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Title page

# An approach to discovering novel muscarinic $M_1$ receptor positive allosteric modulators with potent cognitive improvement and minimized gastrointestinal dysfunction

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Running title page

**Running Title:**

A Novel Screening Strategy for $M_1$ PAMs

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**Abbreviations:**

ACh, acetylcholine

AChE-I, acetylcholinesterase inhibitor

AD, Alzheimer's disease

BSA, bovine serum albumin

BQCA, benzyl quinolone carboxylic acid

CHO, Chinese hamster ovary

Compound A,

3-fluoro-2-((2-(4-(1-methyl-1H-pyrazol-4-yl)benzyl)-3-oxo-2,3-dihydro-1H-isoindol-4-yl)oxy)benzonitrile

Compound B,

7-(((1S,2S)-2-hydroxycyclohexyl)oxy)-2-(4-(1-methyl-1H-pyrazol-3-yl)benzyl)isoindolin-1-one

Compound C,

3-((1S,2S)-2-hydroxycyclohexyl)-6-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl)benzo[h]quinazolin-4(3H)-one

Compound D,

2-(2-fluorophenyl)-5-(4-(1H-pyrazol-1-yl)benzyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quino

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lin-3-one

Compound E,

2-(4-(1-methyl-1H-pyrazol-4-yl)benzyl)-7-(2-(piperidin-1-yl)ethoxy)isoindolin-1-one

Compound F,

2-(4-(1-methyl-1H-pyrazol-4-yl)benzyl)-7-(1H-pyrazol-5-yl)isoindolin-1-one

DMSO, dimethyl sulfoxide

EFS, electric field stimulation

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GI, gastrointestinal

IP, inflection point

KO, knock-out

$M_1R$, muscarinic $M_1$ receptor

PAM, positive allosteric modulator

PD, pharmacodynamic

PI, phosphoinositide

PK, pharmacokinetic

PQCA,

1-((4-cyano-4-(pyridine-2-yl)piperidin-1-yl)methyl-4-oxo-4H-quinolizine-3-carboxylic

acid

RT-PCR, reverse transcription-polymerase chain reaction

TLZ, telenzepine

WT, wild-type

**Recommended section assignment:**

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## ABSTRACT

Activation of muscarinic $M_1$ receptor ($M_1R$) is a promising approach for improving cognitive impairment in Alzheimer's disease. However, an $M_1$ selective positive allosteric modulator (PAM), benzyl quinolone carboxylic acid (BQCA), at 30 mg/kg, induced diarrhea in wild-type mice, but not in $M_1R$ knock-out mice. Moreover, BQCA (0.1–1000 nM) augmented electric field stimulation (EFS) –induced ileum contraction in an in vitro Magnus assay. Thus, we decided to establish a drug-screening strategy to discover novel $M_1$ PAMs producing potent cognitive improvement with minimized gastrointestinal (GI) dysfunction. We assessed PAM parameters of various $M_1$ PAMs with ≥ 100-fold selectivity over other muscarinic receptor subtypes by using in vitro binding and functional analysis. Evaluation of these $M_1$ PAMs in the Magnus assay revealed a significant correlation between % of ileum contractions at 1 μM and their α-value, a PAM parameter associated with the binding cooperativity between acetylcholine and $M_1$ PAM. $M_1$ PAMs with lower α-value showed lower impact on EFS-induced ileum contraction. Next, we characterized in vivo profiles of two $M_1$ PAMs: compound A (log α = 1.18) and compound B (log α = 3.30). Compound A, at 30 mg/kg, significantly improved scopolamine-induced cognitive deficits without prominent signs of diarrhea at up to 1000 mg/kg in mice. In contrast, compound B, at

10 mg/kg, showed both significant improvement of scopolamine-induced cognitive deficits and severe diarrhea. Thus, fine adjustment of the α-values could be a key to discovering $M_1$ PAMs yielding potent cognitive improvement with a lower risk of GI effects.

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## Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized mainly by irreversible cognitive and memory decline. Cholinergic hypofunction contributes to the cognitive deterioration in patients with AD (Auld et al., 2002; Mufson et al., 2003). Consistent losses in cholinergic neurons, as well as a decrease in choline acetyltransferase activity, were commonly observed in the basal forebrain of patients with AD (Giacobini, 2003; Craig et al., 2011). Thus, restoring cholinergic function has been a primary strategy for improving the cognitive deficits in AD. In fact, four of the five U.S. Food and Drug Administration–approved drugs are acetylcholinesterase inhibitors (AChE-Is) (Schneider et al., 2014). AChE-Is provide mild symptomatic relief; however, they all produce undesirable side effects such as nausea, vomiting and diarrhea. Therefore, novel drugs with better efficacy and safety profiles are being sought.

Several lines of evidence indicate that activators of muscarinic $M_1$ receptor ($M_1R$) can improve cognitive deficits in AD (Fisher et al., 1996; Bodick et al., 1997). The $M_1R$ is expressed predominantly in the cerebral cortex and hippocampus, and is also, to some extent, expressed in peripheral tissues. The expression level of $M_1R$ is relatively unchanged in patients with AD (Levey, 1996; Tsang et al., 2006). Importantly, the orthosteric agonists of $M_1R$ improved cognition in phase 2 and 3 studies (Fisher et al.,

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1996; Bodick et al., 1997). In line with clinical results, $M_1$R activators improved cognitive dysfunction in animal models of AD (Medeiros et al., 2011; Jiang et al., 2014). For example, both xanomeline and an $M_1$ positive allosteric modulator (PAM), benzyl quinolone carboxylic acid (BQCA), improved learning and memory in various cognitive tasks (Bartolomeo et al., 2000; Cui et al., 2008; Ma et al., 2009; Shirey et al., 2009; Si et al., 2010; Davie et al., 2013). Another $M_1$ PAM, 1-((4-cyano-4-(pyridine-2-yl)piperidin-1-yl)methyl-4-oxo-4H-quinolizine-3-carboxylic acid (PQCA), also improved cognition in rats and monkeys (Uslaner et al., 2013; Vardigan et al., 2014). Therefore, activation of $M_1$R is an attractive approach to treating cognitive deficiency related to AD.

Although orthosteric ligands of $M_1$R showed compelling efficacy, they failed at some stages of clinical development because of severe adverse effects such as gastrointestinal (GI) symptoms, sweating, and frequent urination (Fisher et al., 1996; Bodick et al., 1997). The undesirable cholinergic side effects by orthosteric agonists have been considered due to their lack of $M_1$R selectivity over the other subtypes, especially $M_2$ and $M_3$R (Iga et al., 1998). Unfortunately, discovery of selective $M_1$R orthosteric agonists has failed because of the high structural similarity at the acetylcholine (ACh) binding site among all subtypes. Therefore, development of an

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$M_1$R-selective PAM has received attention as an alternative approach. However, BQCA tended to induce salivation in rats (Chambon et al., 2012). Furthermore, in this study, because we found that BQCA caused diarrhea in wild-type (WT) mice, but not in $M_1$R knock-out (KO) mice, the adverse-event profiles of $M_1$ PAMs should be carefully considered.

Allosteric modulators, including $M_1$ PAMs, bind to sites that are distinct from the orthosteric site, and are thought to stabilize receptors in specific conformational states. They can therefore modulate their affinity for the endogenous ligands at the orthosteric binding pocket, or they can affect the intrinsic efficacy of the endogenous ligands to engage downstream signaling mechanisms (Conn et al., 2009). Moreover, the extracellular environment, such as the ACh concentration around $M_1$R, might differ between the brain and peripheral tissues. Some $M_1$ PAMs could potentially have a wider margin between cognitive efficacy and induction of adverse events. Several mechanistic analysis methods help in the understanding of the mechanism of allosteric modulation for G-protein–coupled receptors (Canals et al., 2012; Abdul-Ridha et al., 2013; Mistry et al., 2013). In the allosteric ternary–complex model, the effect of an allosteric modulator is characterized as an alteration of orthosteric ligand affinity to the receptor (Leach et al., 2007). Several PAM parameters can be identified through use of the

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binding and functional analysis for $M_1R$. We focused on the inflection point (IP), α-value, and β-value, which represent the PAM potency, the binding cooperativity between ACh and PAM, and the magnitude of the allosteric effect of PAM on the signaling efficacy of ACh, respectively.

This study establishes a strategy to screen $M_1$ PAMs for a reduced risk of diarrhea based on the relationships between PAM parameters and ileum contraction with various $M_1$ PAMs. Our approach provides the ability to discover novel $M_1$ PAMs with a greater therapeutic window between cognitive improvement in AD and cholinergic side effects.

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## Materials and Methods

**Ethics Statement.** The care and use of the animals and the experimental protocols used in this research were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited.

**Animals.** Male ICR mice were supplied by CLEA Japan Inc. (Tokyo, Japan), and were used at 6–17 weeks of age. C57BL/6-*Chrm-1*tm1 Stl/J WT mice and KO mice were obtained from the Massachusetts Institute of Technology (Cambridge, MA), and used at 8 months old. These animals were used for experiments after at least 1 week of acclimation. All mice were housed in a light-controlled room (12-h light/dark cycle with lights on at 7:00). Food and water were provided ad libitum.

**Reagents.** BQCA was purchased from Key Organics Ltd. (Cornwall, UK). 3-fluoro-2-((2-(4-(1-methyl-1H-pyrazol-4-yl)benzyl)-3-oxo-2,3-dihydro-1H-isoindol-4-yl)oxy)benzonitrile (compound A; Fig. 4A), 7-(((1S,2S)-2-hydroxycyclohexyl)oxy)-2-(4-(1-methyl-1H-pyrazol-3-yl)benzyl)isoindolin-1-one (compound B; Fig. 5A), 3-((1S,2S)-2-hydroxycyclohexyl)-6-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl

)benzo[h]quinazolin-4(3H)-one (compound C), 2-(2-fluorophenyl)-5-(4-(1H-pyrazol-1-yl)benzyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (compound D), 2-(4-(1-methyl-1H-pyrazol-4-yl)benzyl)-7-(2-(piperidin-1-yl)ethoxy)isoindolin-1-one (compound E), and 2-(4-(1-methyl-1H-pyrazol-4-yl)benzyl)-7-(1H-pyrazol-5-yl)isoindolin-1-one (compound F) were synthesized by Takeda Pharmaceutical Company Limited (Kanagawa, Japan). [$^3$H]-Pirenzepine was from PerkinElmer (Waltham, MA). Other reagents were purchased from Tocris Bioscience (Minneapolis, MN), unless otherwise noted. All $M_1$ PAMs were suspended in 0.5% (w/v) methylcellulose in distilled water and administered orally. Scopolamine hydrobromide was dissolved in saline and administered subcutaneously. All compounds used in vivo studies were dosed in a volume of 10 or 20 mL/kg body weight for mice. For the in vitro Magnus assay, compounds were dissolved in dimethyl sulfoxide (DMSO).

**Magnus Assay.** After overnight fasting, mice were sacrificed by decapitation. The ileum was quickly dissected out and suspended in ice-cold Krebs solution with the following composition (in mM): NaCl, 120.7; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2;

$NaHCO_3$, 15.5; $NaH_2PO_4$, 1.2; and glucose, 11.5. Longitudinal segments of ileum (10–15 mm long) were detached of mesentery and adipose tissue, and mounted in organ baths containing 10 mL of Krebs solution aerated with 95% $O_2$/5% $CO_2$. Bath temperature was maintained at 37°C. The isolated ileum was given a passive load of 0.5 g. The contractile response of the isolated ileum was continuously recorded with an isometric transducer (MLT050/A, ADInstruments, Bella Vista NSW, Australia) and a recorder (PowerLab 8/30 ML870 and Octal Bridge Amp ML228, ADInstruments).

Platinum electrodes (3–20 mm apart; Iwashiya Kishimoto Medical Instruments, Kyoto, Japan) were mounted on both sides of the isolated ileum. The electrodes were connected to an electric stimulator (SEN-3401; Nihon Kohden, Tokyo, Japan) and an amplifier (SEG-3104; Nihon Kohden) for electric field stimulation (EFS) with square wave pulses (20 V over the electrodes, 50 ms duration, and 20 Hz frequency); pulse trains lasted for 10 seconds with a 20-second pause.

The EFS produced sustained tonic contraction followed by relaxation. The contraction was measured as the maximum strength from the baseline. The effects of experimental substances on the maximum contraction were measured. At the beginning of each experiment, a series of control EFS cycles was tested for more than 1 hour to observe the stability of the contractile response. The ileum, with a steady contractile

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response elicited by control EFS during the 1 hour was used to investigate the effect of the test compounds.

After responses to the control EFS reached a steady state, the compounds were added to the Magnus bath cumulatively, without washing between the subsequent doses. The interval between two adjacent doses was always at least 5 minutes; thus, the isolated preparations received at least 10 pulses in each dose of the compounds. After all doses of the compounds, cumulatively, the bath was washed three times, and the preparations were allowed to rest for a further 30 minutes.

The effect of each concentration of the compounds on contractions induced by EFS was calculated from the average value of 10 responses of the maximum strength. Then the ileal contractile response was expressed as a ratio of the mean maximum contraction to that obtained with the pretreated DMSO (solvent). For the construction of concentration-response curves, we used normalized values of the ratio by DMSO treatments, expressed by percentages. Since the relaxations were so small, we did not analyze the effects of the compounds on them.

**Binding Assay.** Binding assay was performed in a 96-well plate format. Cell membranes from FreeStyle$^{TM}$ 293 cells transiently expressing human $M_1R$ were

incubated with various concentrations of test compounds, varying concentrations of ACh, and 4 nM [$^3$H]-pirenzepine in an assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 0.1% fatty acid free bovine serum albumin [BSA]). After 2 hours of incubation at room temperature, the cell membranes were transferred to GF/C filter plates (PerkinElmer) using a cell harvester (PerkinElmer) and washed five times with 300 µL of 50 mM Tris-HCl. The GF/C plates were dried at 42°C. Then, 25 µL of MicroScint™ 0 (PerkinElmer) was added and the radioactivity was measured by TopCount® (PerkinElmer). Non-specific bound was defined in the presence of 10 µM atropine.

**$Ca^{2+}$ Flux Assay.** Chinese hamster ovary (CHO-K1) cells stably expressing human $M_1$Rs ($hM_1$R-CHO) were placed in black-walled, clear-bottomed, 384-well plates (5000 cells/well) and cultured overnight at 37°C, in the presence of 5% $CO_2$ in Ham's F-12 medium supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. The following day, the medium was removed and incubated with an assay buffer (Hank's balanced salt solution with 20 mM HEPES, 0.1% fatty acid free BSA) containing 2.5 µg/mL Fluo-4 AM and 1.25 mM probenecid for 30 minutes at 37°C in the presence of 5% $CO_2$. After 30 minutes of incubation at room temperature, the cells were

stimulated with the compounds. In this experiment, ACh and each PAM were added to the cells simultaneously. Calcium flux was measured using a fluorescence imaging plate reader (FLIPR) Tetra® System (Molecular Devices, Sunnyvale, CA).

**Data Analysis of PAM Parameters.** All data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Radioligand binding data were globally fitted to the following allosteric ternary complex model, as previously reported (Lazareno and Birdsall, 1995):

$$Y = \frac{\frac{[C]}{K_C} + \frac{\alpha_{BC}[B][C]}{K_B K_C}}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[C]}{K_C} + \frac{\alpha_{AB}[A][B]}{K_A K_B} + \frac{\alpha_{BC}[B][C]}{K_B K_C}}$$

where Y is the percentage of specific binding; [A], [B], and [C] are the concentrations of ACh, the allosteric modulator, and [$^3$H]-pirenzepine, respectively; $K_A$, $K_B$, and $K_C$ denote the equilibrium dissociation constants of ACh, the allosteric modulator, and [$^3$H]-pirenzepine, respectively; and $\alpha_{AB}$ and $\alpha_{BC}$ are the cooperativities between the allosteric modulator and ACh or [$^3$H]-pirenzepine, respectively. Values of cooperativity greater than 1 denote positive cooperativity (allosteric enhancement of orthosteric ligand binding), whereas values of cooperativity less than 1 denote negative

cooperativity (allosteric inhibition of orthosteric ligand binding). The functional interactions between $M_1$ PAMs and ACh were globally fitted to the following operational model of allosterism (Leach et al., 2007; Conn et al., 2009):

$$E = \frac{E_{\max}(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}$$

where $E$ is the pharmacological effect and $E_{max}$ denotes the maximum possible effect; [A] and [B] are the concentrations of ACh and PAM, respectively; $K_A$ and $K_B$ denote the equilibrium dissociation constants of ACh and PAM, respectively; $\tau_A$ and $\tau_B$ represent the operational measures of ACh and PAM efficacy, respectively; $\alpha$ is the binding cooperativity between ACh and PAM; $\beta$ is the magnitude of the allosteric effect of PAM on the signaling efficacy of ACh; $n$ denotes the slope factor. For the analysis of functional data, the equilibrium dissociation constant of each PAM and the cooperativity between ACh and each PAM were fixed to those values determined from the binding experiments.

**Assessment of Cholinergic Side Effects.** Mice were randomly divided into four groups ($n$ = 6), and transferred to the individual cages and allowed to acclimate for more than 1 hour. Each mouse was treated with either vehicle (10 mL/kg) or BQCA (30, 100 or

300 mg/kg p.o.). The animals were monitored, and we scored the severity of cholinergic side effects at 10 minutes and 0.5, 1 and 2 hours after administration. To assess the severity of loose or mucous stool, we counted the number of mice in which swollen or mucous feces was induced. For the severity of diarrhea, we counted the number of mice in which severe watery diarrhea was induced. To measure lacrimation, we counted the number of mice in which moderate to marked discharge (lacrimation was greater than fluid around the eyes) was induced. For salivation, we counted the number of mice in which severe salivation—not just noticeable wetness around the mouth—was induced.

**Assessment of Diarrhea in Detail.** On the day of the experiment, mice were randomly divided into four groups ($n$ = 6–9). Mice were transferred to individual cages and allowed to acclimate for more than 1 hour. Each mouse was treated with either vehicle (10 or 20 mL/kg) or a single dose of each compound, including BQCA (30 mg/kg p.o.), compound A (10, 30, 100, 300, or 1000 mg/kg p.o.) or compound B (3, 10, or 30 mg/kg p.o.). The animals were monitored, and the severity of diarrhea was scored at 0.5, 1, 2, 4 and 6 hours after dose administration. Stool was assessed using an arbitrary scoring scale ranging from 0 to 3: 0 = normal pellets; 1 = wet but formed feces; 2 = swollen or mucous feces; 3 = severe watery diarrhea. The highest score during the observation was

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adopted.

**Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis.** For in vivo pharmacodynamic (PD) marker analysis, mice were killed by decapitation at 90 minutes after administration of compound A or compound B. Hippocampal tissues were isolated from the brain. For the expression analysis of $M_1R$, mice were sacrificed and the whole brain and GI tract (stomach, small intestine, and large intestine) were isolated. The tissues were stored at –80°C until RNA extraction. Total RNA from individual tissues was extracted using QIAzol Lysis Reagent and the RNeasy® kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RT-PCR was carried out using ABI PRISM 7900HT® Sequence Detection System (Life Technologies, Bedford, MA) and TaqMan® reagents (Eurogentec, Seraing, Belgium). RNA quantities were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA transcripts per the manufacturer's instructions. The following primers were used for mouse *Chrm1* analysis: forward primer, 5'-AGTGGTGATCAAGATGCCTATGG-3'; reverse primer, 5'-TGGGCCTCTTGACTGTATTTGG-3', TaqMan probe, 5'-AGGCACAGGCACCCACCAAGCAG-3'. The following primers were used for

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mouse *Arc* analysis: forward primer, 5'- AGCTGAAGCCACAAATGCAG-3'; reverse primer, 5'-CTGAGTCACGGAGCTGAGC-3', TaqMan probe, 5'-AGACCTGACATCCTGGCACCTCCTGG-3'. The primers used for mouse *Gapdh* analysis were TaqMan Rodent GAPDH Control Reagent, VIC probe, purchased from ABI (Applied Biosystems-Life Technologies, Waltham, MA). Mouse *Chrm1* mRNA expression levels were represented as a relative ratio to *Gapdh* mRNA expression levels. For mouse *Arc* expression analysis in the hippocampus, *Arc* mRNA expression levels were normalized by *Gapdh* mRNA expression levels, which were represented as percent changes from a vehicle-treated group.

**Y-Maze Task in Mice.** The Y-maze apparatus is a three-arm maze with equal angles between all arms and made of black acrylic. Each arm was 40 cm long, 4 cm wide, and had walls 12 cm high. The Y-maze was located in a sound-attenuated room and illuminated at 10 lux. The experiment was performed to measure spontaneous alternation behavior as described previously (Hughes, 2004), with minor modification. Compound A (10 or 30 mg/kg) and compound B (1, 3, or 10 mg/kg) were pretreated orally, and 30 minutes after the treatment, memory impairment was induced by administering scopolamine (0.3 or 1 mg/kg, as hydrobromide salt, s.c.). Thirty minutes

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after the administration of scopolamine, the mice were placed in one of three arms of the Y-maze and allowed to explore the maze for 5 or 8 minutes. Alternations and total numbers of arm choices were recorded visually. A mouse was considered to have entered an arm of the maze when all four paws were positioned in one third of the arm runway. Spontaneous alternations were defined as successive entries into a new arm that differed from the previous two choices, and alternation ratio, expressed as a percentage, referred to the ratio of actual (total alternations) to possible alternations (total arm entries – 2) × 100. Each animal underwent one trial. Mice were excluded from the analyses if they had low exploratory activity, defined as fewer than 10 total arm entries. The dose of scopolamine (0.3 or 1 mg/kg) was regulated so that the alternation ratio significantly decreased.

**Statistical Analysis.** Experimental results were expressed as the mean ± standard error of the mean (S.E.M.). The statistical significance of the differences between the two groups was assessed by Aspin-Welch's *t*-test with an alpha level of 0.05. In the experiments examining multiple doses of test compounds, the effects were analyzed using Bartlett's test, which was used for testing the homogeneity of variance, followed by the two-tailed Williams' test (Williams, 1971) (for parametric data, $P > 0.05$ by

Bartlett's test) or the two-tailed Shirley-Williams test (Shirley, 1977) (for nonparametric data, $P \leq 0.05$ by Bartlett's test). Data were analyzed using EXSUS (Ver.8.0.0, CAC EXICARE Corporation, Tokyo, Japan) and statistical significance was set at $P \leq 0.05$.

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## Results

**BQCA Induced Diarrhea Through Activation of the $M_1$ Receptor in Mice.** We examined whether BQCA, a highly selective $M_1$ PAM, induced cholinergic adverse effects such as diarrhea, lacrimation, and salivation in mice. BQCA caused diarrhea at 30 mg/kg in mice ($P \leq 0.01$; Fig. 1, Table 1). In contrast, it did not cause lacrimation and salivation up to 300 mg/kg (Table 1). To determine whether BQCA caused diarrhea through $M_1$R activation, BQCA (30 mg/kg) was administered orally to $M_1$R KO mice. The BQCA-induced severe diarrhea was not observed in $M_1$R KO mice ($P \leq 0.01$; Fig. 1). Thus, BQCA might induce diarrhea by $M_1$R activation.

**BQCA Caused Ileum Contraction via $M_1$ Receptor Activation In Vitro.** We hypothesized that the activation of peripheral $M_1$R, especially in the GI tract, by BQCA caused diarrhea in mice. We measured mRNA levels of $M_1$R in the stomach, small intestine, and large intestine, and compared with those in whole brain. In accordance with the previous reports (Caulfield, 1993; Levey, 1996), $M_1$R was predominantly expressed in the brain (Fig. 2A). $M_1$R was also expressed in the GI tract; the expression level of $M_1$R in the large intestine was approximately 15% of that in the brain (Fig. 2A). We next examined the effects of BQCA on ileum contraction by using the in vitro

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Magnus assay. The application of periodic EFS to the isolated ileum caused a corresponding contractile response. Stimulation of the isolated ileum with BQCA strengthened the EFS-induced contraction in a concentration-dependent manner (Fig. 2, B and C). This effect of BQCA was antagonized by pretreatment with telenzepine (1 nM), an $M_1$R selective antagonist ($P \leq 0.01$ at 1 μM of BQCA; Fig. 2C). Therefore, BQCA potentiated the ileum contraction via activation of $M_1$R in the GI tract.

**Strength of Ileum Contraction Correlated with *α*-Value of $M_1$ PAMs.** The extracellular environment, such as the ACh concentration around $M_1$R, may be different between the brain and peripheral tissues, and each $M_1$ PAM has different characteristics that can exert distinct effects from tissue to tissue within the body. We therefore decided to explore the key $M_1$R modulation parameters associated with the ileum contraction. At first, seven $M_1$ PAMs with ≥ 100-fold selectivity over other muscarinic subtypes were selected for in vitro functional analyses (Table 2). We assessed various $M_1$ PAM parameters of these $M_1$ PAMs with the in vitro binding modulation assay (Tables 2 and 3). Next, we examined the effects of these compounds on the ileum contractile response by means of the Magnus assay. The ileum contraction levels extended from 93% to 116% compared with control conditions (Table 3). By using these results, the

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correlation between the ileum contractile response and the various $M_1$ PAM parameters was explored. The results of a Pearson's correlation analysis between the pIP, log α, or log β, and the ileum contraction augmentation at 1 μM are shown in Fig. 3. Both the pIP and log β did not show the correlation with the augmented level of ileum contraction ($r$ = 0.4979, $P$ = 0.2555; Fig. 3A, $r$ = -0.5505, $P$ = 0.2004; Fig. 3C). Interestingly, log α was significantly correlated with the ileum contractile responses with a correlation coefficient greater than 0.80 ($r$ = 0.8075, $P$ = 0.0281; Fig. 3B).

**Compound A, an $M_1$ PAM with a Low α-Value, Enhanced Cognitive Function Without Inducing Diarrhea in Mice.** To assess the detailed profiles of an $M_1$ PAM with lower α-value, we selected compound A with log α of 1.18 as a representative compound (Fig. 4A, Table 3). At up to 1 μM, compound A had no effect on EFS-induced ileum contractions (Fig. 4B). As expected from this result, compound A, at up to 1000 mg/kg, did not induce severe diarrhea (Fig. 4, C and D); note that this compound lacked pharmacokinetic (PK) linearity at higher doses, and its plasma concentration at 1000 mg/kg was 3.6-fold higher than that at 30 mg/kg (Supplemental Table 2A). To guide the dose selection for cognitive tasks, we used induction of *Arc* mRNA as a PD marker for $M_1$R activation; BQCA has been reported to increase *Arc*

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mRNA expression level in the brain through $M_1R$ activation (Ma et al., 2009). Compound A, at 10–100 mg/kg, showed an approximate 2-fold increase in *Arc* mRNA expression levels in mouse hippocampus (Fig. 4E). From this result, doses of 10 and 30 mg/kg were selected for the evaluation of cognitive efficacy in scopolamine-induced memory impairment in mice. Y-maze tasks have been widely used to assess cognitive function, especially spatial working memory in rodents. Since rodents typically prefer to explore new environments, over the course of multiple arm entries, they usually show a tendency to enter the arms one after the other. Thus, the alternation ratio is considered to show the ability of the rodent's spatial working memory (Hughes, 2004). Untreated mice are reported to typically exhibit an alternation ratio of 60–70% (Belzung, 1999). In fact, the alternation ratio in control mice was within this range (67.9 ± 1.9% in Fig. 4F). Treatment with scopolamine significantly decreased the alternation ratio ($P \leq 0.01$; Fig. 4F). Importantly, compound A, at 30 mg/kg, significantly mitigated scopolamine-induced reduction of the alternation ratio in the Y-maze task in mice under 5-minute measurement conditions ($P \leq 0.05$; Fig. 4F). Thus, an $M_1$ PAM with low α-value could improve cognitive deficits without inducing diarrhea.

**Compound B, an $M_1$ PAM with a High α-value, Had No Margin Between Cognitive**

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**Improvement and Induction of Diarrhea in Mice.** We selected compound B (Fig. 5A) as a representative high–α-value compound; compound B has a log α of 3.30 (Table 3). As expected, compound B (0.1 nM–1 μM) caused concentration-dependent augmentation of the EFS-induced ileum contraction in the in vitro Magnus assay, and this enhancement was suppressed by 1 nM of telenzepine (Fig. 5B). In line with this observation, compound B at 10 and 30 mg/kg caused severe diarrhea in mice ($P \leq 0.05$; Fig. 5C). Compound B enhanced the expression level of *Arc* mRNA in a dose-dependent manner, and showed a significant increase at 30 mg/kg in mice ($P \leq 0.05$; Fig. 5D). We then assessed cognitive improvement using scopolamine-induced cognitive deficits in the Y-maze task in mice. Under 5-minute measurement conditions, scopolamine did not cause a significant reduction of the alternation ratio, and compound B, at 10 mg/kg, showed a trend to improve the scopolamine-induced cognitive deficits ($P = 0.06$; Fig. 5E). When we prolonged the measurement time to 8 minutes, scopolamine treatment induced prominent cognitive deficits, and compound B, at 10 mg/kg, significantly mitigated the scopolamine-induced decrease in the alternation ratio in the Y-maze task in mice ($P \leq 0.05$; Fig. 5F).

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## Discussion

One of the neuropathological hallmarks of AD is progressive loss of presynaptic cholinergic neurons from the basal forebrain. Treatment with AChE-Is provides only limited efficacy because their potency primarily depends on ACh release from presynaptic terminals which undergo early degeneration in AD (Raina et al., 2008). In contrast, postsynaptic neurons containing $M_1R$ are considered to be relatively intact in AD (Levey, 1996). Therefore, $M_1R$ activation could be therapeutically more advantageous than the inhibition of AChE, and the combination of these approaches could also be a promising treatment strategy even when an AChE-I alone no longer exhibits sufficient efficacy. In fact, $M_1R$ activators such as xanomeline and cevimeline have shown measurable efficacy in clinical trials (Fisher et al., 1996; Bodick et al., 1997; Nathan et al., 2013). However, drug developments were discontinued because of severe adverse effects, especially GI related. Although these adverse effects were attributed to the lack of selectivity for $M_1R$, we demonstrated here that selective $M_1R$ activation can be associated with diarrhea induction because of the following: (1) the expression of $M_1Rs$ was confirmed in the GI tract (Fig. 2A); (2) BQCA, a highly selective $M_1$ PAM, enhanced the EFS-mediated ileum contraction in a concentration-dependent manner (Fig. 2, B and C); (3) the BQCA-induced ileum

contraction was suppressed by the $M_1R$ antagonist telenzepine (Fig. 2, B and C); (4) BQCA caused diarrhea (Table 1, Fig. 1); and (5) BQCA-induced diarrhea was not observed in $M_1R$ KO mice (Fig. 1). These results suggest that BQCA caused diarrhea due to the contractile augmentation of the GI tract through the activation of $M_1R$. In this study, the maximum change in ileum contraction by each $M_1$ PAM was approximately 116% compared with vehicle, which was a relatively small effect. However, this effect could be sufficient for our purpose of this study, an establishment of a drug-screening strategy for $M_1$ PAMs with minimized GI dysfunction. Instead of the ileum, other GI regions such as the colon may display more marked response to $M_1$ PAMs; however, further investigation will be needed.

EFS caused the contractile response in the isolated ileum through the release of neurotransmitters including ACh from postganglionic nerve terminals, and this response was almost completely inhibited by either tetrodotoxin or atropine (Fosbraey and Johnson, 1980; Hayashi et al., 1985; Takeuchi et al., 2005); thus, muscarinic receptors were generally considered to regulate the contractile response in GI smooth muscle. Most, but not all, of the studies indicate that the contraction is mediated exclusively by $M_3R$ activation, and $M_2R$ may also have a direct role in inducing contraction in gastric and ileal smooth muscle (Eglen et al., 1996; Matsui et al., 2002; Unno et al., 2006). In

contrast, the contribution of $M_1R$ to GI contractile response seems to be controversial (Ehlert et al., 2012). The coupling of $M_3R$ to phosphoinositide (PI) hydrolysis in intestinal smooth muscle is the fundamental mechanism of action underlying the elicitation of contraction. In the longitudinal muscle of the ileum in $M_1R$ KO mice, only a 15% loss of muscarinic receptor–stimulated PI hydrolysis was observed (Tran et al., 2006), and $M_1R$ expression in peripheral tissues was reported to be low (Ito et al., 2009). In this study, we showed that, in mice, $M_1R$ expression in the intestine was approximately 20% of that in the brain (Fig. 2A). On the other hand, $M_1R$ has been demonstrated to be a key regulator of ileal smooth muscle contraction (Glaza et al., 2011), and $M_1R$ was predominantly expressed in myenteric and submucosal neurons, both cholinergic and nitrergic, in the human colon, suggesting to be involved in neural reflexes underlying colonic motor activity (Harrington et al., 2010). This discrepancy may be explained by differences in species or in isolated regional sections. In addition, $M_1R$, $M_2R$ and $M_4R$, expressed on enteric nerve terminals, act as autoreceptors indirectly modulating reflexes by inhibition or facilitation of neuronal transmission (Tobin et al., 2009). The study using muscarinic receptors KO mice revealed that both $M_2R$ and $M_4R$, not $M_1R$, mainly mediated the muscarinic autoinhibition of ACh release in the mouse ileum (Takeuchi et al., 2005). Overall, it could be reasonable that $M_1R$ is

involved in ileal contractile response via stimulation of myenteric neurons. In the current study, we used various $M_1$ PAMs to help us understand a key parameter in avoiding diarrhea (Table 3). BQCA showed the log α of 1.98, which is in line with the reported value of 2.60 (Mistry et al., 2013); note that the experimental conditions, such as the radioligands used for the experiments ([$^3$H]-pirenzepine or [$^3$H]-N-methylscopolamine), were different.

Importantly, we found that the extent of contractile potentiation (% ileum contraction at 1 μM) was strongly and positively correlated to log α, while it showed no correlation to either pIP or log β (Fig. 3, A, B, and C). In fact, compound A, with a low α-value (log α of 1.18), did not change the ileum contraction level in vitro, while compound B, with a high α-value (log α of 3.30), augmented ileum contraction in a concentration-dependent manner (Fig. 4B and Fig. 5B). Therefore, among the PAM properties, enhancement of ACh affinity to $M_1$R would play a key role in ileum contraction. The precise mechanism of action should be further characterized, but $M_1$ PAMs with high α-value may decrease the threshold of ACh levels to activate $M_1$R, which may lead to an increase in the number of activated $M_1$Rs in the enteric neurons of the GI tract and cause synchronized and synergistic ileum contraction.

Diarrhea is generally caused by not only increased GI motility, but also increased

intestinal secretion (John and Chokhavatia, 2017). The secretory action of ACh on the intestinal epithelium was reported (Browning et al., 1977), and cholinergic intestinal ion transport was predominantly controlled via activation of $M_1R$ and $M_3R$ on neurons and enterocytes, respectively (Hirota and McKay, 2006). However, the activation of neural muscarinic receptors is reported to make a relatively small contribution to the overall secretory response in the guinea-pig ileum (Carey et al., 1987). Therefore, the contribution of intestinal secretion by $M_1$ PAMs to induction of diarrhea might be small, although further detailed examination is needed.

We examined the effects of compound A and compound B on scopolamine-induced amnesia in the Y-maze task, which is generally accepted as a paradigm evaluating spatial working memory (Hughes, 2004). Scopolamine is a nonselective muscarinic receptor antagonist, which is known to serve as a useful pharmacological tool in producing a model of AD (Bartus, 2000). In this study, we selected an optimal dose of scopolamine (0.3 or 1 mg/kg), and measurement time (5 or 8 minutes) for each trial, to achieve a significant decrease in the alternation ratio ($P \leq 0.01$) by scopolamine in the Y-maze task, because the degree of scopolamine-induced cognitive deficits was unstable and varied from trial to trial (Supplemental Table 1). Under these conditions, compound A significantly improved cognitive function at

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30 mg/kg (Fig. 4F). Compound A did not significantly increase the diarrhea score even at 1000 mg/kg (Fig. 4, C and D). Therefore, compound A has a ≥ 3.6-fold safety margin between cognitive improvement and adverse effects at a plasma concentration level; $C_{max}$ at 1000 mg/kg was 0.97 μg/mL and at 30 mg/kg was 0.27 μg/mL (Supplemental Table 2A). In contrast, compound B significantly induced both cognitive improvement and severe diarrhea at 10 mg/kg (Fig. 5, C and F). Thus, compound B had no safety margin between cognitive efficacy and diarrhea induction. In addition, we examined Kp-values, an index representing penetration of a compound into brain tissue from plasma, of both compound A and compound B, and found that compound A showed almost a 2.5-fold higher brain penetration than compound B (Supplemental Table 2B), indicating a more beneficial profile of compound A as a central nervous system–targeting drug. These results suggest that even if the brain penetration of the compounds is taken into consideration, compound A would have a > 1.4-fold broader safety margin than compound B.

The current study supports the idea that lower α-value is a key to discovering safer $M_1$ PAMs for the treatment of cognitive deficits related to AD. Because of compound A's poor potential and PK profile, we could not explore the true safety margin of $M_1$ PAMs with lower α-value. However, by using this strategy, we discovered a novel $M_1$

PAM TAK-071 that has lower risks of adverse effects, including diarrhea. Detailed characteristics of TAK-071 will be discussed elsewhere. TAK-071 is currently being clinically evaluated for the treatment of AD (ClinicalTrials.gov Identifier: NCT02769065).

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## Authorship Contributions

*Participated in research design:* Kurimoto, Matsuda, Shimizu, Sako, Mandai, Sakamoto, Kimura.

*Conducted experiments:* Kurimoto, Matsuda, Shimizu, Sako, Mandai.

*Contributed new reagents or analytic tools:* Sugimoto, Sakamoto.

*Performed data analysis:* Kurimoto, Matsuda, Shimizu, Sako, Mandai, Kimura.

*Wrote or contributed to the writing of the manuscript:* Kurimoto, Kimura.

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## Footnotes

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**Figure legends**

**Fig. 1.** Effects of benzyl quinolone carboxylic acid (BQCA) on diarrhea in wild-type (WT) and $M_1R$ knock-out (KO) mice. BQCA (30 mg/kg) was administered orally to both WT and $M_1R$ KO mice. After treatment with BQCA or vehicle, mice were observed for 90 minutes. Results represent the mean ± S.E.M. of nine (WT) or seven (KO) mice in each group. Statistical significance between vehicle-treated and BQCA-treated group in WT mice was determined using Aspin-Welch's *t*-test ($^{**}P \leq 0.01$), and the effect of BQCA in KO mice was statistically analyzed using Aspin-Welch's *t*-test ($^{\#\#}P \leq 0.01$; versus BQCA-treated group in WT mice). S.E.M., standard error of the mean.

**Fig. 2.** Effects of benzyl quinolone carboxylic acid (BQCA) on mouse ileum contraction in the Magnus assay. (A) mRNA transcripts of $M_1R$ in mouse gastrointestinal tracts (stomach, small intestine and large intestine) and the brain were quantified by TaqMan PCR, and normalized by *Gapdh* mRNA. Results represent the mean ± S.E.M. ($n = 7$ in each group). (B and C) The augmentation of ileum contraction by BQCA treatment was quantified by using the isolated mouse ileum in the presence or absence of 1 nM

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telenzepine (TLZ) under the conditions with electric field stimulation (EFS). Results represent the mean ± S.E.M. ($n$ = 7¬11 in each group). Statistical significance was determined using Aspin-Welch's $t$-test ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$; versus the groups without TLZ treatment). S.E.M., standard error of the mean.

**Fig. 3.** Relationship between ileum contraction and $M_1$ positive allosteric modulator (PAM) parameters. Scatter plots showing the correlation between the potentiation of ileum contraction and pIP value (A), an $M_1$ PAM activity, log α (B), a parameter associated with the binding cooperativity between ACh and PAM, or log β (C), a parameter associated with the magnitude of the allosteric effect of PAM on the signaling efficacy of ACh. Results represent the mean ± S.E.M. ($n$ = 3¬11 in each group). Parameters used for this figure are shown in Tables 1 and 2, and the data are from at least three independent experiments. The $r$ (correlation coefficient) and $P$-values were calculated by Pearson's correlation test (A; $r$ = 0.4979, $P$ = 0.2555, B; $r$ = 0.8075, $P$ = 0.0281, C; $r$ = –0.5505, $P$ = 0.2004). ACh, acetylcholine; IP, inflection point; S.E.M., standard error of the mean.

**Fig. 4.** Effects of a low α-value $M_1$ PAM (compound A) on cognition and diarrhea in

mice. (A) Chemical structure of compound A. (B) The augmentation of ileum contraction by compound A treatment (0.1 nM–1 μM) in isolated mouse ileum with EFS. TLZ means 1 nM telenzepine pretreatment. Results represent the mean ± S.E.M. ($n$ = 7 in each group). (C and D) Diarrhea induction with oral treatment of compound A (10–1000 mg/kg). Results represent the mean ± S.E.M. ($n$ = 6 in each group). (E) The change of *Arc* mRNA expression level in mouse hippocampus by oral treatment of compound A (10–100 mg/kg) at 90 minutes after treatment was detected by TaqMan PCR. Results represent the mean ± S.E.M. ($n$ = 5–6 in each group). (F) Effects of compound A on scopolamine-induced decrease in alternation ratio during 5-minute measurement in the Y-maze task. Compound A was administered orally 60 minutes prior to testing, and scopolamine was treated subcutaneously 30 minutes before testing. Results represent the mean ± S.E.M. ($n$ = 11 in each group). Statistical significance between vehicle-treated and scopolamine-treated groups was determined using Aspin-Welch's $t$-test ($^{**}P \leq 0.01$). Dose-dependent effects of compound A were statistically analyzed using the two-tailed Shirley-Williams test ($^{\#}P \leq 0.05$; versus vehicle-scopolamine-treated group). EFS, electric field stimulation; PAM, positive allosteric modulator; S.E.M., standard error of the mean; TLZ, telenzepine.

**Fig. 5.** Effects of a high α-value $M_1$ PAM (compound B) on cognition and diarrhea in mice. (A) Chemical structure of compound B. (B) The augmentation of ileum contraction by compound B treatment (0.1 nM–1 μM) in the isolated mouse ileum with EFS. TLZ means 1 nM telenzepine pretreatment. Results represent the mean ± S.E.M. ($n$ = 3–4 in each group). (C) Diarrhea induction by oral treatment with compound B (3–30 mg/kg). Results represent the mean ± S.E.M. ($n$ = 6 in each group). Dose-dependent effects of compound B were statistically analyzed using the two-tailed Williams' test ($^{\#}P \leq 0.05$; versus vehicle-treated group). (D) The change of *Arc* mRNA expression level in mouse hippocampus by oral administration of compound B (3–30 mg/kg) at 90 minutes after treatment was detected by TaqMan PCR. Results represent the mean ± S.E.M. ($n$ = 6 in each group). Dose-dependent effects of compound B were statistically analyzed using the two-tailed Shirley-Williams test ($^{\#}P \leq 0.05$; versus vehicle-treated group). (E and F) Effect of compound B on scopolamine-induced decrease in alternation ratio in the Y-maze task under the conditions of measurement recorded for 5 minutes (E) and 8 minutes (F). Compound B was administered orally 60 minutes prior to testing, and scopolamine was given subcutaneously 30 minutes before testing. Results represent the mean ± S.E.M. ($n$ = 7–8 in each group). Statistical significance between the vehicle-treated and scopolamine-treated groups was determined using Aspin-Welch's

*t*-test (E; $P = 0.08$, F; $^{**}P \leq 0.01$). Dose-dependent effects of compound B were statistically analyzed using the two-tailed Williams' test (E; $P = 0.06$, F; $^{\#}P \leq 0.05$; versus vehicle-scopolamine-treated group). EFS, electric field stimulation; PAM, positive allosteric modulator; S.E.M., standard error of the mean; TLZ, telenzepine.

**TABLE 1**

**Cholinergic Adverse Effects of BQCA Treatment in Mice**

Cholinergic adverse effects such as loose stool, diarrhea, lacrimation, and salivation were assessed after oral administration of BQCA (30–300 mg/kg) in mice. Results represent the incidence rates of each severe symptom (out of a total of 6 mice).

BQCA, benzyl quinolone carboxylic acid.

| BQCA (mg/kg p.o.) | Gastrointestinal symptom | | Lacrimation | Salivation |
|---|---|---|---|---|
| | Loose or Mucous Stool | Diarrhea | | |
| 30 | 2/6 | 2/6 | 0/6 | 0/6 |
| 100 | 1/6 | 5/6 | 0/6 | 0/6 |
| 300 | 0/6 | 6/6 | 0/6 | 0/6 |

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**TABLE 2**

**Functional Parameters, Agonistic and PAM Activity, and Selectivity for $M_1R$ of $M_1$ PAMs**

$EC_{50}$, eliciting 50% of the maximal response of an endogenous ligand, ACh, reflects the agonistic activity for $M_1R$ under conditions without ACh. Inflection point (IP) reflects the PAM potency for $M_1$-$M_5R$, which is determined from titration of PAM in the presence of a low concentration of ACh, eliciting 20% of the maximal ACh response ($EC_{20}$). Results of the in vitro functional parameters for $M_1R$ represent the mean and S.E.M. of three independent experiments. ACh, acetylcholine; $M_1R$, $M_1$ muscarinic receptor; N/A, not available; PAM, positive allosteric modulator; S.E.M., standard error of the mean.

| Compound | $M_1R$ Agonistic Activity $pEC_{50}$ | | PAM Activity Against Muscarinic Receptor Subtypes pIP | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $M_1R$ | | $M_2R$ | $M_3R$ | $M_4R$ | $M_5R$ |
| | Mean | S.E.M. | Mean | S.E.M. | | | | |
| BQCA | 6.16 | 0.04 | 7.13 | 0.06 | < 5.00 | < 5.00 | < 5.00 | < 5.00 |
| Compound A | 6.17 | 0.10 | 7.39 | 0.08 | < 5.00 | < 5.00 | < 5.00 | < 5.00 |
| Compound B | 7.14 | 0.20 | 7.99 | 0.09 | < 5.00 | < 5.00 | 6.00 | < 5.00 |
| Compound C | 8.79 | 0.24 | 8.68 | 0.19 | < 5.00 | < 5.00 | < 5.00 | < 5.00 |
| Compound D | 6.66 | 0.19 | 8.07 | 0.11 | < 5.00 | < 5.00 | < 5.00 | < 5.00 |
| Compound E | 5.68 | 0.32 | 7.10 | 0.10 | < 5.00 | < 5.00 | < 5.00 | < 5.00 |
| Compound F | < 5.00 | N/A | 6.56 | 0.19 | < 5.00 | < 5.00 | < 5.00 | < 5.00 |

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**TABLE 3**

**Binding Parameters of $M_1$ PAMs, and Ileum Contractile Response at 1 μM in the In Vitro Magnus Assay**

Allosteric effects of $M_1$ PAM on the binding and signaling of the orthosteric ligand were reflected by α- and β-values, respectively. $K_B$-value means the affinity of the PAM to the free $M_1R$. $\tau_B$ represents intrinsic agonistic efficacy in the system. The augmentation of ileum contraction by PAM treatment (1 μM) in isolated mouse ileum with EFS was described in the Magnus assay. Results of the in vitro binding parameters represent the mean and S.E.M. of three independent experiments. Results of the in vitro Magnus assay represent the mean and S.E.M. $n$ = 3–11 per group.

EFS, electric field stimulation; $M_1R$, $M_1$ muscarinic receptor; N/A, not available; PAM, positive allosteric modulator; S.E.M., standard error of the mean.

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| Compound | $M_1$ PAM Parameters | | | | | | | | Magnus Assay | |
|---|---|---|---|---|---|---|---|---|---|---|
| | log α | | log β | | $pK_B$ | | log $\tau_B$ | | Ileum Contraction at 1 μM (% of pretreatment) | |
| | Mean | S.E.M. | Mean | S.E.M. | Mean | S.E.M. | Mean | S.E.M. | Mean | S.E.M. |
| BQCA | 1.98 | 0.05 | 0.16 | 0.04 | 5.04 | 0.08 | 0.59 | 0.06 | 110 | 2.3 |
| Compound A | 1.18 | 0.09 | 0.58 | 0.07 | 6.01 | 0.14 | -0.10 | 0.01 | 97 | 2.1 |
| Compound B | 3.30 | 0.20 | 0.13 | 0.05 | 4.58 | 0.23 | 2.06 | 0.23 | 116 | 6.3 |
| Compound C | 3.25 | 0.04 | -0.39 | 0.02 | 6.54 | 0.03 | 1.49 | 0.03 | 104 | 3.5 |
| Compound D | 2.67 | 0.16 | -0.23 | 0.03 | 5.51 | 0.12 | 0.65 | 0.09 | 106 | 6.5 |
| Compound E | 1.54 | 0.01 | 0.87 | 0.05 | 5.04 | 0.04 | 0.15 | 0.01 | 102 | 2.1 |
| Compound F | 0.56 | 0.13 | 0.85 | 0.04 | 4.83 | 0.21 | N/A | N/A | 93 | 2.4 |

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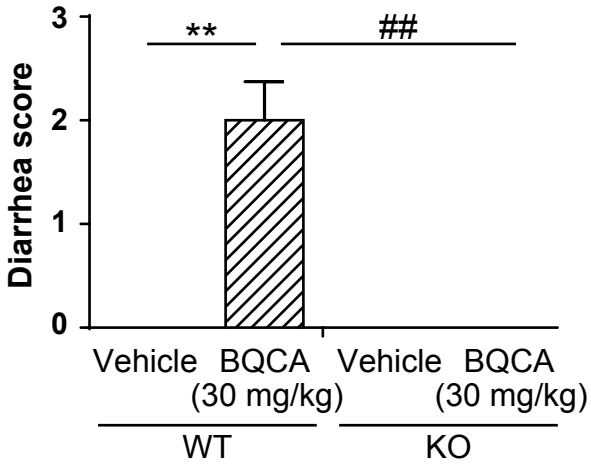




Figure 1

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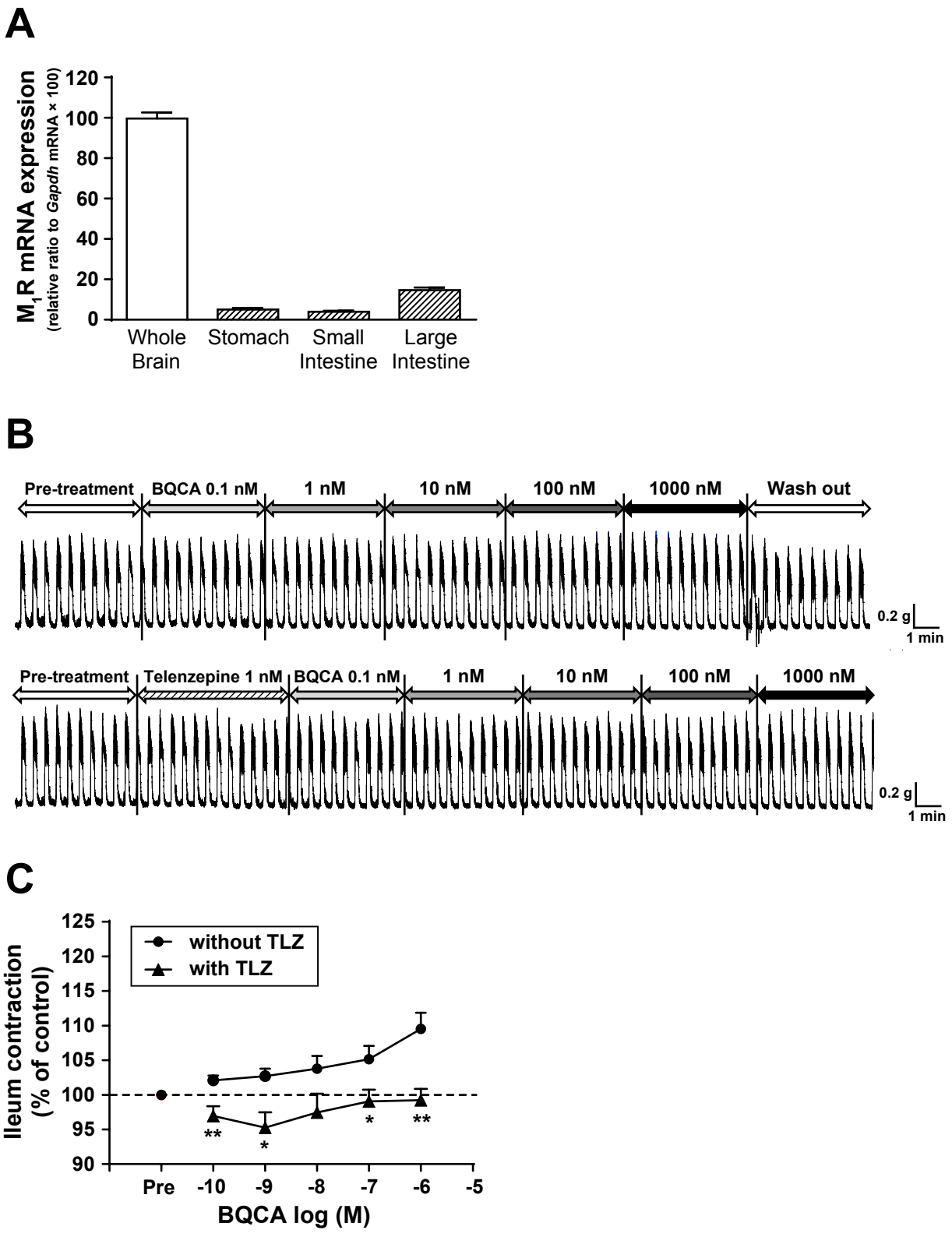


Figure 2

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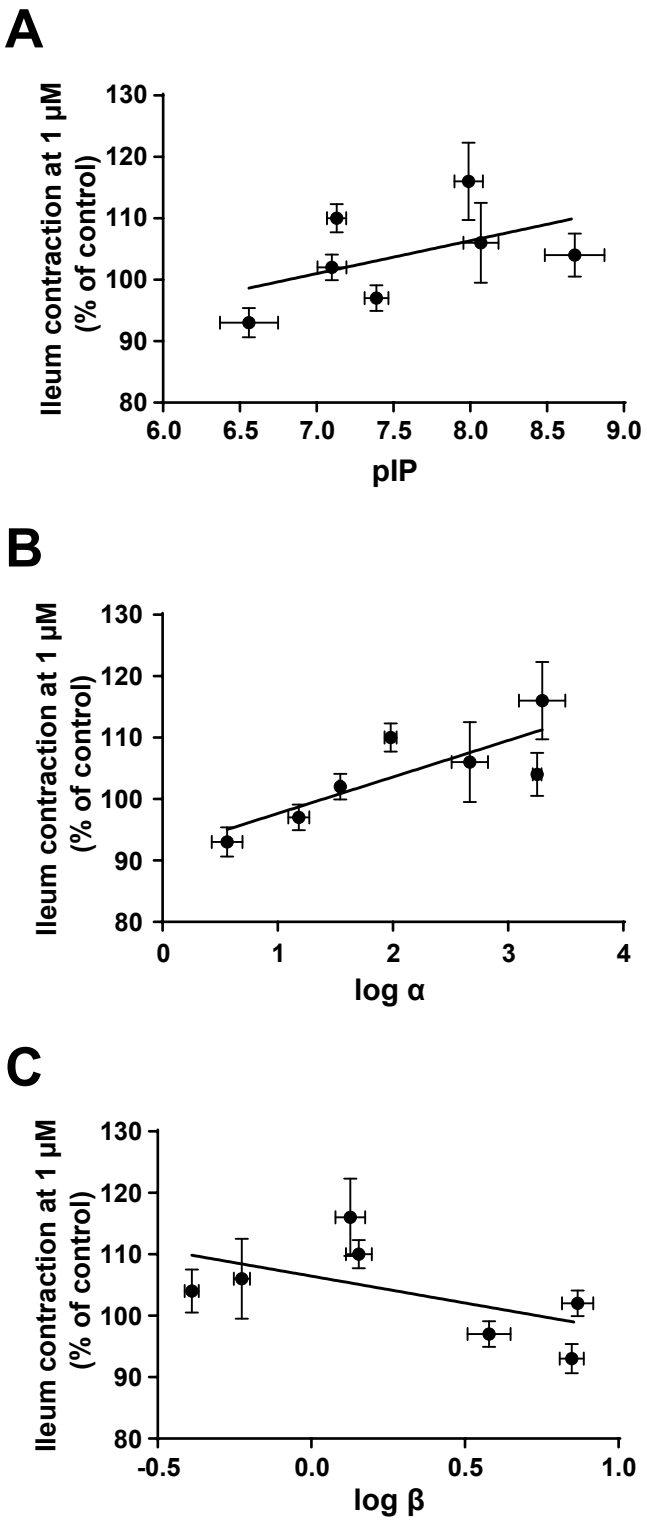


Figure 3

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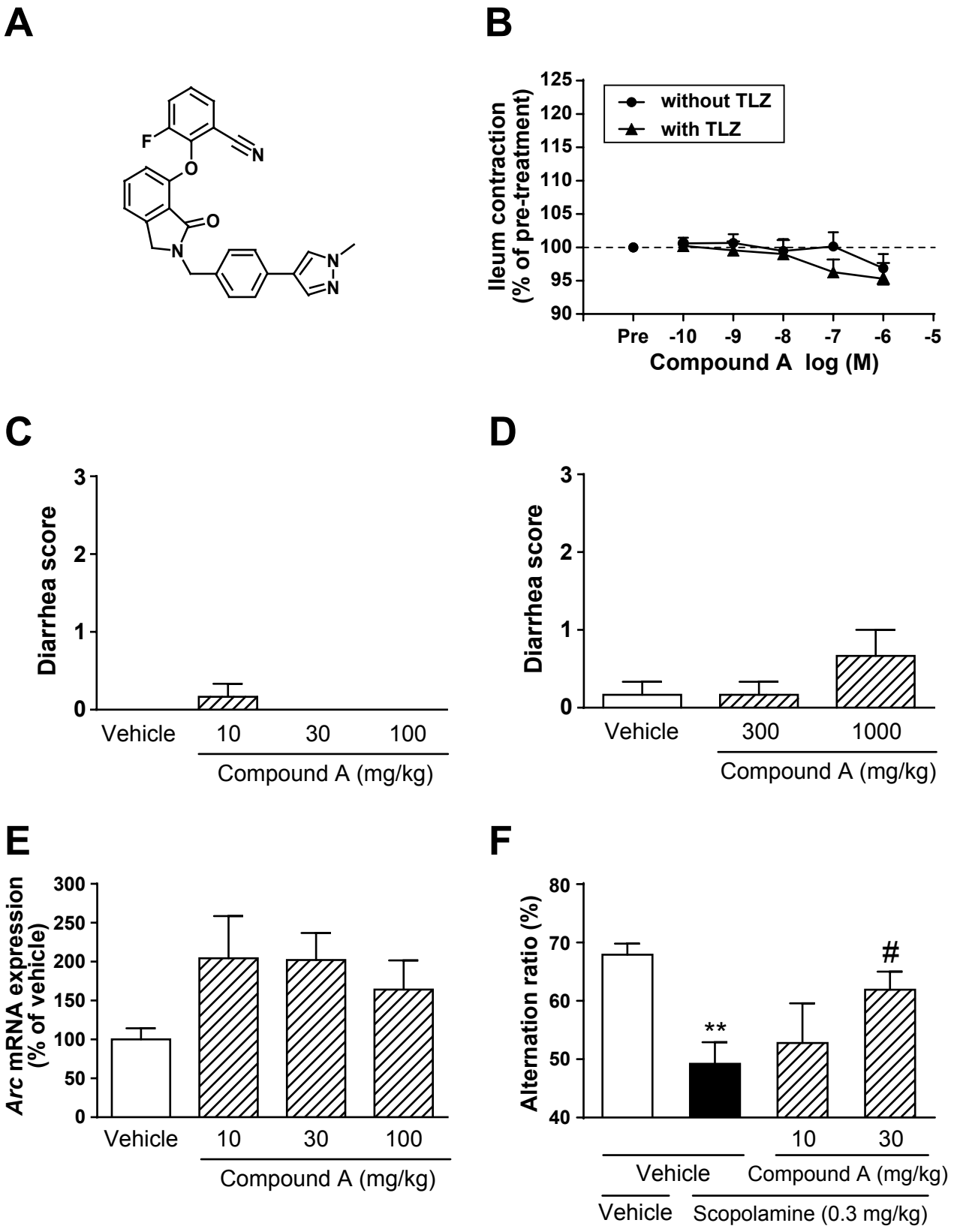


Figure 4

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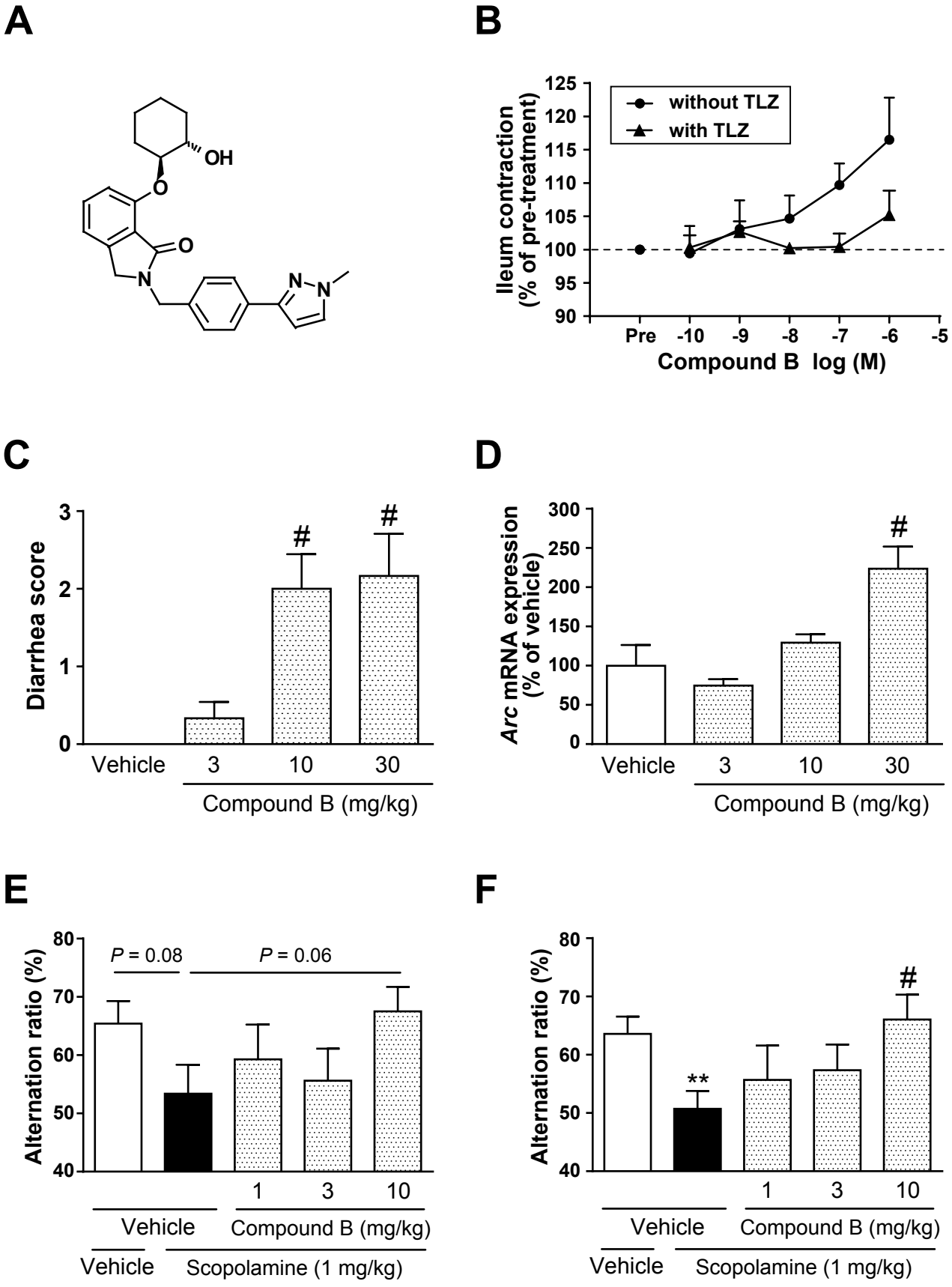


Figure 5

**An approach to discovering novel muscarinic $M_1$ receptor positive allosteric modulators with potent cognitive improvement and minimized gastrointestinal dysfunction**

Emi Kurimoto, Satoru Matsuda, Yuji Shimizu, Yuu Sako, Takao Mandai, Takahiro Sugimoto, Hiroki Sakamoto, and Haruhide Kimura

*Journal of Pharmacology and Experimental Therapeutics*

**Supplemental Table 1**

| Measurement time | Trial No. | Group | n | Total entries | Alternation ratio (%) | Criterion |
|---|---|---|---|---|---|---|
| 5 min | 1 | Vehicle | 10 | 24 ± 2 | 67 ± 3 | - |
| | | Scopolamine (0.3 mg/kg) | 10 | 24 ± 2 | 55 ± 5 * | Satisfied |
| | 2 (Compound A) | Vehicle | 11 | 25 ± 1 | 68 ± 2 | - |
| | | Scopolamine (0.3 mg/kg) | 11 | 23 ± 2 | 49 ± 4 ** | Satisfied |
| | 3 | Vehicle | 8 | 24 ± 2 | 66 ± 2 | - |
| | | Scopolamine (0.3 mg/kg) | 8 | 24 ± 2 | 54 ± 7 | Not satisfied |
| | | Scopolamine (1 mg/kg) | 8 | 22 ± 3 | 46 ± 8 * | Satisfied |
| | 4 (Compound B) | Vehicle | 8 | 27 ± 1 | 65 ± 4 | - |
| | | Scopolamine (1 mg/kg) | 8 | 22 ± 2 ** | 53 ± 5 | Not satisfied |
| | 5 | Vehicle | 8 | 27 ± 1 | 66 ± 5 | - |
| | | Scopolamine (1 mg/kg) | 7 | 28 ± 2 | 47 ± 4 * | Satisfied |
| | 6 | Vehicle | 10 | 25 ± 1 | 64 ± 3 | - |
| | | Scopolamine (1 mg/kg) | 10 | 26 ± 2 | 59 ± 4 | Not satisfied |
| | 7 | Vehicle | 10 | 26 ± 1 | 70 ± 3 | - |
| | | Scopolamine (1 mg/kg) | 10 | 26 ± 2 | 55 ± 3 ** | Satisfied |
| | 8 | Vehicle | 11 | 22 ± 1 | 66 ± 4 | - |
| | | Scopolamine (1 mg/kg) | 11 | 22 ± 3 | 52 ± 5 * | Satisfied |
| | 9 | Vehicle | 10 | 25 ± 1 | 67 ± 4 | - |
| | | Scopolamine (1 mg/kg) | 10 | 23 ± 2 | 64 ± 5 | Not satisfied |
| 8 min | 3 | Vehivle | 8 | 35 ± 2 | 64 ± 2 | - |
| | | Scopolamine (0.3 mg/kg) | 8 | 39 ± 4 | 52 ± 7 | Not satisfied |
| | | Scopolamine (1 mg/kg) | 8 | 38 ± 4 | 49 ± 6 * | Satisfied |
| | 4 (Compound B) | Vehicle | 8 | 39 ± 2 | 64 ± 3 | - |
| | | Scopolamine (1 mg/kg) | 8 | 33 ± 4 | 51 ± 3 ** | Satisfied |
| | 5 | Vehicle | 8 | 38 ± 1 | 64 ± 4 | - |
| | | Scopolamine (1 mg/kg) | 7 | 45 ± 3 | 48 ± 4 * | Satisfied |
| | 6 | Vehicle | 10 | 36 ± 1 | 62 ± 3 | - |
| | | Scopolamine (1 mg/kg) | 10 | 41 ± 2 * | 54 ± 2 * | Satisfied |
| | 7 | Vehicle | 10 | 36 ± 2 | 66 ± 2 | - |
| | | Scopolamine (1 mg/kg) | 10 | 40 ± 3 | 54 ± 3 ** | Satisfied |
| | 8 | Vehicle | 11 | 32 ± 2 | 62 ± 2 | - |
| | | Scopolamine (1 mg/kg) | 11 | 34 ± 4 | 46 ± 5 * | Satisfied |
| | 9 | Vehicle | 10 | 37 ± 2 | 67 ± 3 | - |
| | | Scopolamine (1 mg/kg) | 10 | 37 ± 2 | 60 ± 4 | Not satisfied |

**Supplemental Table 1.** Experimental conditions for inducing prominent cognitive deficits by scopolamine in the Y-maze task in mice. Influence of dosages of scopolamine (0.3 and 1 mg/kg), and of measurement time (5 or 8 minutes) on the induction of significant cognitive deficits in the Y-maze task. Mice were administered vehicle or scopolamine 30 minutes prior to testing. Total entries and spontaneous alternation ratios are represented by the means ±

S.E.M. 'Satisfied' in the criterion column means to achieve the significant decrease in the alteration ratio by scopolamine treatment compared with vehicle-treated group. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ compared with vehicle-treated group using Aspin-Welch's *t*-test. S.E.M., standard error of the mean.

**Supplemental Table 2**

**A**

| PK parameter | Compound A | |
|---|---|---|
| | 30 mg/kg | 1000 mg/kg |
| **Cmax (μg/mL)** | 0.27 | 0.97 |
| **Tmax (h)** | 2.0 | 1.7 |
| **$AUC_{0\text{-}4h}$ (μg·h/mL)** | 0.80 | 3.02 |

**B**

| PK parameter | Compound A | Compound B |
|---|---|---|
| | 10 mg/kg | 10 mg/kg |
| **Kp value** | 0.220 | 0.089 |
| **Brain conc. (μg/g)** | 0.06 | 2.30 |
| **Plasma conc. (μg/mL)** | 0.27 | 25.59 |

**Supplemental Table 2.** Pharmacokinetic parameters after oral administration to mice. (A) Plasma concentration of compound A at 30 and 1000 mg/kg. Plasma samples were collected 0.5, 1, 2, and 4 hours after oral treatment. Results represent the mean. $n = 3$ in each group. (B) Kp-value of compound A and compound B, respectively. Kp-value was a ratio of brain concentration to plasma concentration of the compound, obtained 1 hour after oral treatment. Results represent the mean. $n = 3$ in each group.

## Materials and Methods

**Y-Maze Task in ICR Mice.** The Y-maze apparatus has a three-arm maze with equal angles between all arms and made of black colored acrylic. Each arm was 40 cm in length, 4 cm wide, and had walls 12 cm thick. The Y-maze was located in a sound-attenuated room and illuminated at 10 lux. Memory impairment in an ICR mouse was induced by administering scopolamine (0.3 or 1 mg/kg, as hydrobromide salt, s.c.). At 30 minutes after the administration of scopolamine, mice were placed in the Y-maze and spontaneous alternation performance was assessed visually for 5 to 8 minutes. Each animal underwent one trial. A mouse was considered to have entered an arm when all four paws were positioned in one third of the arm runway. Alternations were defined as successive entries into each of the three arms on overlapping triplet sets, and alternation rate, expressed as a percentage, referred to the ratio of actual (total alternations) to possible alternations (total arm entries – 2) × 100. Mice were excluded from the analyses if they possessed low exploratory activity defined as fewer than 10 total arm entries.

**Plasma and Brain Concentration of Compound A and Compound B in ICR Mice.** Concentrations of compound A and compound B were measured in aliquots of mouse plasma or brain homogenate, which was mixed well with acetonitrile containing the internal standards and then centrifuged. The supernatants were diluted with solvents for LC-MS/MS analysis (mobile phase A: 10 mM ammonium formate/formic acid [100/0.2, v/v], mobile phase B: acetonitrile/formic acid [100/0.2, v/v]). The diluted solutions were injected into an LC-MS/MS (API5000™, AB Sciex, Foster City, CA) that was equipped with Shimadzu (Kyoto, Japan) Shim-pack XR-ODS (2.2 μm, 2.0 × 30 mm) maintained at 50°C. The chromatographic separation was performed with a gradient elution at a flow rate of 0.7 mL/minute. The LC time program for the mouse data was the following: Mobile phase B was held at 5% for 0.1 minute, and increased linearly to 95% for 0.1 minute. After B was held at 95% for another 0.8 minute, it was brought back to 5% B in 0.01 minute, followed by re-equilibration for 0.59 minute. The total cycle time for one injection was 1.6 minutes. The compounds were detected using a multiple reaction monitoring mode using the transition. Analyst® software (version 1.4.2) was used for data acquisition and processing.

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| | 3 | Vehicle | 8 | 24 ± 2 | 66 ± 2 | - |
| | | Scopolamine (0.3 mg/kg) | 8 | 24 ± 2 | 54 ± 7 | Not satisfied |
| | | Scopolamine (1 mg/kg) | 8 | 22 ± 3 | 46 ± 8 * | Satisfied |
| | 4 (Compound B) | Vehicle | 8 | 27 ± 1 | 65 ± 4 | - |
| | | Scopolamine (1 mg/kg) | 8 | 22 ± 2 ** | 53 ± 5 | Not satisfied |
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