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Distribution of acotiamide, an orally active acetylcholinesterase inhibitor, into the *myenteric plexus* of rat and dog stomachs

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ABSTRACT

Aims: Acotiamide is the first-in-class drug for the treatment of functional dyspepsia. Although pharmacological and therapeutic actions of acotiamide are thought to be derived from its inhibitory effects on acetylcholinesterase (AChE), whether the concentration of acotiamide at the site of action is sufficient to inhibit AChE remains unclear. Since major site of acotiamide action is thought to be the cholinergic nerve terminals in gastric *myenteric plexus*, we studied the distribution of [^{14}C]acotiamide into gastric *myenteric plexus*.

Main methods: Distribution of [^{14}C]acotiamide was evaluated using macro- and micro-autoradiography in rats and dogs.

Key findings: The results of macro-autoradiography showed the concentration of radioactivity was 27.9 μM in rat stomach, which was 12 times higher than IC_{50} of acotiamide for rat AChE. Being different from rats, the distribution of radioactivity in the muscular layer was distinguishable from that in the mucosal layer in dog stomach. The concentration of radioactivity in the muscular layer of dog stomach (1.41 μM) was approximately two-times lower than those in the mucosal layer, however, it was approximately 1.2 times higher than IC_{50} of acotiamide for dog AChE. The results of micro-autoradiography also showed the radioactivity distributed homogenously in the muscular layer of rat stomach, suggesting the concentration of radioactivity around the ganglion of *myenteric plexus* is similar to that in the muscular layer of stomach.

Significance: These findings suggest acotiamide distributes to the *myenteric plexus* of stomach, a putative site of acotiamide action, with adequate concentrations to inhibit AChE, in both of rat and dog stomachs.

1. Introduction

Functional dyspepsia (FD) is a common gastrointestinal disorder defined as symptom-based conditions in the absence of organic disease [1]. Symptoms are categorized as postprandial distress and epigastric pain syndromes, which are associated with impaired gastric accommodation and emptying [2,3]. Gastric accommodation and emptying are induced by coordinating motility of gastric fundus, body and antrum, which are regulated by complex nervous systems including cholinergic neurons projected from dorsal motor nucleus of the vagus to the stomach [4,5].

Acotiamide is the first-in-class drug for the treatment of FD [6]. Although some clinical studies have suggested that proton pump inhibitors (*e.g.*, omeprazole) and prokinetics (*e.g.*, itopride or mosapride) are effective, no product except for acotiamide has gotten marketing approval for the treatment of patients with FD [6]. Clinical studies have shown that acotiamide enhances the gastric accommodation reflex and gastric emptying rate [7] and improves meal-related symptoms such as postprandial fullness, upper abdominal bloating and early satiation in patients of FD [8]. These pharmacological and therapeutic effects of acotiamide are thought to be derived from its inhibitory effects on acetylcholinesterase (AChE) which results in the potentiation cholinergic neurons [9,10]. In fact, an *in vitro* animal experiment showed that acotiamide enhanced acetylcholine (ACh)-induced but not carbachol (not hydrolyzed by AChE)-induced contraction of isolated gastric antrum strips of guinea pig [10]. In addition, an *in vivo* animal experiment showed that acotiamide enhanced gastric body contractions induced by electrical stimulation of the vagus nerve in rats, which were completely abolished by classical antagonist of ACh receptors [11].

Acotiamide inhibits AChE with half maximal inhibitory concentrations (IC_{50}) of 3.0, 2.3 and 1.2 μM for human recombinant, and rat and canine gastric AChEs, respectively [10,11]. These values are much larger

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than those of classical inhibitors of AChE, such as neostigmine, however, acotiamide is concentrated into the stomach tissue by carrier-mediated uptake processes, which may account for the selective action of acotiamide for gastric smooth muscle but not for skeletal muscle and central nervous system in rats [12].

Myenteric plexus is a mesh-like system of neurons which provide major motor innervation to the gastrointestinal muscles [13]. Therefore, main target of acotiamide action is thought to be AChE localized around the cholinergic nerve terminals in the *myenteric plexus* which is located in the muscular layer of the stomach. However, whether sufficient concentrations of acotiamide are attained in the *myenteric plexus* of the stomach to inhibit AChE, has not been confirmed in any species yet, although the total concentrations of acotiamide in the homogenate of rat stomach after *in vivo* administration were reported to be higher than *in vitro* IC_{50} value of acotiamide estimated from rat gastric AChE [11].

The aim of the present study was to examine the distribution of acotiamide into the *myenteric plexus* of the stomach after [^{14}C] acotiamide dose enough to exhibit pharmacological action, using macro- and micro-autoradiographs, and to estimate whether the sufficient concentration of acotiamide is attained in the *myenteric plexus* of the stomach, a putative site of acotiamide action, to inhibit AChE in rat and dog stomachs.

2. Materials and methods

2.1. Chemicals

N-[2-[bis(1-methylethyl)amino]ethyl]-2-[(2-hydroxy-4,5-dimethoxybenzoyl)amino]thiazole-4-carboxamide monohydrochloride trihydrate (acotiamide hydrochloride, Z-338/YM443, Fig. 1) was synthesized in the central research laboratories of Zeria Pharmaceutical Co., Ltd. (Saitama, Japan). [^{14}C]Acotiamide (2.26 GBq/mmol) was synthesized by GE Healthcare UK Ltd. (Buckinghamshire, England). All other chemicals were of reagent grade.

2.2. Animals

Male Sprague–Dawley rats aged six to seven weeks were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and housed under standard controlled environmental conditions at 23 ± 3 °C and 55 ± 20% humidity with a 12-h light/dark cycle, and food (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan or CE-2; CLEA Japan Inc., Tokyo, Japan) and water available *ad libitum*. Rats were allowed to acclimate to laboratory conditions for at least one week prior to experiments.

Male beagle dogs were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) or the Institute for Animal Reproduction (Ibaraki, Japan) and housed individually in experimental cages where they were acclimated for at least 12 days before entry to the study. Laboratory chow (NVE-10, Nihon Pet Food K.K., Tokyo, Japan; or DS-A, Oriental Yeast Co., Ltd.) was provided once daily and water was given *ad libitum*. Animals were housed under standard controlled environmental conditions at 22 ± 3 °C and 50 ± 20% humidity with a 12-h light/dark cycle.

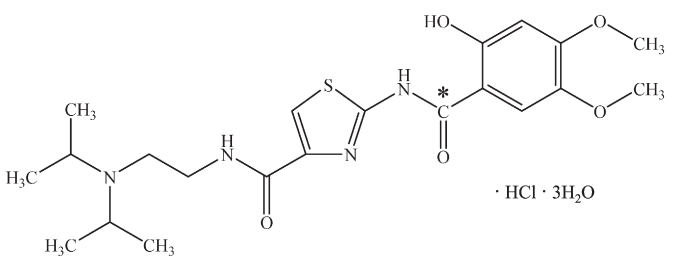

Fig. 1. Structure of acotiamide hydrochloride.

All animal experiments were approved by the Animal Care and Use Committee of the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd., Animal Ethical Committee, Tsukuba Laboratories, Nemoto Science Co., Ltd. (Ibaraki, Japan) and the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. (Wakayama, Japan).

2.3. AChE staining of rat stomach

Rats were exsanguinated *via* the abdominal aorta under anesthesia with diethyl ether and the stomach was excised, washed with saline, ligated at the pylorus, filled with OCT compound through the cardia, ligated at the cardia. Stomach was embedded with OCT compound and frozen in a bath of isopentane and dry ice. Embedded stomach was cut into 6-μm-thick sections using a cryomacrocut CM1900, and immersed in 3 mM copper sulfate/0.05 mM potassium ferricyanide solution containing acetylthiocholine iodide at 37 °C for 90 min. After rinsing in distilled water, sections were immersed in Mayer's hematoxylin for 10 min and then washed with running tap water, dehydrated in ethyl alcohol, and immersed in xylene.

2.4. AChE staining of dog stomach

Dogs were euthanized by intravenous administration of sodium pentobarbital and supersaturated potassium chloride solutions. Stomach was immediately excised, and divided into 2 pieces along the greater curvature. The pieces were rinsed with physiological saline and divided into approximately 1.5 cm × 1.5 cm sections and frozen in liquid nitrogen. Sections were embedded with an OCT compound and frozen in hexane and dry ice. The frozen serial sections were prepared as 8-μm sections using a cryomacrocut CM1900. The sections were immersed as described in AChE staining of stomachs of rats.

2.5. Macro-autoradiography of rat stomach

As any pharmacological effects haven't been reported after oral administration of acotiamide to rats although acotiamide is orally administered for the treatment of FD, rats were subcutaneously administered with [^{14}C]acotiamide (30 mg and 20.6 MBq/kg). These dose and route of acotiamide administration were reported to increase the gastric motility index significantly until 90 min after the administration to rats [11]. Rats were exsanguinated *via* the abdominal aorta under anesthesia with diethyl ether, and the stomach was excised at 30 min after the administration of acotiamide. The excised stomach was washed with saline, ligated at the pylorus, filled with 2% CMC-Na through the cardia, ligated at the cardia, embedded with 2% CMC-Na and frozen in a hexane/dry ice bath. The embedded stomach was cut into 40-μm-thick sections using a cryomicrotome CM3600 (Leica Microsystems GmbH, Wetzlar, Germany). Sections were exposed by contact with an Imaging-Plate BAS-III2040 (Fuji Photo Film, Tokyo, Japan) in a shield box for 16 h. After exposure, the Imaging-Plate was analyzed with a Bio-Imaging Analyzer BAS-2000 (Fuji Photo Film, Tokyo, Japan) to obtain macro-autoradioluminograms and measure radioactivities in the tissues. The concentration of the radioactivity in the tissue was quantified by the absolute calibration method [14,15,16,17]. The calibration curve through the origin ($Y = aX$) was prepared based on the mean value of the intensity of luminescence (PSL-BG/mm^2) of the blood simultaneously collected when stomach was removed and blood concentration of acotiamide determined by a liquid scintillation analyzer LSC-6100 (Aloka, Tokyo, Japan). The background (BG) value was calculated as the mean value of the intensity of luminescence in three positions on the rim of the section. The lower limit of quantification was defined as twice the BG value. Tissue concentration of acotiamide (mean ± standard deviation) was expressed as μM based on the assumption that tissue specific gravity equals 1 mL/g.