Neuropharmacology and analgesia

Effects of novel TRPA1 receptor agonist ASP7663 in models of drug-induced constipation and visceral pain

Ryosuke Kojima a,*, Katsura Nozawa a, Hitoshi Doihara a, Yoshihiro Keto a, Hidetaka Kaku b, Toshihide Yokoyama a, Hiroyuki Itou a

a Pharmacology Research Labs, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan
b Chemistry Research Labs, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan

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ABSTRACT

Constipation is a major gastrointestinal motility disorder with clinical need for effective drugs. We previously reported that transient receptor potential ankyrin 1 (TRPA1) is highly expressed in enterochromaffin (EC) cells, which are 5-hydroxytryptamine (5-HT)-releasing cells, and might therefore be a novel target for constipation. Here, we examined the effects of ASP7663, a novel and selective TRPA1 agonist, in constipation models as well as an abdominal pain model. ASP7663 activated human, rat, and mouse TRPA1 and released 5-HT from QGP-1 cells, and oral but not intravenous administration of ASP7663 significantly improved the loperamide-induced delay in colonic transit in mice. While pretreatment with the TRPA1 antagonist HC-030031 and vagotomy both inhibited the ameliorating effect of oral ASP7663 on the colonic transit, both orally and intravenously administered ASP7663 significantly inhibited colorectal distension (CRD)-induced abdominal pain response in rats. Taken together, these results demonstrate that ASP7663 exerts both anti-constipation and anti-abdominal pain actions, the former is likely triggered from the mucosal side of the gut wall via activation of vagus nerves while the latter is assumed to be provoked through systemic blood flow. We conclude that ASP7663 can be an effective anti-constipation drug with abdominal analgesic effect.

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1. Introduction

Constipation is a common gastrointestinal disorder characterized by such symptoms as straining, hard stool, and infrequent defecation. Population-based studies have shown that the prevalence of constipation is up to 30% of the population in developed countries (Drossman et al., 1993; Everhart et al., 1989; Longstreth et al., 2006; Pare et al., 2001). Current drug treatments for constipation are based on traditional drugs, such as osmotic or secretory laxatives and bulking agents, and fail to offer sufficient improvement in condition due to poor efficacy, late onset, and abdominal side effects such as pain, cramps and bloating (Johnson, 2006; Jones et al., 2002). As such, an anti-constipation drug with good efficacy and no abdominal side effects is awaited.

TRPA1 is a member of the TRP channel family and is activated by various physiochemical stimuli, including cold temperatures and pungent natural chemicals such as aryl isothiocyanate (AITC) and cinnamaldehyde (Bandell et al., 2004; Bautista et al., 2005; Jordt et al., 2004). Functional studies of TRPA1 have mainly focused on its role in diverse sensory processes including cold nociception, hearing and inflammatory pain (Bautista et al., 2006; Kwan et al., 2006; Nagata et al., 2005). We previously reported that TRPA1 is extensively expressed in EC cells of the gastrointestinal tract, and stimulation of TRPA1 activates gastrointestinal motility (Doihara et al., 2009b; Kojima et al., 2009; Nozawa et al., 2009). The above present TRPA1 as a potential novel therapeutic target for constipation.

Here, we investigated the therapeutic potential of (E)-[7-fluoro-1-(2-methylpropyl)-2-oxo-1,2-dihydro-3H-indol-3-ylidene]acetic acid (ASP7663; Fig.1), a newly synthesized TRPA1 agonist, as a potential anti-constipation agent.

2. Materials and methods

2.1. In vitro studies

2.1.1. Current in flux assay

Following a previously described method (Nozawa et al., 2009), we examined the TRPA1 agonist potential of ASP7663 for human, rat and mouse TRPA1. Briefly, HEK293 cells expressing human, rat, or mouse TRPA1 were seeded into black-walled, clear-base 96-well poly-D-lysine-coated plates in 100 μL of DMEM media.
with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at densities of 16,000, 20,000, and 20,000 cells per well, respectively. The cells were cultured overnight at 37 °C in an atmosphere of 5% CO2. On the day of measurement, cells were incubated for 2 h at room temperature in Hank's Balanced Salt Solution containing 20 mmol/L HEPES pH 7.4 and 0.02% CHAPS (HBSS–HEPES–CHAPS) with the addition of the calcium indicator Fluo-4 AM at 2 μmol/L. The cells were washed twice with 80 μL of HBSS–HEPES–CHAPS, and 80 μL of the buffer was added prior to the assay.

Plates were placed into the fluorometric imaging plate reader (FLIPR; Molecular Devices Corporation Japan, Tokyo, Japan) to measure cell fluorescence. After addition of ASP7663, the change in fluorescence was monitored for 30 min, and the differences between maximum and minimum values in the fluorescent signal were noted.

2.1.2. 5-HT release assay
QGP-1 cells obtained from HSRRB (HSRRB JCRB0183) were seeded at 3 × 10^5 cells/mL/well in 24-well plates and cultured for 3 days. The cells were rinsed 3 times with HBSS containing 0.5% FBS and 2 μmol/L fluoxetine (Tocris, Ellisville, MO, USA). The HBSS–FBS–fluoxetine was then removed and replaced with 200 μL HBSS–FBS–fluoxetine containing ASP7663 and incubated for 60 min at 37 °C. The assay buffer was collected and centrifuged for 5 min to remove any detached cells. The supernatants were collected and stored at −80 °C until 5-HT measurement. The 5-HT concentration was measured using an enzyme immunoassay (EIA) kit (Immunotech, Marseille, France) as per the manufacturer’s instructions. Absorbance at 405 nm in each well was measured using a spectrophotometer (SpectraMax® Molecular Devices Corporation Japan, Tokyo, Japan).

2.2. In vivo studies
All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma Inc. The animals were housed under a 12-h light–dark cycle in a controlled-temperature environment (23 ± 2 °C) with food and water available ad libitum.

2.2.1. Drug-induced constipation models
Effects of ASP7663 in drug-induced constipation models were evaluated by the bead transit method described previously (Kojima et al., 2009). Briefly, male ddY mice (Japan SLC, Inc, Shizuoka, Japan; weighing 27–30 g) were fasted overnight with free access to water prior to the experiments. Under light ether anesthesia, a glass bead (3 mm in diameter) was inserted into the distal colon 2 cm above the anus using a silicon-tipped rod. After recovery from the anesthesia, the time required for bead evacuation was measured. Loperamide or clonidine and ASP776 were administered subcutaneously 30 min before and orally 3 min before bead insertion, respectively. In the intravenous administration study, ASP7663 was administered into the tail vein 3 min before bead insertion. In the antagonist study, HC-030010 and ASP7663 were orally administered 45 and 30 min before bead insertion, respectively.

Further characterization of the prokinetic effects of ASP7663 was conducted in vagotomized mice. Vagotomy was performed following the method of Chia et al. with minor modifications (Chia et al., 2006). Mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), and ventral and dorsal truncal branches of the subdiaphragmatic vagi were cut apart. After a one-week recovery period, the mice were used for bead transit experiments.

2.2.2. Abdominal pain
To assess the influence of ASP7663 on abdominal pain, we evaluated the effect on colorectal distension (CRD)-induced pain behavior by the method of Fioramonti et al. with small modifications (Fioramonti et al., 2003). Briefly, male Wistar rats (280–350 g; Japan SLC, Inc.) were lightly anesthetized with 5% isoflurane, and a 5-cm long latex balloon was carefully inserted into the colorectal area via the anus and positioned such that the end of the balloon was 1 cm from the anus. The balloon catheter was then fixed in place by taping to the base of the tail, and the rats were placed in individual cages. After recovery from anesthesia, CRD was loaded using a computerized barostat (DISTENDER SERIES IIR®; G&J Electronics Inc., Ontario, Canada). The balloon was inflated progressively in 15-mmHg steps from 15 to 60 mmHg, with each step of inflation lasting 5 min with 5 min intervals. The number of abdominal contractions during inflation was visually counted for each step. ASP7663 or vehicle was orally or intravenously administered 5 min before the start of the inflation.

2.3. Drugs
ASP7663 and HC-030010 were synthesized at Astellas Pharma Inc. (Tokyo, Japan). Loperamide hydrochloride (loperamide) and clonidine hydrochloride (clonidine) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ASP7663 was dissolved in dimethylsulfoxide (DMSO) and diluted with HBSS–HEPES–CHAPS in the Ca2+ assay or with HBSS–FBS–fluoxetine in the 5-HT release assay. In the animal studies, ASP7663 was dissolved in 10% dimethylformamide and 10% Tween 80/distilled water or 0.5% methylcellulose (MC) in the oral administration, and 5% dimethylformamide and 5% cremophore/saline in the intravenous administration. HC-030010 was suspended in 0.5% MC. Loperamide and clonidine were dissolved in 1% Tween 80/saline and saline, respectively.

2.4. Statistical analyses
The EC50 value of each experiment was calculated by Sigmod–Emax non-linear regression analysis. The geometric mean and the 95% confidence intervals (95% CI) of 4 individual experiments performed in triplicate were calculated. In the animal studies, all data are expressed as the mean ± standard error of the mean (S.E.M.). The significance of differences between two groups was determined using the unpaired Student’s t-test, whereas that among more than two groups was determined using Dunnett’s multiple comparison test. Statistical significance was defined as P < 0.05. In the case of multiple-point comparisons, Bonferroni’s type adjustment was made.
3. Results

3.1. Ca$^{2+}$ influx assay

ASP7663 concentration dependently increased intracellular Ca$^{2+}$ concentration in human, rat, and mouse TRPA1 expressed in HEK293 cells in a similar manner, with respective EC$_{50}$ values (95% confidence interval [CI]) of 0.51 (0.40–0.66), 0.54 (0.41–0.72), and 0.50 (0.41–0.63) μmol/L (Fig. 2). Of note, the increases in intracellular Ca$^{2+}$ induced by 10 μmol/L ASP7663 were abolished by pretreatment with 10 μmol/L HC-030031, a TRPA1 antagonist (Fig. 2).

3.2. 5-HT release assay

ASP7663 concentration-dependently stimulated 5-HT release from QGP-1 cells, a lineage of TRPA1-expressing EC cells (Doihara et al., 2009b), with an EC$_{50}$ value of 72.5 (52.6–99.9) μmol/L (Fig. 3). We previously reported that QGP-1, a human pancreatic endocrine cell line, highly expresses both TRPA1 and tryptophan hydroxylase and releases 5-HT upon stimulation with TRPA1 agonists such as AITC and cinnamaldehyde (Doihara et al., 2009b). These features are in good accordance with those of EC cells (Nozawa et al., 2009), as AITC and cinnamaldehyde (Doihara et al., 2009b). These features indicate that QGP-1 is a good model for investigating the functional significance of the 5-HT-releasing activity of TRPA1 agonists. The 5-HT-releasing activity of ASP7663 observed in the present study indicated that ASP7663 has the potential to release 5-HT from EC cells in the gut wall. Because peripheral 5-HT is mainly synthesized in EC cells and plays an important role in regulating gastrointestinal motility (Gershon, 2003; Spiller, 2007), we investigated ASP7663 effects on gastrointestinal motility in vivo.

We evaluated the effect of ASP7663 on abdominal pain using the CRD model. The number of abdominal contractions was increased with colorectal pressure level (15–60 mmHg). Compared with vehicle treatment, ASP7663 at oral doses of 1 and 3 mg/kg significantly reduced the number of abdominal contractions evoked during CRD at pressures of 30, 45, and 60 mmHg (Fig. 6A). ASP7663 also reduced the number of abdominal contractions by intravenous treatment (Fig. 6B). No behavior indicative of pungency was observed after administration of ASP7663.

3.4. Abdominal pain

In the present experiments, we showed that ASP7663 concentration-dependently increased intracellular Ca$^{2+}$ concentration in human, rat, and mouse TRPA1-expressing HEK293 cells at the same potency, and that the stimulating effects of ASP7663 were abolished by HC-030031. These results indicate that ASP7663 is a potent TRPA1 agonist with no species difference. We also confirmed that ASP7663 had no or only slight affinity to more than 60 receptors, including channels and enzymes like TRPV1 (see Supplemental table S1 and Supplemental figure S1).

We previously reported that QGP-1, a human pancreatic endocrine cell line, highly expresses both TRPA1 and tryptophan hydroxylase and releases 5-HT upon stimulation with TRPA1 agonists such as AITC and cinnamaldehyde (Doihara et al., 2009b). These features are in good accordance with those of EC cells (Nozawa et al., 2009), indicating that QGP-1 is a good model for investigating the functional significance of the 5-HT-releasing activity of TRPA1 agonists. The 5-HT-releasing activity of ASP7663 observed in the present study indicated that ASP7663 has the potential to release 5-HT from EC cells in the gut wall. Because peripheral 5-HT is mainly synthesized in EC cells and plays an important role in regulating gastrointestinal motility (Gershon, 2003; Spiller, 2007), we investigated ASP7663 effects on gastrointestinal motility in vivo.

Although irritable bowel syndrome with constipation (IBS-C) and chronic constipation (CC) are the most common subtypes of functional constipations (Cash et al., 2007), the two differ substantially in terms of gastrointestinal motility, with IBS-C being spastic whereas CC is atonic. We previously characterized loperamide- and clonidine-induced delay in mouse colonic transit models as spastic and atonic constipations (Cash et al., 2007), the two differ substantially in terms of gastrointestinal motility, with IBS-C being spastic whereas CC is atonic. We previously characterized loperamide- and clonidine-induced delay in mouse colonic transit models as spastic and atonic constipation models, respectively (Kojima et al., 2009). In the present study, ASP7663 was effective in both models, suggesting its efficacy against both IBS-C and CC.

While improvement of constipation in mice was observed immediately (3 min) after oral administration of ASP7663, it is unlikely that the compound reached the colon within such a short span time. Further, intravenous administration of ASP7663 did not affect the delay in colonic transit in mice, suggesting ASP7663 effects on colonic propulsion were not evoked via systemic blood flow. These results suggest that orally administered ASP7663 acts on the luminal surface of the upper gastrointestinal, where EC cells
Fig. 4. Effects of orally administered ASP7663 in loperamide- and clonidine-induced constipation models in mice. ASP7663 improves delays in colonic propulsion induced by loperamide (A) and by clonidine (B). All values are represented as mean ± S.E.M (n = 10). #, P < 0.05 compared with the control group (Student’s t-test); *, P < 0.05 compared with the vehicle group (Dunnett’s test).

Fig. 5. Effects of ASP7663 in the loperamide-induced constipation model in mice. (A) Effects of intravenous administration of ASP7663. (B) Effects of oral administration of ASP7663 in vagotomized mice. (C) Effects of oral administration of ASP7663 in HC-030031-pretreated mice. All values are presented as the mean ± S.E.M (n = 5 or 6). #, P < 0.05 compared with the control group (Student’s t-test); *, P < 0.05 compared with the vehicle group (Dunnett’s test); ♭, between loperamide and its vehicle treated groups without ASP7663 treatment (Student’s t-test).; $, P < 0.05 between ASP7663 and its vehicle treated groups with loperamide (Student’s t-test).

Fig. 6. Inhibitory effects of ASP7663 on colorectal distension in rat. ASP7663 decreased the number of abdominal contractions induced by increasing the pressure of colorectal distension. All values are presented as the mean ± S.E.M (n = 6 or 8). #, P < 0.0178 compared with the vehicle group (Dunnett’s test (A) or Student’s t-test (B) with Bonferroni correction).
are abundant (Gershon and Tack, 2007; Rindi et al., 2004), in the constipation models. Further, the efficacy of ASP7663 in mice was abolished by the transection of the vagus nerves, which are known to act as an afferent pathway for the gastrocolic reflex by activating 5-HT from EC cells (Gershon and Tack, 2007; Lang, 1999). We have previously reported that AITC delays gastric emptying in rats and that the effect of AITC is abolished with treatment of a 5-HT3 antagonist or tryptophan hydroxylase inhibitor (Doihara et al., 2009a). These results indicate TRPA1 agonists release 5-HT in the upper gastrointestinal tract. Taken together, these results argue that orally administered ASP7663 stimulates 5-HT release from EC cells located at the luminal surface of the upper gastrointestinal tract, and that the released 5-HT activates the vagal afferent nerve to trigger the gastrocolic reflex.

Abdominal pain or discomfort is a common side effect of currently used anti-constipation agents (Johnson, 2006; Tack et al., 2011). Bulk laxatives increase the water content in stools, whereas osmotic agents attract water from the gastrointestinal wall (Stephen and Cummings, 1979). These properties are supposed to increase the volume of gastrointestinal contents and cause bloating and abdominal cramping. Stimulant laxatives directly stimulate the colonic luminal surface and cause abdominal discomfort and cramps (Bengtsson and Ohlsson, 2005), and IBS-C patients frequently complain of abdominal pain (Saito et al., 2002). Moreover, currently available drugs fail to mitigate abdominal pain in constipation patients (Whitehead et al., 2011). We therefore considered effects on abdominal pain as a criterion when evaluating novel anti-constipation agents. The CRD model in rat is commonly used to evaluate drug effects on abdominal pain and discomfort (Kiso et al., 2001; Liang et al., 2005). In this study, ASP7663 showed a potent analgesic effect in this model which, in addition to its anti-constipation effect, makes ASP7663 an appealing agent.

TRPA1 is known to stimulate sensory nerves and cause pain (Brierley et al., 2009; Jordt et al., 2004), and some reports have shown that AITC, a TRPA1 agonist, causes hyperalgesia in the CRD model (Cattaruzza et al., 2010). In these reports, AITC was administered directly into the colon. Therefore, exposure of the colonic luminal surface to a high concentration of AITC can induce undesirable effects on colonic sensation. In contrast, our present results revealed that systemic (oral or intravenous) administration of a TRPA1 agonist has an analgesic effect in the CRD model. Furthermore, we have observed inhibition potential of orally administrated ASP7663 on acetic acid-induced writhing behavior in preliminary experiments using mice (Supplemental figure 2). Moreover, we observed no painful behavior after the oral or intravenous administration of ASP7663 in the present experiments, suggesting that systemic administration of TRPA1 agonist may have no or little pungency/hyperalgesic activity. Another possible explanation for the difference of ASP7663 and previously reported TRPA1 agonists that induce nociception is the different potential for agonist-evoked desensitization as discussed by Leamy et al. (2011).

Interestingly, in contrast to results in the constipation model in mice, intravenous administration of ASP7663 did inhibit CRD-induced abdominal pain in rats, indicating that the mechanism of the inhibitory action of ASP7663 on abdominal pain differs from that of its actions against constipation. Furthermore, the analgesic effect of ASP7663 is considered to not be mediated by 5-HT3 receptor because a 5-HT3 receptor agonist is reported to not affect the CRD-induced abdominal pain (Kiso et al., 2001). On the other hand, the stimulation of 5-HT4 receptor, which is another well-known 5-HT receptor regulating gastrointestinal function, ameliorate the CRD-induced pain response in rats (Liang et al., 2005). This mechanism can be involved in the effect of ASP7663 on abdominal pain. In addition to the 5-HT mediated mechanisms, direct effect on the afferent nerve may be expected. TRPV1, another member of the TRP family, is well known to be expressed in the peripheral afferent nerve, with desensitization of TRPV1 by agonists inducing analgesic effects (Bhave et al., 2002; Vyklicky et al., 2008). Like TRPV1, TRPA1 is expressed in afferent nerves and reported to contribute to mecano- and chemosensations in the colon (Brierley et al., 2009; La et al., 2011). Therefore, the analgesic effect of ASP7663 on CRD-induced abdominal pain may be mediated by direct desensitization of TRPA1 located on afferent nerve terminal rather than 5-HT-mediated mechanisms. The precise mechanisms underlying the analgesic effect of TRPA1 agonist on abdominal pain remain to be elucidated.

In summary, we showed here that ASP7663 is a potent TRPA1 agonist with 5-HT-releasing efficacy. The compound enhanced colonic motility and exerted analgesic effects against abdominal pain in animal models. Our present results therefore indicate that ASP7663 can be a novel constipation-improving agent with abdominal analgesic effects.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2013.11.020.

References


Characterization of two models of drug-induced constipation in mice and evaluation of mustard oil in these models. Pharmacology 84, 227–233.

TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. Neuron 50, 277–289.

Differences in the expression of transient receptor potential channel V1, transient receptor potential channel A1 and mechanosensitive two pore-domain K⁺ channels between the lumbar splanchnic and pelvic nerve innervations of mouse urinary bladder and colon. Neuroscience 186, 179–187.

Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. J. Neurosci. 25, 4052–4061.


Calcium-dependent desensitization of vanilloid receptor TRPV1: a mechanism possibly involved in analgesia induced by topical application of capsaicin. Physiol. Res. 57 (Suppl. 1), S59–S68.
