

# Potential of Muscarinic M<sub>3</sub> Receptor Activation through a New Allosteric Site with a Novel Positive Allosteric Modulator ASP8302<sup>§</sup>

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## ABSTRACT

Muscarinic M<sub>3</sub> (M<sub>3</sub>) receptors mediate a wide range of acetylcholine (ACh)-induced functions, including visceral smooth-muscle contraction and glandular secretion. Positive allosteric modulators (PAMs) can avoid various side effects of muscarinic agonists with their spatiotemporal receptor activation control and potentially better subtype selectivity. However, the mechanism of allosteric modulation of M<sub>3</sub> receptors is not fully understood, presumably because of the lack of a potent and selective PAM. In this study, we investigated the pharmacological profile of ASP8302, a novel PAM of M<sub>3</sub> receptors, and explored the principal site of amino-acid sequences in the human M<sub>3</sub> receptor required for the potentiation of receptor activation. In cells expressing human M<sub>3</sub> and M<sub>5</sub> receptors, ASP8302 shifted the concentration-response curve (CRC) for carbachol to the lower concentrations with no significant effects on other subtypes. In a binding study with M<sub>3</sub> receptor-expressing membrane, ASP8302 also shifted the CRC for ACh without affecting the binding of orthosteric agonists. Similar shifts in the CRC of contractions by multiple stimulants were also confirmed in isolated human bladder

strips. Mutagenesis analysis indicated no interaction between ASP8302 and previously reported allosteric sites; however, it identified threonine 230 as the amino acid essential for the PAM effect of ASP8302. These results demonstrate that ASP8302 enhances the activation of human M<sub>3</sub> receptors by interacting with a single amino acid distinct from the reported allosteric sites. Our findings suggest not only a novel allosteric site of M<sub>3</sub> receptors but also the potential application of ASP8302 to diseases caused by insufficient M<sub>3</sub> receptor activation.

## SIGNIFICANCE STATEMENT

The significance of this study is that the novel M<sub>3</sub> receptor positive allosteric modulator ASP8302 enhances the activation of human M<sub>3</sub> receptor by interacting with a residue distinct from the reported allosteric sites. The finding of Thr<sup>230</sup> as a novel amino acid involved in the allosteric modulation of M<sub>3</sub> receptors provides significant insight into further research of the mechanism of allosteric modulation of M<sub>3</sub> and other muscarinic receptors.

## Introduction

Muscarinic acetylcholine receptors (mAChRs), a family of G-protein-coupled receptors with five subtypes, mediate a wide range of physiologic effects of acetylcholine (ACh)

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released from parasympathetic nerve terminals (Fetscher et al., 2002; Wess et al., 2003). In the mAChR family, muscarinic M<sub>3</sub> (M<sub>3</sub>) receptors are expressed broadly and are responsible for various cholinergic functions, such as smooth-muscle contraction and secretion from glands (Wess et al., 2007). Although multiple receptor agonists and cholinesterase inhibitors have been used to treat disorders caused by insufficient M<sub>3</sub> receptor activity, currently available agonists have low receptor subtype sensitivity and excessive activation, and their use carries a risk of adverse cholinomimetic side effects (Brown, 2020).

Positive allosteric modulators (PAMs) are a class of drugs that bind to a site spatially distinct site from the orthosteric ligand binding site (i.e., allosteric site), thereby enhancing the responses mediated by receptor activation. Some PAMs

**ABBREVIATIONS:** ACh, acetylcholine; CCh, carbamoylcholine chloride; CI, confidence interval; CRC, concentration-response curve; ECL, extracellular loop; E<sub>max</sub>, maximum efficacy; [<sup>3</sup>H]NMS, [<sup>3</sup>H]-methyl-<sup>3</sup>H]scopolamine; M<sub>3</sub>, muscarinic M<sub>3</sub>; mAChR, muscarinic acetylcholine receptor;  $\alpha$ -MEM,  $\alpha$  minimum essential medium; PAM, positive allosteric modulator; PCR, polymerase chain reaction; TM, transmembrane.

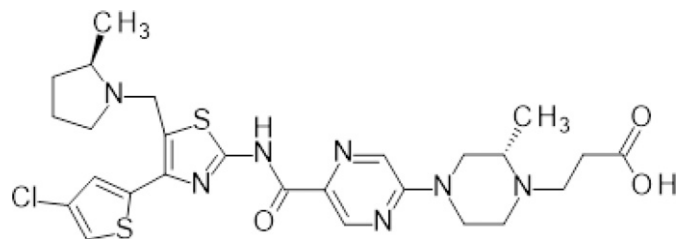
increase the affinity of an agonist to the receptor, whereas others enhance orthosteric agonist efficacy only or enhance both affinity and efficacy mainly because of a structural change in the receptor (Conn et al., 2009). Compared with agonists, PAMs are considered to be advantageous in avoiding side effects by enhancing the effect of agonists only (when they are present) and in obtaining subtype selectivity by not targeting the orthosteric site, which is highly conserved among five subtypes in the case of mAChR (Dror et al., 2013; Kruse et al., 2013).

Considerable progress has been made over the past few decades in our understanding of the mechanisms of allosteric modulations of mAChR subtypes and in identifying allosteric ligands (Burger et al., 2018). Previous studies using mutagenesis have suggested that allosteric ligands for mAChR bind to regions in the extracellular loops (ECLs) and the top of the transmembrane (TM) helices (Gnagay et al., 1999; Krejčí and Tucek, 2001; Huang et al., 2005). Determination of the molecular structures of human M<sub>2</sub> receptors and interaction with allosteric modulators has indicated that key residues and amino acids for the binding and action of allosteric ligands (Gregory et al., 2007) are located in the second and third ECLs and the second and seventh TM regions (Haga et al., 2012; Dror et al., 2013; Kruse et al., 2013). The structure of rat M<sub>3</sub> receptors has also been reported (Kruse et al., 2013). However, details about the allosteric site of M<sub>3</sub> receptors and the mechanism of action of allosteric ligands have not been fully elucidated, presumably because of the low affinity and limited selectivity of the available tools (Bock et al., 2018). To date, only a few studies have reported the discovery of M<sub>3</sub>-preferred PAM through a high-throughput screening and comprehensive structure-activity relationships study (Tanaka et al., 2020, 2021).

ASP8302 (Fig. 1) is a newly developed PAM that targets M<sub>3</sub> receptors. In this study, we investigated the pharmacological profile of ASP8302 in the binding and activation of M<sub>3</sub> receptors as well as the modulation of bladder smooth-muscle contraction, with the latter as a representative biologic function showcasing the role of M<sub>3</sub> receptors (Matsui et al., 2002; Schneider et al., 2004). We also explored the site in human M<sub>3</sub> receptors critical for the PAM effect of ASP8302 using chimeric and mutated receptors.

## Materials and Methods

**Test Substances.** ASP8302 (3-[(2*S*)-4-(5-{[4-(4-chlorothiophen-2-yl)-5-[(2*R*)-2-methylpyrrolidin-1-yl]methyl]-1,3-thiazol-2-yl]carbamoyl]pyrazin-2-yl)-2-methylpiperazin-1-yl]propanoic acid) was synthesized by Astellas Pharma Inc. ASP8302 dihydrochloride was used in the intracellular Ca<sup>2+</sup> mobilization assay except for human M<sub>3</sub>



**Fig. 1.** Chemical structure of ASP8302 (3-[(2*S*)-4-(5-{[4-(4-chlorothiophen-2-yl)-5-[(2*R*)-2-methylpyrrolidin-1-yl]methyl]-1,3-thiazol-2-yl]carbamoyl]pyrazin-2-yl)-2-methylpiperazin-1-yl]propanoic acid).

receptor and the receptor binding assay. ASP8302 dimaleate was used in the intracellular Ca<sup>2+</sup> mobilization assay for the human M<sub>3</sub> receptor and measurement of contractile responses of isolated human bladder strips.

Salts of ASP8302 were dissolved in DMSO for in vitro experiments. Carbamoylcholine chloride (CCh) (Sigma-Aldrich, St. Louis, MO), atropine sulfate monohydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and acetylcholine chloride (Sigma-Aldrich) were dissolved in water. [*N*-Methyl-<sup>3</sup>H]scopolamine (<sup>3</sup>H]NMS) was purchased from PerkinElmer Inc. (Boston, MA) and was diluted with the reaction buffer [50 mmol/l Tris-HCl (pH 7.5), 10 mmol/l MgCl<sub>2</sub>, and 1 mmol/l EDTA] for the binding study.

**Constructions and Cultures of Cells Expressing Human Muscarinic M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub>, M<sub>4</sub>, and M<sub>5</sub> Receptors and Human M<sub>3</sub> Receptor Mutants.** CHO-K1 cells expressing human muscarinic receptors were constructed by Astellas Pharma Inc. Polymerase chain reaction (PCR) products of human M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub>, M<sub>4</sub>, and M<sub>5</sub> muscarinic receptors amplified from human genomic DNA using listed primers (shown in the Supplemental Material) were inserted into pcDNA3.1 expression vectors (Life Technologies Japan Ltd., Tokyo, Japan), and the muscarinic receptor-expressing vectors were transfected into CHO-K1 cells (American Type Culture Collection CCL-61) using LipofectAMINE 2000 reagent (Life Technologies Japan Ltd.). The cells were cultured in  $\alpha$  minimum essential medium ( $\alpha$ -MEM) containing 2 mmol/l glutamic acid, 10% fetal bovine serum, and 2 mg/ml geneticin (Life Technologies Japan Ltd.) for about 4 weeks to acquire drug-resistant clones stably expressing each muscarinic receptor. To convert the signaling pathway of M<sub>2</sub> and M<sub>4</sub> muscarinic receptor subtypes, which are coupled to Gi/o proteins, into a Gq signaling pathway, we also transfected a human G<sub>15</sub> G-protein  $\alpha$  subunit-expressing vector into human M<sub>2</sub>- and M<sub>4</sub>-expressing cells as described above, and drug-resistant clones were selected in the presence of 0.5 mg/ml hygromycin B.

Vectors expressing mutated M<sub>3</sub> receptor were constructed by PCR-based single-site mutagenesis or inverse PCR-based site-directed mutagenesis with or without mega primer polymerase chain reaction. Inverse PCR, digestion of the template plasmid by Dpn I, and self-ligation of PCR products (kinase/ligase) were conducted using a KOD-Plus- Mutagenesis Kit (Toyobo, Osaka Japan) according to the manufacturer's instruction manuals. Sequences of the mutant constructs were confirmed by DNA sequencing. Details are shown in the Supplemental Material. Plasmid DNAs of M1\_TM4\_M3, M3\_TM4\_M1\_TM5\_M3, M1\_TM4\_M3\_TM5\_M1, M3\_ECL2\_F210L\_K213E\_E22\_8Q\_T230I, M3\_ECL2\_F210A, M3\_ECL2\_K213A, M3\_ECL2\_E228A, M3\_ECL2\_T230A, M1\_ECL2\_I187T, and M5\_ECL2\_T192A were constructed using gene synthesis by Thermo Fisher Scientific.

Cells expressing M<sub>1</sub>, M<sub>3</sub>, or M<sub>5</sub> receptor were cultured in  $\alpha$ -MEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 2 mmol/l L-glutamine, and 0.2 mg/ml geneticin at 37°C and 5% CO<sub>2</sub>. Cells expressing the M<sub>2</sub> or M<sub>4</sub> receptor were cultured in  $\alpha$ -MEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 2 mmol/l L-glutamine, 0.2 mg/ml geneticin, and 50  $\mu$ g/ml hygromycin at 37°C and 5% CO<sub>2</sub>.

**Measurement of Intracellular Ca<sup>2+</sup> Mobilization Using Fluorometric Imaging Plate Reader.** The positive allosteric modulating effect of ASP8302 was investigated using a Ca<sup>2+</sup> flux assay described previously (Tanaka et al., 2020) with some modifications. CCh was used instead of the endogenous agonist ACh because of its commonality with ACh in the ligand binding site (and process) (Mokry et al., 2005) and to avoid potential underestimation of receptor activation or variability in the result due to the decomposition of ACh by acetylcholinesterase. ASP8302 and CCh were dissolved in DMSO and water, respectively, and diluted with assay buffer [Hank's balanced salt solution containing 2.5 mmol/l probenecid, 20 mmol/l HEPES (pH 7.5), and 1 mg/ml bovine serum albumin]. The final concentration of DMSO was 1%.

Cells were seeded in tissue culture-treated black-walled clear-bottom 384-well plates. The cells were cultured for 1 day (plates with

12,000 cells per well) or 2 days (plates with 6,000 cells per well). On the day of measurement, the cells were loaded with 3.1  $\mu\text{mol/l}$  Fluo-4 acetoxymethyl (Dojindo, Kumamoto, Japan) in assay buffer and incubated for about 2 hours at room temperature. After they were washed with assay buffer, fluorescence was measured using Fluorometric Imaging Plate Reader–TETRA (Molecular Devices Corporation Japan, Tokyo, Japan) with an excitation wavelength of 470–495 nm and an emission wavelength of 515–575 nm. ASP8302 (0.1, 0.3, and 1  $\mu\text{mol/l}$  for  $M_3$  and  $M_5$  receptors, 10  $\mu\text{mol/l}$  for  $M_1$ ,  $M_2$ , and  $M_4$  receptors) was added to each well. Approximately 4 minutes later, CCh (0.0095–10,000 nmol/l) was added to each well, and the maximal increase induced by CCh was recorded. Data in each single experiment were obtained in duplicate.

Change in intracellular  $\text{Ca}^{2+}$  mobilization induced by CCh in the presence of ASP8302 was expressed as a percentage, with the maximum response at the maximum concentration of CCh in the absence of ASP8302 as 100% and the signal in the absence of CCh and ASP8302 as 0%. The same process was performed for human  $M_3$  receptor mutants.

**Receptor Binding Assay.** Competitive binding studies were performed to examine the displacement of [ $^3\text{H}$ ]NMS binding to human  $M_3$  receptors using the membrane fraction of CHO-K1 cells expressing human  $M_3$  receptors. ACh was used as the agonist in this cell-free system. To calculate the concentrations of ACh, atropine, and ASP8302 showing 50% inhibition of [ $^3\text{H}$ ]NMS binding to the receptor ( $\text{IC}_{50}$  values), 30  $\mu\text{g}$  of cell membrane fraction ([ $^3\text{H}$ ]NMS affinity:  $B_{\text{max}} = 0.41$  pmol/mg protein,  $K_d = 0.092$  nmol/l) was incubated with 0.4 nmol/l of [ $^3\text{H}$ ]NMS for 90 minutes at room temperature in the presence of ACh, atropine, or ASP8302. To evaluate the effect of ASP8302 on the binding affinity of ACh, 30  $\mu\text{g}$  of cell membrane fraction was incubated with 0.4 nmol/l of [ $^3\text{H}$ ]NMS for 90 minutes at room temperature in the presence of ACh and 1, 3, or 10  $\mu\text{mol/l}$  of ASP8302. To determine nonspecific binding, 1  $\mu\text{mol/l}$  of atropine or 1 mmol/l of ACh was added. Cell membranes were then obtained by filtering the cell membrane fraction through glass fiber (GF/C) filters. After drying the GF/C filter, each filter was mixed with 30  $\mu\text{l}$  of liquid scintillation cocktail (Microscint-20, PerkinElmer Inc.) and incubated at room temperature for 1 night. The radioactivity was counted using a TopCount (PerkinElmer Inc.).

**Measurement of Contractile Responses of Isolated Bladder Strips.** Human bladder tissues were obtained from six patients (four males, two females) with bladder cancer undergoing radical cystectomy through the Department of Clinical Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. The study was approved by the Ethics Committee of Kyushu University and Astellas Pharma Inc. All the human bladder tissues were collected after informed consent was obtained from the donors. Isolated human bladder tissues were immediately kept in Krebs-Henseleit solution (composition described below) at 4°C prior to transfer to the laboratory. The cold bladder time ranged from 14 to 16 hours. Bladder smooth-muscle strips (approximately 6 mm in length and 2 mm in width) were prepared from the bladder dome above the trigon after removal of the urothelium and connective tissue under a dissecting microscope.

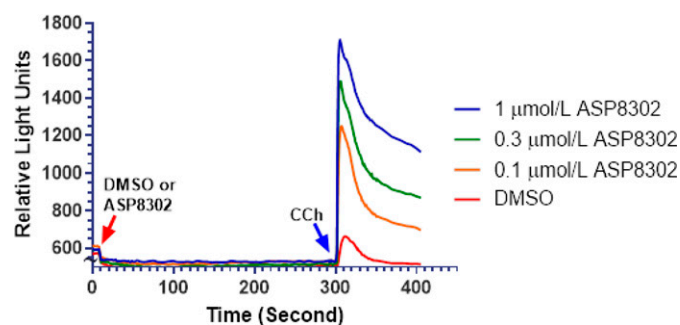
The strip was suspended between a pair of electrodes (Nihon Kohden, Tokyo, Japan) in 10-ml organ baths containing Krebs-Henseleit solution (118.4 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1.2 mmol/l  $\text{KH}_2\text{PO}_4$ , 1.2 mmol/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 25 mmol/l  $\text{NaHCO}_3$ , 11 mmol/l glucose) maintained at 37°C and constantly aerated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Celphines tied with silk ligatures were applied to each end of the bladder strip. One end was attached to a holder and the other to an isometric tension transducer (TB-611T, Nihon Kohden) coupled to a PowerLab system (Power Lab8/30, AD Instruments, Aichi, Japan) to record contractile responses. Resting tension of the strips was adjusted to approximately 1 g, and then the strips were left to equilibrate for approximately 1 hour. Strips were precontracted with KCl (final concentration: 60 mmol/l) and washed out with Krebs-Henseleit solution twice.

Contractions induced by the endogenous agonist ACh as well as those by CCh were evaluated to investigate the effect of ASP8302 on cholinergic contraction. After confirming contraction with 10  $\mu\text{mol/l}$  CCh or 30  $\mu\text{mol/l}$  ACh, the effect of ASP8302 on CCh- or ACh-induced concentration-dependent contraction was evaluated. Concentration-response curves for CCh and ACh were constructed six times by repeating treatments with CCh or ACh (final 0.003  $\mu\text{mol/l}$  to 30  $\mu\text{mol/l}$ ), with a washout time interval of 1 hour between each concentration. ASP8302 (final 1 and 10  $\mu\text{mol/l}$ ) was added 15 minutes before the addition of CCh or ACh. Vehicle for ASP8302 (DMSO final 0.1%) was applied as a time-matched control. Contraction of each strip was expressed as the % of that induced by 10  $\mu\text{mol/l}$  CCh or 30  $\mu\text{mol/l}$  ACh.

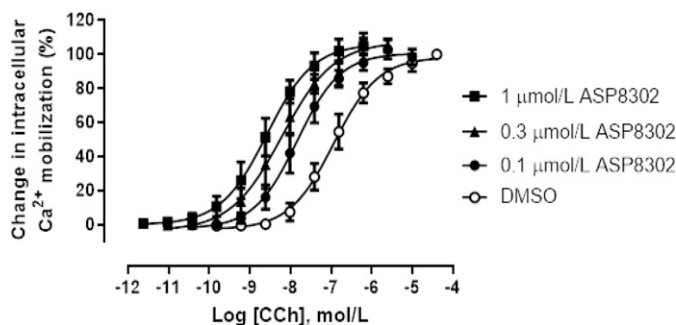
**Data Analysis.** The results are presented as mean  $\pm$  S.E.M.  $\text{EC}_{50}$  and the maximum efficacy ( $E_{\text{max}}$ ) values of the CRC were calculated by Sigmoid- $E_{\text{max}}$  nonlinear regression analysis and expressed as the geometric mean and 95% confidence interval (CI) of three or four independent experiments. The positive allosteric modulating activity of ASP8302 was expressed by a fold shift of CCh-CRC calculated by dividing the  $\text{EC}_{50}$  value of CCh-CRC in the absence of ASP8302 by the  $\text{EC}_{50}$  value of CCh-CRC in the presence of ASP8302. Statistical significance analyses were performed using SAS software (version 9, SAS Institute Japan, Tokyo, Japan) or GraphPad Prism (version 5 to 8, GraphPad Software, Inc., San Diego, CA). In the tissue contraction study, KCl-induced contractions with ASP8302 were compared with control using Dunnett's multiple comparisons test, and a probability value of less than 0.05 was considered significant.

## Results

**Positive Allosteric Modulating Activities of ASP8302 on Human Muscarinic Receptors.** We examined the PAM property and subtype selectivity of ASP8302 by assessing the effect on the CRC for CCh-induced  $\text{Ca}^{2+}$  mobilization in cells expressing human muscarinic receptors. ASP8302 (0.1, 0.3, and 1  $\mu\text{mol/l}$ ) enhanced the response induced by CCh (9.8 nmol/l) in a concentration-dependent manner, whereas ASP8302, per se, did not produce any change in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) up to 10  $\mu\text{mol/l}$ . Representative traces of changes in  $[\text{Ca}^{2+}]_i$  in the cells expressing human  $M_3$  receptors are shown in Fig. 2. ASP8302 shifted the CCh-CRC to the left (low-concentration side) without affecting the  $E_{\text{max}}$  (Fig. 3). The fold shifts of CCh-CRC induced by ASP8302 at 0.1, 0.3, and 1  $\mu\text{mol/l}$  were 7.9, 20.1, and 51.2, respectively (Table 1). In the cells expressing human  $M_5$  receptors, ASP8302 also shifted the CCh-CRC to the left without affecting the  $E_{\text{max}}$ , with fold shifts of 9.5, 22.7, and 79.6 at 0.1, 0.3,



**Fig. 2.** Representative signal traces of the effect of ASP8302 on carbachol-induced changes in intracellular  $\text{Ca}^{2+}$  in cells expressing human  $M_3$  receptors. Representative traces of fluorometric signals in the absence or presence of ASP8302 (0.1, 0.3, or 1  $\mu\text{mol/l}$ ). Control (DMSO, 1%) or ASP8302 was added 10 seconds after the initiation of measurement (red arrow), and CCh (9.8 nmol/l) was added 305 seconds after initiation (blue arrow).



**Fig. 3.** Effects of ASP8302 on carbachol concentration-response curve in CHO-K1 cells expressing human M<sub>3</sub> receptor. Carbachol concentration-response curves in CHO-K1 cells expressing human M<sub>3</sub> receptor in the absence (DMSO, 1%) or presence of ASP8302 (0.1, 0.3, or 1 μmol/l) (mean ± S.E.M., *n* = 4).

and 1 μmol/l, respectively (Table 1). In the cells expressing human muscarinic M<sub>1</sub>, M<sub>2</sub>, and M<sub>4</sub> receptors, the fold shifts of CCh-CRC induced by ASP8302 at 10 μmol/l were 0.5, 0.4, and 1.2, respectively (Table 1). In addition, ASP8302 (10 μmol/l) did not show significant (>50%) inhibition on radioligand binding to any of 57 receptors, ion channels, and transporters tested or on the reactions of three enzymes (shown in the Supplemental Material).

**Enhancement of Binding Affinity of Acetylcholine and Human M<sub>3</sub> Receptor by ASP8302.** To confirm that ASP8302 binds to a site distinct from the orthosteric ligand binding site recognized by the endogenous ligand ACh, a competitive binding experiment was performed using [<sup>3</sup>H]NMS.

Acetylcholine and atropine displaced the binding of [<sup>3</sup>H]NMS in a concentration-dependent manner with IC<sub>50</sub> values of 51 μmol/l and 6.2 nmol/l, respectively. In contrast, ASP8302 did not displace [<sup>3</sup>H]NMS binding up to a concentration of 10 μmol/l (Fig. 4), indicating no interaction on the orthosteric binding site. On the other hand, ASP8302 (1, 3, and 10 μmol/l) concentration-dependently shifted the ACh concentration-inhibition curves for [<sup>3</sup>H]NMS binding to the left (Fig. 5), indicating increased affinity between the receptor and ACh.

TABLE 1

Positive allosteric modulating activity of ASP8302 on carbachol-induced intracellular Ca<sup>2+</sup> changes in CHO-K1 cells expressing human muscarinic receptors EC<sub>50</sub> and E<sub>max</sub> values for the carbachol CRC were calculated by Sigmoid-E<sub>max</sub> nonlinear regression analysis. EC<sub>50</sub> values are expressed as the geometric mean and 95% CI of three or four independent experiments. Each E<sub>max</sub> value is expressed as the arithmetic mean. The fold-shift values were calculated by dividing the EC<sub>50</sub> value of carbachol CRC in the absence of ASP8302 by those in the presence of ASP8302.

Muscarinic Receptors (N)	Test Compound	EC <sub>50</sub> (mol/l)	95% CI (mol/l)	Fold Shift	E <sub>max</sub> (%)
M <sub>1</sub> (2)	DMSO	2.7E-07	ND	NA	105.7
	10 μmol/l ASP8302	5.6E-07	ND	0.5	111.3
M <sub>2</sub> (2)	DMSO	4.7E-07	ND	NA	111.3
	10 μmol/l ASP8302	1.0E-06	ND	0.4	116.2
M <sub>3</sub> (4)	DMSO	1.2E-07	3.1E-08-4.9E-07	NA	97.0
	0.1 μmol/l ASP8302	1.6E-08	3.3E-09-7.4E-08	7.9	100.0
	0.3 μmol/l ASP8302	6.1E-09	1.3E-09-2.8E-08	20.1	104.3
	1 μmol/l ASP8302	2.4E-09	5.4E-10-1.1E-08	51.2	104.8
M <sub>4</sub> (2)	DMSO	5.4E-07	ND	NA	104.4
	10 μmol/l ASP8302	4.5E-07	ND	1.2	105.2
M <sub>5</sub> (3)	DMSO	3.2E-08	2.3E-09-4.3E-07	NA	101.5
	0.1 μmol/l ASP8302	3.3E-09	4.1E-10-2.7E-08	9.5	99.2
	0.3 μmol/l ASP8302	1.4E-09	1.8E-10-1.1E-08	22.7	94.8
	1 μmol/l ASP8302	4.0E-10	5.8E-11-2.7E-09	79.6	93.1

N, number of trials; NA, not applicable; ND, not done.

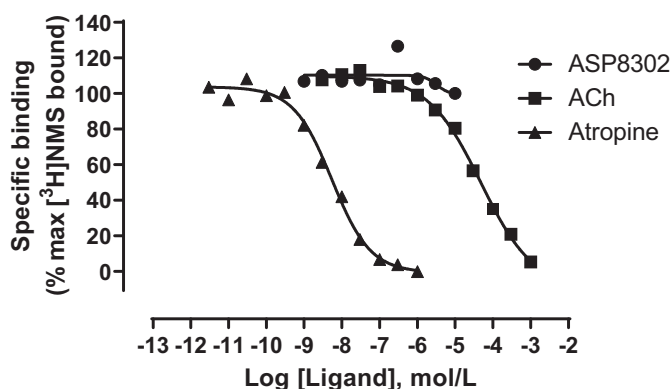
### Determination of the Residue in the Human M<sub>3</sub> Receptor Required for the PAM Activity of ASP8302.

We investigated the principal site in human M<sub>3</sub> receptors required for the PAM activity of ASP8302 by assessing the effect of this compound on CRC for CCh-induced Ca<sup>2+</sup> mobilization using cells expressing the M<sub>3</sub> receptors with various gene sequences transformed. Because ASP8302 did not have PAM activity on M<sub>1</sub> receptors (Table 1), we first located the key area in the M<sub>3</sub> receptor using M<sub>3</sub>-M<sub>1</sub> chimeric receptors with various residues of M<sub>3</sub> receptors replaced with the corresponding parts of M<sub>1</sub> receptors (Fig. 6).

As shown in Table 2, ASP8302 (10 μmol/l) did not show PAM activity in either condition in which we used the cells expressing the C-terminal side of M<sub>3</sub> receptors replaced with those of M<sub>1</sub> receptors from the amino acid next to serine (Ser)<sup>109</sup> (sequence numbering as human M<sub>3</sub> receptors) in TM2 (M3\_TM2\_M1 in Fig. 6) and those of M<sub>3</sub> receptors replaced with M<sub>1</sub> receptors from the amino acid next to alanine (Ala)<sup>201</sup> in TM4 (M3\_TM4\_M1). On the other hand, PAM activity was retained in the cells in which the residues of M<sub>3</sub> receptors next to isoleucine (Ile)<sup>249</sup> in TM5 were replaced with those of M<sub>1</sub> receptors (M3\_TM5\_M1). These results suggested that the principal site required for M<sub>3</sub> PAM might exist between Ala<sup>201</sup> and Ile<sup>249</sup> of M<sub>3</sub> receptors. To further verify the position of the principal site contributing to the PAM activity of ASP8302, we tried to evaluate the effect of ASP8302 using the cells in which the sequence of the N-terminal side was changed. When the N-terminal side until Ile<sup>249</sup> in TM5 was replaced with M<sub>1</sub> receptors (M1\_TM5\_M3), ASP8302 did not show PAM activity. The PAM activity of this compound was, however, observed when the amino-acid sequences of the M<sub>1</sub> receptor were replaced with those of M<sub>3</sub> receptor until Ala<sup>201</sup> in TM4 (M1\_TM4\_M3). Taken together, the principal amino acids are considered to exist in the domain from Pro<sup>202</sup> to Ile<sup>249</sup>, a span that includes the ECL2 and its flanking regions in TM4 and TM5.

We next examined the influence of the region from Pro<sup>217</sup> to Phe<sup>222</sup> on the PAM activity of ASP8302, which was reported to play an important role in the corresponding residue of M<sub>2</sub> receptors (Huang et al., 2005). In cells in which these six amino acids of the M<sub>3</sub> receptor were replaced with the





**Fig. 4.** Competition study of acetylcholine, atropine, and ASP8302 in [<sup>3</sup>H]N-methylscopolamine binding to human M<sub>3</sub> receptor. Competition studies were performed using NMS and membrane fractions expressing human M<sub>3</sub> receptor. Each plot is expressed as the mean of two independent experiments, so the error cannot be shown.

corresponding sequences of the M<sub>1</sub> receptor (Leu<sup>174</sup> to Tyr<sup>179</sup>, M3\_ECL2\_M1), ASP8302 (10 μmol/l) showed PAM activity (Table 2). By contrast, PAM activity of this compound was not observed in cells expressing M<sub>3</sub> receptors, of which all other parts except for Pro<sup>217</sup> to Phe<sup>222</sup> were replaced with the sequences of M<sub>1</sub> receptor (M1\_ECL2\_M3) (Table 2). In addition, ASP8302 did not show PAM activity in cells for which valine (Val)<sup>194</sup> to isoleucine (Ile)<sup>246</sup> of the M<sub>3</sub> receptor was replaced with the equivalent sequence of the M<sub>1</sub> receptor (Leu<sup>151</sup> to Val<sup>203</sup>) (M3\_TM4\_M1\_TM5\_M3) (Table 2). On the other hand, the PAM effect of ASP8302 was maintained in cells expressing M<sub>3</sub> receptors retaining Val<sup>194</sup> to Ile<sup>246</sup> together with replacement of the rest of the sequences with those of M<sub>1</sub> receptor (M1\_TM4\_M3\_TM5\_M1) (Table 2). These results indicate that the critical residue required for the PAM activity of ASP8302 exists in the residues from Pro<sup>202</sup> to Val<sup>216</sup> and/or from Ile<sup>223</sup> to Ile<sup>246</sup> located in or adjacent to ECL2.

**Identification of Threonine 230 as the Principal Amino Acid for the Expression of the PAM Activity of ASP8302.** To identify the principal amino acids in the residues from Pro<sup>202</sup> to Val<sup>216</sup> and from Ile<sup>223</sup> to Ile<sup>246</sup>, we introduced mutations into four amino acids that differ between human M<sub>1</sub> and M<sub>3</sub> receptors in cells used to evaluate the PAM activity of ASP8302. Three of these were common between human M<sub>3</sub> and M<sub>5</sub> receptors (Fig. 7). The PAM activity of ASP8302 (10 μmol/l) was not observed when four specific amino acids of M<sub>3</sub> receptors (Phe<sup>210</sup>, Lys<sup>213</sup>, Glu<sup>228</sup>, and Thr<sup>230</sup>) in cells were replaced with the corresponding amino acids of M<sub>1</sub> receptors (Leu<sup>167</sup>, Glu<sup>170</sup>, Gln<sup>185</sup>, and Ile<sup>187</sup>) (M3\_ECL2\_F210L\_K213E\_E228Q\_T230I) (Table 3). Subsequently, we tried to substitute individual amino acids in the cells with alanine. Among the four conditions, the PAM activity of ASP8302 disappeared only in the cells in which Thr<sup>230</sup> was replaced with alanine (Table 3).

To confirm the role of Thr<sup>230</sup> as a principal amino acid in the PAM activity of ASP8302, we examined the effect of ASP8302 on CRC for CCh-induced Ca<sup>2+</sup> mobilization in cells expressing the mutant M<sub>1</sub> and M<sub>5</sub> receptors in which the amino acids corresponding to Thr<sup>230</sup> (Ile<sup>187</sup> of M<sub>1</sub> receptor, Thr<sup>192</sup> of M<sub>5</sub> receptor) were substituted into alanine and threonine, respectively. In the mutated M<sub>5</sub> receptor (M5\_ECL2\_T192A), the PAM

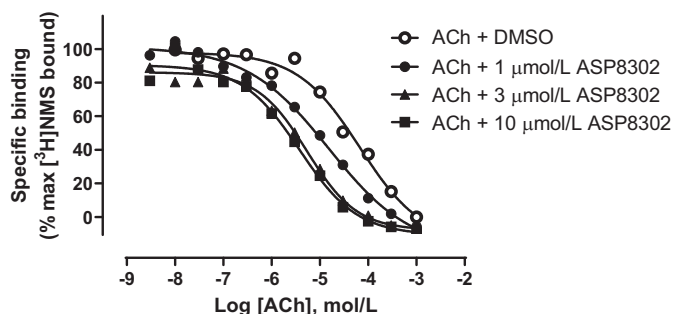
activity of ASP8302 was not observed. On the other hand, ASP8302 showed PAM activity under conditions in which Ile<sup>187</sup> of the M<sub>1</sub> receptor was replaced with threonine (M1\_ECL2\_I187T) (Table 3).

**Potential of ASP8302 on CCh- and ACh-Induced Contraction of Human Isolated Urinary Bladder.** To determine whether ASP8302 functionally modulates cholinergic contractions of the bladder smooth muscle in the most physiologically relevant experimental setting in this study, we investigated the effect of ASP8302 on contractions induced by the endogenous agonist ACh as well as CCh in isolated human bladder smooth-muscle strips. As shown in Fig. 8, ASP8302 (1 and 10 μmol/l) shifted the CRC of CCh and ACh to the left, that is, to the lower concentration side. ASP8302 at 10 μmol/l shifted the EC<sub>50</sub> of CCh-CRC from 0.44 μmol/l to 0.038 μmol/l and the EC<sub>50</sub> of ACh CRC from 0.64 μmol/l to 0.032 μmol/l (Fig. 8, B and C). ASP8302 did not affect either KCl-induced contraction (Fig. 8, D).

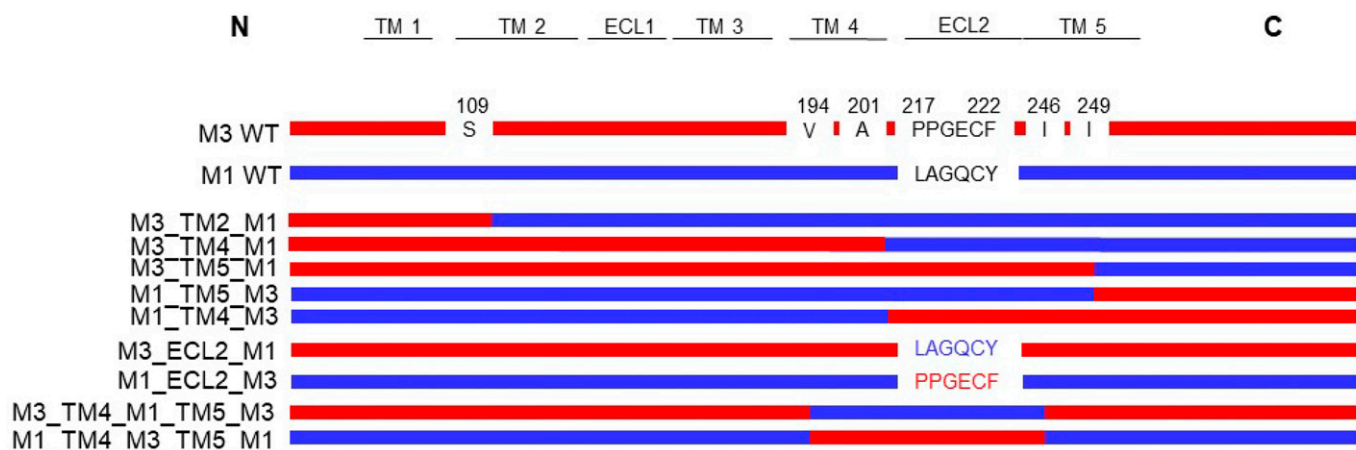
## Discussion

Recent studies on allosteric modulation of muscarinic receptors have developed various PAMs that may potentially overcome the safety concerns of existing cholinergic agents, such as agonists and choline esterase inhibitors, because of their insufficient subtype selectivity and unnecessary activation of the receptor (Urwyler, 2011). However, only a few PAMs have been reported for M<sub>3</sub> receptors (Bridges et al., 2009; Stahl et al., 2011; Tanaka et al., 2020). This may have limited investigations into the mechanistic details underlying allosteric modulation for M<sub>3</sub> receptors and its advantages over existing cholinergic agents. In the present study, we demonstrated that the novel compound ASP8302 possessed potent PAM activity on the M<sub>3</sub> receptor in receptor binding and intracellular Ca<sup>2+</sup> mobilization assays. In addition, using mutagenesis approaches we identified Thr<sup>230</sup> as an essential amino-acid residue for the PAM activity of ASP8302. Furthermore, we confirmed that ASP8302 augmented the human bladder smooth-muscle contraction induced by CCh. To our knowledge, this is the first study to demonstrate that a single amino acid distinct from previously reported allosteric sites (Kruse et al., 2012) plays a critical role in the positive allosteric modulation of M<sub>3</sub> receptor.

In cells expressing M<sub>3</sub> receptors, ASP8302 concentration-dependently shifted the CRC of CCh-induced intracellular



**Fig. 5.** Inhibition of [<sup>3</sup>H]N-methylscopolamine binding to human M<sub>3</sub> receptor by acetylcholine in the absence or presence of ASP8302. Binding studies were performed using NMS and membrane fractions expressing human M<sub>3</sub> receptor. Each plot is expressed as the mean of two independent experiments, so the error cannot be shown.



**Fig. 6.** Composition diagram of the prepared human M<sub>3</sub> receptor mutants. The amino-acid sequence number corresponds to the human M<sub>3</sub> receptor. C, C terminus; M1, muscarinic M<sub>1</sub> receptor; N, N terminus; WT, wild type.

Ca<sup>2+</sup> mobilization to the left (i.e., enhanced efficacy of CCh at lower concentrations). The observed leftward shift by 51.2 times at 1 μmol/l ASP8302 suggests that ASP8302 is more potent than other functionally confirmed M<sub>3</sub> receptor PAMs [no shift with VU0119498 up to 1 μmol/l (Burger et al., 2018) or 10 times shift at 1 μmol/l for compound 9 (Tanaka et al., 2020)]. In addition, ASP8302 produced no change in the E<sub>max</sub> of CCh-induced intracellular Ca<sup>2+</sup> mobilization, whereas amiodarone (Stahl et al., 2011) and VU0119498 (Zhu et al.,

2019) increased it. These findings demonstrate a unique M<sub>3</sub> PAM profile of ASP8302. ASP8302 (10 μmol/l) had no PAM activities on human M<sub>1</sub>, M<sub>2</sub>, or M<sub>4</sub> receptors (Table 1), although ASP8302 did show PAM activity on human M<sub>5</sub> receptors with a similar potency to that on M<sub>3</sub> receptors. Previous studies reported that many known M<sub>3</sub> receptor PAMs had affinity for M<sub>1</sub> receptors (Lazareno et al., 1998, 2002; Bridges et al., 2009; Stahl and Ellis, 2010) and that classic allosteric ligands like gallamine showed PAM activity on both

TABLE 2

Positive allosteric modulating activity of ASP8302 on carbachol-induced intracellular Ca<sup>2+</sup> changes in CHO-K1 cells expressing human wild-type and mutated muscarinic receptors EC<sub>50</sub> values for the carbachol CRC were calculated by Sigmoid-Emax nonlinear regression analysis. EC<sub>50</sub> values are expressed as the geometric mean and 95% CI of three or four independent experiments. The fold-shift values were calculated by dividing the EC<sub>50</sub> value of carbachol conc.-response curve in the absence of ASP8302 by those in the presence of ASP8302.

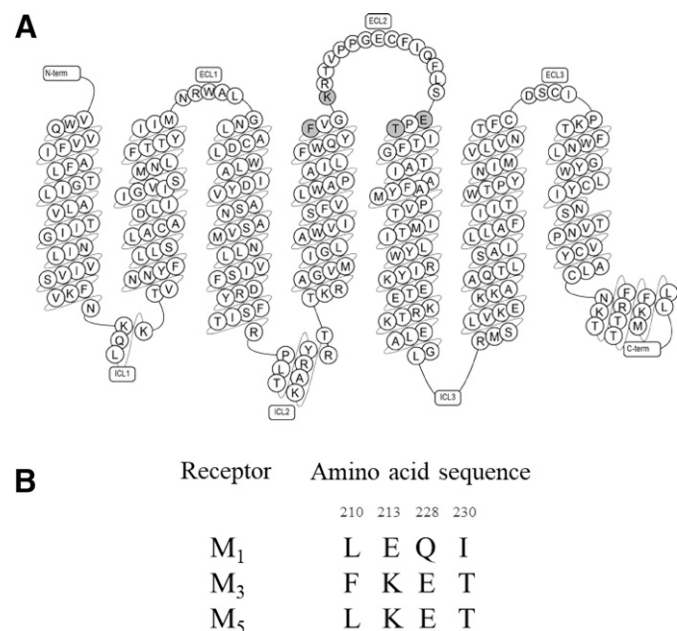
A. Wild type

Subtype (N)	Test Compound	EC <sub>50</sub> (mol/l)	95% CI (mol/l)	Fold Shift
M3 (3)	DMSO	2.4E-07	1.5E-07-3.9E-07	NA
	10 μmol/l ASP8302	1.1E-09	5.2E-10-2.3E-09	218.2
M1 (3)	DMSO	4.2E-08	1.4E-08-1.3E-07	NA
	10 μmol/l ASP8302	5.4E-08	2.6E-08-1.1E-07	0.8

B. Chimeric receptors

Construct (N)	Test Compound	EC <sub>50</sub> (mol/l)	95% CI (mol/l)	Fold Shift
M3_TM2_M1 (3)	DMSO	2.5E-08	1.7E-08-3.6E-08	NA
	10 μmol/l ASP8302	3.5E-08	1.4E-08-8.6E-08	0.7
M3_TM4_M1 (2)	DMSO	1.7E-06	ND	NA
	10 μmol/l ASP8302	3.1E-06	ND	0.5
M3_TM5_M1 (3)	DMSO	4.3E-08	7.6E-09-2.5E-07	NA
	10 μmol/l ASP8302	3.0E-10	4.4E-11-2.0E-09	143.3
M1_TM5_M3 (3)	DMSO	5.9E-08	8.2E-09-4.3E-07	NA
	10 μmol/l ASP8302	5.3E-08	1.3E-08-2.2E-07	1.1
M1_TM4_M3 (3)	DMSO	5.2E-08	1.4E-08-1.9E-07	NA
	10 μmol/l ASP8302	1.1E-09	4.7E-10-2.7E-09	47.3
M3_ECL2_M1 (3)	DMSO	1.5E-07	2.7E-08-8.8E-07	NA
	10 μmol/l ASP8302	1.1E-09	3.5E-10-3.2E-09	136.4
M1_ECL2_M3 (3)	DMSO	5.8E-08	2.2E-08-1.5E-07	NA
	10 μmol/l ASP8302	5.5E-08	1.5E-08-2.0E-07	1.1
M3_TM4_M1_TM5_M3 (3)	DMSO	5.6E-07	1.1E-07-2.8E-06	NA
	10 μmol/l ASP8302	5.6E-07	9.0E-08-1.6E-06	1.5
M1_TM4_M3_TM5_M1 (3)	DMSO	6.4E-08	3.5E-08-1.2E-07	NA
	10 μmol/l ASP8302	1.1E-09	1.0E-10-1.2E-08	58.2

M1, muscarinic M<sub>1</sub> receptor; N, number of trials; NA, not applicable; ND, not done.



**Fig. 7.** Mutation sites of human muscarinic M<sub>3</sub> receptor. (A) Position of four mutated amino acids in the human muscarinic M<sub>3</sub> receptor. Amino acids are indicated by a single letter. The snake plot diagram is from the GPCR database (<https://gpcrdb.org>). The positions of the mutated amino acids are grayed out. ICL, intracellular loop. (B) The three amino acids common to the M<sub>3</sub> and M<sub>5</sub> receptors were K213, E228, and T230. Amino acids are indicated by a single letter. E, glutamic acid; F, phenylalanine; I, isoleucine; K, lysine; L, leucine; Q, glutamine; T, threonine. The amino-acid sequence number corresponds to the human M<sub>3</sub> receptor.

M<sub>2</sub> and M<sub>3</sub> receptors, although their PAM activity on M<sub>3</sub> receptors was less potent than that on M<sub>2</sub> receptors (Gnagey et al., 1999; Jakubík and El-Fakahany, 2010) probably due to a difference in the structure of allosteric sites (Dror et al., 2013; Jakubík et al., 2017). Thus, subtype selectivity of PAM activity on human M<sub>3</sub> receptors with ASP8302 over M<sub>1</sub> and M<sub>2</sub> receptors is considered to be a

unique attribute of this compound compared with previously known M<sub>3</sub> receptor PAMs. In addition, ASP8302 did not show potent binding affinities or inhibition for various receptors, ion channels, transporters, or enzymatic activities (shown in the Supplemental Material).

Although ASP8302 did not displace the binding of [<sup>3</sup>H]NMS, ASP8302 shifted the ACh concentration-inhibition curves for [<sup>3</sup>H]NMS binding concentration-dependently to the left. Although we did not perform the binding study under equilibrium conditions to evaluate the effect on the dissociation of NMS (Jakubík et al., 2017), our results provide evidence that ASP8302 enhances the binding affinity of ACh for human M<sub>3</sub> receptors not by acting on the orthosteric site but by interacting with a site distinct from the orthosteric site, which is a fundamental feature of a PAM.

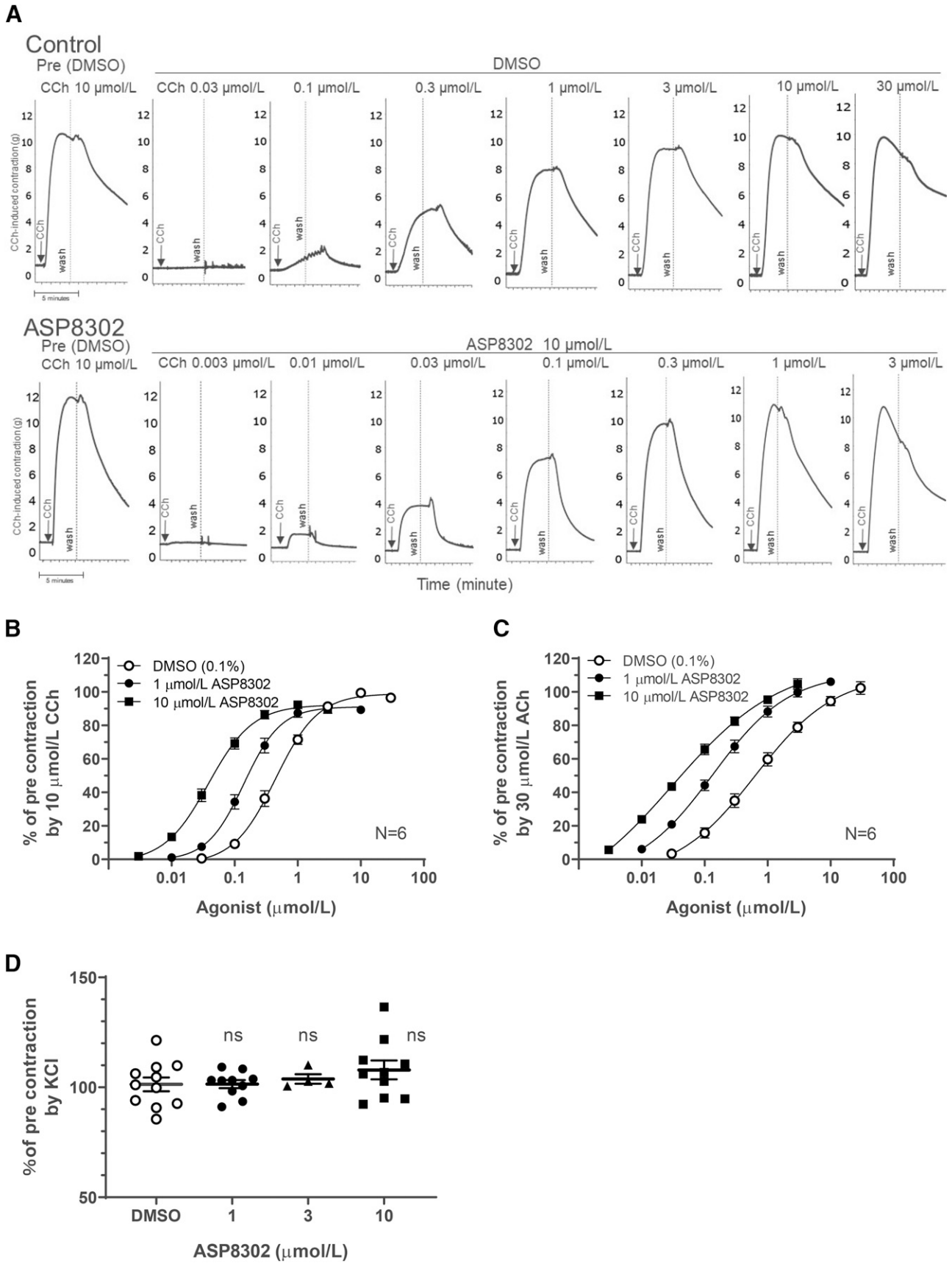
The results of cellular studies suggesting that ASP8302 acts on a site possibly homologous between M<sub>3</sub> and M<sub>5</sub> receptors but presumably different from known allosteric sites led us to determine the key region using a mutagenesis approach. Results using chimeric receptors between M<sub>3</sub> and M<sub>1</sub> at various TMs indicated the importance of ECL2, including some parts of TM4 and TM5. This is not inconsistent with prior findings that the binding site of classic allosteric modulators lies in the space between ECL2 and ECL3 (Jakubík and El-Fakahany, 2020). Surprisingly, however, substitution of the six amino acids in ECL2 (Pro<sup>217</sup> to Phe<sup>222</sup>) corresponding to the charged and acidic EDGE (Glu-Asp-Gly-Glu) motif of M<sub>2</sub> receptors, which is known to be important for allosteric ligand binding (Leppik et al., 1994; Huang et al., 2005) to the relevant part of M<sub>1</sub> receptors [LAGQCY (Leu-Ala-Gly-Gln-Cys-Tyr), nonacidic], did not affect the PAM activity of ASP8302. Therefore, we further investigated the importance of the residues around the boundary between TM4 and ECL2 (from Pro<sup>202</sup> to Val<sup>216</sup>) and between ECL2 and TM5 (from Ile<sup>223</sup> to Ile<sup>246</sup>) using site-directed mutagenesis and identified Thr<sup>230</sup> as the critical amino acid for the PAM activity of ASP8302. Previous studies with mutagenesis in M<sub>2</sub> receptors have determined multiple amino acids important for the interaction with allosteric ligands in the ECL2 and ECL3 and at the top of TM7 [e.g., Tyr<sup>177</sup>, Asn<sup>419</sup>, Trp<sup>422</sup>, Thr<sup>423</sup>

TABLE 3

Positive allosteric modulating activity of ASP8302 on carbachol-induced intracellular Ca<sup>2+</sup> changes in CHO-K1 cells expressing human mutagenetic muscarinic receptors (four amino-acid mutations)  
EC<sub>50</sub> values for the carbachol CRC were calculated by Sigmoid-E<sub>max</sub> nonlinear regression analysis. EC<sub>50</sub> values are expressed as the geometric mean and 95% CI of three or four independent experiments. The fold-shift values were calculated by dividing the EC<sub>50</sub> value of carbachol CRC in the absence of ASP8302 by those in the presence of ASP8302.

Chimeric Receptor (N)	Test Compound	EC <sub>50</sub> (mol/l)	95% CI (mol/l)	Fold Shift
M3_ECL2_F210L_K213E_E228Q_T230I (2)	DMSO	3.7E-07	ND	NA
	10 μmol/l ASP8302	3.3E-07	ND	1.1
M3_ECL2_F210A (3)	DMSO	1.4E-07	3.5E-08-5.9E-07	NA
	10 μmol/l ASP8302	9.4E-10	5.3E-10-1.7E-09	148.9
M3_ECL2_K213A (3)	DMSO	3.4E-07	1.8E-07-6.3E-07	NA
	10 μmol/l ASP8302	2.6E-09	7.2E-10-9.5E-09	130.8
M3_ECL2_E228A (3)	DMSO	3.0E-07	1.2E-07-8.0E-07	NA
	10 μmol/l ASP8302	1.4E-09	1.2E-09-1.7E-09	214.3
M3_ECL2_T230A (3)	DMSO	3.3E-07	1.5E-07-7.6E-07	NA
	10 μmol/l ASP8302	1.1E-07	6.1E-08-1.9E-07	3.0
M1_ECL2_I187T (3)	DMSO	8.6E-08	2.5E-08-2.9E-07	NA
	10 μmol/l ASP8302	3.3E-09	1.7E-09-6.2E-09	26.1
M5_ECL2_T192A (3)	DMSO	2.9E-07	4.4E-08-2.0E-06	NA
	10 μmol/l ASP8302	8.1E-08	2.3E-08-2.8E-07	3.6

M1, muscarinic M<sub>1</sub> receptor; M5, muscarinic M<sub>5</sub> receptor; N, number of trials; NA, not applicable; ND, not done.



**Fig. 8.** Potentiating effect of ASP8302 on CCh- and ACh-induced contraction of human isolated urinary bladder. (A) Representative traces of CCh-induced contraction. (B) Effect of ASP8302 on CCh-induced contraction. Data are shown as the extent (%) of contraction compared with control contraction (precontraction) obtained with the stimulants used in each experiment and expressed as the mean ± S.E.M. of six specimens for CCh or ACh-induced contraction and six specimens (two or three strips per specimen) for KCl-induced contraction. KCl-induced contractions with ASP8302 were compared with control using Dunnett's multiple comparisons test, and a probability value of less than 0.05 was considered significant. N, number of donor specimens; ns, not significant.



(Gnagey et al., 1999; Buller et al., 2002; Voigtländer et al., 2003; Huang et al., 2005; Prilla et al., 2006; May et al., 2007)]. A simulation by Dror et al. (2013) indicated two binding centers for allosteric ligands defined by a pair of aromatic residues (Tyr<sup>177</sup> in ECL2 and Trp<sup>422</sup> in TM7 at center 1, Tyr<sup>80</sup> and Tyr<sup>83</sup> in TM2 at center 2), which form cation- $\pi$  interactions with the ammonium group of allosteric ligands. However, to our knowledge, ASP8302 is the first PAM whose activity is determined by a single nonaromatic amino acid located distinct from known allosteric sites, at least for human M<sub>3</sub> receptors.

The loss- and gain-of-PAM activity of ASP8302 in the Thr<sup>230</sup>Ala mutant of M<sub>3</sub> receptors and Ile<sup>187</sup> Thr of M<sub>1</sub> receptors, respectively, strongly suggests that interaction between ASP8302 and Thr<sup>230</sup> plays a significant role in enhancing receptor activation. Predictions from a database (Kruse et al., 2012) and structural analysis of the corresponding amino acid (Thr<sup>229</sup>) in rat M<sub>3</sub> receptors (Kruse et al., 2012) suggest that Thr<sup>230</sup> [position 5.37 by Ballesteros and Weinstein numbering system (Ballesteros and Weinstein, 1995)] is in the vicinity of ECL2 and could therefore interact with the extracellular vestibule to which allosteric ligands bind. Mutation of Thr<sup>229</sup> in rat M<sub>3</sub> receptors is also reported to impair the activity of the receptor (Schmidt et al., 2003), suggesting the possible involvement of Thr<sup>230</sup> in the modulation of receptor activation. Although threonine is a nonaromatic hydroxyl amino acid that is polar but noncharged, we consider that atomic-level molecular dynamics differing from the above-mentioned cation- $\pi$  interactions play an important role in the drug-receptor interaction between ASP8302 and Thr<sup>230</sup>. Structural analysis of the M<sub>3</sub> receptor complex with ASP8302 is required to prove its importance in the PAM activity of ASP8302 and to further investigate the role of Thr<sup>230</sup> in the allosteric regulation of receptor activity.

Our finding on the augmentation of contractions induced by CCh and the endogenous ligand ACh with ASP8302 in isolated human bladder strips supports previous results obtained with muscarinic receptor agonists/antagonists and genetically modified animals that M<sub>3</sub> receptors play a key role in modulating bladder smooth-muscle contraction (Igawa et al., 2004), although the contribution of other mAChRs, such as M<sub>1</sub>, M<sub>2</sub>, and M<sub>4</sub> receptors, may not completely be excluded. Of the two muscarinic receptor subtypes expressed predominantly in bladder smooth muscle, M<sub>3</sub> receptors have been considered as the major subtype responsible for producing bladder contraction (Matsui et al., 2000; Chess-Williams et al., 2001; Fetscher et al., 2002), whereas more abundantly expressed M<sub>2</sub> receptors (Wang et al., 1995; Hegde and Eglén, 1999) indirectly contribute to contraction (e.g., suppression of relaxation via  $\beta_3$ -adrenergic receptors) (Chess-Williams et al., 2001; Fetscher et al., 2002). However, the insufficient subtype selectivity of pharmacological agents used in past studies has hampered a conclusive decision on the involvement of M<sub>2</sub> and M<sub>3</sub> receptors in the control of bladder contractility. Thus, further studies are needed to clarify the potential involvement of M<sub>2</sub> receptor in the regulation of bladder smooth-muscle contraction using an M<sub>2</sub>-specific tool, such as the M<sub>2</sub>-specific PAM. In addition, the prejunctional excitatory M<sub>1</sub> and inhibitory M<sub>4</sub> subtypes are also reportedly expressed in the bladder. It has been shown that M<sub>1</sub> and M<sub>4</sub> receptors facilitate and inhibit ACh release from nerve terminals, respectively (Giglio and Tobin, 2009). As ASP8302 showed clear selectivity over M<sub>1</sub> and M<sub>4</sub> receptors (Table 1), it is unlikely that M<sub>1</sub> and M<sub>4</sub> receptors are significantly involved in ASP8302-induced enhancement of CCh-induced bladder smooth-muscle

contraction. Furthermore, although ASP8302 has PAM activity on M<sub>5</sub> receptors, it is unlikely that this effect on M<sub>5</sub> receptors is associated with the enhancement of contraction given that no study has indicated significant expression of M<sub>5</sub> receptors in bladder smooth muscle or its contribution to contraction (Chess-Williams et al., 2001).

The potentiation of CCh and ACh demonstrated by an approximately 10-fold shift in EC<sub>50</sub> also suggests the application of ASP8302 in the treatment of diseases caused by impairment of M<sub>3</sub> receptor-mediated bladder smooth-muscle contraction. Insufficient bladder contractility can cause difficulties in urine voiding and complications caused by incomplete bladder emptying, which is known as underactive bladder (Miyazato et al., 2013). ASP8302 may be useful in overcoming these conditions by enhancing bladder contraction by potentiating the activation of M<sub>3</sub> receptors during micturition only, albeit that further studies in animal disease models are required. Another consideration for the clinical use of ASP8302 is the need for selectivity over M<sub>5</sub> receptors because M<sub>5</sub> receptors are implicated in physiologic processes related to Alzheimer disease, schizophrenia, and addiction (Bender et al., 2019). Further studies are required to address the effect of positive allosteric modulation of the M<sub>5</sub> receptor on physiologic and pathophysiological functions. Identification of selective M<sub>5</sub> receptor modulators may suggest the possibility of chemical modification of ASP8302 to gain selectivity over M<sub>5</sub> receptors (Gentry et al., 2010) and thereby help us address these questions.

In conclusion, our pharmacological characterization of the novel human M<sub>3</sub> receptor PAM ASP8302 demonstrates its potency, selectivity, and augmentation of bladder contractility. These findings suggest that this agent has the potential to enhance M<sub>3</sub>-mediated functional responses. In particular, the finding of Thr<sup>230</sup> as a novel amino acid involved in the allosteric modulation of M<sub>3</sub> receptors provides significant insight into further research of the mechanism of allosteric modulation of M<sub>3</sub> and other muscarinic receptors.

#### Authorship Contributions

*Participated in research design:* Okimoto, Ino, Ishizu, Takamatsu, Yuyama, Fuji, Someya, Ohtake, Ishigami, Masuda, Takeda, Kajioka, Yoshimura.

*Conducted experiments:* Okimoto, Ino, Ishizu, Takamatsu.

*Performed data analysis:* Okimoto, Ishizu, Takamatsu.

*Wrote or contributed to the writing of the manuscript:* Okimoto, Ishizu, Sakamoto, Ohtake, Masuda, Takeda.

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