

# Migrastatin Acts as a Muscarinic Acetylcholine Receptor Antagonist

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**Abstract** Migrastatin and its analogs have various biological activities such as inhibition of cell migration and anchorage-independent growth of cancer cells. Although its biosynthesis and chemical synthesis have been under investigation, little is known about the biological target of migrastatin. Here, we found that migrastatin inhibited intracellular calcium mobilization induced by carbachol in neuroblastoma SK-N-SH cells without affecting  $\text{Ca}^{2+}$  mobilization and cAMP accumulation induced by ligands of other receptors. The binding of [ $^3\text{H}$ ] *N*-methylscopolamine, an antagonist for muscarinic receptor was also inhibited by migrastatin. Functionally, migrastatin inhibited  $\text{Ca}^{2+}$  mobilization induced by carbachol in primary cultures of smooth muscle cells of rat bladder. This study reveals that migrastatin acts as a muscarinic acetylcholine receptor antagonist.

**Keywords** migrastatin, antagonists,  $\text{Ca}^{2+}$  mobilization, muscarinic acetylcholine receptor

## Introduction

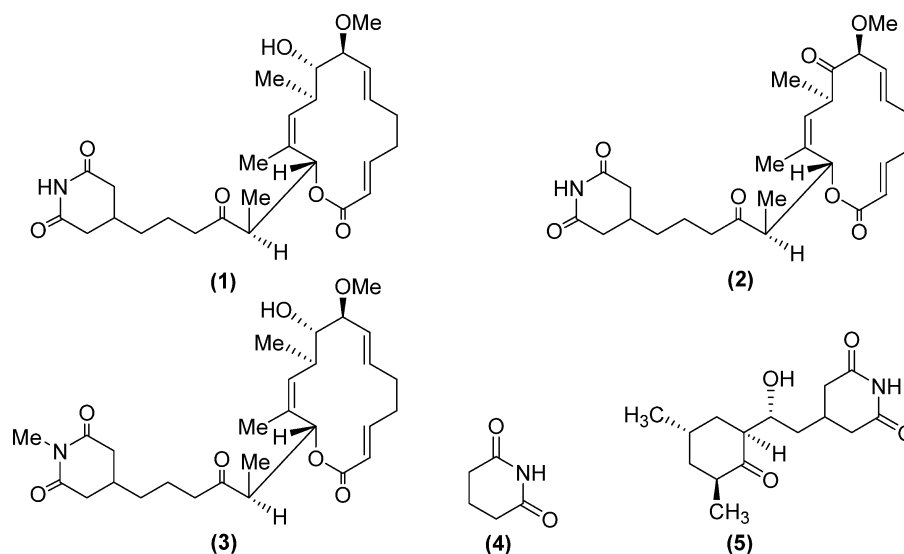
Migrastatin (Fig. 1, **1**) was first isolated as a cell migration inhibitor from a cultured broth of *Streptomyces* sp. [1]. It was also found to inhibit anchorage-independent growth of human small cell lung carcinoma Ms-1 cells without affecting the biosyntheses of DNA, RNA and proteins [2]. The structure of migrastatin was determined to be a 14-membered ring macrolide containing a glutarimide, and

that work was followed by total synthesis [3–5]. Studies on the structure-activity relationship for cell migration led to the potent derivative, the migrastatin core which consists of macrolide moiety without side chain with an  $\text{IC}_{50}$  value of 22 nM [6] and migrastatin core macroketone and the core macrolactam had the anti-metastatic activity against breast tumor in a mouse model [7]. Biosynthesis studies have revealed the DNA sequence that encodes the synthesis of migrastatin, and related polyketides and derivatives have been isolated [8–10]. Although several studies have explored the biological activity of migrastatin, a biological target molecule of migrastatin has not yet been identified.

The muscarinic acetylcholine receptor (mAChR) is a member of the superfamily of G protein-coupled receptors (GPCRs) and consists of five genetically distinct subtypes ( $\text{M}_1\sim\text{M}_5$ ) [11, 12]. The various approaches that have contributed to the elucidation of the role of these receptors *in vitro* and *in vivo* are summarized herein. An expression-profiling study revealed that mAChRs are expressed in many tissues [13]. A pharmacological approach using broad and selective mAChR agonists and antagonists clarified that mAChRs regulate many fundamental functions such as smooth muscle contractility, exocrine gland secretion and pupil dilation [11, 14]. In addition, antagonists of muscarinic receptors such as oxybutynin and scopolamine were known to cause a dry mouth [15, 16]. Mutant mice deficient in specific mAChRs, for example  $\text{M}_3$ -knockout mice, showed that the  $\text{M}_3$  receptor plays a critical role in the control of salivation, and rodents with insufficient salivation showed increased prandial drinking

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**Fig. 1** Structures of migrastatin (1), 9-oxomigrastatin (2), *N*-methylmigrastatin (3), glutarimide (4) and cycloheximide (5).

[17, 18].

In the present study, we tried to assess prandial drinking in mice and examined the effect of migrastatin on mAChRs *in vitro* with a  $\text{Ca}^{2+}$  mobilization assay and a receptor binding assay. This study reports the identification of a novel effect of migrastatin.

## Materials and Methods

### Assessment of Prandial Drinking

All animal handling procedures in this study were approved by the Animal Care and Use Committee of the Microbial Chemistry Research Center. Female ICR mice (9-week-old,  $n=12$ ) were fasted overnight with free access to water. At noon, each 6 mouse was injected intravenously with 0.2 ml volume of migrastatin (33 mg/kg) or saline for 30 minutes and were fed pelleted food (FR-1, Funabashinojo, JAPAN). Eating behavior was observed for 2 hours, recorded with a video camera (GZ-MG50 Victor, JAPAN), the number of approaches to the water nozzle were counted. Migrastatin was dissolved in saline including 10% of ethanol and 10% of Cremophor EL.

### Expression Profiling of mAChRs in SK-N-SH Cells

Total cellular RNA was isolated from neuroblastoma SK-N-SH cells and purified by an acid guanidium thiocyanate-phenol-chloroform method using TRIZOL (Invitrogen, U.S.A.) as an extraction reagent. The RNA (5.0  $\mu\text{g}$ ) was reverse-transcribed with Superscript II reverse transcriptase

in the presence of random hexamers and an oligo (dT) primer (Invitrogen, U.S.A.). PCR analysis was carried out in a volume of 20  $\mu\text{l}$  containing 20 ng of cDNA as a template and 0.75  $\mu\text{M}$  of each primer using the Acupower PCR kit (Bioneer, Korea). Human mAChRs primer sequences were 5'-CCCCACGGAGCTCCCCAAATAC-3' and 5'-AAGCAGCAGCAGGCGAAAGGTG-3' ( $M_1$ ), 5'-TGCAGCTGCCTGGGTCCTCTCT-3' and 5'-TGTTGTGCTCCAGGCCATCGTC-3' ( $M_2$ ), 5'-TGTCCTTTGGGCTCCTGCCATC-3' and 5'-GGCAGCGGCCATACTTCCTCCT-3' ( $M_3$ ), 5'-GGCACAGCCATTGCTGCCTTCT-3' and 5'-ACAGCTCTGTGGCTGGGCGTTC-3' ( $M_4$ ) and 5'-GGCACTGCCATTGCTGCCTTCT-3' and 5'-GGCGGGCTTGTCCTCATCCTCT-3' ( $M_5$ ). PCR condition was as follows: Samples were subjected to denaturing conditions at 94°C for 10 seconds. After annealing at 63°C for 30 seconds, genes were amplified at 72°C for 30 seconds (30 cycles).

### $\text{Ca}^{2+}$ Mobilization Assay Using a FDSS6000 Fluorimeter

$\text{Ca}^{2+}$  loading buffer was prepared by mixing 2  $\mu\text{M}$  Fluo-3 AM (Dojindo, Japan) and 0.02% Pluronic F-127 (Molecular Probes, U.S.A.) in the  $\text{Ca}^{2+}$  assay buffer (17 mM HEPES pH 7.4, 0.1% BSA, 1 mM probenecid, Hank's balanced salt solution). Cells were incubated in the  $\text{Ca}^{2+}$  loading buffer at 37°C for 60 minutes, and then washed with  $\text{Ca}^{2+}$  assay buffer. The drug concentrations were 200 nM sulprostone (Cayman Chemical, U.S.A.) in K562 cells, 2  $\mu\text{M}$  ATP, 30 nM Neuropeptide Y (NPY) (Peptide Institute Inc., Japan), 40 nM [ $\text{Pyr}^1$ ]-Apelin-13 (Peptide

Institute Inc., Japan) in HEL cells and 30 nM capsaicin (Sigma, U.S.A.) in rat dorsal root ganglion neurons (DRG neurons), respectively. Fluorescence emission at 480 nm induced by the ligands was measured using a FDSS6000 fluorimeter (Hamamatsu Photonics, Japan).

### General

NMR data were recorded on a JEOL JNM-ECA600 spectrometer. Mass spectra were obtained with a JEOL JMS-T100LC spectrometer.

### Preparation of 9-oxomigrastatin (2)

Compound **2** was prepared by the treatment of migrastatin (10.9 mg) with Dess-Martin periodinate (15.2 mg) in dichloromethane (1 ml) at room temperature for 3 hours [27]. After dilution with CH<sub>2</sub>Cl<sub>2</sub>, the solution was washed with water, dried over MgSO<sub>4</sub>, and filtered. The filtrate was evaporated to dryness and purified by preparative TLC (toluene : acetone = 2 : 1) to give **2** (6.5 mg, yield 59%). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): δ = 1.12 (d, 3H, *J* = 7.0 Hz), 1.25 (d, 3H, 7.0 Hz), 1.31~1.40 (m, 2H), 1.59~1.65 (m, 2H), 1.90 (d, 3H, *J* = 1.5 Hz), 2.10~2.16 (m, 2H), 2.22~2.30 (m, 3H), 2.38~2.44 (m, 1H), 2.48~2.55 (m, 3H), 2.68~2.73 (m, 2H), 2.93 (dq, 1H, *J* = 10.5, 7.0 Hz), 3.28 (s, 3H), 3.82~3.88 (m, 1H), 4.37 (d, 1H, *J* = 8.5 Hz), 5.12 (dd, 1H, *J* = 15.5, 8.5 Hz), 5.15 (d, 1H, *J* = 10.5 Hz), 5.58 (d, 1H, *J* = 16.0 Hz), 5.72~5.78 (m, 1H), 5.83 (dd, 1H, *J* = 10.0, 1.5 Hz), 6.61 (ddd, 1H, *J* = 16.0, 9.0, 8.0 Hz), 7.95 (br s, 1H); <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>): δ = 13.4, 19.5, 20.1, 26.4, 29.9, 30.3, 31.7, 34.1, 37.7, 37.7, 40.0, 41.3, 51.4, 56.3, 76.5, 84.5, 123.0, 126.9, 127.4, 133.8, 137.8, 148.2, 164.0, 171.8, 171.8, 206.9, 210.5; HRMS (ESI): [M+Na]<sup>+</sup> calcd for 510.2409, found 510.2457.

### Preparation of *N*-Methylmigrastatin (3)

**3** was obtained from migrastatin (7.5 mg) by treatment with iodomethane (44 μl) and potassium carbonate (6.7 mg) in dimethylformamide (1 ml) at room temperature for 44 hours. Water and EtOAc were added to the mixture. The EtOAc layer was evaporated to dryness and purified by preparative TLC (toluene : acetone = 2 : 1) to give **3** (4.3 mg, yield 56%). <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ = 0.96 (d, 3H, *J* = 7.0 Hz), 1.12 (d, 3H, *J* = 7.0 Hz), 1.28~1.34 (m, 2H), 1.58~1.62 (m, 2H), 1.86 (d, 3H, *J* = 1.5 Hz), 2.01~2.08 (m, 1H), 2.17~2.24 (m, 2H), 2.25~2.32 (m, 2H), 2.40~2.46 (m, 2H), 2.48 (t, 2H, *J* = 7.0 Hz), 2.75~2.81 (m, 2H), 2.79 (br s, 1H), 2.90~2.95 (m, 1H), 2.97 (dq, 1H, *J* = 10.5, 7.0 Hz), 3.13 (s, 3H), 3.31 (s, 3H), 3.47 (dd, 1H, *J* = 8.5, 4.5 Hz), 3.50 (d, 1H, *J* = 8.5 Hz), 5.19 (d, 1H, *J* = 10.5 Hz), 5.24 (dd, 1H, *J* = 15.5, 4.5 Hz), 5.49~5.55 (m, 1H), 5.59 (dd, 1H, *J* = 16.0, 1.5 Hz), 5.65 (dd, 1H, *J* = 16.0, 1.5 Hz),

6.49 (ddd, 1H, *J* = 16.0, 10.0, 3.5 Hz); <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>): δ = 13.4, 13.4, 20.1, 26.0, 26.4, 29.4, 30.1, 31.1, 32.0, 34.3, 38.7, 38.7, 40.1, 51.2, 57.0, 77.0, 78.0, 82.5, 122.2, 128.1, 130.6, 131.2, 133.1, 149.9, 163.9, 172.2, 172.2, 210.8; HRMS (ESI): [M+Na]<sup>+</sup> calcd for 526.2722, found 526.2768.

### Receptor Binding Assay

Human M<sub>1~5</sub> CHO membranes (PerkinElmer, U.S.A.) were added into a polypropylene 96-well plate in binding buffer (50 mM Tris-HCl pH 7.4 10 mM MgCl<sub>2</sub> 1 mM EDTA). A final concentration of 0.3 nM of [<sup>3</sup>H]-labeled *N*-Methyl Scopolamine (NMS) (Amersham Pharmacia, U.K.) and migrastatin was added and incubated for 1 hour at room temperature. Binding assay mixture was filtered through Multiscreen-MAFB (Millipore, U.S.A.) and each filter was transferred into a scintillation vial and the scintillant was added. Radioactivity remaining on the filter was counted in a liquid scintillation by Tri-Carb 2100TR (PerkinElmer, U.S.A.). Non-specific binding was measured using excess amounts of atropine (10 μM). Inhibition constants (K<sub>i</sub>) were obtained using the Cheng-Prusoff equation, K<sub>i</sub> = IC<sub>50</sub> / (1 + ([<sup>3</sup>H]NMS/K<sub>D</sub>)), where K<sub>D</sub> is the equilibrium dissociation constant of [<sup>3</sup>H]NMS. The K<sub>D</sub> value of [<sup>3</sup>H]NMS for M<sub>1~5</sub> is 0.16, 0.23, 0.12, 0.11 and 0.97 nM, respectively.

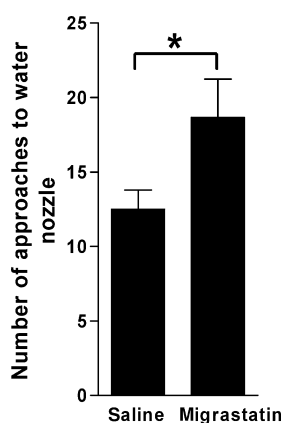
### Ca<sup>2+</sup> Mobilization Assay Using a Confocal Microscopy

Primary cultures of bladder smooth muscle cells were prepared from female rat (8 weeks, SD strain). Urinary bladder was isolated from adjacent tissues and the epithelium was removed after opening of the bladder. The bladder was cut into pieces (approx. 1 × 1 mm) that were explanted into a 6 well plate and incubated with SmGM-2 medium (Sanko Junyaku, Japan) for 1 week. Primary cultures of bladder smooth muscle cells were plated in glass bottom dishes coated with collagen type I (MatTek, U.S.A.). The procedure with reference to loading of Fluo-3 was identical to the one used for the Ca<sup>2+</sup> mobilization assay using a FDSS6000 fluorimeter. Fluorescence images were obtained at an excitation wavelength of 488 nm using a laser scanning confocal microscope (LSM510, Zeiss) and emission was collected through a band pass filter of 505~550 nm. Cells were imaged on the stage of an inverted microscope (Axiovert 100M) with a 20X Zeiss Plan-Neofluar objective.

## Results

### Effects of Migrastatin on Prandial Drinking in Mice

For the assessment of the effect of migrastatin on drinking behavior, we observed prandial drinking in mice. As shown in Fig. 2, the number of approaches to the water nozzle were  $18.7 \pm 2.6$  and  $12.5 \pm 1.3$  during 2 hours in migrastatin and saline administered mice, respectively. These results showed that the administration of migrastatin increased the frequency of water intake during feeding.



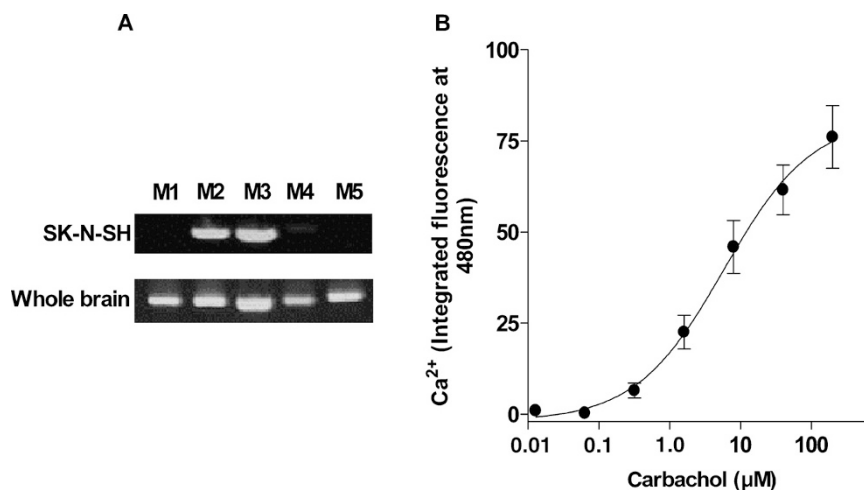
**Fig. 2** Prandial water intake in mice.

Approaches to the water nozzle for 2 hours were counted in migrastatin-administrated mice (33 mg/kg i.v. n=6) and saline-administrated mice (i.v. n=6). Data represents mean  $\pm$  S.E.M. Statistical significance was analyzed by Student's *t* test: \*  $p < 0.05$ .

### Effects of Migrastatin on the Muscarinic Receptor in $Ca^{2+}$ Mobilization

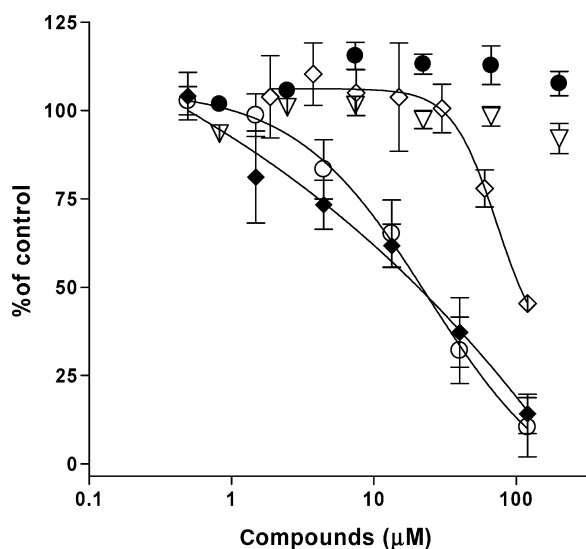
Increased prandial drinking is similarly observed in  $M_3$ -knockout mice and administration of mAChR antagonists caused a dry mouth [15, 16, 18]. We therefore evaluated the effect of migrastatin *in vitro* on mAChRs. For the establishment of cell-based assays for mAChRs signaling, we first examined the expression profile of mAChRs at the RNA level in SK-N-SH cells. PCR analysis revealed that  $M_2$  and  $M_3$  were expressed in SK-N-SH cells (Fig. 3A). The functional role of mAChRs was also examined by measuring the effect of muscarinic agonist carbachol on  $Ca^{2+}$  mobilization in SK-N-SH cells. Carbachol induced  $Ca^{2+}$  mobilization in a dose-dependent manner (Fig. 3B). Next, we examined whether migrastatin showed a functional inhibitory activity in SK-N-SH cells. As shown in Fig. 4, migrastatin inhibited  $Ca^{2+}$  mobilization in a dose-dependent manner with an  $IC_{50}$  value of  $28 \mu M$ .

To investigate the activity of migrastatin derivatives, 9-oxomigrastatin **2** and *N*-methylmigrastatin **3** were synthesized. We evaluated the effect of these derivatives together with the 14-membered ring macrolides clarithromycin and telithromycin, the 15-membered ring macrolide azithromycin and the 16-membered ring macrolide josamycin on  $Ca^{2+}$  mobilization. We also examined the effect of glutarimide related compounds (glutarimide and cycloheximide) because migrastatin possesses glutarimide moiety. While 9-oxomigrastatin showed similar inhibitory activity to migrastatin, the potency of *N*-methylmigrastatin was about 4 times weaker than that of migrastatin. The others compounds did not inhibited  $Ca^{2+}$  mobilization at a concentration of up to  $120 \mu M$  (Table 1).



**Fig. 3** The expression profiling of  $M_1$ – $M_5$  mAChRs and pharmacological activity in SK-N-SH cells.

(A) RT-PCR detection of  $M_1$ – $M_5$  mAChR in SK-N-SH cells and human whole brain. (B) Dose-response of  $Ca^{2+}$  mobilization in SK-N-SH cells induced by carbachol. Data represent means  $\pm$  S.E.M. of 3 independent experiments.



**Fig. 4** The effects of compounds on  $\text{Ca}^{2+}$  mobilization induced by  $1 \mu\text{M}$  of carbachol.

Data represents mean  $\pm$  S.E.M. of 3~4 independent experiments.  $\circ$ : migrastatin,  $\blacklozenge$ : 9-oxomigrastatin,  $\diamond$ : *N*-methylmigrastatin,  $\nabla$ : glutarimide,  $\bullet$ : cycloheximide.

**Table 1** The effect of various compounds on carbachol induced  $\text{Ca}^{2+}$  mobilization

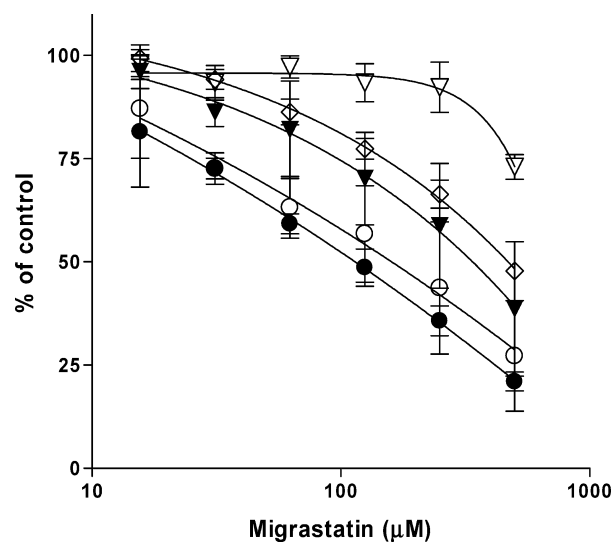
Compounds	$\text{IC}_{50}$ ( $\mu\text{M}$ )
Migrastatin	28
9-Oxomigrastatin	35
<i>N</i> -Methylmigrastatin	110
Glutarimide	>120
Cycloheximide	>120
Clarithromycin	>120
Josamycin	>120
Telithromycin	>120
Azithromycin	>120
Atropine	0.0002

### Effects of Migrastatin on Other Receptors in $\text{Ca}^{2+}$ Mobilization and cAMP Accumulation

The selectivity of migrastatin for the various ligands of other G-protein coupled receptors and ion channels was further evaluated. We evaluated the effect of migrastatin on second messenger variation,  $\text{Ca}^{2+}$  mobilization induced by sulprostone (prostaglandin  $\text{EP}_3$  agonist), SLIGRL- $\text{NH}_2$  (Protease-activated receptor 2 agonist), [ $\text{Pyr}^1$ ]-Apelin-13 (APJ receptor agonist), ATP (purinergic receptor agonist), Neuropeptide Y (neuropeptide Y receptor agonist) and capsaicin (transient receptor potential vanilloid 1 agonist), and finally cAMP accumulation induced by iloprost

**Table 2** The effect of migrastatin on various stimuli

Ligand (cell, readout)	Migrastatin $\text{IC}_{50}$ ( $\mu\text{M}$ )
Carbachol (SK-N-SH, $\text{Ca}^{2+}$ )	28
Sulprostone (K562, $\text{Ca}^{2+}$ )	>120
SLIGRL- $\text{NH}_2$ (A431, $\text{Ca}^{2+}$ )	>120
NPY (HEL, $\text{Ca}^{2+}$ )	>120
[ $\text{Pyr}^1$ ]-Apelin-13 (HEL, $\text{Ca}^{2+}$ )	>120
ATP (HEL, $\text{Ca}^{2+}$ )	>120
Iloprost (HEL, cAMP)	>120
Capsaicin (rat DRG neuron, $\text{Ca}^{2+}$ )	>120



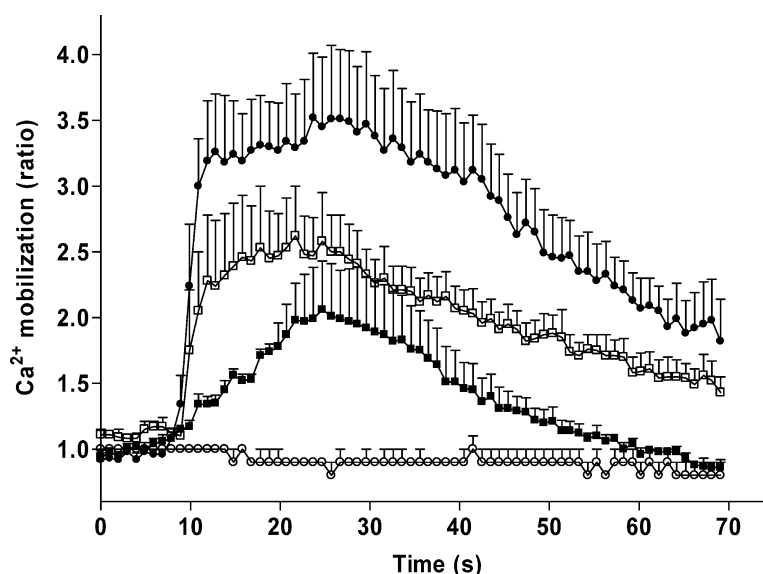
**Fig. 5** The effect of migrastatin on the binding of [ $^3\text{H}$ ]-*N*-methylscopolamine to  $\text{M}_1\sim\text{M}_5$  mAChR expressing CHO membrane.

Data represents mean  $\pm$  S.E.M. of 3 independent experiments.  $\nabla$ :  $\text{M}_1$ ,  $\diamond$ :  $\text{M}_2$ ,  $\bullet$ :  $\text{M}_3$ ,  $\circ$ :  $\text{M}_4$ ,  $\blacktriangledown$ :  $\text{M}_5$ .

(prostaglandin receptor agonist). Migrastatin did not inhibit these receptor activities in concentrations of up to  $120 \mu\text{M}$  (Table 2). These results suggest that migrastatin selectively inhibits  $\text{Ca}^{2+}$  mobilization induced by mAChR agonist.

### Effects of Migrastatin on *N*-Methylscopolamine Binding to $\text{M}_1\sim\text{M}_5$ Receptors

To investigate whether migrastatin directly affects mAChRs and to clarify the selective affinity of migrastatin for each mAChR ( $\text{M}_1\sim\text{M}_5$ ), the effect of migrastatin on the binding [ $^3\text{H}$ ]NMS to  $\text{M}_1\sim\text{M}_5$  expressing CHO membrane was evaluated. Migrastatin inhibited the binding of [ $^3\text{H}$ ]NMS to each receptor in a dose-dependent manner (Fig. 5). The



**Fig. 6** Carbachol-induced  $\text{Ca}^{2+}$  mobilization in primary cultured rat bladder smooth muscle cells and the effect of 10 and 30  $\mu\text{M}$  of migrastatin on this response.

Cells are observed under confocal laser microscopy. The means of data points determined in quintuple are shown. ●, carbachol; □, carbachol+migrastatin 10  $\mu\text{M}$ ; ■, carbachol+migrastatin 30  $\mu\text{M}$ ; ○, none.

$K_i$  value of migrastatin for  $M_{1-5}$  is >200, 200, 31, 43, and >200  $\mu\text{M}$ , respectively. These results suggest that migrastatin acts as a mAChR antagonist.

#### Effect of Migrastatin on $\text{Ca}^{2+}$ Mobilization in Primary Cultures of Rat Bladder Smooth Muscle Cells

Since bladder smooth muscle cells are known to express mAChRs, especially  $M_2$  and  $M_3$  receptors, a pharmacological study was carried out using primary cultures of bladder smooth muscle cells [13]. We evaluated the effect of migrastatin on  $\text{Ca}^{2+}$  mobilization in primary cultures of rat bladder smooth muscle cells. As shown in Fig. 6, carbachol induced  $\text{Ca}^{2+}$  mobilization and migrastatin prevented the effect of carbachol at a concentration of 10 and 30  $\mu\text{M}$ , respectively. These results clearly show that migrastatin is functionally effective in bladder smooth muscle cells.

## Discussion

Behavioral assessment of mice initially indicated a possible pharmacological effect of migrastatin. Intravenous administration of migrastatin increased the frequency of water intake during feeding. It seemed likely that migrastatin affected mAChRs because  $M_3$  knockout mice showed an increase of prandial drinking and clinically-used mAChR antagonists caused a dry mouth [16, 18]. Of course our observation could not exclude other possible

mechanism because several drugs are known to evoke dipsogenic behavior as a side effect [19, 20]. Therefore, pharmacological activities of migrastatin were examined in detail *in vitro*. SK-N-SH cells were reported to express mAChRs [21] and we observed that migrastatin inhibited  $\text{Ca}^{2+}$  mobilization induced by carbachol in SK-N-SH cells dose-dependently, although migrastatin did not affect second messengers induced by other GPCRs and ion channel ligands. These cell-based assays revealed that migrastatin selectively inhibited the signaling of mAChRs. Moreover, receptor-binding experiments with *N*-methylscopolamine and mAChRs suggest that migrastatin is an antagonist of mAChRs.

Migrastatin was first identified as a cell migration inhibitor of human esophageal cancer EC17 cells [1]. We have demonstrated that migrastatin inhibits the cell migration of SK-N-SH cells with a similar concentration that inhibits carbachol induced  $\text{Ca}^{2+}$  mobilization, whereas a broad muscarinic receptor antagonist, atropine did not inhibit cell migration (data not shown). These results indicated the possibility that migrastatin not only interacts with mAChRs but also with other target molecules. Recently, it was reported that synthetic migrastatin analogues, migrastatin core macroketone and core macrolactam, inhibit Rac activation and lamellipodia formation [7]. This observation may provide another approach to investigate the actions of migrastatin.

Furthermore, the effect of other macrolides on  $\text{Ca}^{2+}$

mobilization induced by carbachol was evaluated because migrastatin is classified as a 14-membered ring macrolide. Macrolides are known to have various biological activities such as anti-microbial activity, anti-inflammatory properties and anti-metastatic activity [22~24]. Four other 14~16 membered macrolides in this study did not inhibit  $\text{Ca}^{2+}$  mobilization or prevent cell migration of SK-N-SH cells. In addition, migrastatin did not show antibacterial activity against Gram-positive and Gram-negative bacteria at a concentration of least  $100\ \mu\text{M}$  (data not shown). These results suggest that the biological property of migrastatin is different from other macrolides. Migrastatin also has a structurally characteristic glutarimide group. Glutarimide and cycloheximide, a typical glutarimide-related compound, showed no effect on  $\text{Ca}^{2+}$  mobilization. Cycloheximide is known to have antifungal activity and can interfere with protein synthesis. However, migrastatin showed no antifungal activity at a concentration of up to  $100\ \mu\text{M}$ . Together with the finding that the activity of **3** on  $\text{Ca}^{2+}$  mobilization is attenuated approximately 4 times compared to migrastatin, it can be concluded that both the macrolide and glutarimide portion of migrastatin are critical moieties in the inhibitory effect on mAChRs of migrastatin.

Acetylcholine plays an important role in diseased voiding, such as an overactive bladder as well as in normal physiological voiding. mAChR antagonists have been used clinically because acetylcholine regulates direct contraction of bladder smooth muscle cells [14, 25]. Accordingly, the effects of muscarinic receptor antagonists in bladder smooth muscle cells were well investigated and reflected the results of *in vivo* experiments [26]. We confirmed that migrastatin is pharmacologically active in rat bladder smooth muscle cells so that migrastatin, or more potent derivatives of migrastatin, could be expected to be effective in the regulation of bladder function.

Our present results demonstrate that migrastatin interacts with mAChRs and this result may prove to help further biological and chemical investigations of migrastatin and its derivatives.

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