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Mashiningan improves opioid-induced constipation in rats by activating cystic fibrosis transmembrane conductance regulator chloride channel

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ABBREVIATIONS:

MNG, mashiningan; CPH, codeine phosphate; CFTR, cystic fibrosis transmembrane conductance regulator; CFTRinh-172, CFTR-specific inhibitor; ClC-2, type-2 chloride channel; DW, distilled water; PO, oral; IP, intraperitoneal; PBS, phosphate-buffered saline containing 0.3% dimethyl sulfoxide; SEM, standard error of the mean.

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Abstract

Opioid receptor stimulants are analgesics used in patients with and without cancer; however, they often cause constipation, resulting in poor adherence and deterioration of the quality of life. Hence, suitable treatments for constipation are required. In this study, we investigated the pharmacological mechanisms of action of mashiningan (MNG), a Kampo medicine used to treat constipation, and evaluated the effect of MNG on opioid-induced constipation in rats. MNG (100 or 300 mg/kg) was orally administered to normal or codeine phosphate (CPH)-induced constipation in rats, and its effect was evaluated on the basis of fecal counts, characteristics, and weight. Small intestinal fluid secretion was measured after treatment with MNG alone or co-administration with a cystic fibrosis transmembrane conductance regulator (CFTR) -specific inhibitor (CFTRinh-172). The effects of MNG on the CFTR and type-2 chloride channel were determined using patch clamp or short-circuit current experiments, respectively. MNG increased the fecal weight and proportion of soft feces in normal rats. CPH-induced constipation in rats decreased fecal counts and weight, whereas MNG prevented these effects and increased the proportion of soft feces. MNG increased the electronic chloride current, and this effect was inhibited by the CFTRinh-172 in the CFTR assay. Furthermore, MNG increased small intestinal fluid

secretion, and this effect was abolished by co-administration with the CFTRinh-172. MNG improved opioid-induced constipation in rats, and this improvement may have been mediated by increasing intestinal fluid secretion via CFTR chloride channel activation. Therefore, MNG is expected as a medicine of the treatment for constipation in patients taking opioids.

Introduction

Constipation is a common disorder of the digestive tract observed in 12-19% of people (Gras-Miralles and Cremonini, 2013), especially in the elderly aged 65 years or older in Asia (Kurniawan and Simadibrata, 2011). The number of patients with cancer is currently increasing gradually, and these patients need pain relief at all stages of their disease. The mainstay of the management of cancer pain is opioid-based pharmacotherapy (Canadian Agency for Drugs and Technologies in Health, 2014). However, the use of opioids in the treatment of chronic pain in patients with and without cancer causes bowel disorders as an adverse effect. The most frequent of these disorders is constipation, which often results in the discontinuation of opioid therapy (McNicol et al., 2003; Gyawali et al., 2015; Sani and Mahan, 2015; Nelson and Camilleri, 2016). Therefore, opioid-induced constipation compromises pain management.

For severe constipation, proactive medical treatment is required to improve the quality of life of patients and increase their satisfaction with therapy (LoCasale et al., 2016). Currently, various medications are available for treating constipation, such as laxatives and enemas, depending on the symptom and type (Prichard et al., 2016). Additionally, chloride channel activators have drawn attention as new medicines that are

associated with intestinal fluid secretion. It has been reported that phenylquinoxalinone, a cystic fibrosis transmembrane conductance regulator (CFTR) activator, normalizes stool output in a mouse model of opioid-induced constipation (Cil et al., 2016a; Cil et al., 2016b). Another agent, lubiprostone, increases osmotic pressure in the intestinal gut lumen by transporting chloride ions and promoting the secretion of intestinal fluid (Jakab et al., 2012), although the mechanism of lubiprostone-induced type-2 chloride channel (ClC-2)-mediated chloride secretion remains controversial. Linaclotide, a guanylate cyclase-C (GC-C) receptor agonist, activates CFTR chloride channel, leading to the transport of chloride ions (Cl^-), bicarbonate ions (HCO_3^-), and water to the intestinal lumen (Vaandrager et al., 1998; Sindic and Schlatter, 2006; Bryant et al., 2010). It improved constipation by softening the stool by increasing its water content, which promotes intestinal transportation (Busby et al., 2010; Sharma et al., 2013; Yu and Rao, 2014). However, the use of these medicines, including laxatives, requires caution because of possible side effects such as habit formation, abdominal pain, and nausea (Izzy et al., 2016).

Mashiningan (MNG), a Kampo medicine composed of six crude drugs, is widely prescribed to patients with abdominal sensations such as lumpy or hard stools and symptoms of frailty after illness or associated with aging in Japan and other Asian

countries (Iizuka and Hamamoto, 2015; Nakae et al., 2016). The efficacy and safety of MNG in alleviating functional constipation symptoms have also been reported in randomized double-blind, placebo-controlled studies in China (Cheng et al., 2011; Zhong et al., 2013). However, its precise mechanism of action has not been fully understood. Therefore, in the present study, we investigated the pharmacological mechanisms of action of MNG and evaluated the effect of MNG on opioid-induced constipation in rats.

Materials and Methods

Animals

Six-week-old male Sprague–Dawley rats (weight, 160-210 g) were purchased from Japan Charles River, Inc., (Kanagawa, Japan). All the animals were housed in stainless steel cages in a room with controlled ambient temperature (23 ± 3°C), humidity (50 ± 20%), and lighting (12-h light:dark cycle) conditions. The animals were provided water *ad libitum* and a standard laboratory animal diet (MF; Oriental Yeast Co. LTD, Tokyo, Japan). Experiments were performed using free-fed rats except where otherwise mentioned. All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals and approved by the Laboratory Animal Committee (Permit Nos. 15-021, 15-036, 16-044, and 16-053) of Tsumura & Co., (Tokyo, Japan).

Drugs and treatments

Codeine phosphate (CPH) was purchased from Takeda Pharmaceutical Co., Ltd., (Osaka, Japan). MNG was supplied by Tsumura & Co. (Tokyo, Japan) in the form of a powdered extract obtained by spray-drying a hot water extract mixture of the following six crude drugs: *Cannabis Fructus* (5.0 g), *Armeniacae Semen* (2.0 g), *Paeoniae Radix* (2.0 g), *Rhei Rhizoma* (4.0 g), *Magnoliae Cortex* (2.0 g), and *Aurantii Fructus*

Immaturus (2.0 g). CPH and MNG were dissolved in distilled water (DW) prior to oral (*per os*, PO) administration. CFTR-specific inhibitor (CFTRinh-172, Tocris, MO, USA) was dissolved in phosphate-buffered saline containing 0.3% dimethyl sulfoxide (PBS) prior to intraperitoneal (IP) administration. The other analytical reagents used, including the commercially available products, were of the highest purity. Treatments were administered to unanesthetized and lightly hand-restrained rats at 5 mL/kg (PO) or 1 mL/kg (IP) doses.

Experimental protocols

Effect of MNG on defecation in rats

MNG (100 or 300 mg/kg) or DW were orally administered to normal rats 2 h before the dark cycle commenced and the fecal count was evaluated 16 h after administration. We chose MNG doses (100 and 300 mg/kg) that did not influence the food intake of the free-fed rats in this experiment.

The feces were dried for more than 1 day at approximately 45°C, the weight was measured, and the weight per count was calculated. The fecal characteristics were macroscopically assessed based on criteria modified in a previous report and described as follows (Bhol and Schechter, 2007):

Normal feces: well-formed pellets, rigid as normal; soft feces: formed pellet with moisture, soft feces that retains its shape; and diarrhea: loose feces with abnormal form, much moisture, and softer shapeless form.

Effect of MNG on opioid-induced constipation in rats

Rats were administered (PO) the vehicle or CPH (12 mg/kg) 2 h before the dark cycle onset. MNG (100 or 300 mg/kg) or DW was then administered (PO) 0.5 h after the CPH treatment. The feces were collected for 16 h, counted, and then the characteristics were assessed. After drying for more than 1 day at approximately 45°C, the fecal weight was measured.

Measurement of small intestinal fluid secretion

The effect of MNG on the small intestinal fluid secretion in rats was measured. DW or MNG (100, 300, and 1000 mg/kg) was administered to 24-h fasted normal rats, and 1 h later, the rats were anesthetized with isoflurane to collect intestinal fluid. The initial portion of the duodenum (pylorus) and cecum was clamped using forceps, excised, and then the intestinal fluid content between the duodenum and cecum was measured. In a parallel experiment, 24-h fasted rats were IP injected with a

CFTR-specific inhibitor (CFTRinh-172, 1 mg/kg) 30 min before PO administration of DW or MNG (1000 mg/kg), and then the fluid content of the intestine was measured as described above. The dose of CFTRinh-172 was determined with reference to previous studies (Thiagarajah et al., 2004; Akiba et al., 2005; Mizumori et al., 2009).

Measurement of chloride channel activity

To measure the CFTR chloride channel activity, cryopreserved primary normal human bronchial epithelial cells (NhBE, LifeLine Cell Technology, Frederick, MD, USA) were expanded and transferred to Snapwell^{TM} inserts to form an epithelium. Cells were plated on the Snapwell^{TM} permeable inserts and were grown for at least 21 days in differentiation medium exposed to an air-liquid interface from day 4 to promote differentiation. Polarized NhBE epithelial cells were differentiated on a permeable support exposed to an air-liquid interface, transferred to Ussing chambers containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered physiological saline HB-PS, and the transepithelial potential was voltage clamped to 0 mV. Apical membrane currents from the epithelial sodium channel were blocked with benzamil (final concentration, 10 μmol/L, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Forskolin (final concentration, 10 μmol/L, Sigma-Aldrich) was applied to

epithelia to activate CFTR channels. After 30 min, when the CFTR peak current had decayed to a plateau level, the vehicle or MNG was cumulatively applied (50, 100, 200, and 400 μg/mL) and short-circuit currents (n = 3) were measured using a previously reported method with slight modification (Bijvelds et al., 2009; Yang et al., 2011; Cil et al., 2016a). CFTRinh-172 (20 μmol/L, Tocris or Sigma-Aldrich) was added at the end to confirm the presence of the CFTR-dependent current.

The effect of MNG on cloned human ClC-2 chloride channels (stably expressed as human CLCN2 in Chinese hamster ovary cells) was evaluated using the automated patch-clamp method (QPatch HT, Sophion Bioscience A/S, Ballerup, Denmark). After applying the vehicle to naïve cells using the QPatch robot pipetting system, MNG (400 μg/mL) or the positive control (cadmium chloride, $CdCl_2$; 100 μmol/L, Sigma-Aldrich) was applied at a minimum of 3-min intervals (n = 3).

Binding affinity for opioid μ receptors

To clarify the involvement of opioid μ (OP_3, MOP) receptors, the binding affinity was examined. For the radioligand binding assay of OP_3 receptors, CHO-K1 cells stably transfected with a plasmid encoding the human OP_3 were homogenized in modified Tris-hydrochloride (HCl) buffer and aliquots were added to MNG

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(200 μg/mL) and incubated for 60 min at 25°C with 0.60 nmol [^{3}H] diprenorphine. Nonspecific binding was estimated in the presence of 10.0 μmol naloxone.

Statistical analysis

All values are presented as the mean ± standard error of the mean (SEM). Statistical analyses of the 2 groups were performed using the Student's *t*-test. The differences between the mean values of multiple groups were determined using a one-way analysis of variance (ANOVA), followed by the Dunnett or Steel *posthoc* test. For all tests, statistical significance was set at *P*-values < 0.05.

Results

Effect of MNG on defecation in normal rats

As shown in figure 1a, the fecal count for 16 h was 38.7 ± 1.5 (N) in the control group administered DW, which was not significantly different from that of the MNG (100 or 300 mg/kg)-treated group. The fecal weight was 3.8 ± 0.1 g in the control group, whereas it was 4.9 ± 0.2 g and 5.7 ± 0.4 g in the MNG 100 and 300 mg/kg-treated groups, respectively (figure 1b). MNG significantly increased the fecal weight of rats in a dose-dependent manner. In addition, the results in figure 1c reveal that the fecal weight per count of the MNG-treated group was higher than that of the control group (100 and 300 mg/kg MNG, 0.13 ± 0.01 and 0.14 ± 0.01 g, $P < 0.05$ and 0.001, respectively vs. control, 0.10 ± 0.01 g, Dunnett's test). No difference in food intake was observed between DW and the MNG (100 or 300 mg/kg)-treated groups.

The fecal characteristics shown in table 1 were all normal in the control and MNG 100 mg/kg groups. In contrast, 17.2% soft feces were observed in the MNG 300 mg/kg group ($P < 0.01$ vs. control, Steel's test). Furthermore, diarrhea was not observed in any of the groups.

Effect of MNG on opioid-induced constipation in rats

The fecal excretion 16 h after administration of CPH was evaluated and the typical examples of the feces are shown in figure 2. The CPH + DW-treated group exhibited decreased defecation compared to that of the other groups. Fecal counts were lower in the CPH + DW-treated group (25.6 ± 1.3, $P < 0.001$, Steel's test) than in the control group treated with DW alone (42.3 ± 2.4). The CPH + MNG-treated group showed significantly increased fecal counts compared to those of the CPH + DW-treated group (100 and 300 mg/kg MNG, 34.0 ± 1.9 and 34.0 ± 2.4, respectively, both $P < 0.05$, Steel's test; figure 3a).

The fecal weight significantly decreased in the CPH + DW-treated group (2.5 ± 0.1 g, $P < 0.01$, Steel's test) compared with that of the control group (3.5 ± 0.2 g), and this effect was recovered by co-administration of MNG (100 mg/kg MNG; 3.0 ± 0.1 g, 300 mg/kg MNG; 3.1 ± 0.3 g, $P < 0.05$, Steel's test, figure 3b). Food intake did not differ significantly between groups. The evaluation of the fecal characteristics revealed that 5.0% of the total feces was soft in the CPH + MNG 300 mg/kg group ($P < 0.01$ vs. control, Steel's test), but that of the other groups was normal (table 2). In addition, no diarrhea was observed in any group.

Effect of MNG on small intestinal fluid secretion in normal rats

The effect of MNG on the small intestinal secretion in 24-h fasted rats was assessed. As shown in figure 4a, the fluid content of the small intestine 1 h after administration was 0.62 ± 0.07 g in the DW-treated control group. MNG at doses of 300 and 1000 mg/kg significantly increased the amount of the small intestinal fluid in rats (300 and 1000 mg/kg, 1.01 ± 0.11 and 1.21 ± 0.08 g, $P < 0.05$ and $P < 0.001$, respectively).

The effect of MNG on the small intestinal fluid was abolished by IP injection of the CFTR inhibitor CFTRinh-172 (DW+ PBS group, 0.55 ± 0.07 g; MNG + PBS group, 1.27 ± 0.10 g, $P < 0.001$; and MNG + CFTRinh-172 group, 0.63 ± 0.06 g, $P < 0.001$, figure 4b). In DW-treated rats, the injection of CFTRinh-172 had no effect on the small intestinal fluid (figure 4c).

Effects of MNG on chloride channel activities and opioid receptors

To clarify the mechanisms of action of MNG, its effects on the CFTR chloride channel activity in NhBE cells were evaluated using the Ussing chamber assay. The short circuit current was negatively changed in a dose-dependent manner by the addition of MNG (50-400 μg/mL) to the cell culture. Treatment with CFTRinh-172 (20 μmol/L), a CFTR-specific inhibitor, blocked the response of MNG-treated cells,

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which was restored to the levels of the vehicle-treated cells (table 3). On the other hand, when the activity of the ClC-2 chloride channel was presented as percent inhibition, although an agonist effect is denoted as a negative inhibition value, it was 60.1 ± 9.4% in epithelial cells treated with 400 μg/mL MNG.

The effect of MNG on the OP_3 receptor was evaluated using a radioligand binding assay, and no effect was observed at 200 μg/mL on the binding activities of this receptor (data not shown).

Discussion

In this study, we found that administration of MNG improved opioid-induced constipation in rats. The effect of MNG was likely mediated by an increase in the intestinal fluid secretion via CFTR chloride channel activation. First, we clarified the pharmacological effects of MNG on defecation in normal rats, and the results showed that the administration of MNG significantly increased the fecal weight, but not the count. Next, we elucidated the pharmacological effects of MNG on defecation in a CPH-treated rat model of opioid-induced constipation. The decrease in fecal count and weight in the CPH-treated rats was partially prevented by MNG administration.

Increased fecal output (frequency and volume) is one of the important factors in constipation therapy (Chokhavatia et al., 2016). Dry and hard stool causes difficult intestinal transit (Fredericks et al., 2010) and, therefore, it is desirable for feces to be softened by treatment for constipation (Costilla and Foxx-Orenstein, 2014; Canadian Agency for Drugs and Technologies in Health, 2014). In this study, the analysis of fecal characteristics revealed that MNG administration increased the incidence of soft feces in both normal and CPH-treated rats. Our previous study demonstrated that MNG increased the water content and size of feces in rats with fiber-free diet-induced constipation (Harada et al., 2016). Therefore, MNG may have facilitated defecation by

softening the feces in both normal and CPH-treated rats. The present study demonstrated that MNG had no binding affinity for the OP_3 receptor, suggesting that MNG improved constipation without interfering with the action of opioids.

The enhancement of the small intestinal fluid secretion is important for promoting defecation (De Lisle, 2012; Cil et al., 2016a). The increased fluid secretion in the bowel lumen softens the feces and promotes bowel transit (Lacy and Levy, 2007; Chokhavatia et al., 2016). Recently, stimulation of fluid secretion by pharmacologically opening chloride channels in the epithelium of the intestinal mucosa has been considered a new approach in constipation therapy (Schiller, 2004). The selective chloride channel activator lubiprostone enhances the secretion of the small intestinal fluid and promotes the small bowel movement, leading to increased colon transit (Camilleri et al., 2006; Lacy and Levy, 2007). The GC-C receptor agonist linaclotide also increases chloride secretion and thereby induces cellular movement of sodium and water into the intestinal lumen (Jiang et al., 2015). Furthermore, it promotes the synthesis of intracellular cyclic guanosine monophosphate (cGMP) and subsequently stimulates the cGMP-dependent protein kinase II to phosphorylate CFTR (Vaandrager et al., 1998; Sindic and Schlatter, 2006; Bryant et al., 2010). Additionally, GC-C/cGMP activation plays a role in relieving abdominal pain (Corsetti and Tack,

2013). This new approach improves the difficulty or pain associated with intestinal transit by promoting bowel movements and, therefore, may be beneficial for patients with constipation (Chokhavatia et al., 2016).

The present study demonstrates that MNG enhanced the small intestinal fluid secretion. Moreover, it may be involved in promoting bowel movements by softening feces. Therefore, to clarify the underlying mechanism of action, the effects of MNG on the chloride channels, ClC-2 and CFTR, were examined *in vitro*. The results revealed that MNG exhibited agonistic activity on CFTR; CFTR channels are distributed in the apical membranes of intestinal epithelial cells (Matthews, 2002; Field, 2003; Yang et al., 2011), and MNG acted locally in the intestinal tract rather than systemically. The concentration of MNG used *in vivo* was 30 or 60 mg/mL. Although the strict concentration of MNG delivered to the small intestinal lumen is unknown, we considered that MNG could reach the levels (50-400 μg/mL) used in the Ussing chamber experiments. Furthermore, the increase in the small intestinal fluid content induced by MNG treatment was completely abolished by co-administration with the CFTR-specific inhibitor, CFTRinh-172. These results suggest that MNG enhanced secretion of the small intestinal fluid by activating CFTR chloride channels. Conversely, MNG did not activate ClC-2 channel. Recent studies demonstrated that

ClC-2 is mostly located in proximity to tight junctions, intercellular membranes, and basolateral membranes, and its distribution varies from species to species (Jin and Blikslager, 2015). It suggests that the ClC-2 chloride channel has a critical role in the regulation of intestinal barrier function, colonic NaCl absorption, and cell volume, rather than secretion.

MNG contains multiple components, but the active ingredient mediating its efficacy was not identified in this study. Some studies have demonstrated the pharmacological effects of crude drugs contained in MNG. *Cannabis Fructus* contains a large amount of fatty acid oils, including olein, linolein, and linolenin, which affect the small intestinal fluid secretion (Slota et al., 1983). Naringenin, an ingredient of *Aurantii Fructus Immaturus*, increased the production of cGMP by inhibiting phosphodiesterase activity (Orallo et al., 2005). These findings suggest the activation of CFTR chloride channel through cGMP signal transduction. On the other hand, *Rhei Rhizoma*-containing drugs are widely used as laxatives (Iizuka and Hamamoto, 2015; Hirose et al., 2016). MNG also contains *Rhei Rhizoma*; however, it improved constipation without inducing diarrhea in rats in this study. In addition, the Kampo medicine, daioukanzoto, which is composed of *Rhei Rhizoma* and *Glycyrrhizae radix*, did not have CFTR activity (data not shown). This observation suggested that *Rhei*

Rhizoma was not likely to be involved in the increase in small intestinal fluids induced by MNG. However, *Rhei Rhizoma* enhances intestinal movement (Iizuka and Hamamoto, 2015; Hirose et al., 2016). It is well known that sennosides A and B, which are present in *Rhei Rhizoma*, are metabolized by intestinal bacteria to become rheinanthrone, which has strong laxative activity. Increased myoelectric activity and contractility in the intestine and colon by *Rhizoma*-containing drugs suggests that this may contribute to the reversal of constipation by promoting motility and accelerating transit. Magnolol and honokiol, which are constituents of *Magnoliae Cortex*, promote gastrointestinal motility (Zhang et al., 2005; Yang et al., 2008; Miao et al., 2013). Peoniflorin, a constituent of *Paeoniae Radix*, is known to relieve pain and may have a beneficial effect on abdominal pain in patients with constipation (Zhang et al., 2008; Zhang et al., 2009; Xu et al., 2013). These beneficial effects that improve symptoms of patients with constipation are also likely to be synergistically involved in the efficacy of MNG. However, we did not identify the active ingredient of MNG and its specific detailed mechanism of action for correlating opioid receptors and CFTR, therefore, further investigation is required.

In the present study, we used a rat model in which constipation was induced by codeine, a relatively weak opioid agonist. It has been reported that the CFTR activator

phenylquinoxalinone, normalized stool output and water content in a mouse model of constipation induced by the more potent opioid agonist loperamide (Cil et al., 2016a). These findings suggested that CFTR-mediated stool softening overcame opioid-induced constipation. Moreover, a clinical study of MNG on functional constipation symptoms demonstrated that most patients tolerated MNG well and no serious adverse effect was found. However, further investigation is needed to assess the safety of MNG, particularly in the elderly, who often have multiple co-morbidities, and patients undergoing chemotherapy.

In summary, this study provided evidence to support an undoubtedly major role for MNG in improving constipation by, at least in part, increasing the intestinal fluid secretion through CFTR chloride channel activation.

In conclusion, we demonstrated that MNG increased the fecal output in opioid-induced constipation in rats. Furthermore, our results suggested that the effects of MNG are mediated by increasing the small intestinal fluid secretion by CFTR chloride channel activation. Therefore, MNG could be used for the treatment of constipation in patients taking opioids.

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Authorship Contribution

Participated in research design: Y Harada, N Fujitsuka

Conducted experiments: Y Harada, S Iizuka, Y Saegusa, S Mogami

Performed data analysis: Y Harada, Y Saegusa

Wrote or contributed to the writing of the manuscript: Y Harada, N Fujitsuka, T Hattori

Y Harada, S Iizuka, Y Saegusa, S Mogami, N Fujitsuka, T Hattori are employed by Tsumura & Co. The all authors declare that they have no conflict of interest.

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Figure legend

Figure 1 Effects of mashiningan (MNG) on defecation in normal rats Distilled water (DW) or MNG (100 or 300 mg/kg) was orally (PO) administered to normal rats 2 h before onset of dark cycle. (a) Fecal count and (b) weight were measured over 16 h, and (c) weight per fecal count was also calculated. Data are mean ± standard error of the mean (SEM). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. DW-treated normal rats using Dunnett's test (n = 10).

Figure 2 Representative example of feces in codeine phosphate (CPH)-induced constipation rat model (a) DW only, (b); CPH + DW, (c), CPH + MNG 100 mg/kg, and (d) CPH + MNG 300 mg/kg, DW or MNG were orally (PO) administered 0.5 h after treatment with (a) DW or (b-d) CPH 12 mg/kg (PO). Feces were collected over 16 h after CPH administration. DW, distilled water; MNG, mashiningan.

Figure 3 Effects of mashiningan (MNG) on codeine phosphate (CPH)-induced constipation rat model (a) Fecal count and (b) weight were measured for 16 h after CPH administration. Data are mean ± standard error of the mean (SEM). *P < 0.05, ** P < 0.01 and ***P < 0.001 vs. CPH + DW-treated rats using Dunnett's test or Steel's

test (n = 10).

Figure 4 Effects of mashiningan (MNG) on small intestinal fluid secretion a) Small intestinal fluid secretion was measured 1 h after oral (OP) administration of DW or MNG in 24 h-fasted normal rats (*P < 0.05 and ***P < 0.001 vs. DW-treated group by Dunnett's test, n = 4). (b) Vehicle or CFTR-specific inhibitor (CFTRinh-172, 1 mg/kg) was intraperitoneally (IP) injected into rats 0.5 h before DW or MNG administration ($^{***}P$ < 0.001 vs. MNG + PBS group by Dunnett's test, n = 10). (c) Vehicle or CFTRinh-172 1 mg/kg was IP injected 0.5 h before DW (n = 6). Data are mean ± standard error of the mean (SEM). N.S. by student's *t*-test, n = 10). DW, distilled water; CFTR, cystic fibrosis transmembrane conductance regulator. N.S.; Not significant

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Table 1 Effects of mashiningan (MNG) on fecal characteristics in normal rats

Fecal characteristics	Incidence (%)		
	DW	MNG (100 mg/kg)	MNG (300 mg/kg)
Normal	100	100	83 **
Soft	0	0	17 **
Diarrhea	0	0	0

Distilled water (DW) or MNG (100 or 300 mg/kg) was orally (PO) administered to normal rats 2 h before onset of dark cycle. Fecal characteristics were assessed for 16 h after administration. Data are the mean of incidence (%). $^{**}P < 0.01$ vs. DW-treated normal rats using Steel's test (n = 10).

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Table 2 Effects of mashiningan (MNG) on fecal characteristics of codeine phosphate (CPH)-induced constipation rat model

Fecal characteristics	Incidence (%)			
	Control	CPH + DW	CPH + MNG(100 mg/kg)	CPH + MNG(300 mg/kg)
Normal	100	100	100	95 **
Soft	0	0	0	5 **
Diarrhea	0	0	0	0

Distilled water (DW) or MNG was orally (PO) administered 0.5 h after DW or CPH 12 mg/kg treatment. Fecal characteristics were assessed for 16 h after CPH administration. Data are the mean of incidence (%). ** $P < 0.01$ vs. CPH + DW-treated rat using Steel's test (n = 10).

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Table 3 Effects of mashiningan (MNG) on cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel activity evaluated using Ussing chambers assay

Test article (μg/mL)	Short-circuit current ($\mu A/cm^2$)
Vehicle	0.02 ± 0.13
+ CFTRinh-172	16.81 ± 1.31
MNG 50	- 3.04 ± 0.26 ***
100	- 5.21 ± 0.44 ***
200	- 6.73 ± 0.32 ***
400	- 9.85 ± 0.17 ***
+ CFTRinh-172	23.10 ± 0.69

Short-circuit current was changed dose-dependently by treatment with MNG (50-400 μg/mL), and the response was blocked by a CFTR-specific inhibitor (CFTRinh-172, 20 μmol/L). ***$P < 0.01$ vs. Vehicle by Dunnett's test. Data are mean ± standard error of the mean (SEM, n = 3).

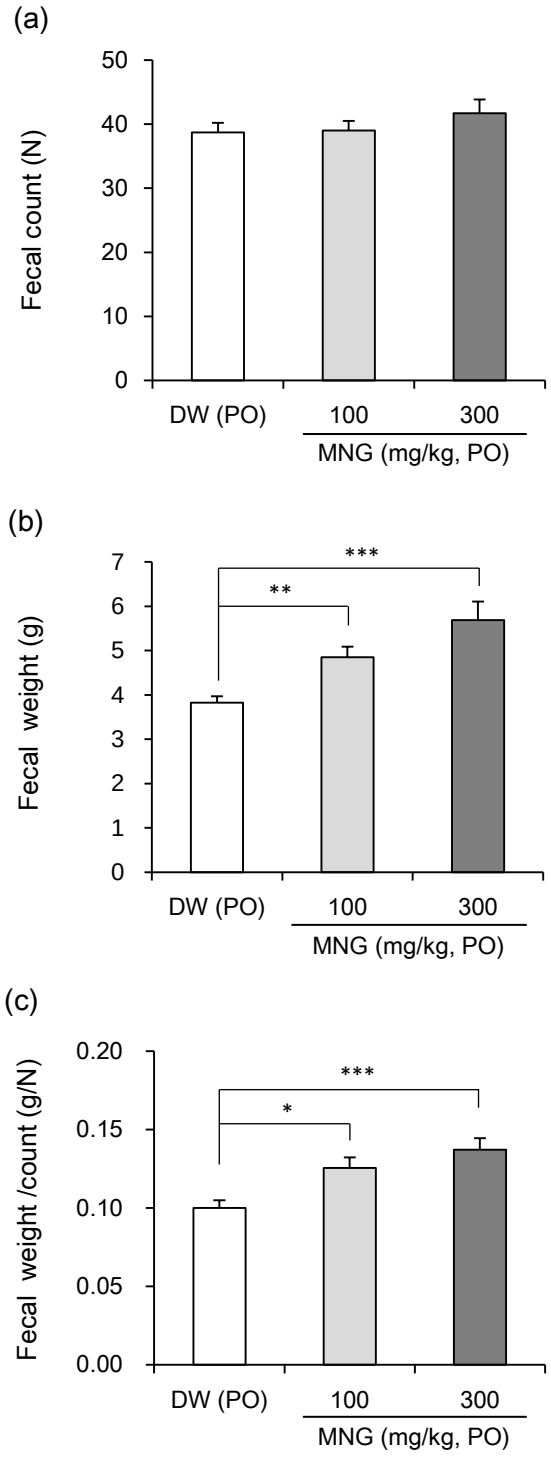
(a)
Fecal count (N)
50
40
30
20
10
0
DW (PO)
100
300
MNG (mg/kg, PO)
(b)
Fecal weight (g)
7
6
5
4
3
2
1
0
***
**
DW (PO)
100
300
MNG (mg/kg, PO)
(c)
Fecal weight /count (g/N)
0.20
0.15
0.10
0.05
0.00
***
*
DW (PO)
100
300
MNG (mg/kg, PO)

Figure 1

(a) DW

(b) CPH + DW

(c) CPH + MNG 100 mg/kg

(d) CPH + MNG 300 mg/kg

Figure 2

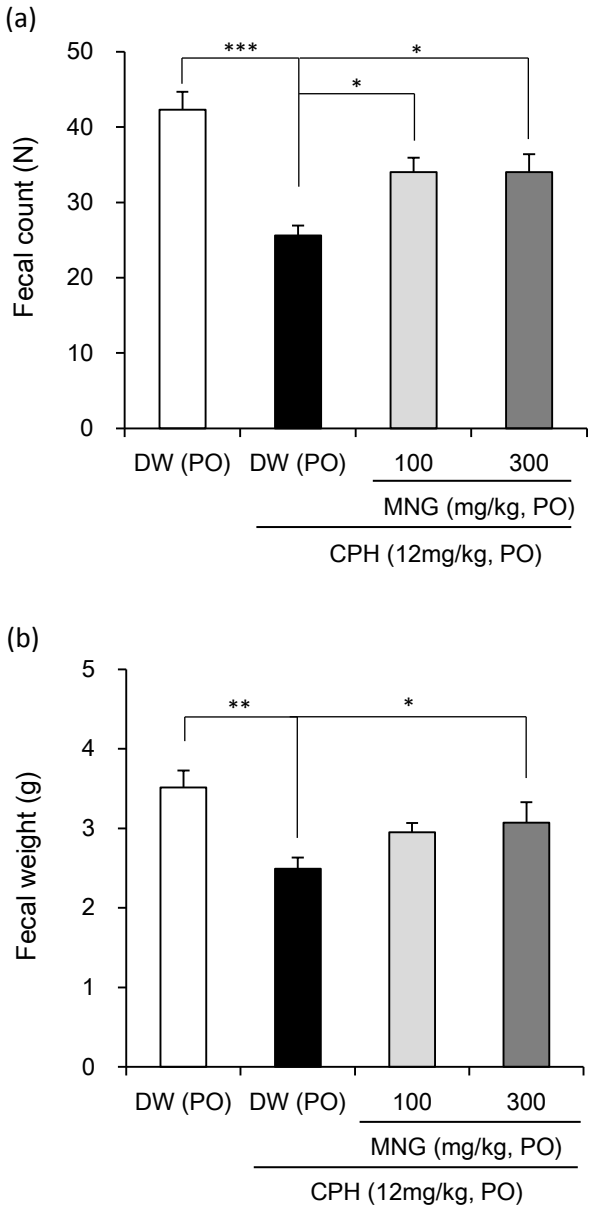
(a)
Fecal count (N)
50
40
30
20
10
0
***
*
*
DW (PO)
DW (PO)
100
300
MNG (mg/kg, PO)
CPH (12mg/kg, PO)
(b)
Fecal weight (g)
5
4
3
2
1
0
**
*
DW (PO)
DW (PO)
100
300
MNG (mg/kg, PO)
CPH (12mg/kg, PO)

Figure 3

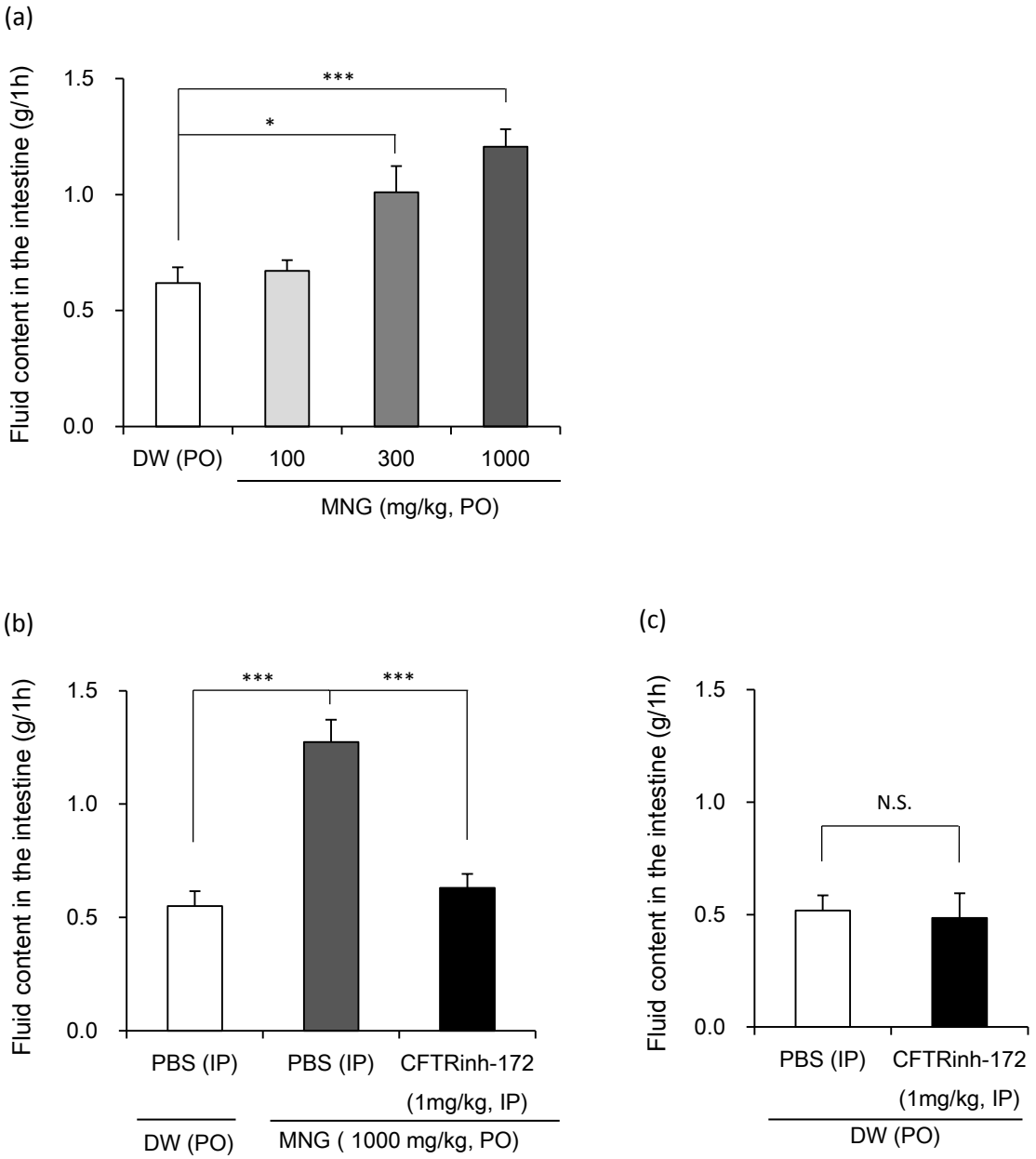
(a)
Fluid content in the intestine (g/1h)
1.5
1.0
0.5
0.0
***
*
DW (PO)
100
300
1000
MNG (mg/kg, PO)
(b)
Fluid content in the intestine (g/1h)
1.5
1.0
0.5
0.0
***
***
PBS (IP)
PBS (IP)
CFTRinh-172
(1mg/kg, IP)
DW (PO)
MNG (1000 mg/kg, PO)
(c)
Fluid content in the intestine (g/1h)
1.5
1.0
0.5
0.0
N.S.
PBS (IP)
CFTRinh-172
(1mg/kg, IP)
DW (PO)

Figure 4