited the activity of chicken gizzard MLCK with an IC_{50} value of 9.2 μM. The inhibition was dependent on time of preincubation of MS-347a with the enzyme, suggesting irreversible inhibition. It is likely that the inhibitor binds to the catalytic domain of MLCK, since the compound inhibited not only calmodulin-dependent but also calmodulin-independent activity of MLCK. Calmodulin-dependent cyclic nucleotide phosphodiesterase, cAMP-dependent protein kinase and cGMP-dependent protein kinase were not inhibited by 150 μM MS-347a at all, although the compound inhibited protein kinase C with an IC_{50} value of 16 μM. MS-347b, a minor component was also isolated from the same culture broths. This minor component at 150 μM did not inhibit the activity of MLCK.

Myosin light chain kinase (MLCK) is a regulatory enzyme in smooth muscle contraction[1,2]. It is widely accepted that hormonal and neural signals for contraction induce increases of cytoplasmic Ca^{2+} concentrations in smooth muscle cells *via* receptor-mediated pathways. The rise of cytoplasmic Ca^{2+} is transduced to calmodulin, a ubiquitous Ca^{2+} binding protein, which activates MLCK to catalyze transfer of γ-phosphate of ATP to Ser-9 of the 20 kDa myosin light chain. Smooth muscle myosin, when phosphorylated in its light chain, can interact with actin and generate contractile force. Since smooth muscle cells are distributed in arteries and bronchi, inhibitors of MLCK would be potential vasodilators and bronchodilators.

During the course of our screening work, we found that a fungal strain *Aspergillus* sp. KY52178 produced inhibitors of MLCK, designated as MS-347a and MS-347b. In this article, we will describe production, isolation, and biological properties of these compounds.

Materials and Methods

Microorganism and Taxonomy

The fungal strain KY52178 was isolated from soil sample collected in Yamaguchi Prefecture, Japan. Colonies on 2% malt extract agar are 52 to 57 mm in diameter after culturing at 25°C for 7 days. The surface of a colony is velutinous and white at the marginal area and pale grayish green at the center. The color of the reverse of the colony is yellow. Soluble pale yellowish pigment is produced in this medium.

Colonies on potato-glucose agar are 59 to 63 mm in diameter after culturing at 25°C for 7 days. The surface of a colony is velutinous and white at marginal area and grayish blue at the center. The reverse of the colony is dark reddish brown. Soluble dark reddish brown pigment is produced in this medium.

Colonies on Czapek yeast extract agar are 74 to 78 mm in diameter after culturing at 25°C for 7 days.

[†] Present address: Tsukuba Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 2 Miyukigaoka, Tsukuba-city, Ibaraki 305, Japan

The surface of a colony is similar to that on 2% malt extract agar. The reverse of the colony is cream yellow.

Colonies on Czapek yeast extract agar with 20% sucrose are 78 to 80 mm in diameter after culturing at 25°C for 7 days. The surface of a colony is velutinous and white at marginal area and dark grayish blue at the center. The reverse of the colony is pale yellowish purple.

Smooth and colorless hyphae are developed on Czapek yeast extract agar. The hyphae are septate and well-branched, but not synnematous. The smooth and colorless conidiophores are formed on hyphae, and are aseptate, *ca.* 100 μm in length and 5 to 9.5 μm in width. Conidial heads are columnar. A vesicle is colorless and spherical or oval with 15 to 21 μm in diameter. The upper three quarters of the vesicle abundantly bears flask-shaped phialides which is 5 to 6.5 μm in length and 1.5 to 2.5 μm in width at the widest part. Metulae are not developed. The conidial ontology is enteroblastic. The phialidic conidia, formed in chains on the top of phialides, are single-celled and spherical or oval with 2 to 2.5 μm in diameter. Conidia are slightly rough at the surface and colorless, or are blue-green when they gather. No teleomorph was observed in this strain.

From the characteristics mentioned above, the fungal strain (KY52178) was identified as *Aspergillus* sp[3]. The fungus has been deposited at the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Japan, as FERM BP-3163.

Fermentation

A 50-ml culture tube containing 10 ml of a seed medium composed of dextrin 3.0% and V8 vegetable juice 20% (pH 6.5 before sterilized) was inoculated with the conidia of the organism grown on 2% malt extract agar. The inoculated tube was incubated for 4 days on a reciprocating shaker at 25°C. A 5-ml portion of the culture was transferred into a 300-ml Erlenmeyer flask containing 50 ml of the seed medium and the flask was incubated for 2 days on a rotary shaker at 25°C. A 5-ml portion of the second seed culture was further transferred into a 300-ml Erlenmeyer flask containing 50 ml of the following fermentation medium: glucose 2.0%, mashed potato powder 2.0%, peptone 0.5%, KH_2PO_4 0.5% and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05%. The flask was incubated for 5 to 6 days on a rotary shaker at 25°C. The growth of the fungus was monitored during fermentation by the measurement of crude nucleic acid content as described previously[4]. Detection of MS-347a and MS-347b in isolation procedures was carried out on a silica gel TLC plate (Merck, No. 5715) developed with chloroform-methanol (9:1) and visualized under UV light.

Enzyme Assay

The activity of MLCK was measured as described previously[5]. For monitoring the active components during fermentation and isolation, 10 μl of culture supernatant or methanol solution of partially purified materials was added to the reaction mixture containing, in a final volume of 0.25 ml, Tris-HCl 25 mM (pH 7.5), $MgCl_2$ 4 mM, $CaCl_2$ 0.2 mM, calmodulin 2.6 nM, peptide substrate 24 μM, MLCK 1.5 nM, and ATP 400 μM, and incubated for 30 minutes at 28°C. For precise analysis of the inhibition mechanism of MS-347a and MS-347b, the solution of the inhibitor (5 μl) was preincubated with the reaction mixture without ATP for 10 minutes at 28°C. The reaction was started by the addition of ATP, and the mixture was incubated for 30 minutes. The reaction was terminated by the addition of 0.1 ml of 10% acetic acid, and the reaction mixture was directly analyzed by HPLC as described[5].

Calmodulin-independent MLCK was prepared by partial digestion with trypsin as described.[6] The enzyme activity of calmodulin-independent enzyme was measured in the same reaction mixture except that EGTA 0.1 mM was added instead of $CaCl_2$ and calmodulin. The assay conditions of other enzymes were described previously[6].

Materials

MLCK was isolated from chicken gizzard smooth muscle as described[7]. Calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine brain cortex, cGMP-dependent protein kinase from porcine lung and protein kinase C from rat brain were prepared as described[8]. Peptide substrate for MLCK (KKRPQRATSNVFS-NH_2) was purchased from Peninsula Lab. Inc., U.S.A. Trypsin (type II-S, from soybean) and the catalytic subunit of cAMP-dependent protein kinase (from bovine heart) were obtained from Sigma Chemical Co. All other reagents were of HPLC or analytical grade.

Results

Fermentation

Aspergillus sp. KY52178 was cultured in 300-ml Erlenmeyer flasks containing the fermentation medium as described under Materials and Methods. Production of myosin light chain kinase inhibitors, growth of the fungus, and pH of the medium were monitored during fermentation (Fig. 1). The active components were produced mainly in mycelia, and increased as the fungus grew.

Isolation and Purification

Purification procedure of the active components is outlined in Fig. 2. The culture broth (5.5 liters) was centrifuged to obtain the mycelia, and the active components were extracted from the mycelia with methanol. The methanol solution was diluted with an equal volume of water, and then applied to a Diaion HP-20 resin column (1 liter). The column was washed with water followed by 50% methanol-water. The active components were eluted from the column with methanol. The eluate was concentrated *in vacuo*, and the resulting oil was partitioned between ethyl acetate and water. The ethyl acetate layer was then concentrated *in vacuo* to yield a brown oil (4.7 g). The oil was treated with a small volume of methanol, and the materials insoluble in methanol were applied to a silica gel column. The column was eluted sequentially with chloroform solutions containing 0%, 0.5%, 1%, 2%, 3% and 5% methanol. MS-347a was eluted in the 0.5% methanol-chloroform fraction, which contained most of the inhibitory activity against MLCK, and was purified further by crystallization in acetone solution. MS-347a was obtained as yellow needles (428.2 mg). MS-347b was obtained as a yellow powder (267.5 mg) by evaporation of the solvent from the 2% and 3% methanol-chloroform fractions.

Physico-chemical Properties

Physico-chemical properties of MS-347a and MS-347b are summarized in Table 1. The structures of both compounds (Fig. 3) were determined from their physico-chemical properties and spectral data as will be described in a separate paper[9]. MS-347b was identified as sydowinin B reported by HAMASAKI *et al.*[10,11], while MS-347a was found to be a new compound whose structure was closely related to that of sydowinin B.

Biological Properties

MS-347a inhibited Ca^{2+} and calmodulin-dependent activity of chicken gizzard MLCK in a concentration-dependent manner (Fig. 4). The maximal inhibition of the activity of MLCK was 90% and was attained around 30 μM of MS-347a. The concentration of MS-347a needed to inhibit the enzyme activity by 50% (IC_{50}) was 9.2 μM. In contrast, MS-347b at 150 μM did not inhibit the enzyme activity at all (Fig. 4).

Fig. 1. Time course of production of MLCK inhibitors in *Aspergillus* sp. KY52178.

Inhibition % of MLCK activity caused by culture supernatant (○) and cell extract (●), pH of culture broth (■), growth monitored with crude nucleic acid extraction (▲).

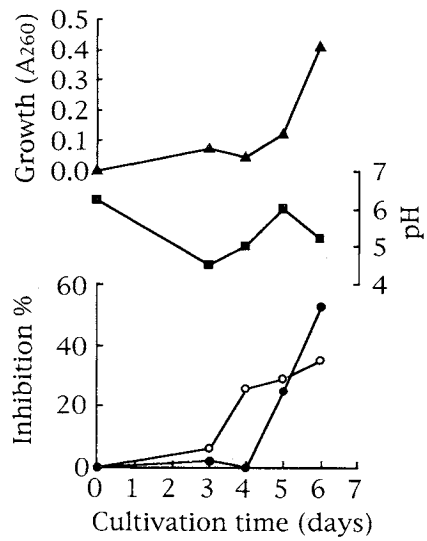


Fig. 2. Purification procedure of MS-347a and MS-347b.

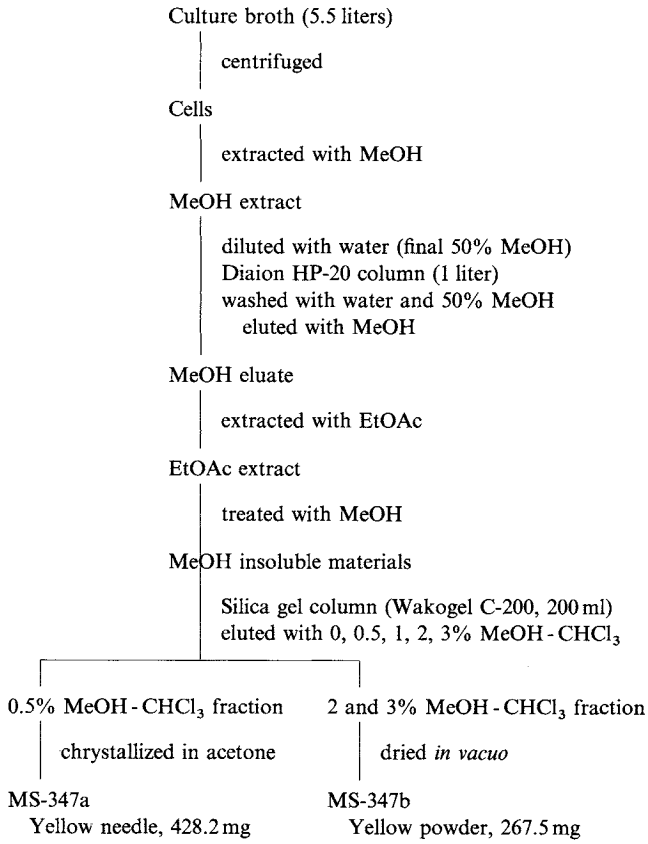


Table 1. Physico-chemical properties of MS-347a and MS-347b.

	MS-347a	MS-347b
Appearance	Yellow needle	Yellow powder
Color reaction		
Positive	Anisaldehyde, I_2, GIBB's reaction	I_2
Negative	Aniline-phthalate, Ninhydrin, Rydon-Smith reaction	
Solubility		
Soluble	DMSO, MeOH, $CHCl_3$, acetone, EtOAc, benzene	DMSO, MeOH, acetone, EtOAc
Insoluble	H_2O, hexane	H_2O
TLC, Rf		
$CHCl_3$ - MeOH, 9 : 1[a]	0.63	0.52
$CHCl_3$ - acetone, 9 : 1[a]	0.17	—[c]
70% MeOH - H_2O[b]	0.55	0.52
UV λ_{max}^{MeCN} nm (ε)	344 (3,600), 280 (27,000), 216 (19,000)	380 (6,100), 292 (9,700), 262 (34,800), 236 (26,400)

[a] Silica gel $60F_{254}$ (Merck, No. 5628).
[b] RP-$18F_{254}$S (Merck, No. 13724).
[c] Not tested.

Fig. 3. Structures of MS-347a and MS-347b.

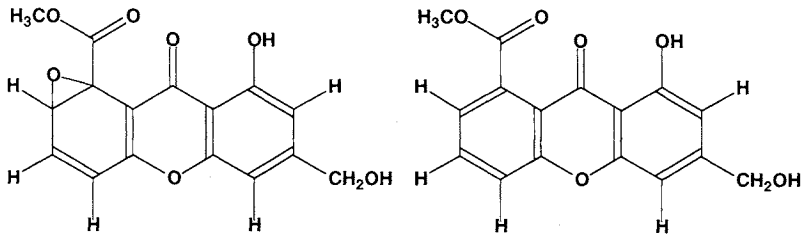


Fig. 4. Effects of MS-347a and MS-347b on the activity of MLCK.

Indicated concentrations of MS-347a (○) or 150 μM of MS-347b (●) were preincubated with the reaction mixture without ATP for 10 minutes at 28°C. The reaction was started by the addition of ATP, and the reaction mixture was incubated for further 30 minutes at 28°C. Means and standard errors of MLCK activity were shown.

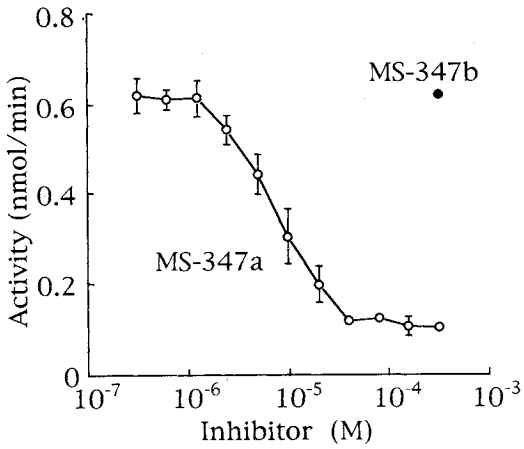
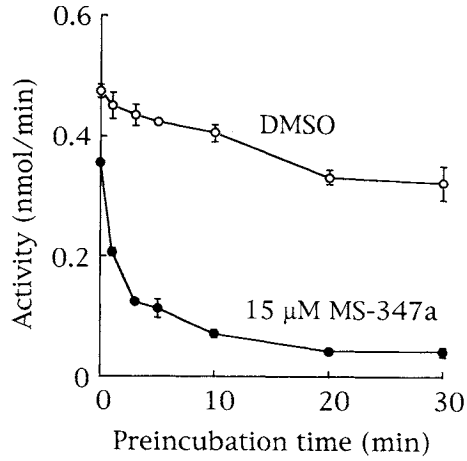


Fig. 5. Effect of preincubation time on susceptibility of MS-347a to inhibit the activity of MLCK.

MS-347a (15 μM, ●) or DMSO (2%, ○) were preincubated with the reaction mixture without ATP for indicated times. The reaction time was 30 minutes. Means and standard errors of MLCK activity were shown.



The inhibition of MLCK activity by MS-347a was dependent on preincubation time. MS-347a at 15 μM inhibited MLCK activity by 17%, when the activity was measured without preincubation (Fig. 5, time = 0). The inhibition was enhanced by increasing the preincubation time and reached maximum with 20 minutes of preincubation (Fig. 5).

The calmodulin-independent activity of MLCK which was generated by partial tryptic digestion of the enzyme was inhibited by MS-347a, but not by MS-347b. The IC_{50} values of both compounds for various enzyme activities including calmodulin-dependent and -independent activities of MLCK are summarized in Table 2. MS-347a inhibited protein kinase C as it did MLCK. MS-347b had no effect on all the enzyme activities tested so far.

The antibacterial spectrum of MS-347a is summarized in Table 3. MS-347b had no antibacterial activities at 100 μg/ml against microorganisms listed in Table 3.

Table 2. Effects of MS-347a and MS-347b on various enzyme activities.

Enzyme	IC_{50} (μM)	
	MS-347a	MS-347b
MLCK		
Calmodulin-dependent	9.2	>150
Calmodulin-independent	31	>150
Calmodulin-dependent cyclic nucleotide phosphodiesterase	>150	>150
cAMP-dependent protein kinase	>150	>150
cGMP-dependent protein kinase	>150	>150
Protein kinase C	16	>150

Table 3. Antibacterial activity of MS-347a.

Test organism	MIC (μg/ml)
Staphylococcus aureus ATCC 6538P	3.0
Streptococcus faecium ATCC 10541	3.0
Bacillus subtilis No. 10707	0.8
Escherichia coli ATCC 26	>100
Klebsiella pneumoniae ATCC 10031	>100
Proteus vulgaris ATCC 6897	0.4
Shigella sonnei ATCC 9290	>100
Salmonella typhi ATCC 9992	>100
Pseudomonas aeruginosa BMH No. 1	>100
Candida albicans ATCC 10231	>100

Discussion

We have isolated MS-347a and MS-347b from the culture broths of *Aspergillus* sp. KY52178, and demonstrated that MS-347a is a potent inhibitor of MLCK and that MS-347b is an inactive homologue of MS-347a. It is suggested from the differences in chemical structures of both compounds that the epoxide group of MS-347a is essential for its inhibitory activity against MLCK.

MLCK is known to have two domain structures: a calmodulin-binding domain and a catalytic domain[12,13]. Trypsin can cut out the catalytic domain[12], which is fully active and no longer calmodulin-dependent. MS-347a inhibited both calmodulin-dependent activity of the native enzyme and calmodulin-independent activity of the tryptic fragment of the enzyme in a similar concentration range (Table 2). Increasing the concentration of calmodulin in the assay mixtures did not restore the inhibition of calmodulin-dependent activity of MLCK by MS-347a (data not shown). These data suggest that MS-347a interacts with the catalytic domain of MLCK, not with calmodulin-binding domain nor calmodulin itself, to inhibit the enzyme activity. Preincubation of MLCK with MS-347a enhanced the inhibition of the enzyme activity, suggesting the effect of MS-347a on MLCK is irreversible. Taken together, MS-347a inhibits the enzyme activity possibly by covalent binding to the catalytic domain of MLCK *via* its epoxide group.

Several compounds from various sources have been reported as MLCK inhibitors: ML-9 synthesized chemically[14], kaempferol a plant product[15], KT5926 a chemical derivative of a microbial product[16], thyroid hormones[17], and wortmannin isolated from a fungus[6]. The potency of MS-347a to inhibit MLCK is intermediate compared with that of the other MLCK inhibitors in our assay system[5]. These compounds including MS-347a are diverse in their ability to inhibit various enzyme activities. Most of these compounds inhibit other protein kinases with more or less the same potency that they do MLCK. MS-347a does not inhibit cAMP-dependent protein kinase, cGMP-dependent protein kinase and calmodulin-dependent cyclic nucleotide phosphodiesterase, but inhibits protein kinase C as well as MLCK. MS-347b whose structure is homologous with MS-347a does not inhibit MLCK and other enzymes tested. Since their chemical structures are different from those of other MLCK inhibitors reported, MS-347a and MS-347b will be used as active and inactive control compounds, respectively, to study the functions of MLCK.

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