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MS-271, A Novel Inhibitor of Calmodulin-Activated Myosin Light Chain Kinase from *Streptomyces* sp.—I. Isolation, Structural Determination and Biological Properties of MS-271

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Abstract—A novel cyclic peptide, MS-271, was isolated from the culture broth of an actinomycete, *Streptomyces* sp. M-271 as an inhibitor of smooth muscle myosin light chain kinase (MLCK). MS-271 inhibited the MLCK from chicken gizzard with an IC_{50} value of 8 μM. MS-271 did not inhibit cyclic AMP-dependent protein kinase, protein kinase C or calcium/calmodulin-dependent cyclic nucleotide phosphodiesterase at concentrations up to 400 μM. The primary structure of MS-271 was identical to that of siamycin I, an anti-HIV peptide isolated from a microbial source.

Introduction

Myosin light chain kinase (MLCK) is thought to play an important role in smooth muscle contraction.[1,2] There is numerous evidence which shows that hormonal and neural signals for contraction induce increases in intracellular Ca^{2+} concentrations in smooth muscle cells via receptor-mediated pathways. The rise of cytoplasmic Ca^{2+} is transduced to calmodulin, an ubiquitous Ca^{2+}-binding protein. The calmodulin bound to Ca^{2+} can activate MLCK, which catalyzes the transfer of the γ-phosphate of ATP to Ser-9 of the 20 kDa myosin light chain. When the myosin light chain is phosphorylated, smooth muscle myosin can interact with actin to generate contractile force. Since smooth muscle cells are distributed in arteries and bronchi, inhibitors of MLCK could be therapeutically useful as potential vasodilators and bronchodilators.

In the course of our screening work to obtain inhibitors of MLCK from a microbial source, we found an inhibitor of MLCK, designated as MS-271, in the culture broth of an actinomycete, *Streptomyces* sp. M-271. In this paper, we will describe the isolation, primary structure determination and biological properties of MS-271.

Results

Production of MS-271 by fermentation

The time course of MS-271 production in a 2-L flask is shown in Figure 1. The production of MS-271 in the culture broth was initiated on day 2 and the amount of MS-271 reached a maximum on day 3.

Isolation and purification

The isolation procedure for MS-271 is outlined in Figure 2. MS-271 was purified from mycelia obtained by filtration of the fermentation broth. The mycelial cake was extracted with methanol. The extract was diluted with water, and applied to a Diaion HP-20 column. The column was washed with 60% methanol, and absorbed material was eluted with methanol. Fractions containing MS-271 were pooled and con-

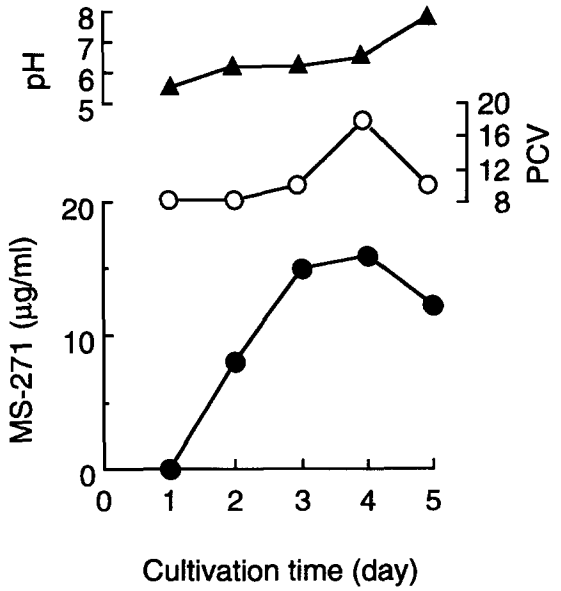

Figure 1. Time course of MS-271 production in a 2-liter Erlenmyer flask. ● MS-271, ○ growth (PCV), ▲ pH.

Key words: Myosin light chain kinase, inhibitor, *Streptomyces*, primary structure, anti-HIV.

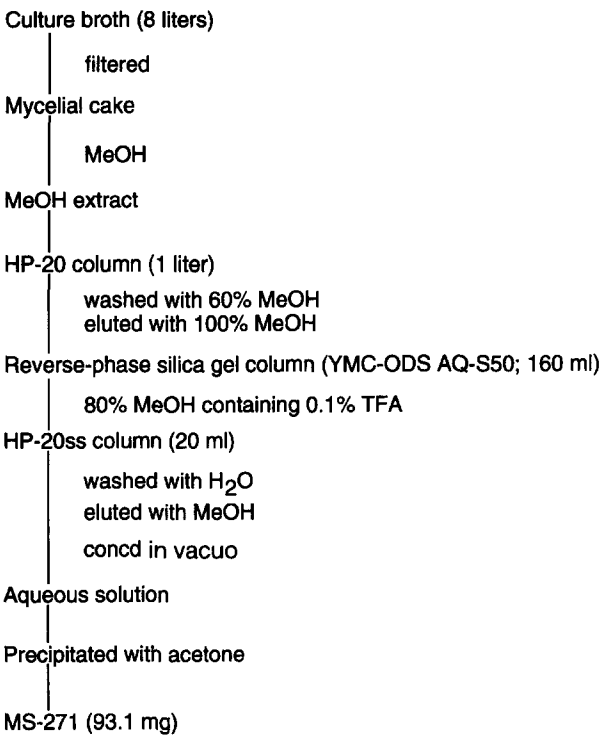

Figure 2. Purification of MS-271.

centrated in vacuo to yield crude MS-271. The crude MS-271 was applied to a reversed-phase silica gel column (YMC ODS AQS50, 160 mL). The chromatography was developed with 80% methanol containing 0.1% trifluoroacetic acid. Active fractions were diluted with the equal amount of water, and loaded on to a Diaion HP-20ss column. The column was washed with water and eluted with methanol. The eluate was concentrated in vacuo to aqueous solution. By the addition of acetone to the aqueous solution, MS-271 (93.1 mg) was precipitated as a colorless powder.

Physico-chemical properties of MS-271

Physico-chemical properties of MS-271 are summarized in Table 1. MS-271 is readily soluble in methanol, dimethylsulfoxide and virtually insoluble in acetone. The molecular formula of MS-271 was determined to be $C_{97}H_{131}N_{23}O_{26}S_4$ on the basis of high-resolution FAB mass spectrum (HRFABMS).

Table 1. Physico-chemical properties of MS-271

Appearance	Colorless amorphous
Molecular formula	$C_{97}H_{131}N_{23}O_{26}S_4$
Color reaction	
positive	H_2SO_4, I_2, Anisaldehyde
negative	Ninhydrin, Rydon–Smith
TLC, R_f[a]	
MeOH	0.73
Solubility	
soluble	MeOH, DMSO
insoluble	Acetone
High resolution FABMS	
observed	2163.8596
calculated	2163.8597
	(as $C_{97}H_{132}N_{23}O_{26}S_4$ $[M+H]^+$)

[a]Silica gel $60F_{254}$ plate (Merck Art. 5628).

Primary structure determination

The amino acid composition of MS-271 is shown in Table 2. Although an amino acid analysis shows that MS-271 is a 21-amino acid peptide, N-terminal amino acid analysis using Edman degradation was unsuccessful. FABMS and an amino acid analysis suggest that MS-271 has two disulfide bonds and an extra amide bond within the molecule. As MS-271 could not be digested with thermolysin or chymotrypsin, partial hydrolyses were attempted to obtain internal peptide fragments. The glycyl–serine bond in MS-271 was cleaved by vapor phase hydrolysis with trifluoroacetic acid, and sequence analysis of the resulting peptide elucidated the amino acid sequence from Ser-6 to the C-terminus. The other peptides obtained by partial hydrolysis with NaOH gave the information of the sequence in the N-terminus and the two pairs of disulfide bridges.

The C-terminal amino acid of MS-271 was determined as D-tryptophan by an amino acid analysis after vapor phase hydrazinolysis. The configuration of the other amino acids is L. The existance of a free carboxyl group in the C-terminus shows that cyclization may not be formed between the α-amino group of the N-terminal residue and the carboxyl group of the C-terminal residue, suggesting the involvement of the β-carboxyl group of Asp-9. The primary structure of MS-271 was determined as shown in Figure 3. This primary structure was confirmed by NMR analysis described in the following paper.

Table 2. Amino acid composition of MS-271

Amino acids	Experimental
Asx	1.7 (2)
Glx	— (0)
Cys	4.1 (4)
Ser	0.9 (1)
Gly	4.1 (4)
His	— (0)
Arg	— (0)
Thr	— (0)
Ala	2.0 (2)
Pro	— (0)
Tyr	1.0 (1)
Val	2.1 (2)
Met	— (0)
Ile	0.9 (1)
Leu	1.1 (1)
Phe	1.9 (2)
Lys	— (0)
Trp	1.2 (1)

Experimental figures are uncorrected values as determined by amino acid analysis after acid hydrolysis (see Experimental). In parentheses are indicated the values calculated from the sequence of MS-271 (Fig. 3).

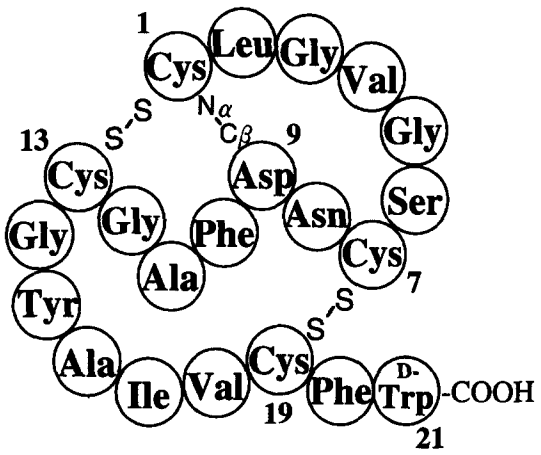

Figure 3. The primary structure of MS-271.

Biological properties

MS-271 inhibited chicken gizzard MLCK with an IC_{50} value of 8 μM (Table 3). The IC_{50} value of MS-271 against MLCK activity did not change when higher concentrations of calmodulin, ATP, or peptide substrate were added to the assay mixture (Fig. 4). No inhibition of the catalytic domain generated by tryptic digestion of MLCK by MS-271 was observed at concentrations up to 180 μM (Table 3).

Double-reciprocal plots of ATP or peptide substrate concentration versus reaction rate suggest that the mode of inhibition of MS-271 may be noncompetitive or mixed type (Fig. 5).

The IC_{50} values against several enzyme activities were summarized in Table 3. No effect was seen on cyclic AMP-dependent protein kinase, or protein kinase C at concentrations up to 400 μM.

The antimicrobial activity of MS-271 is shown in Table 4. MS-271 has activity against *Bacillus subtilis*, *Enterococcus faecium* and *Staphylococcus aureus*.

Discussion

In this paper, we demonstrate that an unique cyclic peptide, MS-271 isolated from *Streptomyces* sp., selectively inhibits chick gizzard MLCK. MS-271 inhibited MLCK with an IC_{50} value of 8 μM whereas the peptide was not effective on cyclic AMP-dependent protein kinase and protein kinase C, suggesting that MS-271 may be a selective inhibitor for MLCK.

Table 3. The effects of MS-271 on various enzymes

Enzymes	IC_{50} (μM)
MLCK	
intact	8
trypsin-digested	>180
Cyclic AMP-dependent protein kinase	>400
Protein kinase C	>400

IC_{50} values against MLCK activity were not affected by increase in the concentration of calmodulin, ATP or peptide substrate. A double-reciprocal plot of ATP or peptide substrate concentration versus reaction rate showed that MS-271 may compete with neither ATP nor peptide substrate. These results showed that MS-271 may bind to a site or sites distinct from where calmodulin, ATP, or peptide substrate binds on the MLCK. Moreover, no inhibition of MS-271 on the trypsin-digested MLCK, on which the sensitivity of calmodulin was abolished, was observed. Thus, it is possible that MS-271 may bind to a site on the domain which was digested by trypsin but different from that of calmodulin. Further studies are needed to determine the precise site where MS-271 acts on the MLCK.

Several compounds from various sources have been reported as MLCK inhibitors: ML-9, kaempferol, KT5926, thyroid hormones, wortmannin, MS-347a,

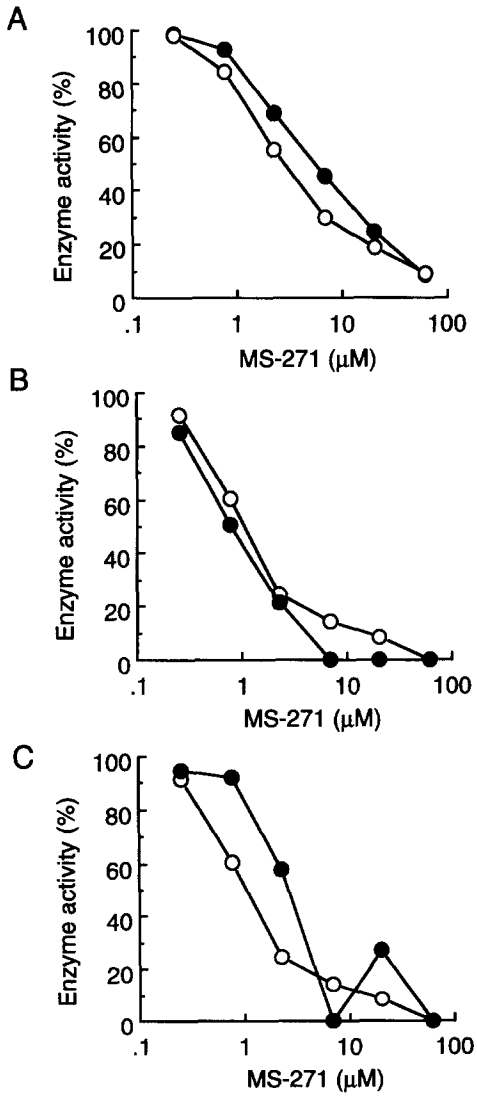

Figure 4. Inhibition by MS-271 of MLCK. MLCK activity was measured as described in the Experimental in the presence of (A) 2.6 (●), 52 (○) nM of calmodulin, (B) 50 (●), 400 (○) μM of ATP, or (C) 3 (●), 24 (○) μM of peptide substrate. All the experiments were carried out in duplicate.

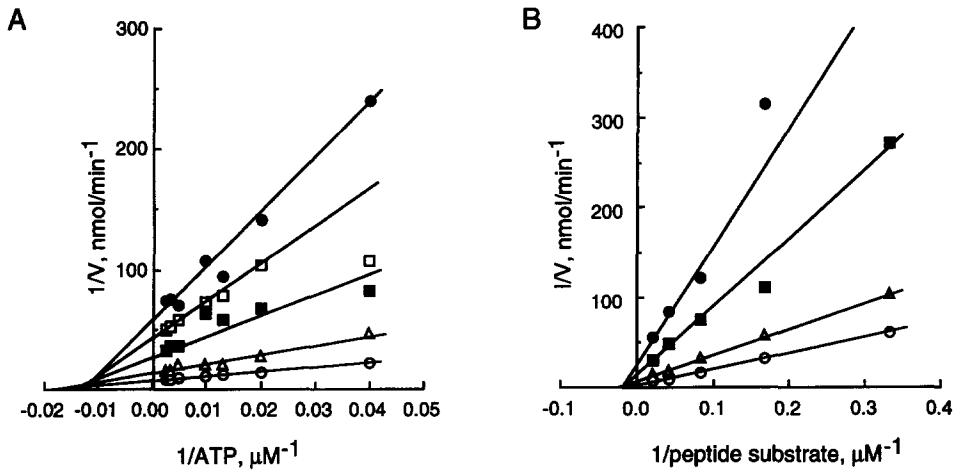

Figure 5. Kinetic analysis of inhibition of MLCK by MS-271. The enzyme activity was assayed with (A) ATP or (B) peptide substrate as variable substrate in the absence (○) or in the presence of 18.5 (●), 9.2 (□), 4.6 (■), 1.9 (△) μM of MS-271. All the experiments were performed in duplicate.

MS-282a and MS-282b.[3-9] The potency of MS-271 to inhibit MLCK is intermediate compared with that of the other MLCK inhibitors in our assay system. The mode of action of many other MLCK inhibitors on MLCK may be competitive against ATP, the peptide substrate, or calmodulin. Wortmannin, an irreversible inhibitor, which shows noncompetitive inhibition against intact MLCK, however, inhibits trypsin-digested MLCK activity. Therefore, MS-271 is thought to act on MLCK in a different manner from that of the other MLCK inhibitors due to the reasons described above.

The primary structure of MS-271 is identical to that of siamycin I, an anti-HIV peptide isolated from a microbial source.[10] Structurally similar compounds, RP71955[11,12] and siamycin II,[10] have also been reported as anti-HIV peptides, however the potency to inhibit MLCK has not been reported on these compounds so far. It has been reported that anantin, an atrial natriuretic peptide receptor antagonist,[13] and RES-701-1, an endothelin type B receptor antagonist,[14] have this unique cyclic form. The solution conformation of RP71955 is quite unusual.[12] The unique tertiary structure of MS-271 will be described in the following paper. Structural information on these compounds may be available for investigation of the structure–function relationship for anti-HIV and/or MLCK inhibitory activities.

Table 4. Antibiotic activity of MS-271

Test microorganisms	MIC (μg ml^{-1})
Candida albicans	>83
Enterococcus faecium	5.2
Pseudomonas aeruginosa	>83
Staphylococcus aureus	5.2
Escherichia coli	>83
Bacillus subtilis	5.2
Proteus vulgaris	>83
Shigella sonnei	>83
Salmonella typhosa	>83
Klebsiella pneumoniae	>83

Experimental

Materials

MLCK was isolated from chick gizzard smooth muscle as described in ref. 15. Calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine brain cortex, and protein kinase C from rat brain were prepared as described in ref. 16. 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 chemicals were analytical grade.

Microorganism

The producing microorganism, *Streptomyces* sp. M-271, was isolated from a soil sample collected under a pine tree at Hinatamura in Machida-city, Tokyo, Japan.

Culture and medium conditions

A loopful of spores of *Streptomyces*, grown on a Hickey and Tresner's agar slant, was inoculated into 10 mL of seed medium composed of 1% glucose, 1% soluble starch, 0.5% bacto tryptone, 0.5% yeast extract, 0.3% meat extract and 0.5% $CaCO_3$ (pH 7.2 before sterilization) in a test tube (21 i.d. × 200 mm). The inoculated tube was incubated at 28 °C for 2 days. A 30 mL portion of the culture was transferred to a 2-L Erlenmeyer flask containing 300 mL of the fermentation medium composed of 4% soluble starch, 1% soy bean meal, 0.5% corn steep liquor, 0.5% dry yeast (Oriental), 0.05% KH_2PO_4, 0.05% $Mg_3(PO_4)_2 \cdot 8H_2O$, 0.001% $ZnSO_4 \cdot 7H_2O$, 0.0001% $CoCl_2 \cdot 6H_2O$, 0.0001% $NiSO_4$ and 10% Diaion HP-20 (pH 7 before sterilization) and incubated at 28 °C for 5 days on a rotary shaker (200 rpm). The growth was monitored by packed cell volume (PCV) measurement. MS-271 was produced in mycelia and its production was traced by HPLC. For this measurement, 5 mL

of the culture broth was sampled and centrifuged. The precipitated mycelium was extracted with 5 mL of methanol. 10–20 μL of the extract was provided for HPLC assay.

Determination of MS-271 by HPLC

MS-271 produced was determined by a HPLC system equipped with a Shimadzu LC9A pump, Shimadzu SPD-6A UV detector and Shimadzu C-R4AX Chromatopac. MS-271 extracted from mycelia was injected on to an octadecylated silica gel column (AQ-312, 6 i.d. × 150 mm, YMC) and developed with 68% acetonitrile solution containing 0.1% trifluoroacetic acid at a flow rate of 1 mL min^{-1} at 40 °C, monitoring absorbance at 220 nm.

Structural determination

All mass spectra were acquired with a JEOL JMS HX/HX-110A tandem four sector mass spectrometer, which was operated at 10 kV accelerating potential.

Amino acid analysis was carried out with a manufacturer's protocol on a Waters Pico-Tag amino acid analyser. Precolumn derivatization with (+)-1-(9-fluorenyl)ethyl-chloroformate (Aldrich) was performed to distinguish between D- and L-amino acids.[17] Amino acid sequencing was performed on a Shimadzu PPSQ-10 protein sequencer (Japan) with a standard procedure.

Partial hydrolysis with 1.0 N NaOH was carried out at 110 °C for 2 h. Vapor-phase hydrolysis with neat trifluoroacetic acid was performed at 90 °C for 4 h. The resulting peptides were separated on a reversed-phase HPLC with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid, and analyzed by a protein sequencer described above.

Carboxyl terminal amino acid was identified with a vapor-phase hydrazinolysis[18] followed by precolumn derivatization with (+)-1-(9-fluorenyl)ethyl-chloroformate described above.

Enzyme assay

The activity of MLCK was measured as described previously.[19] For monitoring the active components during isolation, 10 μL of 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 at 28 °C for 30 min. The reaction was terminated by the addition of 10% acetic acid, and the reaction mixture was directly analyzed by HPLC.

Calmodulin-independent MLCK was prepared by partial digestion with trypsin as described.[3] The 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.[15]

Antimicrobial activity test

The antimicrobial activity of MS-271 was tested in the culture medium containing 3 g L^{-1} bacto tryptone, 3 g L^{-1} beef extract, 1 g L^{-1} yeast extract, 1 g L^{-1} glucose and 16 g L^{-1} agar (pH 7.0). The minimum inhibitory concentration (MIC) of several microorganisms was determined by the agar dilution method.

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