MS-282a AND MS-282b, NEW INHIBITORS OF CALMODULIN-ACTIVATED MYOSIN LIGHT CHAIN KINASE FROM *Streptomyces tauricus* ATCC 27470

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MS-282a and MS-282b were isolated from the culture broth of *Streptomyces tauricus* ATCC 27470 as inhibitors of smooth muscle myosin light chain kinase (MLCK). MS-282a and MS-282b inhibited the activity of chicken gizzard MLCK with IC_{50} values of 3.8 μM and 5.2 μM, respectively. Cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase and protein kinase C were not inhibited by 150 μM MS-282a at all. It is likely that MS-282a blocks MLCK activity by antagonizing calmodulin since 1) the compound inhibited calmodulin-dependent but not calmodulin-independent activity of MLCK; 2) the inhibition of MLCK was antagonized by increasing concentrations of calmodulin, and 3) the compound inhibited calmodulin-dependent cyclic nucleotide phosphodiesterase.

Myosin light chain kinase (MLCK) is a regulatory enzyme that participates in smooth muscle contraction[1,2]. It is widely accepted that hormonal and neural signals for contraction induce increases in 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 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.

During the course of our screening work, we found that Streptomyces tauricus ATCC 27470 produced inhibitors of MLCK, designated as MS-282a and MS-282b. In this article, we describe the production, isolation and biological properties of these inhibitors.

Materials and Methods

Fermentation

A 250-ml Erlenmeyer flask containing 50 ml of a seed medium composed of glucose 1%, soluble starch 1%, peptone 0.5%, yeast extract 0.5%, meat extract 0.3%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.2% (pH 7.1 before sterilized) was inoculated with the mycelia of *Streptomyces tauricus* Gause, Preobrazhenskaya, Sveshnikova, Terekhova and Maximova 1983[3], KCC-S 0837, ATCC 27470 (FERM BP-3716) grown on a Hikey-Tresner agar slant. The inoculated flask was incubated for 3 days at 28°C. A 25 ml portion of the culture was transferred to a 2-liter Erlenmeyer flask containing 300 ml of the seed medium, and the flask was incubated for 2 days at 28°C. Six flasks of the second seed culture (total 1.8 liters) were further transferred into a 200-liter tank fermentor containing 100 liters of the seed medium,

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and the culture was incubated for 2 days at 28°C with agitation of 150 rpm and aeration of 60 liters/minute. For isolation of MS-282a, the third seed culture was transferred into a 2,000-liter tank fermentor containing 1,000 liters of the following fermentation medium: maltose 4.0%, glucose 0.5%, soluble vegetable protein 4.0%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$, 0.05%, $CaCO_3$ 2.0% (pH 6.5 before sterilization). Fermentation was performed for 7 days at 28°C with agitation of 150 rpm and aeration of 400 liters/minute. For the isolation of MS-282b, the second seed culture (1.8 liters) obtained as described above was transferred into a 200-liter tank fermentor containing 100 liters of the following fermentation medium: maltose 4.0%, glucose 0.5%, soybean meal 1.5%, soluble vegetable protein 1.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$, 0.05%, $CaCO_3$ 2.0% (pH 7.0 before sterilization). The growth of the microorganism was monitored during fermentation by the measurement of packed cell volume (PCV). Detection of MS-282a and MS-282b in the isolation procedures was carried out on a silica gel TLC plate (Merck, No. 5715) developed with EtOAc-MeOH-conc. NH_4OH (88 : 10 : 2) and visualized with I_2.

Enzyme Assay

The activity of MLCK was measured as described previously[4]. 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-282a, 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 10% of acetic acid, and the reaction mixture was directly analyzed by HPLC as described[4].

Calmodulin-independent MLCK was prepared by partial digestion with trypsin as described[5]. 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[6].

Materials

MLCK was isolated from chicken gizzard smooth muscle as described[6]. 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[7]. 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

Streptomyces tauricus ATCC 27470 was cultured in 300-ml Erlenmeyer flasks containing the fermentation medium composed of glucose 0.5%, maltose 3.0%, soluble vegetable protein 1.5%, soybean meal 1.0%, yeast extract 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05%. Production of myosin light chain kinase inhibitors, growth of the microorganism, and pH of the medium were

Fig. 1. Time course of the production of MLCK inhibitors by *S. tauricus* ATCC 27470.

Inhibition % of MLCK activity by culture supernatant (○) and cell extract (●), pH of the culture broth (■), and packed cell volume (▲) were indicated.

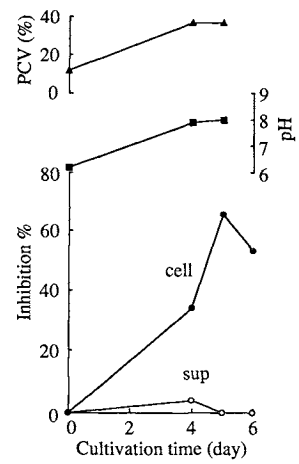


Table 1. Effect of nitrogen source on production of MLCK inhibitors by *S. tauricus* ATCC 27470.

Exp. No.	1	2	3	4	5	6	7	8
Nitrogen source (%)								
Soybean meal	1.0	0	1.0	1.0	1.0	0	1.0	0
Soluble vegetable protein	1.5	1.5	0	0.5	3.0	3.0	1.5	1.5
Yeast extract	0.5	0.5	0.5	0.5	0.5	0.5	0	0
Inhibition %	33	48	30	39	75	74	12	33

S. tauricus ATCC 27470 was cultured for 8 days at 28°C in fermentation media containing maltose 3.0%, glucose 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% and indicated concentrations of soybean meal, soluble vegetable protein and yeast extract. Active materials were extracted from the mycelia with MeOH, and the inhibition of MLCK activity was measured in the presence of the extracts.

monitored during fermentation (Fig. 1). The active components were produced mainly in mycelia, and the inhibitory activity reached maximum on the 5th day.

The production of the active components was greatly affected by the nitrogen source in the fermentation medium. Fermentation was carried out at 30°C for 8 days in 300-ml Erlenmeyer flasks containing the media described in Table 1. The amount of soluble vegetable protein added in the fermentation medium was the most important factor for the production of the active components (Exp. No. 1, 3~5, Table 1). Yeast extract also affected the production of the activity in this experiment (Exp. No. 1 and 7, and 2 and 8). However, the effect of yeast extract was not observed when 4% of soluble vegetable protein was added to the medium (data not shown). Soybean meal showed inhibitory or no effects on the production of the active components (Exp. No. 1 and 2, 5 and 6, and 7 and 8).

Fig. 2. Purification procedure of MS-282a.

Culture broth (1,000 liters)
| filtered
Mycelia
| extracted with MeOH (1,100 liters)
| filtered
MeOH extract
| diluted with H_2O (final 1,400 liters)
Diaion HP-20 column (50 liters)
| washed with 80% MeOH, 150 liters
| washed with 90% MeOH, 500 liters
| eluted with 95% MeOH, 400 liters
Active fractions (elution volume 50 ~ 300 liters)
| concentrated *in vacuo*
| extracted with EtOAc (4 liters)
EtOAc layer
| concentrated *in vacuo*
Silica gel column (Wakogel C-200, 6 liters)
| eluted with EtOAc, 25 liters
Active fractions (elution volume 18 ~ 25 liters)
| concentrated *in vacuo*
Silica gel column (Wakogel C-200, 2 liters)
| washed with MeOH, 10 liters
| eluted with 85% MeOH/0.5% AcOH/H_2O, 3 liters
Active fractions (elution volume 2 ~ 2.8 liters)
| dried *in vacuo*
| dissolved in MeOH
Active carbon column (100 ml)
| eluted with MeOH, 1,000 ml
| dried *in vacuo*
Colorless oily solid (9.3 g)
|
Aliquot (450 mg x 5 times)
|
Silica gel column (Develosil Lop60, 45 x 490 mm)
| eluted with EtOAc-MeOH-concNH_4OH (85:15:1)
Active fractions (elution volume 2,420 ~ 2,640 ml)
|
MS-282a (90 mg)

Isolation and Purification

Purification procedures of MS-282a and MS-282b are outlined in Figs. 2 and 3, respectively. The crude materials extracted from the mycelia of the microorganism contained active compounds which are structurally similar. It was difficult to separate and isolate all these active compounds because of their similarity in physico-chemical properties. However, we did isolate and purify two components, MS-282a and MS-282b from two fermentation batches.

Physico-chemical Properties

Physico-chemical properties of MS-282a and MS-282b are summarized in Table 2. The structures of both compounds (Fig. 4) were determined from their physico-chemical properties and spectral data which

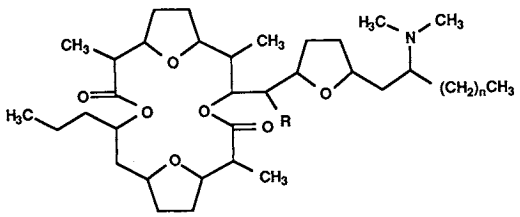
Fig. 3. Purification procedure of MS-282b.

Culture broth (100 liters)
| filtered
Mycellia
| extracted with EtOAc, 100 liters x 2 times
EtOAc extract
| concentrated *in vacuo*
| washed with H_2O
EtOAc layer
|
Silica gel column (Wakogel C-200, 3 liters)
| washed with $CHCl_3$, 15 liters
| washed with $CHCl_3$-MeOH (8:2), 9 liters
| eluted with $CHCl_3$-MeOH-concNH_4OH (90:10:5), 12 liters
Active fractions (elution volume 9.2 ~ 11.6 liters)
| concentrated *in vacuo*
Silica gel column (Wakogel C-200, 2.5 liters)
| washed with EtOAc, 2.5 liters
| eluted with upper layer of EtOAc-concNH_4OH (10:1), 14 liters
Active fractions (elution volume 4.0 ~ 8.8 liters)
| concentrated *in vacuo*
Silica gel column (Wakogel C-300, 300 ml)
| washed with $CHCl_3$, 300 ml
| washed with $CHCl_3$-MeOH (10:2), 1,000 ml
| eluted with $CHCl_3$-MeOH-concNH_4OH (76:20:3), 1,400 ml
Active fractions (elution volume 780 ~ 900 ml)
| dried *in vacuo*
Colorless oily solid (25 mg)
| dissolved in 79% MeOH/0.07% AcOH/H_2O, 1 ml
Aliquot (0.1 ml x 10 times)
|
Reversed phase silica gel column (STR ODS-H, 4.6 x 250 mm)
| eluted with 70% MeOH/0.1% AcOH/H_2O
Active fractions (retention time 6.9 ~ 7.2 min)
|
MS-282b (2.2 mg)

Table 2. Physico-chemical properties of MS-282a and MS-282b.

	MS-282a	MS-282b
Appearance	Colorless oily solid	Colorless oily solid
Color reaction		
Positive	Anisaldehyde, H_2SO_4, I_2, molybdophosphate, Dragendorff	Anisaldehyde, H_2SO_4, I_2, molybdophosphate, Dragendorff
Negative	Bromocresolgreen, 2,4-dinitrophenylhydrazine, Ninhydrin	Bromocresolgreen, 2,4-dinitrophenylhydrazine, Ninhydrin
Solubility		
Soluble	MeOH, $CHCl_3$, EtOAc, Et_2O	MeOH, $CHCl_3$, EtOAc, Et_2O
Insoluble	*n*-Hexane, H_2O	*n*-Hexane, H_2O
TLC, Rf		
$CHCl_3$-MeOH-conc NH_4OH	0.67	0.68
EtOAc-MeOH-conc NH_4OH	0.59	0.62
UV	End absorption	End absorption
High resolution EI-MS		
Observed	636.4828 $(M+H)^+$	635.4718 $(M)^+$
Calculated	636.4839 (as $C_{37}H_{66}NO_7$)	635.4757 (as $C_{37}H_{65}NO_7$)

Fig. 4. Structures of MS-282a and MS-282b.



	R	n
MS-282a	CH_3	4
MS-282b	C_2H_5	3

Fig. 5. Effect of MS-282a and MS-282b on MLCK activity.

○: MS-282a; ●:MS-282b. Data are means of three separate experiments.

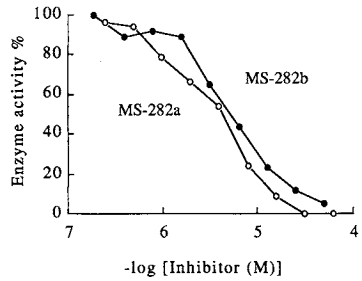


Table 3. Effects of MS-282a and MS-282b on various enzyme activities.

Enzyme	IC_{50} (μM)	
	MS-282a	MS-282b
MLCK		
Calmodulin-dependent	3.8	5.2
Calmodulin-independent	102	NT[a]
Calmodulin-dependent cyclic nucleotide phosphodiesterase	4.2	NT
cAMP-dependent protein kinase	>150	NT
cGMP-dependent protein kinase	>150	NT
Protein kinase C	>150	NT

[a] Not tested.

Fig. 6. Effect of calmodulin on susceptibility of MS-282a to inhibit MLCK activity.

MLCK activity was measured in the reaction mixture with 2.6 nM (●) and 52 nM (○) calmodulin (CaM) in the presence of indicated concentrations of MS-282a. Data are means of three separate experiments.

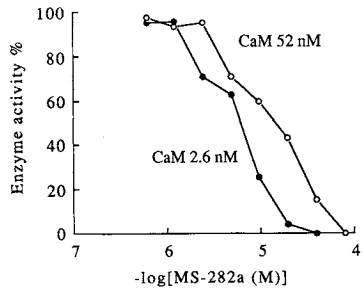


Table 4. Antibiotic activity of MS-282a.

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	50
Proteus vulgaris ATCC 6897	50
Shigella sonnei ATCC 9290	>100
Salmonella typhi ATCC 9992	>100
Pseudomonas aeruginosa BMH No. 1	>100
Candida albicans ATCC 10231	0.8

will be described in a separate paper[8].

Biological Activities

MS-282a and MS-282b inhibited Ca^{2+} and calmodulin-dependent activity of chicken gizzard MLCK in concentration-dependent manner (Fig. 5). The concentrations of MS-282a and MS-282b needed to inhibit the enzyme activity by 50% (IC_{50}) were 3.8 μM and 5.2 μM, respectively (Table 3).

The IC_{50} values of MS-282a against several enzyme activities are summarized in Table 3. MS-282a had no effect on cAMP-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C at concentrations up to 150 μM. The calmodulin-independent activity of MLCK, which was generated by partial tryptic digestion of the enzyme, was inhibited by MS-282a with an IC_{50} value of 102 μM. Calmodulin-dependent cyclic nucleotide phosphodiesterase and calmodulin-dependent activity of MLCK were also inhibited by MS-282a. These data suggest that MS-282a, and probably MS-282b as well, inhibited calmodulin itself or calmodulin-dependent mechanism of the enzyme activation. In order to

confirm this hypothesis, we tested whether the inhibitory effect of MS-282a on calmodulin-dependent activity of MLCK could be overcome by increasing the calmodulin concentration in the assay mixture. As shown in Fig. 6, the inhibition curve of MS-282a was shifted towards the right when the calmodulin concentration was increased 20-fold in the MLCK assay mixture.

The antibacterial spectrum of MS-282a is listed in Table 4. MS-282a inhibited growth of Gram-positive bacteria and *C. albicans*, but not that of Gram-negative bacteria.

Discussion

We have isolated MS-282a and MS-282b from the culture broth of *Streptomyces tauricus* ATCC 27470, and demonstrated that these compounds are inhibitors of MLCK. The following data suggest that the inhibitory effect of MS-282a on MLCK was achieved by antagonizing calmodulin which binds to the regulatory domain of MLCK to activate this enzyme. 1) The calmodulin-independent activity of MLCK generated by partial tryptic digestion was not inhibited by MS-282a in the concentration range similar to that needed to inhibit calmodulin-dependent activity of MLCK. 2) Increased concentrations of calmodulin reduced the potency of MS-282a to inhibit MLCK. This idea is supported by the observations that MS-282a inhibited calmodulin-dependent cyclic nucleotide phosphodiesterase, but not calmodulin-independent protein kinases.

MS-282a and MS-282b are new compounds, the structure of which are close to that of pamamycins produced by *Streptomyces alboniger*[9~11]. It has been reported that pamamycin-607 inhibits growth of *Cochliobolus miyabeanus*, *Diaporthe citri*, *Bacillus subtilis* and *Bacillus cereus*, and that pamamycin-607 and pamamycin-635B induce aerial mycelia of an aerial mycelium-less mutant of *S. alboniger*[10,11]. The antibiotic activity and the aerial mycelium-inducing activity of pamamycins were distinguishable since pamamycin-635A, pamamycin-649A and pamamycin-649B inhibited growth of the mutant of *S. alboniger*, but did not induce aerial mycelia of the mutant[11]. We have shown that MS-282a has an antibacterial spectrum similar to that of pamamycin-607, but we have not tested aerial mycelium-inducing activity of MS-282a. NATSUME *et al.* speculated from the difference in structures of five pamamycins that methyl group, but not ethyl group, at R position (Fig. 4) was important for aerial mycelium-inducing activity of pamamycins[11]. It is, therefore, probable from their speculation that MS-282a which has methyl group at R position could induce aerial mycelia of the mutant *S. alboniger*, but not MS-282b which has ethyl group at R position. There are no reports about inhibition of MLCK or other calmodulin-dependent enzymes by pamamycins.

Several compounds from various sources have been reported as MLCK inhibitors: ML-9, kaempferol, KT5926, thyroid hormones, wortmannin and MS-347a[5,12~16]. There are many more diverse structures and sources of calmodulin inhibitors reported (see ref 17 and the references therein). The potency of MS-282a and MS-282b to inhibit MLCK is intermediate compared with that of the other MLCK inhibitors and calmodulin inhibitors in our assay system[3]. It is believed that despite the structural diversity of known calmodulin inhibitors, their mutual feature is that they contain hydrophobic and often basic groups in their structures; MS-282a and MS-282b share this feature.

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