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 has been reported to improve meal-related symptoms of functional dyspepsia in clinical studies. Here, we examined the gastroprokinetic effects of acotiamide and its anti-acetylcholinesterase 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. Further, 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 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 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 (MAP) duration, QT interval, or corrected QT interval (QTc) 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 which has been listed in the Rome III classification (Tack et al., 2006), is defined as chronic or recurrent epigastric pain or burning (EPS: epigastric pain syndrome), postprandial fullness, or early satiation (PDS: 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 20-40% of patients (Talley et al., 2001; Sarnelli et al., 2003; Lorena et al., 2004; Pallotta et al, 2005). Patients with FD have also shown postprandial gastric antral hypomotility (Kusunoki et al., 2000).

Gastrointestinal motor activity is mainly regulated by neural and hormonal system (Ormsbee et al, 1979; Rogers et al, 1996;). The major excitatory neuron involving in the gastrointestinal motor activity is considered to be the cholinergic neuron, because most of the gastrointestinal contractions are strongly inhibited by atropine, a muscarinic receptor antagonist (Ormsbee and Mir, 1978; Shiba et al, 1995; Furuichi 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 gastropokinetic agents and their ability to enhance gastrointestinal motility. In a recent meta-analysis, Hiyama et al., (2007) reported that these gastropokinetics are significantly more effective than placebo in the treatment of FD. However, concern has been expressed at the ability of dopamine D₂ receptor antagonists to also induce extrapyramidal syndrome and increase plasma prolactin levels (Albibi and McCallum 1983; Tonini et al., M 2004). Further, the clinical use of cisapride, a widely used
A gastroprokinetic drug that mainly activates serotonin 5-HT₄ receptors (Talley, 1992), has now been restricted owing to its effect of prolonging 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 which 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 (Ogishima et al, 2000; Nakajima et al., 2000). Recently, Seto et al. (2008) reported that acotiamide improves 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 (Matsueda et al, 2010; Tack et al, 2009).

The primary aim of this study was to examine the gastroprokinetic effects of acotiamide in conscious dogs. We also examined its anti-acetylcholinesterase 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 9–11 kg and male beagles weighing 9.6–12.9 kg were purchased from Kasho Co., Ltd. (now Toyota Tsusho Corporation, Tokyo, Japan) and Nosan Corporation (Yokohama, Japan), respectively. The total number of dogs is 60 used in this study. Male Hartley guinea pigs weighing 250–300 g were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were acclimated for more than one 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-hr 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 pellet (LRC4, 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 (Hokuriku Seiyaku Co., Ltd.; now Abbott Japan Co., Ltd., Fukui, Japan), Gasmotin® powder (Dainippon Pharmaceutical Co., Ltd.; now Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), and Risamol® granules (Yoshitomi Pharmaceutical; now Mitsubishi Tanabe Pharma Corp., Osaka, Japan), respectively. Part of the itopride and mosapride were 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 in a few drops of acetic acid followed by dilution with isotonic glucose solution; and mosapride 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 Inc., St Louis, MO) were dissolved in purified water. Acetylcholine chloride (Ovisot® for injection, Daiichi Pharmaceutical Co., Ltd.; now Daïichi Sankyo Co., Ltd., Tokyo, Japan) was dissolved in distilled water. Atropine sulfate (Atropine Sulfate Injection 0.5 mg, Tanabe Pharmaceutical Co., Ltd.; now Mitsubishi Tanabe Pharma Corp.) was dissolved in physiological saline.

**Gastric motor activity during the digestive state in dogs**

Contractile force of the circular muscle in dogs was measured using the method of Itoh et al. (1977). Briefly, strain gauge force transducers (F-12IS, Star Medical Inc., Tokyo, Japan) were chronically implanted onto the upper gastrointestinal tract under pentobarbital anesthesia (30 mg/kg, i.v., Nembutal Injection, Dainippon Pharmaceutical Co., Ltd.; now Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) by suturing onto the gastric antrum (3 cm proximal to the pylorus) and duodenum (facing the main pancreatic duct). A catheter (2.0 mm OD ×1.0 mm ID Silascon® Medical Grade Tubing, Kaneka Medics., Osaka, Japan) was chronically introduced into the right external jugular vein for the intravenous administration of drugs. The lead wires of the transducers and the chronic catheter were protected with a protective jacket. The experiments were performed about 2 weeks after surgery. For 3 days after surgery, the dogs were maintained with an intravenous infusion of a maintenance solution including antibiotic
agent (Solita-T3G; Shimizu Pharmaceutical Co., Shimizu, Japan, now Ajinomoto Pharmaceuticals Co., Ltd., Tokyo, Japan, 500 ml/day, and 0.5 g/day of kanamycin sulfate injection; Meiji Seika Kaisha Ltd., Tokyo, Japan). There were no signs indicating serious distress and wound infection.

The contractile activity of the gastroduodenum in the conscious state was measured with a multi-telemetry system (WEB-5000, Nihon Kohden Corp., Tokyo, Japan) and recorded with a pen-writing recorder (RTA-1200, Nihon Kohden Corp.). The gastric motility was recorded on a computer (PC-9801RX, NEC, Inc., Tokyo Japan) after digitization with the Data Collection system (MCHDDWHD, V.51 and DSPDDW, V.1.1.9, Nihon Kohden Corp.) and an interface unit (ADX-98E, Canopus Co.; now 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). Gastric motility index was evaluated using a motor analyzing system (DSSDDWHD, V.30 and DSSFFT, V.21, Nihon Kohden Corp.) to measure the area under the contraction curve in the gastric antrum, expressing as a contractile power consisting of the contractile amplitude and the contracting time.

For intravenous administration, experiments were performed during the digestive state at 2–3 hr after feeding (DS, Oriental Yeast Co. Ltd.). 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 at a constant contraction, but 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., Hackettstown, NJ). Drugs were administered orally 30 minutes before feeding with 10 ml of physiological saline, and the motility index was calculated as gastric motility for 120 min after feeding.

The wash-out period is at least two days during the experiments of intravenous administration, and at least 4 days during the experiment of 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/hr for 20 min was started immediately after a bolus injection at 0.05 mg/kg. This dose of atropine has been confirmed to completely inhibit bethanechol-stimulated contraction in dogs (Shiba et al., 1995). Acotiamide at 1 mg/kg was then administered intravenously at 10 min after the starting 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 hr after feeding to suppress gastrointestinal motility. In the preliminary study, clonidine at 10 and 20 µg/kg dose-dependently decreased contractile activity in the gastroduodenum during the digestive state in dogs. When 20 µg/kg of 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 have chosen the dose of clonidine at 10 µg/kg was determined 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®, Fujisawa Pharmaceutical Co., Ltd.; now Astellas Pharma Inc., Tokyo, Japan; 20% protein, 15% fat, 59% carbohydrate, trace amounts of vitamins and minerals) 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. Plasma acetaminophen concentration was determined by a modification of the method of Ameer et al. (1981) by high performance pressure chromatography (HPLC System, 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 due to 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-8K, 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 hr, and then ACh (1×10⁻⁵ M) was added at 20-min intervals. During each 20-min interval, the wash-out was performed 3 times. This procedure was repeated until stable contractile responses were obtained. ACh (1×10⁻⁸-1×10⁻⁴ M) or CCh (1×10⁻⁹-1×10⁻⁶ 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 starting the cumulative addition of ACh or CCh. 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 Inc.) was dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 mg/ml bovine serum albumin (BSA, Sigma-Aldrich Inc.). 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] (DTNB) 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 seconds after the addition of acetylthiocholine iodide (ATC)
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 the following
equation:

\[
\frac{v}{\Delta OD_{412}/\text{min}} = \frac{V_{\text{max}} \cdot S}{K_m + S}
\]

where \(v\) and \(V_{\text{max}}\) were AChE activity and maximum reaction velocity (\(\Delta OD_{412}/\text{min}\)),
respectively, \(S\) was ATC concentration in the medium (\(\mu\)M), and \(K_m\) was the Michaelis
constant (\(\mu\)M). The above equation was fit to the AChE activity data sets by the nonlinear
least squares method using the WinNonlin Professional application, ver. 5.2 (Pharsight
Corporation, Mountain View, CA).

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

\[
\frac{v}{\Delta OD_{412}/\text{min}} = \frac{V_{\text{max}} \cdot S}{K_m \cdot \left(\frac{1}{K_{i1}} + 1\right) + S \cdot \left(\frac{1}{K_{i2}} + 1\right)}
\]

\[
\frac{v}{\Delta OD_{412}/\text{min}} = \frac{V_{\text{max}} \cdot S}{K_m \cdot \left(\frac{1}{K_i} + 1\right)}
\]

If \(K_{i1}\) was similar to \(K_{i2}\), the manner of inhibition was considered noncompetitive and
\(K_i\) was estimated with Equation (3).
To elucidate whether the inhibitory effects of acotiamide on AChE activity were reversible, AChE inhibitory effect was estimated by dialyzing the reaction mixture. One of the samples was dialyzed (Slide-A-Lyzer\textsuperscript{®}3.5K, Thermo Fisher Scientific Inc.) at 6.1 to 7.1 °C for 24 hours 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 hours without dialysis (non-dialyzed solution). Enzymatic activities in the dialyzed and non-dialyzed solutions were then measured. The activity inhibition rate was calculated using dialyzed or non-dialyzed 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 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 System v.8.2 (SAS Institute Inc., Cary, NC) to obtain estimates of the 50% inhibitory concentration (IC\textsubscript{50}). The inhibition ratio was calculated according to the following equation:

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

**Inhibition of canine stomach-derived AChE activity in vitro**

A frozen tissue of 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. 0.1 M sodium phosphate buffer (pH 6.9) containing 10 mM ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt dihydrate (EDTA) was added.
to the crushed stomach, which was then homogenized. The precipitate of this homogenate obtained by centrifugation at 100,000×g 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 mM N-ethylmaleimide, 50 μL of stomach-derived AChE containing 2 mM tetraisopropyl pyrophosphoramide (iso-OMPA; butyrylcholinesterase inhibitor), test compound solution, and 30 μL of 10 mM DTNB were added to EDTA-supplemented sodium phosphate buffer (pH 7.6). AChE activity and the IC₅₀ 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 D₂S receptor, serotonin 5-HT₄ receptor, and α₂-adrenoceptor were determined through commercial radioligand binding assays by Cerep (Celle l’Evescault, France) according to their standard assay protocols. These radioligand binding assays of each drug were examined using the following target tissues, cell lines, recombinant receptors, incubation condition, non-specific compound and radio-labeled ligands: D₂S receptor, 0.3 nM [³H]spiperone, 60 min/22°C, 100 μM (+)butaclamol, human recombinant (CHO cell); 5-HT₄ receptor, 0.1 nM [³H]GR 113808, 30 min/22°C, guinea-pig striatum; 5-HT₄c receptor, 0.2 nM [³H]GR 113808, 30 min/37°C, human recombinant (CHO cell); 5-HT₄d receptor, 0.2 nM [³H]GR 113808, 30 min/37°C, human recombinant (CHO cell); 5-HT₄e receptor, 0.3 nM [³H]GR 113808, 30 min/37°C, human recombinant (CHO cell); and α₂-adrenoceptor, 0.5 nM [³H]RX821002, 30 min/22°C, 100 μM (-)epinephrine, rat cerebral cortex, respectively. Acotiamide (0.3-100
µM), itopride (0.1-30 µM) and mosapride (0.3-100 µM) were tested in D₂S receptor assay. Acotiamide (100 µM), itopride (100 µM) and mosapride (0.01-10 µM) were tested in each 5-HT₄ receptor assay. These testing were performed in duplicate and each assay repeated 3 times. Acotiamide (1, 10, and 10 µM) was tested in α₂-adrenoceptor assay in duplicate. Following 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 unlabelled ligand. The results are expressed as a percent of control specific binding ((measured specific binding/control specific binding) x 100) and as a percent inhibition of control specific binding (100-((measured specific binding/control specific binding) x 100)) obtained in the presence of drugs. The IC₅₀ values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting. The inhibition constants (Kᵢ) were calculated using the Cheng-Prusoff equation Kᵢ = IC₅₀/(1+(L/Kₐ)), where L is the concentration of radioligand in the assay, and Kₐ the affinity of the radioligand for the receptor.

Myocardial monophasic action potential (MAP) duration, QT interval, and corrected QT interval (QTc) in dogs

MAP duration, QT interval, and QTc were examined. After induction of anesthesia with thiamylal sodium (Isozol, Welfide 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, USA) was inserted into the femoral vein and introduced into the right ventricle with monitoring of 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, Nihon Kohden Corp.) via a dedicated pre-amplifier for MAP (EP Technologies™, Boston Scientific Corp.) and an ECG amplifier (AC-601G, Nihon Kohden Corp.).

At the same time, body surface ECG was determined using an ECG amplifier (AC-601G, Nihon Kohden Corp.), and aortic blood pressure (BP) was measured using a transducer (Spectramed P23XC, Grass Instrument Co., Quincy, MA, USA) and amplifier for determination of blood pressure (AP-641G, Nihon Kohden Corp.) via a catheter inserted into the femoral artery.

MAP was determined before and 1, 3, 5, 10, and 15 min after administration. In the case of cisapride, MAP duration was additionally determined at 5-min intervals because changes lasted for more than 15 min after administration. MAP duration was recorded for about 6 sec at a chart speed of 25 mm/sec 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 min was calculated by multiplying the heart rate determined for 6 sec 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...
Statistics

All data are expressed as the mean ± standard error (S.E.). Gastric motility was analyzed by the paired t-test or one-way analysis of variance followed by 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 the 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 non-parametric Dunnett’s multiple comparison test (two-tailed) when not homogenous. A P-value of 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 to 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 the control groups showed a constant motility index. Typical changes in response to acotiamide of antral motor activity after feeding 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 to 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 to the respective control. 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. Except for itopride, the gastroprokinetic agents tested in this study exhibited no observable side-effects. After administration of itopride at 100 mg/kg, twitching and collapse were observed in two and one of six dogs, respectively.

**Clonidine-induced hypomotility in dogs**

Administration of clonidine induced hypomotility in the gastric antrum, with a decrease in gastric motility index to 36.6% of the baseline value in the control group. Acotiamide at 0.2, 0.5 and 1 mg/kg increased the motility index in a dose-dependent manner (Fig. 4), with a significant effect at the 0.5 and 1 mg/kg doses which almost completely restored antral contractility to the normal value. Cisapride at 0.2 mg/kg and itopride at 5 mg/kg also significantly increased the motility index. In contrast, mosapride was ineffective against clonidine-induced hypomotility.
Clonidine-induced delayed gastric emptying in dogs

In clonidine-induced gastroparesis dogs, the plasma acetaminophen concentration of the clonidine-treated (control) group was decreased for 45 min compared to the normal group. In the serial analysis of AUC0-45, the control AUC0-45 was significantly decreased compared to the normal group. Acotiamide recovered the decrease in AUC0-45 by clonidine 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 AUC0-45 (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/hr), 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 1×10^{-6} M significantly enhanced ACh-induced contraction compared with the vehicle-treated group at ACh concentrations from 3×10^{-6} to 3×10^{-5} M, shifting the ACh concentration-response curve to the upper left. Moreover, acotiamide at 3×10^{-6} M significantly increased contractions at ACh concentrations from 3×10^{-8} to 3×10^{-5} M, shifting the ACh concentration-response curve to the upper left (Fig. 7A). The EC_{50} value of ACh was 5.8 (95% confidence interval: 5.3-6.4) ×10^{-7} M. The EC_{50} values of ACh in the presence of acotiamide (1×10^{-7}, 3×10^{-7}, 1×10^{-6}, and 3×10^{-6} M) were 5.8 (95% confidence interval: 5.0-6.8) ×10^{-7} M, 4.4 (95% confidence interval: 4.0-5.0) ×10^{-7} M, 2.0
(95% confidence interval: 1.7-1.2) ×10^{-7} \text{ M}, and 6.6 (95% confidence interval: 5.6-7.8) ×10^{-8} \text{ M}, respectively. In contrast, acotiamide (1×10^{-6}, 3×10^{-6}, and 1×10^{-5} \text{ 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) ×10^{-8} \text{ M}. The EC_{50} values of CCh, in the presence of acotiamide (1×10^{-6}, 3×10^{-6}, and 1×10^{-5} \text{ M}) were 9.1 (95% confidence interval: 8.5-9.8) ×10^{-8} \text{ M}, 9.2 (95% confidence interval: 8.0-10.6) ×10^{-8} \text{ M}, and 8.6 (95% confidence interval: 7.2-10.4) ×10^{-8} \text{ 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_{i1} (competitive inhibition) and K_{i2} values (noncompetitive inhibition) of acotiamide were 6.1×10^{-7} \text{ M} and 2.7×10^{-6} \text{ M}, respectively. IC_{50} values for AChE of acotiamide, itopride, mosapride, neostigmine, and physostigmine were 3.0×10^{-6}, 1.2×10^{-6}, >5.0×10^{-5}, 2.1×10^{-7}, and 2.4×10^{-7} \text{ M}, respectively. IC_{50} values for BuChE of acotiamide, itopride, mosapride, neostigmine, and physostigmine were >1.0×10^{-3}, 4.3×10^{-4}, 2.1×10^{-5}, 2.4×10^{-6}, and 2.1×10^{-7} \text{ M}, respectively. Acotiamide inhibited AChE selectively, and the ratio of the IC_{50} value for BuChE to AChE was more than 330 (Table 2).

To confirm the reversibility of AChE inhibition by acotiamide, the inhibition ratio of acotiamide for AChE was measured by dialyzing reaction media. Results showed a decrease to 1.0±1.0% compared to the non-dialyzed values (75.1±1.4%). The manner of inhibition of acotiamide on AChE activity was considered reversible.
Inhibition of canine stomach-derived AChE activity (\textit{in vitro})

The inhibitory effects of acotiamide, itopride, and neostigmine on canine stomach-derived AChE activity exhibited a concentration-dependent inhibition with estimated IC$_{50}$ values of 1.2$\times$10$^{-6}$ M, 1.2$\times$10$^{-6}$ M, and 3.6$\times$10$^{-7}$ M, respectively (Table 3). In contrast, mosapride showed only 8.3±1.3% inhibition at maximum concentration (5$\times$10$^{-5}$ M).

Receptor binding assays

Acotiamide (1$\times$10$^{-4}$ M) had no affinity for $\alpha_2$-adrenoceptor, dopamine D$_{2S}$ receptor, or serotonin 5-HT$_4$ receptors, including 5-HT$_{4c}$, 5-HT$_{4d}$, or 5-HT$_{4e}$ receptors. In contrast, itopride, a dopamine D$_2$ receptor antagonist, exhibited a $K_i$ value of (3.7±0.8) $\times$10$^{-6}$ M (Hill coefficient: 1.0±0.1) for dopamine D$_{2S}$ receptor. $K_i$ values (M) of mosapride, a serotonin 5-HT$_4$ receptor agonist, for 5-HT$_4$, 5-HT$_{4c}$, 5-HT$_{4d}$, and 5-HT$_{4e}$ receptors were (6.7±0.8) $\times$10$^{-8}$ M (Hill coefficient: 1.0±0.0), (1.8±0.3) $\times$10$^{-7}$ M (Hill coefficient:1.1±0.1), (1.3±0.2) $\times$10$^{-7}$ M (Hill coefficient:0.8±0.1) and (1.4±0.2) $\times$10$^{-7}$ M (Hill coefficient:1.0±0.1), respectively.

Myocardial monophasic action potential (MAP) duration, QT interval, and QTc in dogs

Acotiamide had no apparent effects at 1 or 3 mg/kg but slightly prolonged MAP duration at 10 mg/kg. This prolongation by acotiamide was no greater than 11%, however, and was not significant compared to the control at any time point. Cisapride at 0.3 mg/kg significantly prolonged MAP duration at all time points by 19.5%-24.8%.

Acotiamide did not affect QT interval or QTc at any dose level. Cisapride at 0.3 mg/kg significantly prolonged QT interval and QTc at all time points. Changes in QT
interval and QTc by cisapride were 31.5%-39.6% and 31.1%-42.6%, respectively (Table 4).

Discussion

This study provides several lines of evidence for the gastroprokinetic activity of acotiamide and its possible mechanism of action. The results demonstrate that acotiamide stimulates postprandial gastric motor activity and gastric emptying by inhibiting AChE without binding to dopamine D₂ or serotonin 5-HT₄ receptors. These findings suggest that acotiamide may represent a new gastroprokinetic agent in the treatment of FD involving gastric motility dysfunction.

Both intravenous and oral administration of acotiamide dose-dependently stimulated gastric motor activity during the digestive state in conscious dogs. The motility index of acotiamide given intravenously at doses of 1 and 3 mg/kg were 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 3 of 6 dogs. The toxicity of itopride at dose of 100 mg/kg and metoclopramide at dose of 10 mg/kg, a dopamine D₂ receptor antagonist, has been reported that a reduced-activity, tremor and abnormal behavior have been 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 gastroprokinetic 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 $\alpha_2$-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 (Colucci et al., 1998; Tack and Wood, 1992; Taylor and Mir, 1982). 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 $\alpha_2$-adrenoceptors, because this drug has no affinity for $\alpha_2$-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 gastroprokinetic agent for clinical use.

It is generally accepted that gastrointestinal motor activity is mainly regulated by neural and hormonal system (Ormsbee et al, 1979; Rogers et al, 1996). The major excitatory neuron involving in the gastrointestinal motor activity is considered to be the
cholinergic neuron, because most of the gastrointestinal contractions are strongly inhibited by atropine, a muscarinic receptor antagonist (Ormsbee and Mir, 1978; Shiba et al, 1995; Furuichi et al, 2001). Stimulation of peripheral dopamine D₂ receptors located on the post-ganglionic 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 post-ganglionic cholinergic nerves enhances ACh release in the gastrointestinal tract (Kilbinger and Wolf, 1992; Leclere 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.

In this study, acotiamide clearly enhanced ACh-induced, but not CCh-induced, contraction of gastric antrum 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 with pretreatment of atropine, a muscarinic receptor antagonist. In addition, acotiamide had no affinity for dopamine D₂ or serotonin 5-HT₄ receptors. These results suggest that acotiamide enhances gastric antral contraction mainly by inhibiting AChE activation in the gut. Unlike itopride, which showed a noncompetitive type of inhibition of AChE activity, acotiamide showed a mixed type of inhibition. Nevertheless, these differences in AChE inhibition patterns may correlate poorly with gastroprokinetic activity in vivo in the present study. Acotiamide showed a selective inhibition of AChE activity, but not of BuChE activity. In general, BuChE is mainly localized in the plasma and liver as a detoxification enzyme (Çokuğraş, 2003). Although the physiological role(s) of BuChE in
the gut is 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 M₂ receptors in guinea pig stomach (Ogishima et al., 2000). This effect of increasing ACh release may also be involved in the gastroprokinetic effects of higher doses of acotiamide in vivo. Given that the Kᵢ₁ value of the competitive AChE inhibition activity of this drug (6.1×10⁻⁷ M) was about 10 times lower than those for muscarinic M₁ and M₂ receptors, at 8.4×10⁻⁶ and 9.4×10⁻⁶ 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 20-40% of FD patients (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). Gastroprokinetics agents are likely useful for the treatment of FD, and symptoms do subside with an improvement in gastric motility dysfunction (Allescher et al., 2001; Mizuta et al., 2006; Talley, 1995). 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 (Matsueda et al, 2010; Tack et al, 2009). The clinical effects of acotiamide in FD patients 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.

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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, Tack, and Itoh
References


involvement of presynaptic alpha2-adrenoceptors or imidazoline receptors? Naunyn Schmiedebergs Arch Pharmacol 357: 682-691.


effect of a new benzamide derivative itopride and its action mechanisms in conscious

Kilbinger H and Wolf D (1992) Effects of 5-HT4 receptor stimulation on basal and
electrically evoked release of acetylcholine from guinea-pig myenteric plexus.
Naunyn Schmiedebergs Arch Pharmacol 345: 270-275.

toxicity study on HSR-803 with 4-week recovery test in dogs (in Japanese). Preclin

Real-time ultrasonographic assessment of antroduodenal motility after ingestion of
solid and liquid meals by patients with functional dyspepsia. J Gastroenterol Hepatol
15: 1022-1027.

Leclere PG and Lefebvre RA (2002) Presynaptic modulation of cholinergic

emptying and intragastric distribution of a solid meal in functional dyspepsia:

dose-dependent therapeutic efficacy of acotiamide hydrochloride (Z-338) in patients
with functional dyspepsia - 100 mg t.i.d. is an optimal dosage. Neurogastroenterol
Motil 22: 618-e173.

effect of mosapride citrate and existing 5-HT4 receptor agonists on gastrointestinal


Footnotes

Citation of meeting abstracts

Parts of this study were presented at the 100th Annual Meeting of the American Gastroenterological Association, Orlando, FL, 1999 (Ueki S et al., Gastroenterology 116, No. 4, Part 2, A1094, 1999) and the 71st Annual Meeting of the Japanese Pharmacology Society, Kyoto, 1998 (Matsunaga Y et al. and Ueki S et al., Jpn J Pharmacology 79, Suppl. 1, 290, 1998)

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**Figure Legends**

Fig. 1. Structural formula of acotiamide

Fig. 2. Effects of intravenous administration of acotiamide and reference drugs on gastric antral motor activity during the digestive state in dogs. Each drug was administered intravenously during the digestive state. The motor index for the 10-min period after administration was expressed as a percentage of that for the 10-min period before administration. Mean ± S.E. of 6 dogs. * p<0.05 vs. control. (Dunnett’s test: one-tailed).

Fig. 3. Enhancement of postprandial gastric antral motor activity by oral administration of acotiamide and reference drugs in dogs. Changes in gastric antral motor activity observed after oral administration of acotiamide at 10 mg/kg in the same dog (A). Effects of acotiamide and reference drugs on the gastric motor index. The motor index for the 2-hr period after the feeding is shown (B). Each drug or vehicle was administered p.o. 30 min before the meal. Mean ± S.E. of 6 dogs, *p<0.05 vs. control. (paired t-test).

Fig. 4. Effects of acotiamide and reference drug on the gastric antral hypomotility induced by clonidine during the postprandial state in dogs. Each drug or vehicle was administered by i.v. 30 min after treatment with clonidine (10 μg/kg s.c.). Mean ± S.E. of 8 or 9 dogs. *p<0.05, **p<0.01 vs. control. (Dunnett’s test: two-tailed).

Fig. 5. Effects of acotiamide (A) and cisapride (B) on clonidine-induced delayed gastric emptying of a liquid meal in dogs. Clonidine (10 μg/kg s.c.) was administered 10 min before the meal. Each drug or vehicle was administered by i.v. 5 min after administration of clonidine. The area under the curve (AUC) from 0 to 45 min was calculated at the plasma acetaminophen concentration in dogs. Mean ± S.E. of 6-12 dogs. ##p<0.01 vs. normal (Student’s t-test), *p<0.05 vs. control. (Dunnett’s test: two-tailed).

Fig. 6. Changes in contractile activity of the gastric antrum and duodenum observed after intravenous administration of acotiamide in the absence (upper panel) or presence (lower panel) of atropine during the digestive state in a dog.
Fig. 7. Effects of acotiamide on cumulative concentration-response curves of contraction response to acetylcholine (A) or carbachol (B) in the isolated guinea pig gastric antrum. Mean±S.E. of 6 experiments. *p<0.05, **p<0.01 vs. control. (Dunnett’s test: two-tailed).

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 (μM). v; Acetylcholinesterase activity (ΔOD_{412}/min).
**Tables**

Table 1. Kinetic parameters for recombinant human AChE inhibition by acotiamide, itopride and neostigmine.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acotiamide</th>
<th>Itopride</th>
<th>Neostigmine</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_m (M)</td>
<td>93.2×10^{-6}</td>
<td>90.5×10^{-6}</td>
<td>89.4×10^{-6}</td>
</tr>
<tr>
<td>V_max (ΔOD_{412}/min)</td>
<td>0.0342</td>
<td>0.0341</td>
<td>0.0339</td>
</tr>
<tr>
<td>K_i1 (M)</td>
<td>6.1×10^{-7}</td>
<td>1.3×10^{-6}</td>
<td>9.4×10^{-8}</td>
</tr>
<tr>
<td>K_i2 (M)</td>
<td>2.7×10^{-6}</td>
<td>9.0×10^{-7}</td>
<td>2.6×10^{-7}</td>
</tr>
<tr>
<td>K_i (M)</td>
<td>NC</td>
<td>1.1×10^{-6}</td>
<td>NC</td>
</tr>
<tr>
<td>Inhibition type</td>
<td>Mixed</td>
<td>Noncompetitive</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

NC; Not calculated

Each value represents the mean from 6 individual experiments.
Table 2. Selectivity of inhibitory effects on recombinant human AChE activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition Ratio</th>
</tr>
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<tbody>
<tr>
<td>Acotiamide</td>
<td>&gt; 330</td>
</tr>
<tr>
<td>Itopride</td>
<td>360</td>
</tr>
<tr>
<td>Mosapride</td>
<td>&lt; 0.42</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>11</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Inhibition ratio: IC$_{50}$ value for BuChE activity/IC$_{50}$ value for AChE activity

IC$_{50}$ values were calculated by means of 4 experiments performed in triplicate.

BuChE: Human Globulins Cohn fraction IV-4 BuChE
Table 3. Inhibitory effects of canine stomach AChE activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ value (M)</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>&gt; 5×10⁻⁵</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>3.6×10⁻⁷</td>
</tr>
</tbody>
</table>

IC₅₀ values were calculated by means of 4 experiments performed in triplicate (n=4).

AChE: canine stomach-derived AChE
Table 4. Effects of acotiamide on myocardial monophasic action potential (MAP) duration, QT interval, and corrected QT interval (QTc) in dogs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Drug</th>
<th>Pre</th>
<th>1 min</th>
<th>3 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>100.3±1.7</td>
<td>101.0±1.2</td>
<td>100.5±1.6</td>
<td>97.8±2.9</td>
<td>94.8±1.1</td>
</tr>
<tr>
<td>MAP duration (%)</td>
<td></td>
<td>100</td>
<td>100.1±2.1</td>
<td>100.0±1.9</td>
<td>99.6±2.3</td>
<td>98.8±2.9</td>
<td>96.9±1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>109.3±2.4</td>
<td>111.0±4.1</td>
<td>111.0±4.3</td>
<td>106.3±3.7</td>
<td>100.8±2.0</td>
</tr>
<tr>
<td></td>
<td>Cisapride</td>
<td>100</td>
<td>119.5±8.4*</td>
<td>124.8±10.7**</td>
<td>120.1±8.1*</td>
<td>121.6±6.5**</td>
<td>120.0±4.8**</td>
</tr>
<tr>
<td></td>
<td>(0.3 mg/kg)</td>
<td>100</td>
<td>99.1±2.1</td>
<td>96.9±1.7</td>
<td>97.4±1.9</td>
<td>96.9±1.9</td>
<td>94.4±2.7</td>
</tr>
<tr>
<td></td>
<td>Acotiamide</td>
<td>100</td>
<td>105.4±7.7</td>
<td>103.8±4.0</td>
<td>104.7±5.3</td>
<td>106.3±3.4</td>
<td>105.8±3.5</td>
</tr>
<tr>
<td></td>
<td>(1 mg/kg)</td>
<td>100</td>
<td>102.8±2.2</td>
<td>105.2±2.2</td>
<td>104.4±2.4</td>
<td>102.8±1.9</td>
<td>99.5±1.9</td>
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<td></td>
<td></td>
<td>100</td>
<td>101.8±3.2</td>
<td>109.1±3.4</td>
<td>106.0±5.5</td>
<td>104.3±5.0</td>
<td>100.7±3.3</td>
</tr>
<tr>
<td></td>
<td>(3 mg/kg)</td>
<td>100</td>
<td>133.9±17.1*</td>
<td>136.4±17.1*</td>
<td>134.4±16.4*</td>
<td>139.6±13.5**</td>
<td>131.5±6.9**</td>
</tr>
<tr>
<td></td>
<td>Cisapride</td>
<td>100</td>
<td>99.7±3.6</td>
<td>95.9±3.2</td>
<td>97.6±3.6</td>
<td>98.0±3.6</td>
<td>96.7±2.3</td>
</tr>
<tr>
<td></td>
<td>(0.3 mg/kg)</td>
<td>100</td>
<td>107.0±8.5</td>
<td>102.2±4.7</td>
<td>103.0±8.0</td>
<td>107.5±7.2</td>
<td>109.7±6.3</td>
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<tr>
<td></td>
<td>Acotiamide</td>
<td>100</td>
<td>101.4±1.3</td>
<td>101.6±2.6</td>
<td>102.2±2.7</td>
<td>100.7±2.7</td>
<td>101.4±3.4</td>
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<tr>
<td></td>
<td>(3 mg/kg)</td>
<td>100</td>
<td>96.7±2.8</td>
<td>102.6±1.6</td>
<td>100.4±3.9</td>
<td>99.5±5.1</td>
<td>101.6±4.2</td>
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<tr>
<td></td>
<td>Cisapride</td>
<td>100</td>
<td>142.6±24.6*</td>
<td>141.1±20.4*</td>
<td>137.8±20.6*</td>
<td>137.5±20.2*</td>
<td>131.1±11.3**</td>
</tr>
<tr>
<td></td>
<td>(0.3 mg/kg)</td>
<td>100</td>
<td>128.6±24.6*</td>
<td>127.8±20.6*</td>
<td>126.4±20.6*</td>
<td>127.5±20.2*</td>
<td>121.1±11.3**</td>
</tr>
</tbody>
</table>

Parameters obtained before administration of a drug were regarded as 100%.

Mean ± S.E., * p<0.05, ** p<0.01 vs Control (Dunnett’s multiple comparison)
Figure 3

A

Control  ↓  Feeding  ↓
Gastric antrum

Acotiamide 10 mg/kg  Feeding  ↓
Gastric antrum

Time intervals (30 min)

B

**Motility index (units/2 hr)**

- **Control**
- **Acotiamide**

<table>
<thead>
<tr>
<th>Dose (mg/kg, p.o.)</th>
<th>Motility index</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>3</td>
<td>600</td>
</tr>
<tr>
<td>10</td>
<td>800</td>
</tr>
<tr>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>30</td>
<td>1000</td>
</tr>
</tbody>
</table>

*Significant difference from control

- **Control**
- **Itopride**

<table>
<thead>
<tr>
<th>Dose (mg/kg, p.o.)</th>
<th>Motility index</th>
</tr>
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<tr>
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<td>800</td>
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<tr>
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</tr>
<tr>
<td>100</td>
<td>1000</td>
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</tbody>
</table>

*Significant difference from control

- **Control**
- **Cisapride**

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<td>800</td>
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<td>1000</td>
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</tbody>
</table>

*Significant difference from control

- **Control**
- **Mosapride**

<table>
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<th>Dose (mg/kg, p.o.)</th>
<th>Motility index</th>
</tr>
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</tr>
<tr>
<td>1</td>
<td>600</td>
</tr>
<tr>
<td>3</td>
<td>800</td>
</tr>
<tr>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>10</td>
<td>1000</td>
</tr>
</tbody>
</table>

*Significant difference from control
Figure 4

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Figure 5

A

\[ \text{AUC}_{0-45} \text{ (µg/min/ml)} \]

Control \[ \begin{array}{c}
0.5 \\
1 \\
\end{array} \]

Acotiamide (mg/kg, i.v.)

Clonidine (10 µg/kg, s.c.)

Normal

B

\[ \text{AUC}_{0-45} \text{ (µg/min/ml)} \]

Control \[ \begin{array}{c}
0.1 \\
0.2 \\
\end{array} \]

Cisapride (mg/kg, i.v.)

Clonidine (10 µg/kg, s.c.)

Normal
Figure 6

A

Acotiamide, 1 mg/kg, i.v.

Gastric Antrum

Duodenum

B

Acotiamide, 1 mg/kg, i.v.

Atropine, 0.05 mg/kg, i.v. + 0.05 mg/kg/hr

Gastric Antrum

Duodenum

Time intervals (10 min)
Figure 7

A

- Control
- 0.1 (µM)
- 0.3 (µM)
- 1 (µM)
- 3 (µM)

B

- Control
- 1 (µM)
- 3 (µM)
- 10 (µM)
Figure 8

A. Acotiamide
- Control
- 0.5 (µM)
- 1 (µM)
- 2.5 (µM)
- 5 (µM)

B. Itopride
- Control
- 0.5 (µM)
- 1 (µM)
- 2 (µM)
- 3 (µM)

C. Neostigmine
- Control
- 0.05 (µM)
- 0.1 (µM)
- 0.2 (µM)
- 0.4 (µM)