Title manuscript: Tetrahydroberberine, an Isoquinoline Alkaloid Isolated from Corydalis Tuber, Enhances Gastrointestinal Motor Function

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Abstract

As delayed gastric emptying and impaired gastric accommodation are regarded as the pathophysiological mechanisms underlying functional dyspepsia (FD), prokinetic and fundic relaxants have been suggested as a new treatment for FD. We isolated tetrahydroberberine (THB), an isoquinoline alkaloid, (5,8,13,13 α-Tetrahydro-9,10-dimethoxy-6H-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizine) from Corydalis Tuber, and found that it has micromolar affinity for dopamine D2 (pKi 6.08) and 5-HT\textsubscript{1A} (pKi 5.38) receptors, but moderate to no affinity for other relevant serotonin receptors [\textit{i.e.}, 5-HT\textsubscript{1B}, 5-HT\textsubscript{1D}, 5-HT\textsubscript{3}, and 5-HT\textsubscript{4}: pKi < 5.00]. Oral administration of THB not only resulted in significantly accelerated gastric emptying of normal rats in a bell-shaped relationship with a maximal efficacy at the dose of 30 µg/kg, but also restored the delayed gastric emptying caused by apomorphine, which might be mediated by an anti-dopaminergic effect. Data from electromyography indicated enhanced gastro motor function of the upper GI tract by THB, which occurred through strengthening of contractility and shortening of the contraction interval. Furthermore, in rats stressed by repeated restraint, a significantly higher shift in pressure-volume curve by THB (10 µg/kg, P<0.05), which was inhibited by WAY100635, a 5-HT\textsubscript{1A} antagonist and L-NAME, a NOS inhibitor, but not VIP antagonist, was observed. Oral administration of THB resulted in a drastic increase of gastric accommodation in Beagle dogs. Area under the volume versus time curve was significantly increased by THB (30 µg/kg, p<0.01), comparable to that of sumatriptan (3 mg/kg), a potent fundic relaxant. Taken together, our data suggested that THB, with D\textsubscript{2} antagonist and 5-HT\textsubscript{1A} agonist properties, has significant potential as a therapeutic for treatment of FD.
Introduction

Functional dyspepsia (FD) is one of the main syndromes associated with gastrointestinal motor dysfunction (FD) (Tack, 2007). Delayed gastric emptying and impaired gastric accommodation are known to contribute to clinical manifestations, such as postprandial fullness, early satiation, epigastric pain, and burning sensation (Talley et al., 2006). Currently, prokinetics and fundic relaxants appear to be the drugs of choice for treatment of FD (Tack, 2008; Brun and Kuo, 2010). Prokinetics stimulate smooth muscle contractions, leading to enhanced gastric emptying and acceleration of both small and large intestinal transits (Karamanolis and Tack, 2006; Tack, 2008). Fundic relaxants target impaired gastric accommodation of the upper gastrointestinal tract (Kindt and Tack, 2006; Tack, 2008). One of the major categories of prokinetic drugs is the antidopaminergic agent (Tonini et al., 2004). The gastrointestinal tract actually has the ability to produce a substantial amount of dopamine. Enteric dopamine has been known to mediate inhibition of gut motility and decrease antroduodenal coordination in gut muscle, thereby inhibiting acetylcholine release from the cholinergic nerve by activation of neuronal dopamine D₂ receptor (Iwanaga et al., 1990). Domperidone, a D₂ receptor antagonist, has been reported to induce an increase in gastric muscle contraction in guinea pigs (Reddymasu et al., 2007). Itopride is a benzamide derivative antagonising D₂ dopamine receptor, which has demonstrated acceleration effects on gastroduodenal motility and gastric emptying in both animals and humans (Tsubouchi et al., 2003; Holtmann et al., 2006). Another significant category of prokinetic drugs is the serotonergic agent (Tonini and Pace, 2006). The main effects relevant to gastrointestinal motility are mediated by 5-HT₄ receptors (De Maeyer et al., 2008). Tegaserod, an aminooindole compound, is a partial 5-HT₄ receptor agonist that has been shown to induce acceleration of gastric emptying in FD patients as well as healthy volunteers (Degen et al.,
Cisapride is a non-selective 5-HT₄ receptor agonist with a partial weak 5-HT₃ antagonist effect, which exhibits strong prokinetic actions (Mearin et al., 2004). However, tegaserod and cisapride were withdrawn due to incidence of cardiovascular ischemia and serious cardiac arrhythmias, respectively (Wysowski et al., 2001; Pasricha, 2007). On the other hand, several serotonin receptors have been known to regulate gastric accommodation. Buspirone, a non-selective 5-HT₁A receptor agonist has been involved in the gastric accommodation reflex by release of nitric oxide (Coulie et al., 1999) in enteric neurons, resulting in muscle relaxation (Tack J, 1999). In a clinical study, it was reported to be superior to the placebo control in lessening dyspeptic syndromes (Van Oudenhove et al., 2008). Subcutaneous administration of sumatriptan, a 5-HT₁B/D receptor agonist, was shown to restore meal induced relaxation in patients with impaired gastric accommodation (Tack et al., 2000).

Previously, we reported on the strong gastroprokinetic effects of DA-9701, an herb-based novel prokinetic agent formulated with Pharbitidis Semen and Corydalis Tuber (Lee et al., 2008). Administration of DA-9701 not only resulted in accelerated gastric emptying and gastrointestinal transit in normal rats, as well as abnormally induced conditions, but also induced an increase in gastric accommodation in Beagle dogs (Lee et al., 2008). In an effort to determine the active component(s), we performed binding assays for various receptors controlling gastrointestinal motor functions using several compounds isolated from DA-9701. Among them, tetrahydroberberine (THB), an isoquinoline alkaloid, 5,8,13,13a-Tetrahydro-9,10-dimethoxy-6H-benzo[g]-1,3-benzodioxolo[5,6-a]quinolizine (Fig. 1), isolated from Corydalis Tuber, was found to be bound to both dopamine D₂ and 5-HT₁A receptors, but not other relevant serotonin receptors. Here we show that THB works not only as a prokinetic but also a fundic relaxant in animal models, and that it has potential as a therapeutic for...
gastrointestinal motor dysfunctions such as FD.
Methods

Identification of tetrahydroberberine from *Corydalis Tuber*. Tubers of *C. yanhusuo* (10 kg) were extracted with 50% EtOH two times at room temperature. The ethanol extract (250 g) was suspended in distilled water (7.2 L), followed by successive partitioning with *n*-hexane, CHCl₃, EtOAc, and *n*-BuOH, yielding 10, 30, 4, and 26 g, respectively. The CHCl₃ soluble fraction (28 g) was subjected to silica gel column chromatography (230-400 mesh, 600 g) and eluted with CHCl₃-MeOH [15:1 (3.0 L), and 5:1 (3.0 L)] to afford seven fractions [F1, 15:1, 1.0 L; F2, 15:1, 1.0 L; F3, 15:1, 1.0 L; F4, 5:1, 0.5 L; F5, 5:1, 0.5 L; F6, 5:1, 1.0 L; and F7, 5:1, 1.0 L]. F1 (3.5 g) and F2 (10.0 g) were mixed and were subjected to silica gel column chromatography (230-400 mesh, 250 g, *n*-hexane/EtOAc = 3:1) to give seven subfractions [SF1-SF7 (each 1.0 L)]. SF3 (350 mg) was purified by preparative HPLC using a solvent of *n*-hexane/CHCl₃/EtOAc (6:3:5) at a flow rate of 2.0 mL/min (Apollo Silica 5 μm column; 250 × 10 mm; 5 μm particle size; Shodex refractive index detector) to obtain tetrahydroberberine (30 mg, *t*ₚ = 13.5 min).

Chemicals. L-NAME hydrochloride, L-arginine, WAY 100635 maleate, and Penicillin G were purchased from Sigma Chemical Company (St. Louis, MO, USA). Tiletamine/Zolazepam (Zoletil®) was obtained from Virbac Laboratories (Carros, France). Ketamine was purchased from Yuhan Corporation (Seoul, Korea). VIP antagonist (COOH-Lys-Pro-Arg-Arg-Pro-Tyr-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH₂) was obtained from Anaspec (San Jose, CA, USA). G protein-coupled receptor membrane preparations were purchased from PerkinElmer (Waltham, MA, USA). Other chemicals and reagents used were of analytical or reagent grade.

Radioligand competition receptor binding assays. Frozen CHO-K1 and HEK-293 membranes containing cloned human recombinant serotonin 5-HT₁A, 5-HT₃, or dopamine
D_{2S} receptors were purchased from PerkinElmer Life Sciences. Membranes were thawed on ice and re-suspended in assay buffer (Table 1). For serotonin 5-HT_{1A} receptor, binding of \[^{3}\text{H}]8-OH-DPAT (0.25 nM) to CHO-K1 cell membranes expressing the recombinant human 5-HT_{1A} receptor was performed in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgSO_{4}, 0.5 mM EDTA, and 0.1 % ascorbic acid in a total volume of 0.2 mL for 1 hour at 27 °C in the dark. Non-specific binding was determined with 10 μM metergoline. Assays for the compounds, along with the positive control compound, NAN-190, were performed with 10-log unit concentrations (May et al., 2003). For the serotonin 5-HT_{3} receptor binding assay, binding of \[^{3}\text{H}]\text{-GR65630} (0.55 nM, 60.7 Ci/mmol) to HEK-293 cell membranes expressing the recombinant human 5-HT_{3} receptor was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM MgCl_{2}, 1 mM EDTA in a total volume of 0.2 mL for 1 hour at 25 °C in the dark. Non-specific binding was determined with 10 μM MDL-72222. Assays for the compounds, along with the positive control compound, MDL-72222, were performed with 10-log unit concentrations (Boess et al., 1997). For the D_{2S} receptor binding assay, binding of \[^{125}\text{I}]\text{-Iodospiperone} (0.27 nM, 2200 Ci/mmol) to CHO cell membranes expressing the recombinant human D_{2} receptor (short variant) was performed in 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 5 mM MgCl_{2}, and 1 mM EDTA 5 mM MgSO_{4} in a total volume of 0.2 mL for 2 hours at 25 °C in the dark. Non-specific binding was determined with 5 μM haloperidol. Assays for the compounds, along with the positive control compound S(-)-Eticlopride, were performed with 10-log unit concentrations (Stormann et al., 1990). Assays were terminated by rapid vacuum filtration over a GF/C filter previously soaked in 0.5 % polyethyleneimine and a filter washed with ice-cold 50 mM Tris-HCl buffer (pH 7.4) at 25 °C. Radioactivity was measured on a β-counter, and the data were analyzed graphically with inhibition curves and IC_{50} values were derived. \(K_{i}\) values were calculated.
according to the equation $K_i = \frac{IC_{50}}{1 + \left(\frac{C}{K_d}\right)}$, with $C$ as the concentration of each radioligand and $K_d$ as the equilibrium dissociation constant of each radioligand. Binding assays for other serotonin (5-HT$_{1B}$, and 5-HT$_{4}$) receptors were conducted at Ricerca Biosciences (Taipei, Taiwan) and Caliper Lifesciences (Hopkinton, MA, USA).

**Animals.** Male Sprague-Dawley rats (200-220 g) were purchased from OrientBio, Inc (Gapyeung, Korea) and had free access to water and a standard pellet diet. Rats were kept in an environmentally controlled room in groups of 1-2 animals before experiments and kept individually after the animals had been surgically prepared. Beagle dogs were purchased from Central Lab. Animal Inc (Seoul, Korea), individually housed in single, air-conditioned boxes, and given dog food in pellet form (Purina, Dog, Chow®). All experimental procedures were conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals, prepared by the Institutes of Laboratory Animal Resources, National Research Council (http://www.nsa.edu/nrc), and Dong-A Pharm. Research Institute.

**Gastric emptying.** Gastric emptying was measured according to the method of Ozaki and Sukamoto (Ozaki and Sukamoto, 1999). Male Sprague–Dawley rats (200-220 g) were fasted for 24 h prior to the start of all experiments; the animals then received oral administration with test drugs (tetrahydroberberine, or conventional prokinetics as positive controls at the indicated doses) or 3 % (w/v) hydroxypropyl methylcellulose (HPMC) as a vehicle. Normal rats were given 2 mL of semisolid meals by gavages at 45 min after drug administration. After 35 min, animals were sacrificed, and the weight and contents of the stomach were measured for determination of gastric emptying rate. Gastric emptying rate (%) = $\left[1 - \frac{\text{weight of test stomach}}{\text{weight of 0 time control stomach}}\right] \times 100$. For the delayed gastric emptying model, animals were given 2 ml of semisolid meal at 45 min after drug administration, and
simultaneously injected with apomorphine (s.c., 0.05 mg/kg). After 50 min, gastric emptying was determined by the same method described above.

**Electromyography study.** Male Sprague-Dawley rats (10-12 weeks old and weighing 350-450 g) were used. Animals were surgically prepared for chronic electromyography (EMG) under aseptic conditions and under general anesthesia with Tiletamine/Zolazepam (i.m., 12.5 mg/kg). Three pairs of electrodes (Silver, 0.005” bare, 0.007” coated diameter, A-M Systems, Sequim, WA, USA) were implanted into the muscular wall of the antrum and the small intestine 5 and 15 cm distal to the pylorus, as described previously (Rukebusch and Fioramonti, 1975). Electrodes were folded and fixed to the back of the animal to allow free movement in the cage. After the surgical procedure, rats were treated with antibiotic (penicillin G) for 48 h. Electrode position was verified during necropsy. EMG recordings started 7 days after surgery, when rats had recovered completely and exhibited clear MMC. Rats were placed in Böllman cages. Electrodes were unfolded from the back of the animal and protected with a rubber tube that allowed the animal to move freely in the cage throughout the experiment. Electrodes were connected to an EMG preamplifier. The time constant was set at 0.01 s and the low and high cut-off frequencies were set at 10 and 1,000 Hz, respectively. Before the experiment, rats were fasted (24 h). After 90 min of recording a clear MMC pattern, either the test drug or vehicle was administered intravenously. Recordings lasted for at least another 90 min after treatment.

**Impaired gastric accommodation.** Male Sprague-Dawley rats weighing 250-300 g were used. Animals had free access to water and a standard pellet diet. Prior to experiments, rats were kept in an environmentally controlled room in groups of 4. For restraint stress-induced impaired gastric compliance, rats were restrained in a plastic cage for 4 h per day for 4 days. Rats were fasted for 24 h prior to the start of the experiment (water was available *ad libitum*).
At the beginning of the experiment, rats were sedated with a single dose of ketamine (50 mg/kg, i.m., Yuhan, Seoul, Korea). Thereafter, a dose of 25 mg/kg was administered intramuscularly every hour in order to maintain sedation. During the experiment, the animals were always positioned lying down on their right side. Ketamine sedation allowed the rats to tolerate swallowing of the finely folded polyethylene balloon that was adherent to the polyethylene tube and intubation of the tube during the experiment while spontaneous breathing was preserved. The bag was positioned in the stomach via a guide-wire. A heating pad was used throughout the experiments in order to maintain the body temperature of the animal at 37 °C. Gastric pressure-volume relationships were studied using a gastric barostat (Distender series II; G and J Electronics, Ontario, Canada). The system consists of an ultrathin polyethylene balloon (10 mL maximal capacity, Mui Scientific, Ontario, Canada), which was finely folded, and can be inserted through the mouth into the proximal stomach, and has infinite compliance at the volumes used for distention of the rat stomach. A polyethylene balloon was adhered to a single lumen polyethylene tube (1.2 mm diameter, Natsume, Tokyo, Japan). The tube was connected to a pressure recording port and an air inflation port at a computer driven barostat for recording of volume changes while the pressure was kept constant. Prior to the start of the experiment, the balloon was connected to the barostat and the intrabag pressure was raised to 10 mm Hg. Monitoring of a constant intrabag volume ensured that there was no leak (Janssen et al., 2004). Drugs were administered after the intragastric volume maintained a stable baseline value during a period of at least 10 min at the constant pressure used. A single dose of tetrahydroberberine was administered per experiment. Different doses (1, 3, 10, 30, or 100 μg/kg, i.v.) of tetrahydroberberine were randomly divided over different experiments. The doses of drugs
(WAY 100635, L-NAME, L-arginine and VIP antagonist) used for mechanism studies were chosen based on the previous publications (Takahashi and Owyang, 1997; Zhou et al., 2008).

**Canine gastric accommodation.** Experiments were performed on 4 adult female Beagle dogs (7-9 kg body weight). Dogs were trained to stand quietly in a sling without sedation. Experiments were performed on conscious dogs after allowing at least 15 days for recovery after surgery. Before each experimental session, the dogs were fasted for at least 18 h; water was available *ad libitum*. Between consecutive experimental sessions with the same animal, a washout period of at least 72 h was allowed. Dogs were observed throughout the experiment, and any sign of discomfort or anomalous behavior was noted. The gastric cannula was opened, and after verifying that the stomach did not contain any food residues, the bag of the barostat was introduced into the proximal stomach (position of the bag checked radiographically). Before and at the end of the *in vivo* tests, the bag was checked for air leaks by increasing the pressure to 20 mmHg (Chen et al., 2009). To determine the influence of 5-HT$_1$ receptor antagonists in mediation of canine gastric accommodation induced by tetrahydroberberine, WAY-100635 (0.1 mg/kg) was tested vs. 30 μg/kg tetrahydroberberine and administered i.v. 10 min prior to tetrahydroberberine.

**Data analysis.** Results were expressed as mean ± S.E.M. Differences in the data were evaluated using paired *t*-test for comparison of two groups or one-way ANOVA followed by Dunnett’s test for multiple comparisons. A difference was considered significant if *p*<0.05.
Results

Identification and characterization of THB. In an effort to determine active component(s) from DA-9701, we have fractionated the ethanol extract of *Corydalis Tuber* according to polarity and purified several compounds by column chromatography, as described in the Methods section. We have screened and chased the affinities of the compounds for relevant receptors involved in control of gastrointestinal motor function, which included various serotonergic receptors, dopamine D<sub>2</sub> receptor, by radioligand competition binding assay, according to the conditions presented in Table 1. We found that a compound known as SF-2 has a micromolar affinity for dopamine D<sub>2</sub> receptor (pKi 6.08) and 5-HT<sub>1A</sub> (pKi 5.38), but moderate to no affinity for other relevant serotonin receptors [*i.e.,* 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>3</sub> and 5-HT<sub>4</sub>: pKi < 5.00], as presented in Table 2. SF-2 showed similar affinity for D<sub>2</sub> receptor to itopride (in this study), while 1,434 fold lower affinity than domperidone, compared with values previously reported (Freedman et al., 1994). In terms of 5-HT<sub>1A</sub> affinity, SF-2 showed 132 times lower affinity than buspirion (pKi 7.50). The structure of SF-2 was identified by NMR and mass spectroscopy. Spectroscopic data, including <sup>1</sup>H-NMR and <sup>13</sup>C-NMR, are as below. Yellow powder; m.p. 167 °C; FAB-MS: m/z 340 [M + H]<sup>+</sup>; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): δ 6.87 (1H, d, J = 8.5 Hz, H-12), 6.80 (1H, d, J = 8.5 Hz, H-11), 6.74 (1H, s, H-1), 6.60 (1H, s, H-4), 6.08 (2H, s, -OCH<sub>2</sub>O-), 4.27 (1H, d, J = 15.5 Hz, H-8), 3.86 (6H, s, OCH<sub>3</sub>-9, OCH<sub>3</sub>-10), 3.58 (2H, d, J = 15.5 Hz, H-8, H-14), 3.25 (1H, m, H-6), 3.22 (1H, m, H-13), 3.14 (1H, m, H-5), 2.86 (1H, dd, J = 15.0, 12.0 Hz, H-13), 2.68 (1H, m, H-6), 2.65 (1H, m, H-5); <sup>13</sup>C-NMR (125MHz, CDCl<sub>3</sub>): δ 150.2 (C-10), 146.1 (C-2), 145.8 (C-3), 145.0 (C-9), 130.6 (C-14a), 128.5 (C-8a), 127.7 (C-4a), 127.6 (C-12a), 123.8 (C-12), 110.9 (C-11), 108.3 (C-4), 105.4 (C-1), 101.0 (-OCH<sub>2</sub>O-), 59.9 (OCH<sub>3</sub>-9), 59.5 (C-14), 55.8 (OCH<sub>3</sub>-10), 53.8 (C-8), 51.3 (C-6), 36.3 (C-13), 29.4 (C-5). Spectral data for

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tetrahydroberberine was determined to be identical to that of the source material (Trademax, China). SF-2 is identified as tetrahydroberberin (THB), an isoquinolin alkaloid. The chemical structure is shown in Fig. 1. To the best of our knowledge, there have been no reports on serotonin receptors, although it has been known as antidopaminergic agent (Niwa et al., 1991). Based on the above data, we performed testing in order to determine whether THB has potential as a prokinetic as well as a fundic relaxing agent.

**Effects of THB on gastric emptying.** In order to estimate the prokinetic effects of THB, we performed gastric emptying experiments using semi-solid meals. In normal rats, compared to the control group, THB induced significant acceleration of gastric emptying. As shown in Fig. 2A, data from the control group indicated that only 28.1±1.1 % was emptied; however, the decrease in residual meal by THB was significant. The effect was a bell-shaped relationship, with a maximal efficacy at the dose of 30 µg/kg. The residual percentage of the meal was 50.3±1.2 % (p<0.05), and the maximal effect of THB was comparable to that achieved with cisapride at the dose of 10 mg/kg (p.o.; 54.8±1.5, n=8, p<0.05). Next, we harbored the delayed models of gastric emptying in order to determine whether THB, known to function as a D₂ antagonist (Wu and Jin, 1996; Wu and Jin, 1997), is capable of correcting abnormally depressed gastric emptying. Apomorphine has inhibitory effects on gastric emptying through its action as a dopamine agonist (Blancquaert et al., 1982). Administration of apomorphine (s.c., 0.05 mg/kg) resulted in a marked delay of gastric emptying of a semisolid meal by approximately 50%, compared with naïve rats (37.8±2.4 vs 68. 9±7.4). Delayed gastric emptying was restored by THB. At doses of 10 µg/kg and 100 µg/kg, the gastric emptying rate was 48.8±5.2 (p<0.05) and 55.9±3.7 (p<0.01), respectively. The effect of THB 100 µg/kg was comparable to that of itopride at a dose of 30 mg/kg (Fig. 2B). These results suggested that THB could induce acceleration of gastric emptying under normal
conditions, as well as apomorphine-induced abnormally delayed gastric emptying.

**Effects of THB on motor activity in the upper GI tract.** To further evaluate the question of whether THB enhances gastric motor function, we measured contractility using electromyography. Rats received surgical implantation with 41 pairs of electrodes in the antrum, duodenum, and jejunum. The effects of THB on gastrointestinal contractile activity upon intravenous injection under fasting conditions are presented in Figure 3. Control rats injected with saline showed a typical pattern of motor activity; however, injection of THB (10 µg/kg, i.v.) resulted in significant enhancement of contractile force in the GI tract, and an overall increase in the motor activity index was observed. As shown in Fig. 3A, peak count was significantly increased by THB, not only in the gastric antrum, but also the duodenum and jejunum, compared with the control. Amplitude and integral areas showed a significant increase, as shown in Figs. 3B and 3C, respectively, indicating that THB induced stronger contractility, compared with the control. The effects were prominent in the jejunum, resulting in approximately 2-fold increases, when compared with the control. Furthermore, the interval of migrating motor complex (MMC) was measured from the end of one activity to the end of the next one. The MMC occurred at regular intervals of approximately 10.1±1.3 min in the control group. Injections of THB resulted in significant shortening of the interval between the MMC in the duodenum (7.3±1.1 min, p<0.05) and jejunum (7.4±0.4 min, p<0.01), while significant effects were not observed in the antrum (Fig. 3D). These results suggested that THB could enhance gastro motor function in the upper GI tract through strengthening of the contractility and shortening of the contraction interval.

**Fundic relaxing effects of THB.** Because DA-9701 not only enhanced gastric accommodation in Beagle dogs, but we also determined the binding affinity of THB on the 5HT₁A receptor (Table 2), we attempted to determine whether THB has the ability to perform
fundic relaxant activities, using two animal models. We first harbored rats with impaired gastric compliance by repeated restraint stress. Rats were restrained in small plastic bags for 4 h per day for 4 consecutive days; balloons connected with a barostat were then inserted into the stomach for measurement of the volume-pressure relationship. A significant shift (p<0.05) of pressure-volume curve was observed in stressed rats towards the lower volume, compared with control rats, indicating impaired gastric compliance, resulting in a significantly lower maximal gastric volume (data not shown). Intravenous administration of THB after maintenance of the intragastric volume at a stable baseline value resulted in a significantly higher shift in pressure-volume curve in rats treated with the doses of 10 µg/kg (Fig. 4C) and 30 µg/kg (Fig. 4D), while doses below 10 µg/kg and 100 µg/kg did showed no significant effects. At ½Pmax, the gastric volume was 9.6±0.8 mL (p<0.05, 10 µg/kg treated rats) vs. 7.6±0.8 mL (control), and in 30 µg/kg treated rats, the value was 9.5±1.0 mL (p<0.05) vs. 6.8±0.9 mL (control). The maximum delta volume (approximately 2 mL) at ½Pmax was obtained in rats treated with 10 µg/kg of THB (Fig 4F). In addition, in an effort to understand the mode of action for gastric relaxation, we conducted an antagonist study. WAY100635, a 5HT1A receptor antagonist, was chosen based on data from the receptor binding assay of THB on the 5HT1A receptor, and, additionally, other antagonists of signaling molecules, including NO and VIP, were also used (Desai et al., 1991). WAY100635 was administrated (0.1 mg/kg, i.v.) 10 min before administration of THB (10 or 30 µg/kg, i.v.) at doses presenting significant efficacy. THB induced an increase of gastric volume that was significantly inhibited by WAY 100635. Delta volume at ½Pmax was lowered to 0.02 mL (Fig. 5). Administration with L-NAME (10 mg/kg), a NOS inhibitor, also resulted in significant inhibition of delta volume at ½Pmax (0.2±0.1 mL vs. 1.5±0.3 mL, p<0.05). In addition, in order to exclude nonspecific effects of L-NAME, we used L-arginine, an NO precursor.
When L-arginine (100 μM/kg) was administered, L-NAME restored the gastric volume to the levels induced by THB, indicating involvement of NO. However, administration of VIP antagonist (30 nmol/kg) did not result in any changes in gastric volume induced by THB. As shown in Fig. 5, Delta volume at ½Pmax was 1.0±0.3 mL. We then assessed the effects of THB on gastric accommodation by oral administration in Beagle dogs surgically implanted with a barostat. Postprandial gastric volume was evaluated by three time intervals; 10-35 mins, 35-60 mins, and 10-60 mins after each meal (Fig. 6A). In dogs who received 30 μg/kg of THB, postprandial volume was significantly higher than that of the control for each time interval (p<0.01), which was comparable with those of sumatriptan (3 mg/kg, i.v.), a potent fundic relaxant. Area under the volume versus time curve (AUC) was significantly increased in dogs treated with THB (Fig. 6B). Taken together, these data suggested the potential of THB as a fundic relaxant for increase of gastric accommodation.
Discussion

This study began from DA-9701, an herb-based gastroprokinetic agent formulated with *Pharbitis Semen* and *Corydalis Tuber* (Lee et al., 2008). A phase III clinical trial of DA-9701 for FD has recently been completed, and we are anticipating DA-9701 as a novel natural medicine for treatment of FD in Korea. In this study, we provided several pieces of evidence to show that tetrahydroberberine (THB), an isoquinoline alkaloid isolated from *Corydalis Tuber*, is one of the active component(s) responsible for gastroprokinetic and fundic relaxing activity. THB has been known as an antidopaminergic agent; however, for the first time, we recognized THB as an agonist for the 5-HT$_{1A}$ receptor, and explored the possibility of its possible use for treatment of FD. We demonstrated that THB stimulates gastric emptying and gastrointestinal contractility, as well as enhances gastric accommodation in conscious animals, which might be mediated by dopamine D$_2$ and/or serotonin 5-HT$_{1A}$ receptors. These findings suggested that THB may have potential as a novel agent in treatment of FD associated with gastric motor dysfunction.

The extent of dopaminergic innervations and its roles in the gut are not completely understood; however, dopamine is known to cause potent inhibition of motility, reduced lower oesophageal sphincter tone, reduced gastric tone and intragastric pressure, and decreased antroduodenal GI motility. Stimulation of peripheral dopamine D$_2$ receptor located on the post-ganglionic nerves causes a decrease in acetylcholine release from parasympathetic nerves. Indeed, after worldwide withdrawal of cisapride due to cardiac safety issues, antidopaminergic agents have been exploited clinically for management of gastrointestinal motor dysfunction. Several antidopaminergic agents, including domperidone, metoclopride, and itopride are used primarily for treatment of motility disorders of the upper digestive tract, such as FD, and some agents seem to be beneficial for improvement of
symptoms. However, the toxicity issue is still under consideration. Itopride at a dose of 100 mg/kg and metoclopride at a dose of 10 mg/kg were reported to produce a reduced activity, tremor, and abnormal behavior in dogs (Koizumi H, 1992). Therefore, development of safer and more effective anti-dopaminergic agents for treatment of gastric functional disorders is definitely needed. THB was found to enhance gastric emptying in a bell shaped, dose-dependent manner and the maximal effective dose was 30 μg/kg, while cisapride, a conventional prokinetic used as a control in this experiment, showed an effect comparable to that at a much higher concentration (10 mg/kg), indicating that THB has a superior effect to conventional drugs. Data from a delayed gastric emptying model using apomorphin together with a dopamine D₂ receptor binding assay indicated that THB might propel gastric emptying through dopaminergic antagonism. The effects were prominent at the dose of 10 μg/kg, which was equivalent to those of itopride at the dose of 30 mg/kg. In addition, intravenous injection of THB resulted in significantly increased gastrointestinal motility. The dose of 10 μg/kg of THB was sufficient for a significant increase of contractility from stomach to jejunum. Taken together, THB appears to be a new antidopaminergic prokinetic.

Both intravenous injection and oral administration of THB resulted in significantly increased gastric accommodation in restraint-stressed rats and conscious dogs, respectively. Currently, it has been accepted that fundic relaxation can be achieved by agonists for 5-TH₁ receptor subtypes, including the 5-HT₁A and/or 5-HT₁B/D receptors. Activation of the 5-HT₁A receptor causes release of nitric oxide (NO) for relaxation of gastric fundus and decrease of gastric fundus tone (Desai et al., 1991; Coulie et al., 1999). Sumatriptan, a 5-HT₁B/D receptor agonist, has been reported to induce a fast and profound relaxation of the proximal stomach in both canine models and human studies (Tack et al., 2000); however, its cost and mode of administration appear to be unsuitable for chronic treatment of FD. The non-selective 5-HT₁A
receptor agonist, buspirone, reduces fundic tone and enhances meal-induced relaxation, and enhances gastric accommodation and gastric emptying in humans in a dose-dependent manner; however, due to central side effects, it is not suitable for routine clinical use for treatment of FD (Koizumi H, 1992). THB not only binds the 5-HT$_{1A}$ receptor but also induced a significant increase of gastric volume (at the dose of 10 $\mu$g/kg) in rats stressed by restraint. The relaxing effects were almost completely blocked by WAY 100635, a 5-HT$_{1A}$ receptor antagonist, and we also observed that treatment with NOS inhibitor resulted in significant suppression of THB-induced increase of gastric volume, which was reversed by NO donor, while VIP antagonist was not able to affect the gastric volume increase by THB. In addition, we recognized that oral administration of THB resulted in significantly increased postprandial gastric volume in conscious dogs and that the effects at the dose of 30 $\mu$g/kg were comparable to those of sumatriptan (3 mg/kg). Based on our data, THB is an orally available as well as injectable fundic relaxant acting on the 5-HT$_{1A}$ receptor. For safety, we performed testing to determine whether THB can induce ataxia, a serious side effect of the 5-HT$_{1A}$ receptor agonist, using the rotarod assay (Millan et al., 1996). THB (0.3 mg/kg, i.v) did not show any rotarod performance at 30 min post administration, whereas buspiron (3 mg/kg, i.v.) produced a significant decrease in rotarod performance (data not shown). Taken together, our study suggested that THB has significant potential as a therapeutic for FD, for enhancement of gastric motor function. Although the question of whether and/or how dual activities on dopamine and serotonin by THB work synergically or co-operatively on gastric emptying and gastric accommodation remains to be elucidated, considering the superior effects of THB to the conventional drug and its safety, further research for its development as medicine is clearly needed.
Authorship Contributions

Participated in research design: Miwon Son, Tae Ho Lee, Mirim Jin

Conducted experiments: Ki Hyun Kim, Sung Ok Lee

Performed data analysis: Kang Ro Lee, Mirim Jin

Wrote or contributed to the writing of the manuscript: Mirim Jin
References


Footnotes

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Figure legends

**Figure 1** Structure of tetrahydroberberine

**Figure 2** Effect of tetrahydroberberine on gastric emptying (GE). (A) In normal rats, after 24 h of fasting, animals ($n=8$ for each group) received oral administration with tetrahydroberberine (THB) and cisapride (Cisa) at the indicated doses, or with 3% HPMC as a vehicle (Con). (B) Effects of tetrahydroberberine on delayed gastric emptying. In an apomorphine induced delay model, animals received oral administration with tetrahydroberberine (THB) and itopride (Ito) at the indicated doses, or with 3% HPMC as a vehicle (Con), and were simultaneously injected with apomorphine (s.c., 0.05 mg/Kg). Naïve animals were not injected with apomorphine and received oral administration with the vehicle. % gastric emptying was calculated as described in the Materials and Methods section. *$p<0.05$, **$p<0.01$ vs. control. ‡$p<0.01$ vs. normal (one way ANOVA with post-hoc Dunnet’s test).

**Figure 3** Effect of tetrahydroberberine on gastrointestinal motor activity in conscious rats. Tetrahydroberberine (10 μg/kg, i.v.) versus control (□, saline, i.v.) on the different electromyographic parameters (A, peak count; B, amplitude; C, integral area; D, interval of MMC) from the rat gastric antrum at 5 mm proximal to the pylorus, the duodenum, and the jejunum, respectively, at 5 cm and 15 cm distal to the pylorus. *$p<0.05$, **$p<0.01$ vs. control (one way ANOVA with post-hoc Dunnet’s test)

**Figure 4** Pressure-volume relationship after intravenous administration of vehicle (orus,
tetrahydroberberine (THB, ●) in rats: 1 μg/kg (A), 3 μg/kg (B), 10 μg/kg (C), 30 μg/kg (D), 100 μg/kg (E). Note that tetrahydroberberine shifted the pressure-volume curve toward higher volumes. (F) on the y-axis volume recorded at ½Pmax after tetrahydroberberine administration at the indicated dosage. *p < 0.05 vs. stress-free (one way ANOVA with post-hoc Dunnett’s test). Values indicate means ± SEM (n = 4)

**Figure 5** Effect of L-NAME, L-NAME plus L-arginine, VIP antagonist, and WAY 100635 on mean increase of gastric volume at ½Pmax after administration of tetrahydroberberine (THB; 10 μg/kg, i.v.). Tetrahydroberberine-induced rat gastric relaxation was antagonized by the nitric oxide synthase inhibitor L-NAME and 5-HT1A receptor antagonist and WAY 100635, indicating involvement of nitrergic nerves and 5-HT1 receptor subtypes. *p < 0.05 vs. vehicle

**Figure 6** Effects of tetrahydroberberine (THB, 30 μg/kg, p.o.) on gastric accommodation in Beagle dogs. (A) Intragastric volume as determined with a barostat at MDP ± 2 mmHg. (B) Area under the volume versus time curve (AUC) due to administration of tetrahydroberberine or control during each time interval. *p < 0.05, **p < 0.01 vs. control
Table 1. Experimental conditions for determination of affinities at native and recombinant receptors *in vitro*.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Source</th>
<th>Radioligand (nM)</th>
<th>Kd (nM)</th>
<th>Non-specific (μM)</th>
<th>Inc. buffer¹</th>
<th>Inc. time (min) &amp; temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT₁ₐ</td>
<td>CHO-K1</td>
<td>[³H]-8-OH-DPAT (0.25)</td>
<td>0.29</td>
<td>Metergolin (10)</td>
<td>A</td>
<td>60 min, 27 °C</td>
</tr>
<tr>
<td>5-HT₁₈</td>
<td>SD cerebral cortex</td>
<td>[¹²⁵I]-Cyanopindolol (0.01)</td>
<td>0.19</td>
<td>Serotonin (10)</td>
<td>B</td>
<td>90 min, 37 °C</td>
</tr>
<tr>
<td>5-HT₃</td>
<td>HEK-293</td>
<td>[³H]-GR65630 (0.55)</td>
<td>0.57</td>
<td>MDL-72222 (10)</td>
<td>C</td>
<td>60 min, 25 °C</td>
</tr>
<tr>
<td>5-HT₄</td>
<td>Hartley GP striatum</td>
<td>[³H]-GR113808 (0.70)</td>
<td>0.14</td>
<td>Serotonin (30)</td>
<td>D</td>
<td>30 min, 25 °C</td>
</tr>
<tr>
<td>D₂S</td>
<td>CHO-K1</td>
<td>[¹²⁵I]-Iodospiperone (0.27)</td>
<td>0.12</td>
<td>Haloperidol (5)</td>
<td>E</td>
<td>120 min, 25 °C</td>
</tr>
</tbody>
</table>

¹Buffer A: 50 mM Tris-HCl (pH 7.4), 10 mM MgSO₄, 0.5 mM EDTA, 0.1 % ascorbic acid; Buffer B: 50 mM Tris-HCl (pH 7.4), 154 mM NaCl, 10 μM Pargyline, 30 μM Isoprenaline; Buffer C: 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM EDTA; Buffer D: 50 mM Tris-HCl (pH 7.4); Buffer E: 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 1 mM EDTA.
Table 2 Binding affinities (pKi) for serotonin receptor agonists and antagonists at different receptors involved in GI functions.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>THB</th>
<th>Cisa</th>
<th>Mosa</th>
<th>Suma</th>
<th>Busp</th>
<th>WAY</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
<td>5.38</td>
<td>&lt;6.00</td>
<td>&lt;5.00</td>
<td>6.43</td>
<td>7.50</td>
<td>9.05</td>
<td>7.58</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;1B&lt;/sub&gt;</td>
<td>&lt;5.00</td>
<td>&lt;6.00</td>
<td>&lt;5.00</td>
<td>7.60</td>
<td>&lt;5.00</td>
<td>5.88</td>
<td>9.18</td>
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<tr>
<td>5-HT&lt;sub&gt;1D&lt;/sub&gt;</td>
<td>n.d.</td>
<td>&lt;5.00</td>
<td>n.d.</td>
<td>7.92</td>
<td>5.82</td>
<td>6.48</td>
<td>8.41</td>
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<tr>
<td>5-HT&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>3.69</td>
<td>&lt;6.00</td>
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<td>5-HT&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>n.d.</td>
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<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6.08</td>
<td>3.43</td>
<td>&lt;4.00</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

1Tetrahydroberberine; in this study, 2Cisapride;(Yoshikawa et al., 1998), 3Mosapride;(Kakigami et al., 1998), 4Sumatriptan; (Leysen et al., 1996), 5Buspirone;(Koek et al., 1998), 6WAY 100635;(Gommeren W, 1998), 7GR 127935; (Gommeren W, 1998)
Figure 1
Figure 2

A

Gastric emptying (%)

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Cisa (mg/kg)</th>
<th>THB (μg/kg)</th>
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<tbody>
<tr>
<td>-</td>
<td>25</td>
<td>65</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>90</td>
<td>65</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>70</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>85</td>
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</tr>
<tr>
<td>30</td>
<td>50</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>100</td>
<td>55</td>
<td>95</td>
<td>65</td>
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</tbody>
</table>

B

Gastric emptying (%)

<table>
<thead>
<tr>
<th></th>
<th>Naïve</th>
<th>Con</th>
<th>THB (mg/kg)</th>
<th>Ito (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>60</td>
<td>50</td>
<td>50</td>
<td>50</td>
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<tr>
<td>-</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
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<tr>
<td>30</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>
Figure 3

(A) Peak count (min⁻¹)

(B) Amplitude (mV)

(C) Integral area (mV·sec)

(D) Interval of MMC (min)
Figure 4

A - C: Graphs showing the relationship between gastric volume (mL) and gastric pressure (mmHg) for different doses of THB (1µg/kg, 10µg/kg, 30µg/kg, 100µg/kg) compared to control. Each graph includes error bars indicating variability.

D: Graph showing the differences in gastric volume at half maximum (Δvol at ½Pmax) for different doses of THB (1µg/kg, 3µg/kg, 10µg/kg, 30µg/kg, 100µg/kg). The histogram includes error bars indicating variability.
Figure 6

A

Gastric volume (mL) vs. Time (min)

- Control (vehicle)
- Sumatriptan (3mg/kg)
- THB (30μg/kg)

B

Area under the volume versus time curve (AUC, x10^3 mL/sec)

- Control (vehicle)
- THB (30 μg/kg, p.o.)

Bars represent AUC for different time intervals:
- AUC 10-35min
- AUC 35-60min
- AUC 10-60min