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Characterization of functional effects of Z-338, a novel gastroprokinetic agent, on the muscarinic M_1, M_2, and M_3 receptors expressed in *Xenopus* oocytes

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Abstract

This study characterized the functional effects of a novel gastroprokinetic agent, *N*-[2-(diisopropylamino)ethyl]-2-[(2-hydroxy-4,5-dimethoxybenzoyl)amino]-1, 3-thiazole-4-carboxyamide monohydrochloride trihydrate (Z338), on the muscarinic M_1, M_2, and M_3 receptors expressed in *Xenopus* oocytes using the two-electrode voltage clamp method. Z-338 did not produce by itself any currents in oocytes expressing muscarinic M_1, M_3 receptors or muscarinic M_2 receptors/G protein-gated inward rectifying K^+ channels (Kir3.1 channels). In oocytes expressing muscarinic M_1 receptors, Z-338 inhibited the acetylcholine-induced Ca^{2+}-activated Cl^- current with an IC_{50} of 1.8 μM. In oocytes expressing muscarinic M_2 receptors/Kir3.1 channels, Z-338 inhibited the acetylcholine-induced K^+ currents with an IC_{50} of 10.1 μM, whereas in oocytes expressing muscarinic M_3 receptors, Z-338 did not inhibit the acetylcholine-induced Ca^{2+}-activated Cl^- current in a concentration-dependent manner. These results indicate that Z-338 is a potent antagonist not for muscarinic M_3 receptor but for both muscarinic M_1 and M_2 receptors. Thus, Z-338 is a gastrokinetic agent with a unique profile.

Keywords: Gastrokinetic agent; Muscarinic receptor; Z-338

1. Introduction

N-[2-(diisopropylamino)ethyl]-2-[(2-hydroxy-4,5-dimethoxybenzoyl)amino]-1, 3-thiazole-4-carboxyamide monohydrochloride trihydrate (Z338) is a newly synthesized gastroprokinetic agent which enhances gastrointestinal motility in conscious dogs and gastric emptying in rats and dogs (Ueki et al., 1998). The mechanism underlying the enhancement of gastrointestinal motility has been proposed to be facilitation of acetylcholine release from enteric cholinergic nerve terminals (Ogishima et al., 2000). Z-338 appears to possess an antagonistic effect on muscarinic M_1 and M_2 receptors and inhibits the negative feedback system by blocking muscarinic autoreceptors, which regulate acetylcholine release. Muscarinic M_1 and M_2 receptors located on the presynaptic cholinergic neurons have been reported to modulate the release of acetylcholine from cholinergic nerve terminals (Kawashima et al., 1988; Schwörer, 1988; Kilbinger et al., 1993; Ren and Harty, 1994; Dietrich and Kilbinger, 1995). The released acetylcholine stimulates muscarinic M_3 receptor in gastrointestinal smooth muscle cells and induces muscle contraction (Eglen et al., 1994; Igawa, 2000). Thus, muscarinic M_3 receptor is an important element in the regulation of the contraction of smooth muscle. However, whether Z-338 affects muscarinic M_3 receptor functionally is not known. It also remains to be elucidated how potently Z-338 inhibits muscarinic M_1 or M_2 receptor activation elicited by agonist.

The *Xenopus* oocyte expression system has been used to express a multiplicity of receptors from complementary DNAs (cDNAs) or cRNAs with pharmacological proper-

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ties that mimic those of native receptors. Stimulation of the muscarinic M_1 or M_3 receptor (Gq protein-coupled receptors) expressed in *Xenopus* oocytes leads to the opening of endogenous Ca^{2+}- activated Cl^- channels (Snutch, 1988). Stimulation of the muscarinic M_2 receptor coexpressed with G protein-gated inward rectifying K^+ channels (Kir3.1 channels) in *Xenopus* oocytes induces K^+ conductance mediated by the opening of Kir3.1 channels (Reuveny et al., 1994). Thus, these systems have been characterized and have proven useful for studying the effects of drugs acting on muscarinic M_1, M_2, and M_3 receptors.

In the present study, we investigated the functional effects of Z-338 on the muscarinic M_1, M_2, and M_3 receptors expressed in *Xenopus* oocytes to elucidate the mechanism underlying its gastroprokinetic action.

2. Materials and methods

2.1. Drugs and chemicals

The drugs used were as follows: Z-338 and 11,2-(diethylamino)methyl-1-piperidinyl-acetyl-5,11-dihydro-6H-pyrido-2,3b-1,4-benzodiazepine-6-one (AF-DX 116) were gifts from ZERIA Pharmaceutical (Tokyo, Japan); acetylcholine chloride and pirenzepine dihydrochloride (Sigma, St Louis, MO, USA); 1,1-dimethyl-4-diphentlacetoxypiperidinium iodide (4-DAMP) (TOCRIS, Avonmouth, UK).

2.2. Electrophysiological study using Xenopus oocytes

cDNAs for rat muscarinic M_1 receptor and rat Kir3.1 channel were obtained from Dr. H.A. Lester (Caltech, CA, USA). cDNA for human muscarinic M_2 receptor was from Dr. E.G. Peralta (Harvard University, MA, USA), and cDNA for rat muscarinic M_3 receptor was from Dr. T.I. Bonner (National Institutes of Health, MD, USA). The cRNAs for the muscarinic M_1 receptor, the muscarinic M_2 receptor, and the muscarinic M_3 receptor were synthesized with T7 polymerase, and the cRNA for Kir3.1 channel was synthesized with T3 polymerase using Ambion's MEGAscrip™ (Ambion, Austin, TX, USA) kits.

Guidelines for Institutional Animal Care and Utilization were followed during the experiments. Oocyte preparation and the two-electrode voltage clamp method have been described elsewhere (Nagase et al., 1999). Stages V–VI *Xenopus* oocytes were isolated and defolliculated by gently shaking them at room temperature (21–23 °C) for 60 min in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) containing 0.5 mg/ml collagenase (Yakult, Tokyo, Japan). cRNAs (5 ng for the muscarinic M_1, M_2, and M_3 receptors and 0.2 ng for the Kir3.1 channel) were injected into the oocytes using Picospritzer II (General Valve, Fairfield, NJ, USA), and then the oocytes were incubated at 19 °C in modified Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 10 mM HEPES, pH 7.4) containing 2.5 mM sodium pyruvate and 20 μg/ml gentamycin for 2–4 days. Electrophysiological measurements were performed between 2 and 4 days after cRNA injection, using a two-electrode voltage clamp amplifier (TEV-200, Dagan Instruments, Mineapolis, MN, USA). The oocytes were clamped at −60 mV and continuously superfused with bath solution (ND96 with 1 mM $CaCl_2$). The bath had a volume of 150 μl, and the flow rate was 2 ml/min. Each of the substances was dissolved in the bath solution and superfused at the same flow rate. To obtain the acetylcholine-induced current in oocytes expressing muscarinic M_1 or M_3 receptors, acetylcholine (100 nM) was applied for 15 s after a 15-min equilibrium period from the start of voltage clamp. At 30 min after the initial application of acetylcholine, Z-338 or antagonists (pirenzepine for muscarinc M_1 receptor and 4-DAMP for muscarinc M_3 receptor) were administered 30 s before the second application of acetylcholine. To obtain the acetylcholine-induced current in oocytes expressing muscarinic M_2 receptors/Kir3.1 channels, the bath solution was changed for high K^+ solution (composition, 2 mM NaCl, 96 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES at pH 7.4) after a 15-min equilibrium period from the start of voltage clamp, and then acetylcholine (100 nM)-containing high K^+ solution was perfused continuously. At 5 min after the start of acetylcholine application, a high K^+ solution containing both acetylcholine and Z-338 or AF-DX116 was applied. The acetylcholine concentration of 100 nM was approximately the half-effective dose for each of these receptors expressed in the oocytes.

2.3. Data analysis

All data are presented as means±S.E.M. Concentration–response curves were analyzed by nonlinear regression using Kaleida Graph 3.08 (Synergy Software, Reading, PA, USA). All statistics were generated using StatView 4.0 and SuperANOVA 1.11 (Abacus Concepts, Berkeley, CA, USA). Statistical analysis between control and substance-treated groups was assessed using a one-way analysis of variance (ANOVA) followed by Dunn multiple-comparison test. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Reproducibility of current induction with acetylcholine in oocytes expressing muscarinc M1 or M3 receptors

In a series of our studies, in defolliculated oocytes not injected with cRNA for muscarinic M_1 or M_3 receptor, application of acetylcholine induced no significant currents (Matsumoto et al., 1988; Nagase et al., 1999). To determine the effects of Z-338 on the acetylcholine-induced Ca^{2+}-activated Cl^- currents via muscarinic M_1 or M_3 receptors, it

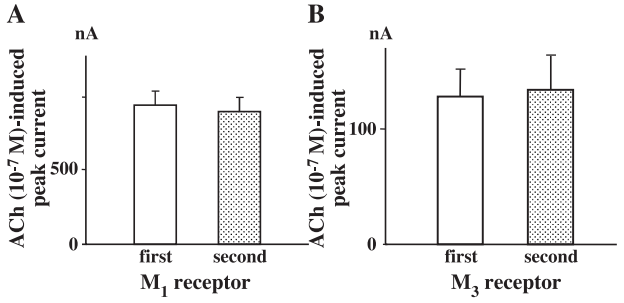

Fig. 1. Reproducibility of the currents induced by two successive applications of acetylcholine to an oocyte expressing muscarinic M_1 or M_3 receptors. Each column is presented as the mean±S.E.M. of the peak amplitude of acetylcholine (100 nM)-induced currents (8 oocytes for muscarinic M_1 receptor, 10 oocytes for muscarinic M_3 receptor) in oocytes expressing muscarinic M_1 (A) or M_3 receptor (B). Firstly, acetylcholine (100 nM) was applied for 15 s after a 15-min equilibrium period from the start of voltage clamp, and the same concentration of acetylcholine was reapplied at 30 min after the first application. There was no significant difference in the peak amplitude between two successive acetylcholine-induced currents in oocytes expressing muscarinic M_1 or M_3 receptor.

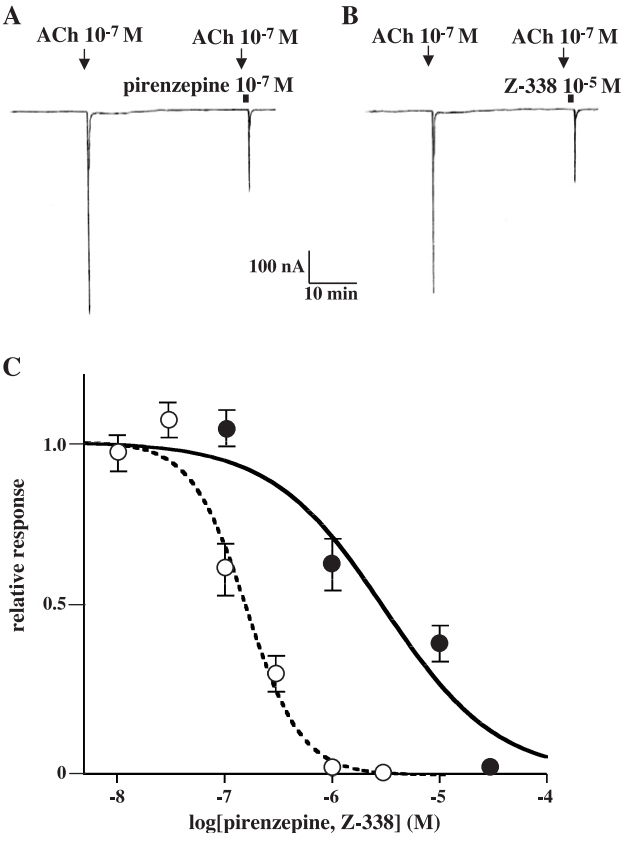

Fig. 2. Effects of pirenzepine and Z-338 on the acetylcholine-induced response in oocytes expressing muscarinic M_1 receptors. Application of acetylcholine to oocytes expressing muscarinic M_1 receptors elicited transient inward currents through the Ca^{2+}-activated Cl^- channels. (A) Pirenzepine (100 nM, 30 s) inhibited activation of the current. (B) Z-338 (1 μM, 30 s) inhibited activation of the current. (C) Pirenzepine (○) and Z-338 (●) inhibited activation of the currents in a concentration-dependent manner. The IC_{50} values were 144.0 nM for pirenzepine and 1.8 μM for Z-338, respectively. Each point is presented as the mean±S.E.M. of independent experiments (n=5–11).

would be ideal to compare the acetylcholine-induced currents with or without Z-338 pretreatment in an oocyte. To eliminate possible desensitizing effects of repeated application of the agonist, we elicited two consecutive Ca^{2+}-activated Cl^- currents with acetylcholine (100 nM) in an oocyte at 30-min intervals. The mean peak sizes for two consecutive applications were 940±110 (first application) and 887±113 nA (second application; n=8) in oocytes expressing muscarinic M_1 receptors and 127±23 (first application) and 133±31 nA (second application; n=10) in oocytes expressing muscarinic M_3 receptors. There were no significant differences between the means of two consecutive responses in oocytes expressing muscarinic M_1 or M_3 receptors (Fig. 1a,b).

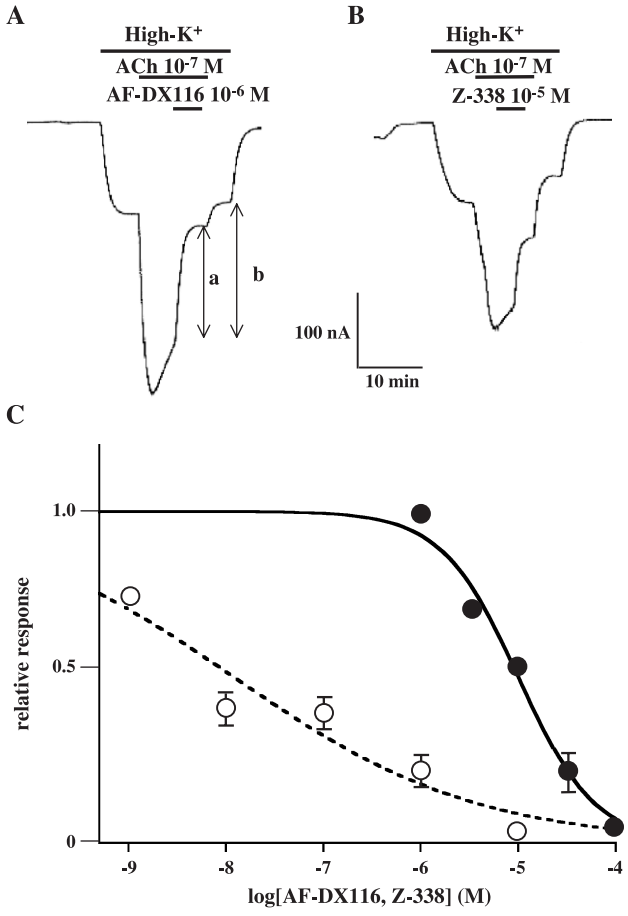

Fig. 3. Effects of AF-DX116 and Z-338 on the acetylcholine-induced response in oocytes coexpressing muscarinic M_2 receptors and Kir3.1 channels. Effects of AF-DX116 (A) and Z-338 (B) on the acetylcholine-induced currents recorded in the high K^+ solution (96 mM KCl and 2 mM NaCl) are shown. In high K^+ solution, inward currents through Kir3.1 channels were observed. During the application of acetylcholine (100 nM), Kir3.1 channel currents were activated. AF-DX116 (1 μM) and Z-338 (10 μM) were applied in the presence of acetylcholine. (C) AF-DX116 and Z-338 inhibited the currents activated by acetylcholine in a concentration-dependent manner. Relative response (y) was obtained from the equation $y=1-a/b$ (a and b are indicated in the representative current trace in panel [A]). The IC_{50} values were 13.2 nM for AF-DX116 and 10.1 μM for Z-338, respectively. Each point is presented as the mean±S.E.M. of independent experiments (n=4–8) for each concentration of AF-DX116 or Z-338.

3.2. Effect of Z-338 on the muscarinic M_1 receptor expressed in Xenopus oocytes

In oocytes expressing muscarinic M_1 receptors, pirenzepine (10 nM to 3 μM) did not produce any currents, while pretreatment with pirenzepine for 30 s attenuated the acetylcholine-induced Ca^{2+}-activated Cl^- current in a concentration-dependent manner (Fig. 2a,c). Application of Z-338 (100 nM to 30 μM) did not produce any currents in oocytes expressing muscarinic M_1 receptors, while pretreatment with Z-338 (100 nM to 30 μM) for 30 s attenuated the acetylcholine-induced current in a concentration-dependent manner (Fig. 2b,c). The IC_{50} values for pirenzepine and Z-338 were 144.0 nM and 1.8 μM, respectively.

3.3. Effect of Z-338 on the muscarinic M_2 receptor expressed in Xenopus oocytes

In oocytes coexpressing muscarinic M_2 receptors and Kir3.1 channels (muscarinic M_2 receptors/Kir3.1 channels), application of acetylcholine (100 nM) produced inward K^+ currents through Kir3.1. AF-DX116 (1 nM to 10 μM) attenuated the acetylcholine-induced inward K^+ current in a concentration-dependent manner (Fig. 3a,c). Z-338 (1 to 100 μM) also attenuated the acetylcholine-induced current in a concentration-dependent manner (Fig. 3b,c). The IC_{50} values for AF-DX116 and Z-338 were 13.2 nM and 10.1 μM, respectively. Application of Z-338 (10 nM to 100 μM) neither produced any currents nor attenuated the basal K^+ conductance in oocytes expressing muscarinic M_2 receptors/Kir3.1 channels (data not shown).

As previously reported (Dascal et al., 1993), the acetylcholine-induced current declined during the application of acetylcholine, and the decline in different oocytes occurred at a different rate (Fig. 3a,b). Inasmuch as we did not take into account the decline to calculate the relative response, the antagonistic effects may be overestimated.

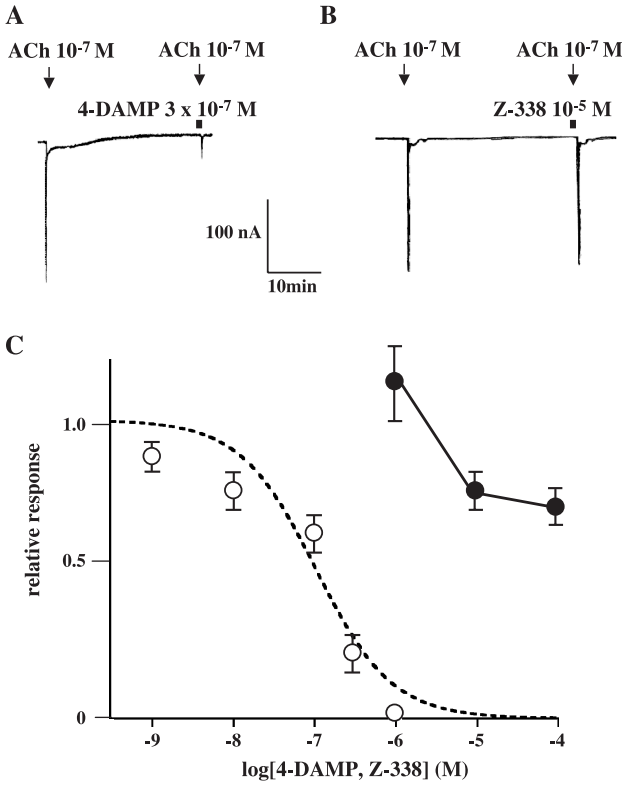

Fig. 4. Effects of 4-DAMP and Z-338 on the acetylcholine-induced response in oocytes expressing muscarinic M_3 receptors. Application of acetylcholine to oocytes expressing muscarinic M_3 receptors elicited transient inward currents through Ca^{2+}-activated Cl^- channels. (A) 4-DAMP (300 nM, 30 s) inhibited activation of the current. (B) Z-338 (10 μM, 30 s) slightly inhibited activation of the current. (C) Z-338 (●) did not inhibit activation of the currents in a concentration-dependent manner (P=0.10, ANOVA). The IC_{50} values were 91.9 nM for 4-DAMP (○). Each point is presented as the mean±S.E.M. of independent experiments (n=4–8) for each concentration of 4-DAMP or Z-338.

3.4. Effect of Z-338 on the muscarinic M_3 receptor expressed in Xenopus oocytes

In oocytes expressing muscarinic M_3 receptors, 4-DAMP (1 nM to 1 μM) did not produce any currents, while pretreatment with 4-DAMP for 30 s attenuated the acetylcholine-induced Ca^{2+}-activated Cl^- current in a concentration-dependent manner with an IC_{50} value of 91.9 nM (Fig. 4a,c). Pretreatment with Z-338 (1 to 100 μM) for 30 s did not show a concentration-dependent inhibition of the Cl^- current (Fig. 4b and c). Application of Z-338 (1 to 100 μM) did not produce any currents in oocytes expressing muscarinic M_3 receptors.

4. Discussion

In the present study, we demonstrated the antagonistic profiles of Z-338, a prokinetic agent, at cloned muscarinic M_1 and M_2 receptors expressed in *Xenopus* oocytes. Stimulation of the muscarinic M_1 and M_3 receptors expressed in oocytes elicited Cl^- currents mediated by opening of the endogenous Ca^{2+}-activated Cl^- channels via the intracellular signal pathway of Gq protein, phospholipase C, inositol-1,4,5-triphosphate, and Ca^{2+} mobilization (Snutch, 1988). Stimulation of muscarinic M_2 receptors coexpressed with Kir3.1 channels in oocytes produces a current mediated by the opening of Kir3.1 channel via βγ subunits of $G_{i/o}$ proteins (Reuveny et al., 1994). These currents were blocked by selective antagonists, respectively. Therefore, the effect of Z-338 on each muscarinc receptor expressed in the oocytes could be analyzed.

Our data showed that Z-338 did not have an agonistic profile but had an antagonistic profile at specific muscarinic receptors expressed in oocytes. Z-338 inhibited the acetylcholine-induced response mediated by stimulation of muscarinic M_1 receptor with an IC_{50} of 1.8 μM although the IC_{50} value of Z-338 was larger than that of pirenzepine, a selective muscarinic M_1 receptor antagonist. Z-338 also inhibited the

acetylcholine-induced response mediated by stimulation of muscarinic M_2 receptor with an IC_{50} of 10.1 μM although the IC_{50} value of Z-338 was larger than that of AF-DX116, a selective muscarinic M_2 receptor antagonist. Z-338 (1 to 100 μM) did not exert a concentration-dependent inhibition of the acetylcholine-induced response mediated by muscarinic M_3 receptor. Our present results are in good agreement with the results of receptor-binding assays using cell membrane preparations (Ogishima et al., 2000). The latter authors showed that Z-338 displaced the specific binding of [^{3}H]pirenzepine to rat cortex membrane (muscarinic M_1 receptor dominant tissues) and [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS) to rat heart muscle (muscarinic M_2 receptor dominant tissues) but did not displace the specific binding of [^{3}H]NMS to rat submaxillary gland (muscarinic M_3 receptor dominant tissues). Therefore, Z-338 may be a useful tool to distinguish muscarinic M_1 and M_2 receptor-mediated responses from muscarinic M_3 receptor-mediated responses when compared with available muscarinic receptors antagonists (Dörje et al., 1990).

Neuronal muscarinic M_1 and/or M_2 receptors appear to be located on cholinergic nerve terminals and to act as autoreceptors which operate as a negative feedback system (Schwörer, 1988; Kilbinger et al., 1993; Ren and Harty, 1994; Dietrich and Kilbinger, 1995; Kawashima et al., 1988). It is accepted that muscarinic M_3 receptor is involved in the contraction of gastrointestinal smooth muscles. Contraction of gastrointestinal smooth muscles is mediated by the release of acetylcholine from cholinergic nerve terminals onto muscarinic M_3 receptors. Therefore, the unique antagonistic profile of Z-338, being a potent antagonist not for muscarinc M_3 receptor but for muscarinic M_1 and M_2 receptors, explains the mechanisms underlying its prokinetic action. The concept is supported by previous data, in which Z-338 facilitated gastric motility due to acceleration of acetylcholine release from cholinergic nerve terminals (Ogishima et al., 2000). Thus, Z-338 may be available for clinical use as a potent gastroprokinetic agent possessing a new mechanism of action.

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