Acotiamide Hydrochloride (Z-338), a New Selective Acetylcholinesterase Inhibitor, Enhances Gastric Motility without Prolonging QT Interval in Dogs: Comparison with Cisapride, Itopride, and Mosapride

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ABSTRACT

Acotiamide hydrochloride (acotiamide; N-[2-[bis(1-methylethyl)amino]ethyl]-2-[(2-hydroxy-4,5-dimethoxybenzoyl)amino]thiazole-4-carboxamide monohydrochloride trihydrate, Z-338) has been reported to improve meal-related symptoms of functional dyspepsia in clinical studies. Here, we examined the gastroprokinetic effects of acotiamide and its antiacetylcholinesterase activity as a possible mechanism of action in conscious dogs. Acotiamide increased postprandial gastric motor activity in conscious dogs with chronically implanted force transducers and, like itopride, mosapride, and cisapride, exhibited gastroprokinetic activity in these dogs. Furthermore, acotiamide improved clonidine-induced hypomotility and delayed gastric emptying. Acotiamide-enhanced postprandial gastroduodenal motility was suppressed completely by pretreatment with atropine, a muscarinic receptor antagonist. In vitro studies, acotiamide enhanced acetylcholine- but not carbachol-induced contractile responses of guinea pig gastric antrum strips. Moreover, like itopride and neostigmine, acotiamide inhibited recombinant human and canine stomach-derived acetylcholinesterase (AChE) activity in vitro. The mode of the AChE inhibitory action of acotiamide was selective and reversible. Unlike itopride or mosapride, acotiamide showed no affinity for dopamine D2 or serotonin 5-HT4 receptors. With regard to cardiovascular side effects, unlike cisapride, acotiamide did not affect myocardial monophasic action potential duration, QT interval, or corrected QT interval in anesthetized dogs. These results suggest that acotiamide stimulates gastric motility in vivo by inhibiting AChE activity without affecting QT interval. Acotiamide thus represents a beneficial new drug for the treatment of functional dyspepsia involving gastric motility dysfunction, with differences from other prokinetic agents.

Introduction

Functional dyspepsia (FD), a common clinical syndrome in gastroenterology practice that has been listed in the Rome III classification (Tack et al., 2006), is defined as chronic or recurrent epigastric pain or burning (epigastric pain syndrome), postprandial fullness, or early satiation (postprandial distress syndrome). Although standard diagnostic tests in patients with FD fail to identify an underlying organic abnormality (Talley et al., 1999), delayed gastric emptying has been described in approximately 20 to 40% of patients (Talley et al., 2001; Sarnelli et al., 2003; Lorena et al., 2004; Pallotta et al., 2005).

ABBRVIATIONS: FD, functional dyspepsia; 5-HT, 5-hydroxytryptamine, serotonin; ACh, acetylcholine; AChE, acetylcholinesterase; MAP, monophasic action potential; QTc, corrected QT interval; AUC, area under the concentration-time curve; CCh, carbachol; BuChe, butyrylcholinesterase; CHO, Chinese hamster ovary; GR 113808, 1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl|methyl 1-methyl-1H-indole-3-carboxylate; RX821002, 2-[[methylsulfonyl]amino]ethyl]-4-piperidinyl|methyl 1-methyl-1H-indole-3-carboxylate.
Patients with FD have also shown postprandial gastric antral hypomotility (Kusunoki et al., 2000).

Gastrointestinal motor activity is mainly regulated by neural and hormonal systems (Ormsbee et al., 1979; Rogers et al., 1996). The cholinergic neuron is considered to be the major excitatory neuron involved in gastrointestinal motor activity, because most gastrointestinal contractions are strongly inhibited by atropine, a muscarinic receptor antagonist (Ormsbee and Mir, 1978; Shiba et al., 1995; Furuchi et al., 2001). Dopamine D2 receptor antagonists (Brogden et al., 1982; Iwanaga et al., 1990, 1996) and serotonin 5-HT4 receptor agonists (Mine et al., 1997) are well known for their use as gastroprokinetic agents, and their ability to enhance gastrointestinal motility. In a recent meta-analysis, Hiyama et al. (2007) reported that these gastroprokinetic agents are significantly more effective than placebo in the treatment of FD. However, concern has been expressed about the ability of dopamine D2 receptor agonists to also induce extrapyramidal syndromes and increase plasma prolactin levels (Alibibi and McCallum 1983; Tonini et al., 2004). Furthermore, the clinical use of cisapride, a widely used gastroprokinetic drug that mainly activates serotonin 5-HT4 receptors (Talley, 1992), has now been restricted owing to its prolongation of QT intervals (Bran et al., 1995). At present, there is no drug approved for the indication of FD. Thus, a new gastroprokinetic drug that does not affect dopamine D2 receptors and QT interval would be beneficial for the treatment of FD involving dysfunction of gastric motility.

ACh is an important regulator of gastrointestinal motility, and the inhibition of AChE activity has been reported to enhance gastric motility (Iwanaga et al., 1990; Ueki et al., 1993). Acotiamide hydrochloride (acotiamide; N-[2-[bis[1-methylethyl]amino]ethyl]-2-[(2-hydroxy-4,5-dimethoxybenzoyl) amino] thiazole-4-carboxamide monohydrochloride trihydrate, Z-338), is a newly synthesized compound that is expected to enhance gastric motility. Acotiamide has been reported to facilitate acetylcholine release from enteric neurons in the stomach of guinea pigs and to enhance electrically stimulated contraction of strips of guinea pig gastric fundus in vitro (Nakajima et al., 2000; Ogishima et al., 2000). Seto et al. (2008) reported that acotiamide improved stress-induced delayed gastric emptying in rats, suggesting the gastroprokinetic activities of acotiamide in vivo. In clinical studies, acotiamide has been reported to improve meal-related symptoms of FD and quality of life in patients with FD (Tack et al., 2009; Matsueda et al., 2010).

The primary aim of this study was to examine the gastroprokinetic effects of acotiamide in conscious dogs. We also examined its antiacetylcholinesterase activity in vitro as a possible mechanism of action of this drug. Finally, to assess the potential risk of drug-induced QT interval prolongation, we also examined the effect of acotiamide on myocardial monophasic action potential (MAP) duration, QT interval, and corrected QT interval (QTc).

Materials and Methods

Animals. This study complied with the “Principle of Ethics in Animal Studies” of Zeria Pharmaceutical Co., Ltd. (Saitama, Japan). Male mongrel dogs weighing 9 to 11 kg and male beagle dogs weighing 9.6 to 12.9 kg were purchased from Toyota Tsusho Corporation (Tokyo, Japan) and Nusan Corporation (Yokohama, Japan), respectively. The total number of dogs used in this study was 60. Male Hartley guinea pigs weighing 250 to 300 g were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were acclimated for more than 1 week before entry into the study. They were housed under standard controlled environmental conditions at 23 ± 3°C and 55 ± 20% humidity, with a 12-h light/dark cycle. Water was available ad libitum. The dogs were individually housed in cages and given dog food (DS; Oriental Yeast Co., Ltd., Tokyo, Japan), and the guinea pigs were given free access to chow pellets (LCR4; Oriental Yeast Co., Ltd.).

Drugs. Acotiamide (Fig. 1) was obtained from Zeria Pharmaceutical Co., Ltd. (Saitama, Japan). Itopride, mosapride, and cisapride were extracted from Ganaton tablets (Abbott Japan Co., Ltd., Fukushima, Japan), Gasmotin powder (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), and Risamol granules (Mitsubishi Tanabe Pharma Corp., Osaka, Japan), respectively. Part of the itopride and mosapride was synthesized at Zeria Pharmaceutical Co., Ltd. Chemical purities were confirmed to be more than 99%. For intravenous studies, acotiamide and itopride were dissolved in isotonic glucose solution, cisapride was developed in a few drops of acetic acid followed by dilution with isotonic glucose solution, and mosapride was developed in a few drops of lactic acid followed by dilution with isotonic glucose solution. For oral administration, the drugs were prepared in gelatin capsules. The control group received vehicle or empty capsules. Neostigmine bromide and physostigmine hemisulfate salt (eserine; Sigma-Aldrich, St. Louis, MO) were dissolved in purified water. Acetycholine chloride (Oxfor for injection; Daiichi Sankyo Co., Ltd., Tokyo, Japan) was dissolved in distilled water. Atropine sulfate (atropine sulfate injection 0.5 mg; Mitsubishi Tanabe Pharmaceutical Co., Ltd.) was dissolved in physiological saline.

Gastric Motor Activity during the Digestive State in Dogs. The contractile force of the circular muscle in dogs was measured using the method of Itoh et al. (1977). In Brief, strain gauge force transducers (F-121S; Star Medical Inc., Tokyo, Japan) were chronically implanted onto the upper gastrointestinal tract under pentobarbital anesthesia (30 mg/kg i.v., Nembutal injection; Dainippon Sumitomo Pharma Co., Ltd.) by suturing onto the gastric antrum (3 cm proximal to the pylorus) and duodenum (faceing the main pancreatic duct). A catheter (2.0 mm o.d. × 1.0 mm i.d. Silason Medical Grade Tubing; Kaneka Medic, Osaka, Japan) was placed in the vena cava superior through a branch vein of the right external jugular vein for the intravenous administration of drugs (Shiba et al., 1995). The lead wires of the transducers and the catheter were protected with a protective jacket. The experiments were performed approximately 2 weeks after surgery. For 3 days after surgery, the dogs were maintained with an intravenous infusion of a maintenance solution including antibiotic agent (500 ml/day Solita-T3G; Ajinomoto Pharmaceuticals Co., Ltd., Tokyo, Japan; and 0.5 g/day of kanamycin sulfate injection; Meiji Seika Kaisha Ltd., Tokyo, Japan). There were no signs indicating serious distress or wound infection. The contractile activity of the gastroduodenum in the conscious state was measured with a multitelemetry system (WEB-5000; Nikon Kohden Corp., Tokyo, Japan) and recorded with a pen-writing recorder (RTA-1200; Nikon Kohden Corp.). The gastric motility was recorded on a computer (PC-9801RX; NEC, Inc., Tokyo, Japan) after digitization with the data collection system (MCHDDWHD, version 51, and DSPDWD, version 1.1.9; Nikon Kohden Corp.) and an interface unit (ADX-98E; Thomson Canopus Co., Ltd., Kobe, Japan).
using an amplifier system, the force transducer was calibrated by standardizing a maximum contractile force of the phase III contractions in the canine stomach (Itoh and Sekiguchi, 1983). The gastric motility index was evaluated using a motor analyzing system (DSS-DDWHD, version 30, and DSSFP, version 21; Nihon Kohden Corp.) to measure the area under the concentration curve in the gastric antrum, expressed as a contractile power consisting of the contractile amplitude and the contracting time.

For intravenous administration, experiments were performed during the digestive state at 2 to 3 h after feeding (DS). Drugs were administered intravenously through the catheter at 30 min after the stabilization of contractile activity in the gastric antrum. The criterion for stabilization of contractile activity was to maintain a constant contraction, not gradually getting weaker or stronger. The motility index for 10 min after the administration of test drugs was expressed as a percentage of that for 10 min before drug administration.

For oral administration, measurement of gastroduodenal motility was started more than 30 min before feeding with chow at 23.5 g/kg (Pedigree; Master Foods Ltd., Hacketstown, NJ). Drugs were administered orally 30 min before feeding with 10 ml of physiological saline, and the motility index was calculated as gastric motility for 120 min after feeding.

The washout period was at least 2 days during the experiments with intravenous administration and at least 4 days during the experiment with oral administration.

**Effect of Atropine on Acotiamide-Induced Motor Activity in Dogs.** In dogs in the digestive state, intravenous infusion of atropine at 0.05 mg/kg/h for 20 min was started immediately after a bolus injection at 0.05 mg/kg. This dose of atropine was confirmed to completely inhibit bethanecol-stimulated contraction in dogs (Shiba et al., 1995). Acotiamide at 1 mg/kg was then administered intravenously at 10 min after the start of atropine infusion.

**Clonidine-Induced Hypomotility in Dogs.** Gastric dysfunction in dogs was induced using clonidine, an α2-adrenergic receptor agonist, as described previously (Tanaka et al., 1998) with a minor modification. Clonidine was dissolved in 10% gelatin solution to prolong its effect and given subcutaneously at 10 μg/kg at 3 h after feeding to suppress gastrointestinal motility. In the preliminary study, clonidine at a 10 and 20 μg/kg dose dependently decreased contractile activity in the gastroduodenum during the digestive state in dogs. When 20 μg/kg clonidine was given, the gastric motor activity was absolutely suppressed for more than 120 min. In addition, the high dose of clonidine had induced emesis or vomiting. From these results, we chose the dose of clonidine to be 10 μg/kg in this study. Each test drug was administered intravenously at 30 min after the administration of clonidine. Contractile activity of the gastric antrum was determined for 45 min from 30 min before the administration of clonidine. The motility index for 15 min after the administration of test drugs was expressed as a percentage of the mean motility index for 15 min before the administration of clonidine.

**Clonidine-Induced Delayed Gastric Emptying in Dogs.** Delayed gastric emptying in dogs was induced using clonidine as described above. A nutritious liquid meal (Besvion, 20% protein, 15% fat, 59% carbohydrate, and trace amounts of vitamins and minerals; Astellas Pharma Inc., Tokyo, Japan) containing acetaminophen (30 mg/kg) was given orally to fasted dogs. Clonidine (10 μg/kg) was given subcutaneously 15 min before the test meal (100 kcal/120 ml/dog), and each test drug was administered intravenously at 5 min before the meal. Serial blood samples were collected at 15-min intervals with sodium citrate after the test meal and centrifuged at 10,000 rpm at 4°C for 10 min to separate plasma. The plasma acetaminophen concentration was determined by a modification of the method of Ameer et al. (1981) by a high-performance liquid chromatography (Irika Industry, Tokyo, Japan) with a reverse-phase column (Unisil Pac 5C18-150A; GL Sciences Inc., Tokyo, Japan). Serial determination of the area under the concentration-time curve up to 45 min (AUC0–45) was obtained.

Acetylcholine/Carbachol-Induced Contraction of Guinea Pig Antrum Strips In Vitro. Guinea pigs were stunned with a small hammer and then killed by bleeding after cutting of the carotid artery. The whole stomach was isolated and placed in Krebs-Henseleit solution (118.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25.0 mM NaHCO3, and 10.0 mM glucose d(+)-glucose). The gastric antrum was cut into circular muscle strips of 5-mm length and 2-mm width, and the mucosa was immediately removed. These preparations were suspended with a resting tension of 0.5 g in an organ bath containing 10 ml of Krebs-Henseleit solution and continuously aerated with a 95% O2/5% CO2 gas mixture at 37°C. Contractile responses were recorded on a thermal stylus recorder (Recti-Horiz-5K; NEC San-ei Instruments, Tokyo, Japan) from an isometric transducer (TB-651T; Nihon Kohden Corp.) via an amplifier (EF-601G or AP-621G; Nihon Kohden Corp.).

The gastric antrum strips were equilibrated for at least 1 h, and then ACh (10−5 M) was added at 20-min intervals. During each 20-min interval, washout was performed three times. This procedure was repeated until stable contractile responses were obtained. ACh (10−5–10−6 M) or CCh (10−5–10−6 M) was cumulatively added, and concentration-response curves to them in the presence or absence of the respective test drug were obtained. The drugs were added 5 min before cumulative addition of ACh or CCh was started. The ACh- and CCh-induced contractile responses were expressed as a percentage of the maximum responses on the concentration-response curves without each test drug.

**Inhibition of Human Cholinesterase Activity.** Enzymatic activity was measured by the method of Ellman et al. (1961) with a minor modification. Recombinant human AChE (Sigma-Aldrich) was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 mg/ml bovine serum albumin (Sigma-Aldrich). The final volume of the reaction medium was set at 1 ml. Fifty microliters of test compound solution (acotiamide, itopride, or neostigmine) or vehicle and 30 μl of 10 mM 5,5′-dithiobis[2-nitrobenzoic acid] were added to 0.1 M sodium phosphate buffer (pH 8.0). The time-dependent increase in absorbance at 412 nm was measured using a spectrophotometer (model U-3310; Hitachi, Ltd., Tokyo, Japan) at 30°C. The change in absorbance per minute (A) was calculated from the profile of change from 35 to 95 s after the addition of acetylthiocholine iodide solution. The change in absorbance measured using 0.1 M sodium phosphate buffer (pH 8.0) in the absence of AChE and test compound solution was regarded as a blank (B), and (A − B) was regarded as AChE activity.

The kinetic parameters for AChE activity were estimated according to eq. 1:

\[ \frac{V_{\text{max}}}{K_m + S} \]

where \( v \) and \( V_{\text{max}} \) are AChE activity and maximum reaction velocity (ΔOD1145/min), respectively, \( S \) is acetylthiocholine iodide concentration in the medium (micromolar), and \( K_m \) is the Michaelis constant (micromolar concentration). Equation 1 was fit to the AChE activity data sets by the nonlinear least-squares method using WinNonLin Professional (version 5.2; Pharsight, Mountain View, CA).

The inhibition constants (\( K_{i1} \) and \( K_{i2} \)) were calculated according to equation 2 from the data obtained by varying the inhibitor concentration (\( I \)) (acotiamide at 0.5, 1, 2.5 and 5 μM; itopride at 0.5, 1, 2 and 3 μM; and neostigmine at 0.05, 0.1, 0.2 and 0.4 μM) in the incubation medium.

\[ \frac{V_{\text{max}}}{K_m + S} = \frac{V_{\text{max}} \cdot I / K_{i1} + 1}{S / K_{i2} + 1} \]

\[ v = \frac{V_{\text{max}} \cdot S / (I / K_{i2} + 1)}{K_m + S} \]

where \( v \) and \( V_{\text{max}} \) are AChE activity and maximum reaction velocity (ΔOD1145/min), respectively, \( S \) is acetylthiocholine iodide concentration in the medium (micromolar), and \( K_m \) is the Michaelis constant (micromolar concentration). Equation 1 was fit to the AChE activity data sets by the nonlinear least-squares method using WinNonLin Professional (version 5.2; Pharsight, Mountain View, CA).

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If $K_i$ was similar to $K_{i2}$, the manner of inhibition was considered noncompetitive, and $K_i$ was estimated with eq. 3.

To elucidate whether the inhibitory effects of acotiamide on AChE activity were reversible, the AChE inhibition effect was estimated by dialyzing the reaction mixture. One of the samples was dialyzed (Slide-A-Lyzer 3.5K; Thermo Fisher Scientific, Waltham, MA) at 6.1 to 7.1°C for 24 h using 500 ml of 0.1 M sodium phosphate buffer (pH 8) as an external dialysate, which was replaced once, and the second was stored at 6.1 to 7.1°C for 24 h without dialysis (nondialyzed solution). Enzymatic activities in the dialyzed and nondialyzed solutions were then measured. The activity inhibition rate was calculated using dialyzed or nondialyzed blank and control solutions.

Selectivity for the AChE inhibition of acotiamide was estimated by comparing the inhibitory effect of acotiamide for AChE and butyrylcholinesterase (BuChE) activity. Human BuChE from Globulins Cohn fraction IV-4 (Sigma-Aldrich) was dissolved in 0.1 M sodium phosphate buffer (pH 7). AChE activity was measured as described above, and BuChE activity was measured using butyrylthiocholine iodide solution as substrate in the same manner as that for AChE activity. For all relationships identified in these tests, the logistic curve was fit to the inhibition study data sets by the nonlinear least-squares method using SAS (version 8.2; SAS Institute Inc., Cary, NC) to obtain estimates of the IC$_{50}$. The inhibition ratio was calculated according to the following equation:

$$\text{Inhibition Ratio} = \frac{\text{IC}_{50} \text{ value for BuChE activity}}{\text{IC}_{50} \text{ value for AChE activity}}$$

(4)

**Inhibition of Canine Stomach-Derived AChE Activity In Vitro.** Frozen tissue from canine stomach was obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA). The full-thickness segment of gastric antrum was frozen and crushed in a cool mill. Then 0.1 M sodium phosphate buffer (pH 6.9) containing 10 mM EDTA was added to the crushed stomach, which was then homogenized. The precipitate of this homogenate obtained by centrifugation at 100,000g and 4°C for 60 min was washed with EDTA-supplemented sodium phosphate buffer (pH 6.9). After treatment with the same buffer containing 1% Triton X-100, the supernatant obtained by centrifugation under the same conditions was used as canine stomach-derived AChE. To measure AChE activity, 10 µl of 1 M N-ethylmaleimide, 50 µl of stomach-derived AChE containing 2 mM tetraisopropyl pyrophosphoramide (butyrylcholinesterase inhibitor), test compound solution, and 30 µl of 10 mM 5,5-dithiobis-[2-nitrobenzoic acid] were added to EDTA-supplemented sodium phosphate buffer (pH 7.6). AChE activity and the IC$_{50}$ value of test compound were measured by the same method as in the inhibition study using recombinant human AChE.

**Receptor Binding Assays.** Binding affinities for human dopamine D$_2$ receptor, serotonin 5-HT$_2$ receptor, and $\alpha_2$-adrenoceptor were determined through commercial radioligand binding assays by Cerep (Celle l’Evescaut, France) according to their standard assay protocols. These radioligand binding assays of each drug were examined using the following recombinant receptors, radiolabeled ligands, incubation condition, nonspecific compound, target tissue, and cell lines: D$_2$ receptor, 0.3 nM [H]$^3$H]apenerone, 60 min/22°C, 100 µM (+)-butaclamol, human recombinant (CHO cell); 5-HT$_2$ receptor, 0.1 nM [H]$^3$H][1-[(methylsulfonyl)-amino]ethyl]-4-piperidinyl)methyl 1-methyl-1H-indole-3-carboxylate (GR 113808), 30 min/22°C, guinea pig striatum; 5-HT$_4$ receptor, 0.2 nM [H]$^3$H][GR 113808, 30 min/37°C, human recombinant (CHO cell); 5-HT$_3$ receptor, 0.1 nM [H]$^3$H][GR 113808, 30 min/37°C, human recombinant (CHO cell); 5-HT$_3$ receptor, 0.3 nM [H]$^3$H][GR 113808, 30 min/37°C, human recombinant (CHO cell); and $\alpha_2$-adrenoceptor, 0.5 nM [H]$^3$H][2-(2-methoxy-1,4-benzo- dioxan-2-yl)-2-imidazoline (RX821002), 30 min/22°C, 100 µM (–)-epinephrine, rat cerebral cortex, respectively. Acotiamide (0.3–100 µM), itopride (0.01–10 µM), and mosapride (0.01–100 µM) were tested in the D$_2$ receptor assay. Acotiamide (100 µM), itopride (100 µM), and mosapride (0.01–10 µM) were tested in each 5-HT$_4$ receptor assay. These tests were performed in duplicate, and each assay was repeated 3 times. Acotiamide (1, 10, and 100 µM) was tested in the $\alpha_2$-adrenoceptor assay in duplicate. After the incubation, the membranes or cells were filtered rapidly under vacuum through glass fiber filters, dried, and then counted for radioactivity in a scintillation counter using a scintillation cocktail. The specific ligand binding to the receptors is defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess of unlabeled ligand. The results are expressed as a percentage of control specific binding (measured specific binding/control specific binding) × 100) and as percent inhibition of control specific binding (100 – (measured specific binding/control specific binding) × 100) obtained in the presence of drugs. The IC$_{50}$ values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients were determined by nonlinear regression analysis of the competition curves generated with mean replicate values using Hill equation curve-fitting. The inhibition constants ($K_i$) were calculated using the Cheng-Prusoff (Cheng and Prusoff, 1973) equation, $K_i = IC_{50}/[1 + (L/K_{i2})]$, where $L$ is the concentration of radioligand in the assay, and $K_i$ is the affinity of the radioligand for the receptor.

**Myocardial MAP Duration, QT Interval, and QTc in Dogs**

MAP duration, QT interval, and QTc were examined. After induction of anesthesia with thiamylal sodium (Isovol; Wellfide Korea Co., Ltd., Hwasung City, Korea), dogs were constrained in the supine position under anesthesia with halothane inhalation (Fluothane; Takeda Pharmaceutical Co. Ltd., Osaka, Japan). A MAP pacing catheter (EP Technologies, Boston Scientific Corp., Natick, MA) was inserted into the femoral vein and introduced into the right ventricle with monitoring of the intracardiac ECG. The electrode attached to the tip was pushed against the surface of the ventricular wall and immobilized at the site where waveforms could be most clearly observed. Waveforms were recorded using a heat-writing recorder (WT-648G; Nikon Kohden Corp.) via a dedicated preamplifier for MAP (EP Technologies, Boston Scientific Corp.) and an ECG amplifier (AC-601G; Nikon Kohden Corp.).

At the same time, the body surface ECG was determined using an ECG amplifier, and aortic blood pressure was measured using a transducer (Spectramed P2X2C; Grass Instruments, Quincy, MA) and amplifier for determination of blood pressure (AP-641G; Nikon Kohden Corp.) via a catheter inserted into the femoral artery.

MAP was determined before and 1, 3, 5, 10, and 15 min after administration. For cisapride, MAP duration was also determined at 5-min intervals because changes lasted for more than 15 min after administration. MAP duration was recorded for approximately 6 s at a chart speed of 25 mm/s at each time point.

At each time point, three standard waveforms were selected, and the action potential duration at 90% of repolarization was determined. Systolic and diastolic blood pressures were determined at one site at each time point, and mean blood pressure was calculated. Heart rate per minute was calculated by multiplying the heart rate determined for 6 s from the ECG by 10. QT intervals were analyzed by selecting three standard waves, and QTc was calculated by correcting for heart rate. When it was impossible to select three standard waves because of noise, QT intervals were analyzed using measurable waves only. The data for MAP duration, QT interval, and QTc were determined as a percentage of the pretreatment baseline.

**Statistics.** All data are expressed as the mean ± S.E. Gastric motility was analyzed by a paired $t$ test or one-way analysis of variance followed by the parametric Dunnett’s multiple comparison test (one-tailed). Clonidine-induced hypomotility was analyzed by one-way analysis of variance followed by the parametric Dunnett’s multiple comparison test (two-tailed). Clonidine-induced delayed gastric emptying was tested by Student’s $t$ test for comparison between the normal and control groups and analyzed by one-way analysis of variance followed by the parametric Dunnett’s multiple
comparison test (two-tailed) for comparison between the experimental and control groups. ACh-induced contractions were analyzed by one-way analysis of variance followed by the parametric Dunnett’s multiple comparison test (two-tailed). Homogeneity of variance was confirmed by Bartlett’s and Levene’s tests. MAP duration, QT interval, and QTc were tested by Bartlett’s test for homogeneity of variance. When the variance was homogenous, the data were analyzed by the parametric Dunnett’s multiple comparison test (two-tailed) and by the nonparametric Dunnett’s multiple comparison test (two-tailed) when the data were not homogeneous. \( p < 0.05 \) was considered statistically significant.

**Results**

**Gastric Motor Activity during the Digestive State in Dogs.** Intravenous administration of acotiamide at doses of 0.3, 1, and 3 mg/kg increased the postprandial gastric motility index in a dose-dependent manner (Fig. 2). Acotiamide at 1 or 3 mg/kg significantly enhanced postprandial gastric motor activity compared with that for the control. Cisapride (0.1, 0.3, and 1 mg/kg), itopride (3 mg/kg) and mosapride (1 and 3 mg/kg) also significantly enhanced gastric motor activity (Fig. 2).

When the test drug was administered orally, all of the control groups showed a constant motility index. Typical changes of antral motor activity after feeding in response to acotiamide are shown in Fig. 3A. Acotiamide at 3, 10, and 30 mg/kg dose dependently increased the motility index with significance at 10 and 30 mg/kg compared with the respective control (Fig. 3B). Cisapride (1 and 3 mg/kg p.o.), itopride (30 and 100 mg/kg p.o.), and mosapride (3 and 10 mg/kg p.o.) significantly increased the motility index compared with the control (paired \( t \) test).
treated (control) group was decreased for 45 min compared with that in the normal group. Acotiamide restored the clonidine-induced decrease in AUC_{0-45} with a significant difference at 1 mg/kg (Fig. 5A). Cisapride at 0.2 mg/kg i.v. also improved the clonidine-induced decrease in AUC_{0-45} (Fig. 5B).

**Effect of Atropine on Acotiamide-Induced Motor Activity in Dogs.** Intravenous administration of acotiamide at 1 mg/kg enhanced antral and duodenal contractions in the digestive state (Fig. 6A). As shown in Fig. 6B, atropine (0.05 mg/kg i.v., thereafter 0.05 mg/kg per h), a muscarinic receptor antagonist, completely suppressed gastroduodenal motor activity in the digestive state. Acotiamide failed to stimulate gastroduodenal motor activity under treatment with atropine.

**Acetylcholine/Carbachol-Induced Contractions of Guinea Pig Antrum Strips.** Acotiamide at 10^{-6} M significantly enhanced ACh-induced contraction compared with that in the vehicle-treated group at ACh concentrations from 3 \times 10^{-8} to 3 \times 10^{-5} M, shifting the ACh concentration-response curve to the upper left. Moreover, acotiamide at 3 \times 10^{-6} M significantly increased contractions at ACh concentrations from 3 \times 10^{-8} to 3 \times 10^{-5} M, shifting the ACh concentration-response curve to the upper left (Fig. 7A). The EC_{50} value of ACh in the presence of acotiamide (10^{-7}, 3 \times 10^{-7}, 10^{-6}, and 3 \times 10^{-6} M) were 5.8 (95% confidence interval: 4.9–6.8) \times 10^{-7}, 4.4 (95% confidence interval: 4.0–5.0) \times 10^{-7}, 2.0 (95% confidence interval: 1.7–2.2) \times 10^{-7}, and 6.6 (95% confidence interval: 5.6–7.8) \times 10^{-8} M, respectively. In contrast, acotiamide (10^{-6}, 3 \times 10^{-6}, and 10^{-5} M) had no effect on CCh-induced contractions (Fig. 7B). The EC_{50} value of CCh was 8.4 (95% confidence interval: 7.5–9.3) \times 10^{-8} M. The EC_{50} values of CCh in the presence of acotiamide (10^{-6}, 3 \times 10^{-6}, and 10^{-5} M) were 9.1 (95% confidence interval: 8.5–9.8) \times 10^{-8}, 9.2 (95% confidence interval: 8.0–10.6) \times 10^{-8}, and 8.6 (95% confidence interval: 7.2–10.4) \times 10^{-8} M, respectively.

**Inhibition of Human Cholinesterase Activity.** Acotiamide, itopride, and neostigmine inhibited recombinant human AChE activity in vitro (Table 1). Acotiamide and neostigmine showed a mixed-type inhibition, and itopride showed a noncompetitive-type inhibition (Fig. 8). K_i (competitive inhibition) and K_{s2} values (noncompetitive inhibition) of acotiamide were 6.1 \times 10^{-7} and 2.7 \times 10^{-6} M, respectively. IC_{50} values for AChE of acotiamide, itopride, mosapride, neostigmine, and physostigmine were 3.0 \times 10^{-6}, 1.2 \times 10^{-6}, and 1.0 \times 10^{-5} M, respectively.
Kinetic parameters for recombinant human AChE inhibition by acotiamide, itopride, and neostigmine

Each value represents the mean from six individual experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acotiamide</th>
<th>Itopride</th>
<th>Neostigmine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{i1}$ (M)</td>
<td>9.32 x 10^{-6}</td>
<td>9.05 x 10^{-6}</td>
<td>8.4 x 10^{-6}</td>
</tr>
<tr>
<td>$V_{\text{max}}$ ($\Delta OD_{412}$/min)</td>
<td>0.0342</td>
<td>0.0341</td>
<td>0.0339</td>
</tr>
<tr>
<td>$K_{i2}$ (M)</td>
<td>6.1 x 10^{-7}</td>
<td>1.3 x 10^{-6}</td>
<td>9.4 x 10^{-8}</td>
</tr>
<tr>
<td>$K_{i3}$ (M)</td>
<td>2.7 x 10^{-6}</td>
<td>9.0 x 10^{-7}</td>
<td>2.6 x 10^{-7}</td>
</tr>
<tr>
<td>Inhibition type</td>
<td>Mixed</td>
<td>Noncompetitive</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

N.C., not calculated.
and itopride at 100 mg/kg and higher than those of mosapride at 3 mg/kg, respectively. The motility index of acotiamide given orally at 30 mg/kg was almost equivalent to those of itopride and mosapride at 3 mg/kg, respectively. The toxicity of itopride at a dose of 100 mg/kg and metoclopramide, a dopamine D2 receptor antagonist, at a dose of 10 mg/kg was reported previously as reduced-activity, tremor, and abnormal behavior observed in dogs (Koizumi et al., 1992). This toxicity is considered to relate to the dopamine D2 receptor antagonistic action. Moreover, acotiamide, unlike cisapride, did not affect MAP duration, QT interval, or QTc in dogs. These results suggest that acotiamide may be a new orally active gastrokinetic agent with a lack of serious adverse reactions, unlike some other agents that have a risk of drug-induced QT interval prolongation.

It is well known that α2-adrenergic agents such as clonidine depress gastrointestinal motor function via the inhibition of ACh release from cholinergic nerve terminals and consequently delay gastric emptying and intestinal transit (Taylor and Mir, 1982; Tack and Wood, 1992; Colucci et al., 1998). Our present study indicated that, like cisapride, acotiamide improved clonidine-induced gastric hypomotility in dogs. Moreover, like cisapride, acotiamide improved delayed gastric emptying induced by clonidine in dogs. Our receptor binding studies suggest that these effects of acotiamide were not due to any direct effect on α2-adrenoceptors, because this drug has no affinity for α2-adrenoceptors.

We used the acetaminophen method with a nutritious liquid meal. Orally administered acetaminophen (paracetamol), which is soluble in weak basic lipid, is absorbed much more rapidly from the small intestine than from the stomach (Brodie, 1964), and its absorption is thus influenced by the rate of gastric emptying (Heading et al., 1973; Clements et al., 1978). Moreover, Dive et al. (1995) demonstrated that erythromycin accelerates gastric emptying, as assessed by the pharmacokinetics of acetaminophen absorption in humans. These findings appear to validate our use of the acetaminophen method in the gastric emptying study. The study using a dog model of clonidine-induced gastric dysfunction successfully demonstrated the enhancement of both antral motor activity and gastric emptying in response to acotiamide, supporting the positive findings observed in the clinical studies that acotiamide represents a gastrokinetic agent for clinical use.

It is generally accepted that gastrointestinal motor activity is mainly regulated by neural and hormonal systems (Ormsbee et al., 1979; Rogers et al., 1996). The cholinergic neuron is considered to be the major excitatory neuron involved in gastrointestinal motor activity, because most gastrointestinal contractions are strongly inhibited by atropine, a muscarinic receptor antagonist (Ormsbee and Mir, 1978; Shibata et al., 1995; Furuichi et al., 2001). Stimulation of peripheral dopamine D2 receptors located on the postganglionic cholinergic nerves causes a decrease in ACh release from parasympathetic nerves (Takahashi et al., 1991). In contrast, stimulation of serotonin 5-HT4 receptors at the pre- and postganglionic cholinergic nerves enhances ACh release in the gastrointestinal tract (Kilbinger and Wolf, 1992; Leclerc and Lefebvre, 2002). The released ACh is inactivated by AChE, and results in the regulation of gastric motility. Thus, an increase in ACh release and inhibition of AChE activity, either alone or in combination, are important in enhancing gastric motility.

TABLE 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acotiamide</td>
<td>1.2 × 10^{-6}</td>
</tr>
<tr>
<td>Itopride</td>
<td>1.2 × 10^{-6}</td>
</tr>
<tr>
<td>Mosapride</td>
<td>6 × 10^{-5}</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>3.6 × 10^{-7}</td>
</tr>
</tbody>
</table>

Almost equivalent to that of cisapride at 1 mg/kg and more than those of itopride and mosapride at 3 mg/kg, respectively. The motility index of acotiamide given orally at 30 mg/kg was almost equivalent to those of cisapride at 3 mg/kg and itopride at 100 mg/kg and higher than those of mosapride at 3 or 10 mg/kg. Acotiamide had no influence on the general behavior of the dogs at the tested doses, whereas itopride at 100 mg/kg caused twitching or collapse behaviors in three of six dogs. The toxicity of itopride at a dose of 100 mg/kg and metoclopramide, a dopamine D2 receptor antagonist, at a dose of 10 mg/kg was reported previously as reduced-activity, tremor, and abnormal behavior observed in dogs (Koizumi et al., 1992). This toxicity is considered to relate to the dopamine D2 receptor antagonistic action. Moreover, acotiamide, unlike cisapride, did not affect MAP duration, QT interval, or QTc in dogs. These results suggest that acotiamide may be a new orally active gastrokinetic agent with a lack of serious adverse reactions, unlike some other agents that have a risk of drug-induced QT interval prolongation.

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Fig. 8. Lineweaver-Burk plots with acetylthiocholine iodide as the substrate at various concentrations in the absence and presence of acotiamide (A), itopride (B), or neostigmine (C). S, concentration of acetylthiocholine iodide (millimolar); v, acetylcholinesterase activity (ΔOD412/min).
In this study, acotiamide clearly enhanced ACh-induced but not CCh-induced contraction of gastric antral strips, suggesting that acotiamide inhibits AChE activity in the gastric antrum because CCh is not hydrolyzed by AChE. Indeed, our enzyme assays using recombinant human and canine stomach-derived AChE revealed that acotiamide inhibits AChE activity in a selective and reversible manner. Moreover, acotiamide-enhanced gastric motor activity was suppressed completely by pretreatment with atropine, a muscarinic receptor antagonist. In addition, acotiamide had no affinity for dopamine D2 or serotonin 5-HT4 receptors.

Moreover, acotiamide-enhanced gastric motor activity was suppressed completely by pretreatment with atropine, a muscarinic receptor antagonist. In addition, acotiamide, unlike cisapride, showed no risk of gastric hypomotility. In general, BuChE is mainly localized in the plasma and liver as a detoxification enzyme (Çökurgâças, 2003). Although the physiological role or roles of BuChE in the gut are not precisely clear, selective inhibition of AChE activity might avoid the side effects arising from the inhibition of BuChE activity.

Acotiamide has been reported to facilitate acetylcholine release from enteric neurons by blocking muscarinic M1 and M3 receptors in guinea pig stomach (Ogishima et al., 2000). This increased ACh release may also be involved in the gastroprokinetic effects of higher doses of acotiamide in vivo. Given that the Kᵣ value of the competitive AChE inhibition activity of this drug (6.1 \times 10⁻⁷ M) was approximately 10 times lower than those for muscarinic M3 and M2 receptors at 8.4 \times 10⁻⁶ and 9.4 \times 10⁻⁶ M, respectively (Ogishima et al., 2000), it is likely that acotiamide stimulates gastric motility mainly by inhibiting AChE activation.

Delayed gastric emptying has been described in approximately 20 to 40% of patients with FD (Talley et al., 2001; Sarnelli et al., 2003; Lorena et al., 2004; Pallotta et al., 2005), and postprandial gastric antral hypomotility has been shown in patients with FD (Kusunoki et al., 2000). Gastroprokinetic agents are probably useful for the treatment of FD, and symptoms do subside with an improvement in gastric motility dysfunction (Talley, 1995; Allescher et al., 2001; Mizuta et al., 2006). However, at present, there is no drug approved for the indication of FD, and the development of a novel drug for the treatment of FD is being demanded. In clinical trials, acotiamide has been reported to improve meal-related symptoms of FD and quality of life in patients with FD (Tack et al., 2009; Matsueda et al., 2010). The clinical effects of acotiamide in patients with FD may be due to its improvement of gastric hypomotility.

In conclusion, we have demonstrated that acotiamide enhances gastric motility and improves both clonidine-induced hypomotility during the postprandial state and delay in gastric emptying in dogs. This gastroprokinetic activity may be due to the inhibition of AChE activation in the stomach. In addition, acotiamide, unlike cisapride, showed no risk of drug-induced QT interval prolongation. Thus, acotiamide may represent a new gastroprokinetic agent for the treatment of FD involving a dysfunction in gastric motility.

Acknowledgments

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Authorship Contributions

**Participated in research design**: Matsunaga, Tanaka, Ueki, Hori, Kawabata, Yoshida, Matsumura, Takei, and Itoh.

**Conducted experiments**: Matsunaga, Tanaka, Ueki, Hori, Eta, Kawabata, Yoshii, Yoshida, and Matsumura.

**Performed data analysis**: Matsunaga, Tanaka, Hori, Eta, Kawabata, Yoshii, Yoshida, and Matsumura.

**Wrote or contributed to the writing of the manuscript**: Matsunaga, Tanaka, Yoshinaga, Ueki, Kawabata, Furuta, Takei, Taek, and Itoh.
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