Transient receptor potential ankyrin 1 agonists improve intestinal transit in a murine model of postoperative ileus

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Key Points
- TRPA1 expresses in enterochromaffin cells (ECC) and may be involved in gut motility.
- Here, we investigated the effect of TRPA1 agonists, including high-quality traditional Japanese medicine daikenchuto (TU-100), in three different systems: (i) a manipulation-induced postoperative ileus model, (ii) in vitro culture of small intestinal (SI) segments and (iii) a model ECC.
- TRPA1 was found to be indispensable for coordinated peristaltic motility and TRPA1 agonists improve SI transit. Further research is required to determine whether ECC are involved.

Abstract
Background Stimulation of transient receptor potential ankyrin 1 (TRPA1), which abundantly expressed in enterochromaffin cells (ECC), has been reported to exert apparently contradictory results in in vitro contractility and in vivo gastrointestinal (GI) transit evaluations. The pharmaceutical-grade Japanese traditional medicine daikenchuto (TU-100) has been reported to be beneficial for postoperative ileus (POI) and accelerate GI transit in animals and humans. TU-100 was recently shown to increase intestinal blood flow via stimulation of TRPA1 in the epithelial cells of the small intestine (SI). Methods The effects of various TRPA1 agonists on motility were examined in a manipulation-induced murine POI model, in vitro culture of SI segments and an ECC model cell line, RIN-14B. Key Results Orally administered TRPA1 agonists, aryl isothiocyanate (AITC) and cinnamaldehyde (CA), TU-100 ingredients, [6]-shogaol (6S) and γ-sanshool (GS), improved SI transit in a POI model. The effects of AITC, 6S and GS but not CA were abrogated in TRPA1-deficient mice. SI segments show periodic peristaltic motor activity whose periodicity disappeared in TRPA1-deficient mice. TU-100 augmented the motility. AITC, CA and 6S increased 5-HT release from isolated SI segments and the effects of all these compounds except for CA were lost in TRPA1-deficient mice. 6S and GS induced a release of 5-HT from RIN-14B cells in a dose- and TRPA1-dependent manner. Conclusions & Inferences Intraluminal TRPA1 stimulation is a potential therapeutic strategy for GI motility disorders. Further investigation is required to determine whether 5-HT and/or ECC are involved in the effect of TRPA1 on motility.

Keywords aryl isothiocyanate, daikenchuto, enterochromaffin cells, shogaol, γ-sanshool.

Abbreviations: 6G, [6]-gingerol; 6S, [6]-shogaol; AITC, allyl isothiocyanate; CA, cinnamaldehyde; ECC, enterochromaffin cell; ECC-TRPA1, TRPA1 on enterochromaffin cells; EN-TRPA1, TRPA1 on enteric neurons; EPC, epithelial cell; EPC-TRPA1, TRPA1 on epithelial cells; GI, gastrointestinal; GS, γ-sanshool; HAS,
hydroxy-α sanshool; IM, intestinal manipulation; NO, nitric oxide; PG, prostaglandin; POI, postoperative ileus; SMC, smooth muscle cells; TRPA1, transient receptor potential ankyrin 1; TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid type 1; TU-100, daikenchuto.

INTRODUCTION

Transient receptor potential ankyrin 1 (TRPA1) was first identified as a cold-sensitive cation channel in mouse sensory neurons and is believed to play a role in pain sensation. Transient receptor potential ankyrin 1 is activated by various compounds such as allyl isothiocyanate (AITC), found in mustard and wasabi, cinnamaldehyde (CA), found in cinnamon, and allicin (found in garlic). In the gastrointestinal (GI) system TRPA1 is predominantly expressed in visceral (vagal, splanchnic and pelvic) afferents especially in nerve endings at peripheral sites where mechanical stimuli are transduced. Transient receptor potential ankyrin 1 is thought to be involved in enhanced visceral hyperalgesia after colitis, and is activated by various compounds such as allyl isothiocyanate (AITC; found in garlic). In the gastrointestinal (GI) system TRPA1 is predominantly expressed in visceral (vagal, splanchnic and pelvic) afferents especially in nerve endings at peripheral sites where mechanical stimuli are transduced. Transient receptor potential ankyrin 1 is thought to be involved in enhanced visceral hyperalgesia after colitis, which might last for an extended duration even until adulthood when induced in neonatal animals. Furthermore, TRPA1 activation has been shown to be directly involved in the maintenance and progression of several experimental inflammatory models via neurogenic inflammation.

Transient receptor potential ankyrin 1 protein has also been detected in other cells in the GI tract such as enteric nerves, enteroendocrine cells, and intestinal epithelial cells (EPCs). These findings suggest a broad range of physiological and pathological roles for TRPA1. We and other groups have shown that TRPA1 on the EPCs play important roles in adrenomedullin-mediated vasodilatation and prostaglandin-mediated anion secretion, respectively. Abundant expression of TRPA1 in enterochromaffin cells and in a subset of enteric neurons strongly suggest that TRPA1 may be involved in the control of GI motility. Although significant research effort has been directed towards elucidating the role of TRPA1 in gut motility, there remain many unanswered questions. The effects of TRPA1 agonists have been found to be different, even sometimes apparently opposing, depending on the species, region of the intestines, conditions of the specimen and methodology used for evaluation (Table 1). Thus, it is still unclear whether TRPA1 is a suitable target for novel therapeutic agents for GI motility disorders.

In our previous study, we demonstrated that a traditional Japanese medicine daikenchuto (TU-100) and its major ingredients gingerols and shogaols (derived from a TU-100 component herb, ginger) stimulated EPC-TRPA1, and resulted in the release of adrenomedullin and an increase in intestinal blood flow. TU-100 is a pharmaceutical-grade traditional Japanese (Kampo) medicine and has been widely used for the treatment of various GI diseases, especially for paralytic postoperative ileus (POI). A double-blind, placebo-controlled study on healthy volunteers in the U.S. has shown that treatment with TU-100 significantly accelerates ascending colon emptying. The efficacy of TU-100 on POI in patients with hepatic resection and 5-HT receptor activation, although its molecular mechanism has not been elucidated. TU-100 is a pharmaceutical-grade traditional Japanese (Kampo) medicine and has been widely used for the treatment of various GI diseases, especially for paralytic postoperative ileus (POI). A double-blind, placebo-controlled study on healthy volunteers in the U.S. has shown that treatment with TU-100 significantly accelerates ascending colon emptying. The efficacy of TU-100 on POI in patients with hepatic resection and 5-HT receptor activation, although its molecular mechanism has not been elucidated. Here, we have investigated whether TRPA1 stimulation is involved in the effects of two TU-100 ingredients, AITC and CA, using the well-established murine POI model and an in vitro motility/5-HT secretion assay.

MATERIALS AND METHODS

Animals

C57Bl/6 mice were from Charles River Laboratories Japan Inc. (Yokohama, Kanagawa, Japan). Transient receptor potential vanilloid type 1 (TRPV1)-deficient B6;129X1-Trpv1<tm1Jul>/? and TRPA1-deficient B6;129P-Trpa1<tm1Kykw>/? mice originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) were maintained in Charles River Laboratories Japan Inc. and transported to the animal facilities of Tsumura Laboratories at 8 weeks old. The animals were allowed free access to water and standard laboratory food, housed at a temperature of 23 ± 2 °C with a relative humidity of 55 ± 10%, and a 12 : 12-h light/dark cycle with lights on from 07:00 to 19:00 daily. All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Tsumura & Co. Ethical approval for the experimental procedures used in this study was obtained from the Laboratory Animal Committee of Tsumura & Co. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (#13-016, 28 August 2013).

POI model

Age-matched male wild-type and TRPA1-deficient mice (9–33 weeks old) were used. However, due to the considerably decreased fertility of TRPA1-deficient mice, it was sometimes difficult to obtain sufficient numbers of age-matched animals. Consequently, it was necessary to use mice with a broad range of ages in some experiments. The adult wild-type and TRPA1-deficient mice used in this study have been found to have similar GC values irrespective of age. The mice were anesthetized with inhaled isoflurane, and then the abdomen was opened by midline laparotomy. The intestine was eventrated and then the colon was gently compressed along its entire length by using moistened sterile cotton applicators. The bowel was repositioned in the
abdominal cavity, and the incision was closed with two layers of continuous sutures. The duration of the surgical procedure was approximately 15 min. Animals moved freely about their cage within 20 min of anesthetic withdrawal.

GI transit

For estimation of GI transit, mice were gavaged with 250 mg/10 mL/kg of fluorescein-labeled dextran of 70 000 MW (FD70; Sigma-Aldrich, St. Louis, MO, USA) and test drugs or vehicle (saline). After 30 min, mice were euthanized by decapitation and the GI tract was immediately excised. Fluorescence was visualized and quantified using the G-box system (Syngene, Cambridge, UK)24 and the geometric center was calculated using the formula: Σ(% FD70 per segment segment number)/100.

Ca²⁺ flux assay

Ca²⁺ flux assay was performed with a FLIPR Calcium 5 Assay Kit (Molecular Devices, Sunnyvale, CA, USA) following the supplied instructions. 293A cells transiently expressing human TRPV1 or TRPA1, which were generated in our laboratory according to the standard procedure, were incubated with Ca²⁺ chelating dye dissolved in Assay Buffer [20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂ and 13.8 mM D-glucose, pH 7.4] at room temperature. After 30 min, the plates were assayed in FlexStation 3 (Molecular Devices). The baseline level of fluorescence was measured 20 s before test ligand application. The response was expressed as percentage activation (% activation = 100 × (RFUmax / C₀ RFUbaseline)/RFUbaseline) / C₀ 100). The dose response curve was fitted to the sigmoidal dose response equation (Top: max response of each ligand) and EC₅₀ values were calculated using GraphPad Prism 3.0 software (Graphpad Software Inc. La Jolla, CA, USA).

Isolation of mouse small intestinal tract and measurement of intraluminal pressure

Mice were fasted overnight and euthanized by decapitation before removing their entire SI. A 2- to 3-cm segment of the small intestine (SI) was placed in an organ bath (100 mL volume), which was continuously perfused with warm Krebs solution (3.5 mL/ min, 34–35 °C). The oral and aboral ends of the SI segment were securely attached with string to saline input and output ports respectively. In order to monitor intraluminal pressure (cmH₂O), a Micro-Tip catheter pressure transducer (SPR-524; Millar

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### Table 1 Summary of the effects of TRPA1 agonists on GI motility

| Agents (dose) | Animals | Sites | Outcome | TRPA1 antagonism | Ref |
|--------------|--------|------|---------|------------------|----|
| **Contractility assay in vitro** | | | | | |
| AITC (100 μM) | ddY mouse | Proximal colon | Contract | NT | 17825279 |
| CA (100 μM) | B6 mouse | Proximal colon | Inhibit | KO | 21689654 |
| AITC (100 μM) | ddY mouse | Distal colon | Contract | NT | 17825279 |
| CA (100 μM) | B6 mouse | Duodenum | No effect | NA | 21689654 |
| AITC (100 μM) | ddY mouse | Jejunum | No effect | NA | 17825279 |
| AITC (300 μM) | Guinea pig | Ileum | No effect | RR | 19211797 |
| AITC (100 μM) | ICR mouse | Ileum | Inhibit | ×HC | 21955242* |
| AITC (200 μM) | Guinea pig | Small intestine | Contract | ×HC | 23438208 |
| CA (100 μM) | B6 mouse | Gastric antrum | No effect | NA | 21689654 |

| Agents (dose) | Administration route | Sites | Outcome | TRPA1 antagonism | Ref |
|--------------|---------------------|------|---------|------------------|----|
| **Contractility in vivo (dog)** | | | | | |
| AITC (1 mg/kg) | p.o. | Gastric antrum | Contract | RR | 19576208 |
| AITC (1 mg/kg) | p.o. | Jejunum | Contract | RR | 19576208 |
| AITC (1 mg/kg) | p.o. | Colon | GMC† | RR | 19576208 |

| Agents (dose) | Administration route | Animals | Outcome | TRPA1 antagonism | Ref |
|--------------|---------------------|--------|---------|------------------|----|
| **Gastric emptying** | | | | | |
| AITC (0.25 μmol/mouse) | p.o. | B6 mouse | No effect | NA | 21689654 |
| CA (10 mg/kg) | p.o. | ICR mouse | Delay | HC, RR | 23990963 |
| Methyl Syringate (10 mg/kg) | p.o. | ICR mouse | Delay | HC, RR | 23990963 |

| Agents (dose) | Administration route | Sites | Outcome | TRPA1 antagonism | Ref |
|--------------|---------------------|------|---------|------------------|----|
| **SI transit** | | | | | |
| AITC (0.25 μmol/mouse) | p.o. | B6 mouse | No effect | NA | 21689654 |
| AITC (10 μg/kg) | p.o. | ICR mouse | Enhance | ×HC | 21955242 |
| AITC (10 μg/kg) | i.p. | ICR mouse | Inhibit | ×HC | 21955242 |
| Cannabichromene (10 μg/kg) | i.p. | ICR mouse | Normalize | ×HC | 22300105‡ |

| Agents (dose) | Administration route | Sites | Outcome | TRPA1 antagonism | Ref |
|--------------|---------------------|------|---------|------------------|----|
| **Colonic transit** | | | | | |
| AITC (0.25 μmol/tat) | Intracolon | B6 mouse | Inhibit | HC, KO | 21689654 |
| ASP7663 (0.3 mg/kg) | p.o. | ddY mouse | Enhance | HC | 24291101§ |
| ASP7663 (1 mg/kg) | i.v. | ddY mouse | No effect | NA | 24291101§ |

* vs, spontaneous, Ach-, EFS-induced contraction. † Giant migrating complex (GMC) was induced. ‡ Drug-induced hypermotility model. § Drug-induced constipation model. Ref are indicated by PubMED ID. B6 mouse, C57Bl/6 mouse; HC, HC030031; KO, gene knockout; NA, not applicable; NT, not tested; RR, ruthenium red; ×HC, not inhibited by HC.
Instruments, Houston, TX, USA) was set in the lumen of the aboral end. Motility was initiated by applying an intraluminal pressure of approximately 4 cmH2O by elevating the drain tube. After an equilibration period of 15 min, contraction had reached a consistent pattern, in terms of amplitude and frequency. The intraluminal pressure waves were evaluated by a data acquisition and analysis system (MP100; BIOPAC Systems Inc., Goleta, CA, USA). The motility was macroscopically observed through video images (PCR-SR87; SONY, Tokyo, Japan). All drug solutions were infused with the input Krebs solution. Peak frequency (PF) was calculated as the mean number of pressure peaks per minute during a defined period. The peak pressure amplitude (PA) was calculated as the mean pressure of peaks during an allotted time period. The %-PF and %-PA of each period were calculated as the ratio to the PF and PA before drug treatment respectively.

Tissue and cell culture procedures

The mouse SI was processed for tissue culture \( n = 3–7 \), from age-matched mice. Briefly, the isolated tract (about 20 cm) was cultured \( 37^\circ C, 5\% CO_2 \) in 48-well tissue culture plates, with each well containing 360 \( \mu L \) of Hank’s Balanced Salt Solution (HBSS) supplemented with 0.1 mmol/L pargyline hydrochloride. The pargyline hydrochloride, a selective MAO inhibitor that blocks the breakdown of 5-HT, was added to evaluate the net production/release of 5-HT from the tissue.25 Aryl isothiocyanate, CA and 6SG were dissolved in HBSS containing 3% DMSO at a concentration of 10 mg/mL and 40 \( \mu L \) of each sample was added per well to the cultures and incubated for 120 min. Culture supernatants were then centrifuged at 14 000 \( g \) for 5 min at 4 \( ^\circ C \). Vehicle (DMSO) did not affect 5-HT release. The supernatants were collected and frozen at \(-80^\circ C\). Rat pancreatic endocrine cell line RIN-14B was cultured in DMEM containing 5% FBS in 24-well tissue culture plates. Test samples were added to the cultures and incubated for 20–60 min and the culture supernatants collected and frozen at \(-80^\circ C\).

Measurement of 5-HT levels

5-HT levels in the culture supernatants were measured by EIA (BA E-9800; Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany) or high performance liquid chromatography (HPLC). The HPLC system (LaChromUltra; HITACHI, Tokyo, Japan) was used with an Inertsil® ODS-3 analytical column (3 mm i.d. \( \times \) 10 mm, 3 \( \mu m \) particles, run at 40 \( ^\circ C \); GL Science Ltd., Tokyo, Japan). The mobile phase consisted of 16% methanol and 84% 0.1 mol/L acetic acid-citric acid buffer (pH 3.5), which contained 200 mg/mL 1-octanesulfonic acid sodium salt and 5 mg/L EDTA-2Na. The flow rate was 0.45 mL/min. The oxidation potential of the electrochemical detector (Shideido Co. Ltd, Tokyo, Japan) was set at +750 mV.

Statistical analysis

Data are expressed as mean \( \pm \) SEM. We performed an unpaired Student’s t-test to analyze differences between two groups of mice (Figs 5–7). Among three or more groups of mice, the Dunnett’s test for multiple comparison for Figs 1C, 3C, D, 8A, B and two-way ANOVA followed by post hoc Tukey’s test for Figs 1A, 2A, B, 8C were used respectively. \( p < 0.05 \) was considered a significant difference.

RESULTS

Oral administration of TU-100 improved the small intestinal transit 24 h after surgical intestinal manipulation (IM; Fig. 1A). The effect of TU-100 was dose-dependent because TU-100 (2700 mg/kg) significantly improved GI transit also in TRPV1-deficient (TRPV1 KO, B) and TRPA1-deficient (TRPA1 KO, C) mice, though the effects appeared slightly weaker. \( ^* p < 0.05, ^{**} p < 0.01, ^{***} p < 0.001 \).

N = 10–11 for each group.
dependent but the statistically significant amelioration was observed at 2700 mg/kg BW (data not shown). An effect was observable on administration at 23.5 h after IM, with inflammation having already been established, suggesting TU-100 has a therapeutic effect on POI. As fluorescence was virtually undetected in the large intestine, the present experimental protocol could evaluate the upper GI transit only. A significant effect on GC by TU-100 was not seen in control (anesthesia only) mice. When TU-100 was administered to TRPV1-deficient mice (Fig. 1B) or TRPA1-deficient mice (Fig. 1C), its ameliorating effect was still observed. However, when the ratio of GC value of TU-100-treated, manipulated mice to that of vehicle-treated, manipulated mice was calculated as the percentages (that is, the average GC in vehicle-treated, manipulated mice was normalized to 100), the ratio was, 124.37 ± 5.85% (p = 0.0062, n = 11), 111.36 ± 4.29% (p = 0.0429, n = 10) and 116.22 ± 5.32% (p = 0.0309, n = 10) in WT, TRPV1-deficient and TRPA1-deficient mice, respectively (p-value represents the statistical difference by unpaired t-test between TU-100-treated manipulated mice and vehicle-treated manipulated mice in each strain). These results suggest both TRPV1 and TRPA1 may be involved in the effect of TU-100. Accordingly, TRPV1 has been reported to be involved in the prokinetic effect of TU-100 or its major ingredients in vivo \(^{26,27}\) and in vitro. \(^{28}\) In contrast, although the involvement of TRPA1 has been reported in the vasodilatory \(^{12}\) and anti-colitis \(^{29}\) effects of TU-100, its impact on motility has not been examined. Therefore, in this study, we have focused our investigations on TRPA1 agonists AITC and CA and the ingredients of TU-100.

Fig. 2 shows the TRPA1 and TRPV1 agonistic activities of these ingredients in vitro. As previously reported, [6]-shogaol [6S], [6]-gingerol [6G], and hydroxyl-α sanshool [HAS] induced Ca\(^{2+}\) influx in both TRPA1- and TRPV1-expressing 293A cells, although the specific activity and maximal response of HAS was very small compared to other compounds including AITC and CA. A Japanese pepper ingredient γ-sanshool (GS) affected TRPV1 only modestly, but strongly stimulated TRPA1. The specific activity and maximal response of GS were comparable to those of 6S and 6G. Thus, subsequent experiments focused on 6S and GS among the TU-100 ingredients.

Aryl isothiocyanate, CA, 6S and GS showed an ameliorating effect on the POI model as shown in Fig. 3A and B. The effects of AITC and CA were dose-dependent but the statistically significant amelioration was observed at 50 mg/kg BW for AITC and 100 and 300 mg/kg BW for CA respectively (data not shown). No effect was observed in TRPA1-deficient mice for AITC, 6S and GS (Fig. 3C and D). The effect of CA was unchanged by TRPA1 deficiency (Fig. 3C). When the agents were administered to control (anesthesia only) mice, AITC, CA and 6S had no significant effect on GC while GS significantly increased GC.

Next, we investigated in vitro peristalsis of isolated SI. Segments of SI were placed in an organ bath that was continuously perfused with Krebs solution. The oral and aboral ends of the segment were securely attached to an input and output port of the solution and intraluminal pressure and motor patterns were then monitored using a pressure transducer and video camera respectively. In the steady state, an intermittent peristaltic movement was seen to commence in conjunction with a pressure peak, which then immediately ceased (Video S1, left panel). This pattern was repeated at regular intervals. As shown in Fig. 4A, although the amplitudes of the pressure peaks were different among the tested segments, the peak frequencies were similar in almost all cases. However, the segments isolated from TRPA1 KO mice had the highest peak frequencies but with little or no periodicity, having a large variation in frequencies among the segments, as shown in Fig. 4B, the histogram of PF values. Quantitative analyses of pressure peak shown in Fig. 5 revealed that the contractions of SI in TRPA1-
deficient mice have the following characteristics compared to WT mice: (i) higher frequency of peaks, (ii) similar peak amplitude of the highest peak, and (iii) lower peak amplitude of the lowest peak. These data clearly indicate that the peak amplitude of the pressure in TRPA1-deficient mice did not change. However, in KO mice the prominent increase in the number of peaks and the downside of the lowest peak decreased the average area under the curve (AUC) of peak amplitude. Video images (Video S1, right panel) demonstrate frequent, irregular and unorganized peristaltic motor activity. These data suggest that regulation of periodic peristaltic motility was lost in TRPA1-deficient mice.

Fig. 6 and Video S1 [right panel] show the effect of addition of TU-100 via a perfusion solution to the oral end of SI segments of wild type mice. After a time lag of around 10 min following commencement of TU-100 infusion, which is thought to be necessary for the concentration of TU-100 to reach an effective dose, the intervals between the peaks reduced [shown by the increased PF] with no effect on the intensity [shown by unaffected PA]. Removal of TU-100 from the perfusion solution resulted in the gradual return to the former control peak profiles [data not shown]. The data of intraluminal pressure in Fig. 6 and video images in Video S2 strongly suggest that TU-100 may enhance SI motility via increasing the frequency of peristaltic motion. It should be noted that, although PF increased, the peaks appeared at even intervals. Addition of TU-100 to the Krebs solution in the bath, outside of the intestinal tract [i.e., from the serosal side] did not give clear effects [data not shown].

Release of 5-HT from SI segments in vitro was also examined. Addition of AITC, CA, or 6S to the in vitro culture of intestinal segments resulted in the elevation of 5-HT concentration in the culture supernatant [Fig. 7A–C]. The effect was absent when using the segments isolated from TRPA1-deficient mice except for CA, which is in good agreement with the result of the GI transit experiments (Fig. 3C). GS could not be examined due to its poor solubility.

Finally, we also investigated the ingredients for stimulation of release of 5-HT from rat RIN14B cells, a
model cell line of ECCs. 6-shogaol and GS induced the release of 5-HT in a dose-dependent manner (Fig. 8A and B). The effects by these TRPA1 agonists were inhibited by a specific TRPA1 inhibitor AP-18 (Fig. 8C).

DISCUSSION

Previous experimental studies have demonstrated that the pathogenesis of the endogenous component of POI can be divided in two distinct phases. 30,31 The first neural phase, activation of inhibitory neural pathways by nociceptive stimuli, leads to the inhibition of propulsive activity, which ends within several hours after closure of the abdomen. TU-100 was found to be effective against hypomotility during this phase using an acute POI model in rat. 23 There then follows a second immunological phase that can last for days, which is caused by formation of an inflammatory infiltrate in the muscular layers of the intestine resulting in the release of nitric oxide [NO] and PGs and prolonged hypomotility. 24,32–37 In the second phase, inflammation is thought to play a vital role. A wide range of anti-inflammatory treatments have been proposed based on experimental models, including mast cell stabilizers, 32 non-steroidal anti-inflammatory drugs, 38 interleukin-10, 39 blocking antibodies to adhesion molecules, 33,40 and inhibitors of inflammatory signaling cascades. 41–43 Furthermore, various therapeutic candidates including matrix metalloproteinase inhibition, 44 carbon monoxide supply, 45,46 nicotinic acetylcholine receptors, 47 and herbal/nutrient therapies 48–53 have been proposed. However, these therapies require pretreatment before development of the inflammatory phase. In the present study, TU-100 and TRPA1 agonists were administered 23.5 h after IM, when the inflammatory changes including neutrophil
infiltration, NO and PG release and hypomotility have already been established. Therefore, the effects of the agents are not prophylactic but therapeutic. If the mechanism of action of these agents is stimulation of ECC-TRPA1 as discussed below, an ECC-TRPA1-targeted approach, and TU-100 therapy, may have the potential to restore intestinal motility even in the established disease state.

The presence of TRPA1 protein in ECCs and enteric neurons strongly suggests that TRPA1 may be involved in the regulation of GI motor function. Considerable research efforts have been exerted in the investigation of the effect of TRPA1 agonists on GI motility. As shown in Table 1, apparently inconsistent results for the effect of TRPA1 agonists have been observed during in vitro and in vivo motility studies. Specifically, for in vitro contractility assays and in vivo SI transit evaluations in mice, enhancement, inhibition, and no-effect were reported.

It is possible that these discrepancies may be due to differences in animal species/strains, administered agonists, administration doses, administration routes, experimental protocols, health conditions (i.e., diseased or not), evaluation methods, etc. Among these possible causes, the difference in administration routes may deserve further investigation. The presence of

Figure 5 The pressure amplitude of the maximum peak [A] and minimum peak [B] during the defined period, and the average values [C] of PF and pressure peak amplitude [PA] and area under curve [AUC] of the isolated SI of WT and TRPA1 KO mice are shown. In KO mice, PF increased and PA and AUC decreased. ***p < 0.001.

Figure 6 The effect of TU-100 on periodic peristaltic motility in the SI of WT mice. [A] Representative changes of intraluminal pressure in the SI of WT mice induced by TU-100 (10 mg/mL). After a certain time lag [around 10 min] post commencement of TU-100 infusion, which allows the concentration of TU-100 to reach an effective dose, the medicine begins to affect the pressure peak patterns. [B] The changes of %PF and %PA induced by TU-100. %PF and %PA were calculated as described in Materials and Methods. ***p < 0.001 vs Pre, by paired t-test.
TRPA1 protein in ECCs is unsurprising considering the physiological role of the ECCs. Specifically, when TRPA1 agonists in the food stimulate ECC-TRPA1 from inside the lumen, ECCs release 5-HT and stimulate submucosal intrinsic primary afferent neurons, which initiate peristaltic reflexes. The loss of periodic motility in TRPA1-deficient mice demonstrated in Fig. 4 and Supplementary Movie M1 indicates the possible involvement of TRPA1 in the periodic generation of peristaltic motor activity in the SI. These assumptions naturally lead to the hypothesis that oral administration of TRPA1 agonists may result in the enhancement of gut motility. Several investigations have analyzed the effect of oral administration of TRPA1 agonists that appear to support this hypothesis; for example, in vivo contractility evaluation in dogs,\(^5^7\) gastric emptying in mice\(^5^8\) and rats\(^5^9\), SI (Ref. 55 and this study) and colonic transit in mice\(^5^7,6^0\) and dogs.\(^6^1\)

It should be noted that AITC i.p.\(^5^5\) inhibited GI transit whereas ASP7663 i.v.\(^6^0\) had no effect despite the fact that these treatments exerted an enhancing effect by oral administration.

The difference in the effect between peroral and parenteral administrations could be explained by the presence of EN-TRPA1. Pool et al.\(^1^6\) have found that functional TRPA1 protein is expressed on the cell surface of inhibitory motoneurons. The presence of TRPA1 in the inhibitory motoneurons in the enteric nervous system readily leads us to the following hypothesis: i.p. or i.v. injection of TRPA1 agonists may exert inhibitory effects on GI motor function. Furthermore, depending on the timing and the relative degrees of activation of ECC-TRPA1 and EN-TRPA1, the effects of administered TRPA1 agonists may range from enhancement to inhibition. This hypothesis also explains the diversity of the results of in vitro...
contractility assays using Magnus tubes. In such assays, TRPA1 agonists may readily access both ECC-TRPA1 and EN-TRPA1, and therefore the results will vary depending on the physicochemical properties of each TRPA1 agonist, which determines permeability to the deeper layers in the intestinal tissue, and on the expression pattern of TRPA1 in each tissue. Accordingly, in the present experiments on *in vitro* SI motility, the different observed TU-100 effects (enhancement vs inhibition/no-effect) depends on the administration route (intraluminal perfusion vs addition to bath solution outside from SI tract). This hypothesis is also compatible with the results of the study by Pool et al. These workers reported that intracolonic administration of AITC inhibited colonic transit in mice. Given that mouse colon has been reported to contain no TRPA1-expressing ECCs, presumably intracolonic AITC predominantly activates EN-TRPA1.

The beneficial effects of AITC, 6S and GS were absent in TRPA1-deficient mice. Therefore, it is highly plausible that TRPA1 is intimately involved in the observed effects. However, whether these TRPA1 agonists interact directly with TRPA1 is still to be determined in the future. In several reports, the effects of AITC were unaffected by HC-030031. We also observed that the effect of HC-030031 varies depending on the experimental settings (unpublished observation, KT, KK, MY). Aryl isothiocyanate has several other bioactivities, such as inhibition of cell cycle/induction of apoptosis on cancer cells and induction of phase 2 proteins through Nrf2 activation, though detailed molecular mechanisms are still unknown. [6]-shogaol, 6G, HAS and GS have both stimulatory and suppressive activities with respect to various enzymes and ion channels. Therefore, TRPA1 may function as a secondary, or a cooperative, but indispensable, transducer of a primary signal for motility. It should be noted that a similar assumption has been made for the role of TRPA1 in mechanical sensitivity.

Previous studies on TU-100 pharmacology and pharmacokinetics suggest this could be a promising TRPA1-targeting prokinetic drug candidate. Moreover, the administration route for TU-100 (oral only), absorption properties and the conjugation/metabolism of TU-100 are also favorable. The major TRPA1 agonists contained in TU-100 are shogaols and gingerols. Both ginger ingredients are stable in stomach juice and are not absorbed from the stomach (unpublished observations, MY). Specifically, these compounds are transported to the SI and stimulate TRPA1 on the intestinal epithelium, i.e., stimulate ECCs and EPCs to release 5-HT and adrenomedullin. 5-HT triggers peristaltic motility and adrenomedullin increases intestinal blood flow. Shogaols and gingerols are both rapidly absorbed from the SI. However, it appears that most of the gingerols are rapidly conjugated in the intestinal EPCs as the gingerols in the portal blood are already conjugated. Shogaols are converted to various metabolites in the EPCs. Thus, free gingerols and shogaols are present only in trace amounts in portal

Figure 8 The effect of 6S and GS on 5-HT release from RIN-14B cells. Dose-dependent effect of 6S (A) and GS (B) on 5-HT release from RIN-14B cells. (C) Inhibition of the effects of 6S and GS by a specific TRPA1 receptor antagonist AP-18. AITC, 6S, and GS induced 5-HT release from RIN-14B cells in a TRPA1-dependent manner. *p < 0.05, ***p < 0.001. N = 3–4 for each group.
and peripheral blood. Consequently, stimulation of TRPA1 located in cells other than ECCs and EPCs by gingerols and shogaols is assumed to be minimal. γ-sanshool, like gingerols and shogaols, showed clear TRPA1 agonist activity and is absorbable. However, the contribution of GS to the overall effect of TU-100 may be limited due to its lower content. The composition of TU-100 thus appears to be suitable for preferential stimulation of intraluminal TRPA1.

In conclusion, we have demonstrated that several TRPA1 agonists, including the active principle ingredients of TU-100, are effective in a standard POI mouse model. The results are in good accordance with the assumption that a selective activation of luminal TRPA1 in ECCs could augment GI motor response by enhancing endogenous 5-HT release although the proof of concept is still a subject to be done in the future. The role of TRPA1 in GI motility deserves further extensive investigation with the aim of developing more efficient therapeutic strategies to treat GI motility disorders.

FUNDING
This project has been executed using funding from Hokkaido University and Tsumura & Co. provided to TK, KM, and YU for this collaborative research.

DISCLOSURE
This project has been executed using funding from Hokkaido University and Tsumura & Co. provided to TK, KM, and YU for this collaborative research. KT, KK, KO, AK, NO, AM, HM, and MY are the employees of Tsumura & Co.

AUTHOR CONTRIBUTION
KT performed experiments estimating GI transit in vivo and 5-HT release from tissues in vitro; KK performed in vitro motility experiments; AM and HM analyzed video imaging; KO generated recombinant cells and performed Ca2+ influx assay; AK and NO performed in vitro 5-HT release assay from RIN14B cells; MY analyzed data and wrote the manuscript; KM, MY, and YU contributed to the experimental design; TK directed the entire study, analyzed data, and wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the supporting information tab for this article:

**Movie S1** Peristaltic motor activity of SI segments of WT (left panel) and TRPA1-KO (right panel) mice. The relationship between motor pattern (upper row) and intraluminal pressure peaks (lower row) is demonstrated. In WT SI, the intraluminal pressure rises and falls in a regular pattern, which is synchronous with motor activity. As the pressure reaches a maximum, several cycles of rapid motor activity occurs before abruptly ceasing as the pressure drops. This periodicity is absent in the TRPA1 KO SI and its motor activity is irregular.

**Movie S2** Peristaltic motor activity of control (left panel) and TU-100-infused (right panel) SI tract. The relationship between motor pattern (upper row) and intraluminal pressure peaks (lower row) is demonstrated. TU-100 increased the frequency of the pressure peaks. However, the pressure peaks maintained their periodicity, that is, appeared at regular intervals.