Effects of YM905, a Novel Muscarinic M₃-Receptor Antagonist, on Experimental Models of Bowel Dysfunction In Vivo

Seiji Kobayashi*, Ken Ikeda, Mami Suzuki, Toshimitsu Yamada and Keiji Miyata
Pharmacology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan

ABSTRACT—We investigated the effects of YM905 [(+)-(1S,3'R)-quinuclidin-3'-yl 1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carboxylate monosuccinate], a new orally active muscarinic M₃-receptor antagonist, on bowel dysfunction in vivo using experimental models that reproduce the symptoms present in irritable bowel syndrome (IBS). YM905 potently inhibited restraint stress-induced fecal pellet output in fed rats (ED₅₀: 4.0 mg/kg) and diarrhea in fasted rats (ED₅₀: 1.7 mg/kg), with similar potencies to the inhibition of bethanechol-, neostigmine- and nicotine-induced fecal pellet output in rats (ED₅₀: 3.3, 7.9 and 4.5 mg/kg, respectively). YM905 also inhibited 5-hydroxytryptamine (5-HT)-, prostaglandin E₂- and castor oil-induced secretory diarrhea in mice (ED₅₀: 5.5, 14 and 6.3 mg/kg, respectively), but showed no significant effect on cholera toxin-induced intestinal secretion in mice. In addition, YM905 (3, 10 mg/kg) reversed morphine-decreased postprandial defecation in ferrets, a model of spastic constipation, whereas remesetron, a 5-HT₃-receptor antagonist, was not effective. The mode of YM905 action was similar to that of darifenacin, a selective M₃-receptor antagonist, with equivalent potencies. By contrast, propantheline, an antimuscarinic drug that has been used for IBS, was much less potent. These results show that YM905 ameliorates a wide spectrum of bowel dysfunctions through the blockade of M₃ receptors, suggesting its therapeutic potential for treating IBS.

Keywords: YM905, Muscarinic M₃ receptor, Irritable bowel syndrome, Restraint stress, Defecation

Irritable bowel syndrome (IBS) is a common functional disorder characterized by symptoms of altered bowel habits, such as frequent stool, diarrhea and/or spastic constipation (1, 2), probably resulting from a dysfunction of the autonomic nerve system (3, 4). In the current therapy, anticholinergic agents have become the standard class of drugs for the control of IBS (5), since they suppress the cholinergic neurotransmission that excitatory regulates gastrointestinal motility by blockade of the muscarinic receptors.

Muscarinic acetylcholine receptors have been categorized into five subtypes (M₁ – M₅) genetically and pharmacologically, although the distribution and biological significance of the M₁ and M₂ subtypes are not yet well understood (6). Smooth muscle tissues heterogeneously express the M₂ and M₃ subtypes with a population of approximately 4:1 (7, 8), and M₃ receptors are known to play a predominant role in contractile responses in intestinal smooth muscle tissues, including the small intestine (9), ileum (10) and colon (11) under physiological conditions. M₃-receptor antagonists that display fewer effects on M₂ receptors would therefore have some therapeutic utility because they scarcely cause M₂-associated side effects.

YM905 [(+)-(1S,3'R)-quinuclidin-3'-yl 1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carboxylate monosuccinate, Fig. 1] is a novel muscarinic M₃-receptor antagonist that shows a higher binding affinity for exogenously expressed human

*Corresponding author. FAX: +81-298-54-1616
E-mail: kobayashi.seiji@yamanouchi.co.jp

Fig. 1. Chemical structure of YM905.
M₁ receptors (Kᵢ: 9.9 nM) than for M₃ and M₂ receptors (Kᵢ: 24 and 120 nM, respectively) as reported previously (12). In the present study, we evaluated the therapeutic potential of YM905 for the treatment of IBS by using experimental animal models that reproduce bowel abnormalities closely similar to those observed in IBS in vivo. YM905 was examined for inhibitory activity on bowel dysfunction models caused by restraint stress in rats and on secretory diarrhea models in mice. YM905 was also tested on morphine-suppressed postprandial defecation in ferrets, an experimental model of spastic constipation. The effects of YM905 were compared with those of darifenacin, a selective M₁-receptor antagonist, and propantheline, an antimuscarinic agent that has been used clinically for the control of IBS.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (190 – 270 g) and male ICR mice (29 – 42 g) were purchased from Japan SLC (Shizuoka). The animals were maintained in a 12-h light-dark cycle at room temperature (23°C) and were provided food and water ad libitum up to the time the experiment began, except when stated otherwise. Male ferrets (0.9 – 1.5 kg) were purchased from Marshall Farms (North Rose, NY, USA). The ferrets were housed under the same conditions as described above and were fed once a day with free access to water. In the present study, all the animals were used for the experiments with approval from the institute’s animal ethics committee at Yamanouchi Pharmaceutical Co., Ltd.

Restraint stress-induced bowel dysfunction in rats

The effects of YM905, darifenacin and propantheline on restraint stress-induced bowel dysfunction models in rats were examined according to the method of Miyata et al. (13). In the first series, a restraint stress-induced fecal pellet output model was produced using rats with no restrictions on food intake before testing. The rats were restrained in individual restraint cages (KN-468, 53 × 47 × 200 mm; Natsume Seisakusho, Tokyo) for 1 h at room temperature. The number of fecal pellets excreted during restraint stress was counted. In the second series, a stress-induced diarrhea model was produced using rats fasted overnight. The animals were stressed for 3 h by the same procedure as described above, and the occurrence of diarrhea was defined as the evacuation of watery or unformed stools during restraint stress. In each experiment, the test drug was given orally 1 h before exposure to restraint stress.

Bethanechol-, neostigmine- and nicotine-induced fecal pellet output in rats

Rats were subcutaneously administered bethanechol (3 mg/kg), neostigmine (0.1 mg/kg) or nicotine (1 mg/kg). A preliminary experiment showed that each agent, at the dosage described above, produces a submaximal increase in fecal pellet output in fed rats, respectively. The animals were then placed in individual cages at room temperature, and the number of fecal pellets excreted in the subsequent 2-h periods was counted. The test drug was given orally 1 h before administration of bethanechol, neostigmine or nicotine.

5-HT₁-, PGE₂- and castor oil-induced diarrhea in mice

According to the previously reported method (13), diarrhea was induced by an injection of 5-hydroxytryptamine (5-HT) (3 mg/kg, i.p.), prostaglandin E₂ (PGE₂) (0.3 mg/kg, i.p.) or castor oil (0.3 ml/animal, p.o.). The animals were then maintained in individual cages and observed for 3 h at room temperature. The occurrence of diarrhea was defined as the evacuation of watery or unformed stools during the observation period. The test drug was given 1 h before administration of 5-HT, PGE₂ or castor oil.

Morphine-induced spastic constipation in ferrets

The ferrets were fed at 10 a.m. on the day of the experiment and treated subcutaneously with morphine (0.3 mg/kg) 90 min later. The test drug was given orally 90 min after the morphine treatment and the number of pellets excreted during the 2-h period following drug administration was counted. The ferrets were used in a randomized crossover design, with a washout period of at least 7 days.

Cholera toxin-induced intestinal fluid secretion in mice

Mice fasted overnight were used in the experiment. Under ether anesthesia, the small intestine was closed by ligating both the pyloric and ileocecal regions, and 100 μg of cholera toxin in a 0.2 ml saline solution was then injected into the ligated intestinal loop. The loop was isolated and weighed 3 h after cholera toxin injection. The net fluid secretion was defined as the difference in the weights of the loop determined before and after drying. The test drug was injected into the loop immediately after cholera toxin injection.

Data analyses

The value for the number of fecal pellets represents the mean ± S.E.M. and the value for diarrhea the percentage of incidences. A comparison of the values for fecal output between the control and each treatment group was determined statistically using an analysis of variance (ANOVA), followed by Dunnét’s multiple range test. The incidences of diarrhea (number of animals with diarrhea/total number of animals tested) were compared by Pearson’s Chi-square test or Fisher’s exact probability test. Probabilities of less than 0.05 were considered significant. A 50% effective dose...
(ED$_{50}$) value with a 95% confidence limit was estimated as a potency for the test drug by linear regression analysis. All the statistical analyses were performed using the SAS statistical software package (SAS Institute, Cary, NA, USA).

**Chemicals**
YM905, darifenacin and ramosetron hydrochloride (YM060) were synthesized at Yamanouchi Pharmaceutical Co., Ltd. (Ibaraki). Propantheline bromide was purchased from Sigma (St. Louis, MO, USA). The test drugs were administered orally in 0.5% (w/v) methylcellulose solution or intravenously in physiologic saline solution. The other reagents used were as follows: bethanechol, neostigmine bromide, PGE$_2$ and cholera toxin (Sigma); nicotine and 5-HT hydrochloride (Nacalai Tesque, Kyoto); castor oil (Kanto Chemical, Tokyo); morphine hydrochloride (Takeda Chemical Industries, Osaka). The doses of the test drugs and reagents are expressed in terms of the free base.

**RESULTS**

**Effects of YM905 on stress-induced defecation and diarrhea**

Figure 2a shows the effects of YM905, darifenacin and propantheline on restraint stress-induced fecal pellet output in fed rats. The number of fecal pellets counted during the observation period was negligible in unrestrained normal rats. Restraint stress caused fecal pellet output with pellet counts of 6.0 ± 0.8 for the control group for YM905 and darifenacin and 4.7 ± 0.6 for the control group for propantheline (n = 10). YM905 at oral doses of 1 – 30 mg/kg...
dose-dependently decreased the number of pellets excreted
with an ED_{50} value (with 95% confidence interval) of 4.0
(1.8 – 7.3) mg/kg, p.o. Darifenacin (0.3 – 10 mg/kg, p.o.)
and propantheline (10 – 300 mg/kg, p.o.) also decreased
the fecal pellet count with ED_{50} values of 4.4 (2.4 – 14)
and 41 (20 – 73) mg/kg, p.o., respectively. In another
experiment using rats fasted overnight, restraint stress caused
diarrhea with an incidence of 100% in the vehicle-control
group. As shown in Fig. 3a, YM905 (0.3 – 10 mg/kg, p.o.),
darifenacin (0.1 – 3 mg/kg, p.o.) and propantheline (10 –
300 mg/kg, p.o.) dose-dependently decreased the incidences
of diarrhea with ED_{50} values of 1.7 (0.6 – 4.8), 0.9 (0.3 –
6.0) and 64 (29 – 160) mg/kg, p.o., respectively.

Effects of YM905 on bethanechol-, neostigmine- and nico-
tine-induced fecal pellet output
Subcutaneous administration of bethanechol (3 mg/kg),
neostigmine (0.1 mg/kg) or nicotine (1 mg/kg) induced
fecal pellet output in fed rats, with pellet counts of 9.2 ±
1.0, 7.3 ± 0.6 and 4.2 ± 0.6 for the control group for YM177
and darifenacin and 8.0 ± 0.9, 8.3 ± 0.7 and 4.7 ± 0.6 for
the control for propantheline, respectively (n = 10). Oral
administration of YM905 (1 – 30 mg/kg), darifenacin (0.3 –
10 mg/kg) and propantheline (10 – 300 mg/kg) dose-
dependently suppressed bethanechol-induced fecal pellet
output, with ED_{50} values of 3.3 (2.1 – 4.8), 2.7 (2.0 – 3.9)
and 67 (48 – 97) mg/kg, p.o., respectively (Fig. 2b). Oral
YM905, darifenacin and propantheline also caused a de-
crease in neostigmine- and nicotine-induced fecal pellet
output dose-dependently. Their ED_{50} values were 7.9
(3.8 – 22), 4.4 (2.1 – 19) and 56 (40 – 79) mg/kg, p.o. for
neostigmine-induced defecation and 4.5 (2.0 – 8.7), 1.7
(0.7 – 4.4) and 97 (52 – 270) mg/kg, p.o. for nicotine-
induced defecation, respectively (Fig. 2: c and d). In the
preliminary experiment, the potencies of YM905 and
darifenacin on bethanechol-induced fecal pellet output
were estimated for the intravenous route as ED_{50} values of
2.3 (1.6 – 3.4) and 0.2 (0.1 – 0.3) mg/kg, respectively (data

Fig. 3. Effects of YM905 (square), darifenacin (circle) and propantheline (triangle) on restraint stress (a)-induced diarrhea in
fasted rats and 5-HT (b)-, PGE_{2} (c)- and castor oil (d)-induced diarrhea in mice. The drugs were administered orally 1 h before
restraint stress or injection of 5-HT (3 mg/kg, i.p.), PGE_{2} (0.3 mg/kg, i.p.) or castor oil (0.3 ml/animal, p.o.). Each point repre-
sents the % inhibition of diarrhea in 7 – 10 animals. *P<0.05, **P<0.01, ***P<0.001 vs control (χ^{2} test).
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Effects of YM905 on 5-HT-, PGE\textsubscript{2} and castor oil-induced diarrhea

The effects of YM905, darifenacin and propantheline on 5-HT-, PGE\textsubscript{2} and castor oil-induced diarrhea in mice are shown in Fig. 3, b–d. Administration of 5-HT (3 mg/kg, i.p.), PGE\textsubscript{2} (0.3 mg/kg, i.p.) or castor oil (0.3 ml/animal, p.o.) caused diarrhea in vehicle-treated control mice with an incidence of 100%. YM905 (0.3–10 mg/kg, p.o.), darifenacin (0.1–3 mg/kg, p.o.) and propantheline (100–1000 mg/kg, p.o.) dose-dependently decreased the incidence of diarrhea caused by 5-HT and PGE\textsubscript{2}, with ED\textsubscript{50} values of 5.5 (2.7–11), 2.6 (1.2–8.0) and 330 (150–1200) mg/kg, p.o. for 5-HT-induced diarrhea and 14 (2.6–330), 3.7 (0.9–20) and 180 (20–440) mg/kg, p.o. for PGE\textsubscript{2}-induced diarrhea, respectively. In contrast, although YM905 and propantheline decreased the incidence of diarrhea caused by castor oil dose-dependently, with ED\textsubscript{50} values of 6.3 (2.6–17) and 290 (71–1200) mg/kg, p.o., respectively, darifenacin did not cause significant inhibition of castor oil-induced diarrhea at doses up to 30 mg/kg, p.o.

Effect of YM905 on morphine-induced spastic constipation

Feeding stimulates colonic motility via the so-called gastrocolonic response under nervous regulation (14), thereby resulting in the occurrence of defecation. In the present study, feeding caused almost all of the ferrets not treated with morphine to defecate 1 or 2 pellets during the 2-h observation periods. Subcutaneously administration of morphine (0.3 mg/kg) significantly decreased feeding-stimulated fecal pellet output in ferrets. In the morphine-treated control group, about half of the ferrets defecated only once during the 2-h periods. Oral doses of YM905 (3, 10 mg/kg) significantly increased the morphine-decreased number of fecal pellets dose-dependently, and the number of pellets for the 10 mg/kg-treated group was approximately equal to that for the group not treated with morphine. Darifenacin (10 mg/kg, p.o.) also significantly increased the morphine-decreased number of pellets. The selective 5-HT\textsubscript{3}-receptor antagonist ramosetron, on the other hand, had no significant effect on morphine-induced constipation at doses up to 0.1 mg/kg, p.o. (Fig. 4).

Effect of YM905 on cholera toxin-induced intestinal fluid secretion in mice

Injection of cholera toxin significantly increased intestinal fluid secretion in mice with net fluid volumes of 1.6 ± 0.1 and 3.1 ± 0.2 ml for the cholera toxin-untreated and treated groups, respectively (P < 0.001, n = 10). YM905, at intraduodenal doses of 3, 10 and 30 mg/kg, showed no significant effect on cholera toxin-induced increase in intestinal fluid secretion with net fluid volumes of 2.9 ± 0.1, 3.0 ± 0.2 and 3.0 ± 0.2 ml, respectively (n = 10). Intraduodenal darifenacin (30 mg/kg) and propantheline (100 mg/kg) also did not affect the intestinal secretion with fluid volumes of 3.0 ± 0.1 (n = 10) and 3.7 ± 0.3 ml (n = 8), respectively.

DISCUSSION

Stress is known to be an important factor in causing IBS, since it significantly alters bowel functions (15). Several

![Fig. 4. Effects of YM905 (left panel), ramosetron and darifenacin (right panel) on morphine-induced spastic constipation in ferrets. The drugs were administered orally 90 min after morphine injection (0.3 mg/kg, s.c.). The results are expressed as the number of pellets excreted during the 2-h period following drug administration. \(*P<0.01, **P<0.001 vs normal group; *P<0.05, **P<0.01, ***P<0.001 vs control group (Dunnett’s multiple range test).\)
rodent models of bowel dysfunction caused by restraint stress have been investigated for pharmacological analysis of a stress-related bowel disorder like IBS (13, 16, 17). Previous studies demonstrated that restraint stress results in an increase in fecal pellet output in fed rats, as well as in diarrhea in food-deprived rats, which are equally mediated through the endogenous activation of the 5-HT$_3$ receptor (13). 5-HT$_3$ receptors are prevalent in enteric neurons that are responsible for a variety of gastrointestinal functions (18), and their activation is thought to enhance cholinergic transmission via the release of acetylcholine from parasympathetic nerve terminals (13), thus resulting in an increase in gastrointestinal motility and fluid secretion. In the present study, oral YM905 potently suppressed both fecal output and diarrhea caused by restraint stress within an equal dose range. YM905 also suppressed cholinergic enhancement of fecal output responses following injection of bethanechol (a muscarinic agonist), neostigmine (an acetylcholine esterase inhibitor) and nicotine (an agent that releases acetylcholine from nerve endings), with similar potencies to those in restraint stress models. In addition, YM905 does not have a significant affinity for any 5-HT-receptor subtypes (K. Ikeda et al., unpublished data). The inhibitory effects of YM905 in restraint stress models are therefore caused by the antimuscarinic action.

The antidiarrheal activity of YM905 was also evaluated using exogenous 5-HT, PGE$_2$ and castor oil-induced diarrhea in mice, which are well-known models for secretory diarrhea (19–21). Although 5-HT, PGE$_2$ and castor oil stimulate diarrhea through distinct mechanisms of action, these diarrhea models are suitable for evaluating the antidiarrheal activity of drugs, since these models are reported to be sensitive to clinically efficacious antidiarrheal drugs such as loperamide (22) and trimebutine (23). In the present study, the antidiarrheal activities of YM905 were similarly observed in these models, and the potencies were almost equivalent to those in both of cholinergically-enhanced and stress-induced bowel dysfunction models. This YM905 activity pattern resembles that of atropine, a non-selective muscarinic antagonist, as reported previously (13). However, YM905 actions are distinguishable from those of trimebutine since trimebutine is not efficacious for inhibiting restraint stress-induced fecal pellets output (23). In addition, YM905 showed no effect on cholera toxin-induced intestinal fluid secretion in vivo, which is mediated via the activation of the cyclic AMP pathway, suggesting that YM905 may have no other antisecretory effect beyond its antimuscarinic action. The antidiarrheal actions of YM905 therefore appear to be predominantly dependent on its inhibitory effects on cholinergic-mediated enteric propulsion of watery stool, rather than its antisecretory action.

Although a large proportion of IBS patients suffer constipation derived from spams of the lower gastrointestinal tract, animal models for spastic constipation have been poorly developed. In the present study, the effect of YM905 on spastic constipation was tested using the morphine-induced constipation model in ferrets, whose gastrointestinal motility pattern resembles that in humans (24). Since morphine, a $\mu$-opioid receptor agonist, causes constipation in vivo by potentiating intermittent tonic contraction and reducing propulsive waves of the colon (25, 26), this model can reproduce the bowel behavior similar to that shown in IBS patients with spastic constipation. Our results showed that YM905 reverses morphine-induced constipation with a similar potency to those shown in improving defecation and diarrhea. The exact mechanism explaining why YM905 blocked the action of morphine remains unknown, since, at least from the in vitro experiments, morphine is generally known as an inhibitor of acetylcholine release from cholinergic nerve terminals in the myenteric plexus (27). In the enteric nervous system, however, $\mu$-opioid receptors are located in not only cholinergic neurons, but also adrenergic neurons that negatively regulate cholinergic tone (28). Moreover, it has been reported that stimulation of the $\mu$-opioid receptor enhances intestinal contraction by preventing the adrenergic inhibition of acetylcholine release from cholinergic neurons (28, 29), providing the putative mechanism whereby morphine induces intestinal spasm. This notion is supported by the previous report showing that morphine-caused colonic hypermotility was abolished by pretreatment with atropine in conscious dogs (30). Taken together, YM905 improved constipation possibly by inhibiting the cholinergically-enhanced intestinal segmental spasms. It has been reported that ramosetron, a selective 5-HT$_3$-receptor antagonist, suppresses restraint stress-induced bowel dysfunction and 5-HT-induced diarrhea (13, 22), and this class of agent holds therapeutic promise for gastrointestinal disorders, including IBS (18). In the present study, ramosetron had no effects on ferret constipation, even at doses sufficient to protect against stress-induced bowel dysfunction. These findings suggest that YM905 may be efficacious for spastic constipation in IBS that is resistant to the 5-HT$_3$-receptor antagonist.

In the present study, the effects of YM905 were compared with those of darifenacin, a selective M$_2$-receptor antagonist, to validate the role of M$_2$ receptors in stress-related bowel dysfunction, because YM905 selectivity for muscarinic receptor subtypes seems not to be sufficient to distinguish the inhibitory action on smooth muscle M$_2$ receptors from that on ganglionic M$_3$ receptors facilitating vagal neurotransmission. Darifenacin displays a more than tenfold selectivity for M$_3$ receptors as compared not only with M$_2$ but also with M$_1$ receptors, as a result of which the compound has been extensively used as a tool for pharmacologically characterizing the smooth muscle M$_3$ recep-
tors (6, 7, 31). In the present study, darifenacin suppressed restraint stress-induced bowel dysfunction dose-dependently, with potencies similar to its antimuscarinic potencies for bethanechol-, neostigmine- and nicotine-induced defecation. The M₃-antagonistic action of darifenacin may be negligible over the dose range that inhibits stress-mediated bowel dysfunction, since darifenacin did not antagonize the M₁-receptor-mediated response in conscious dogs, even at an oral dose of 3 mg/kg (31), a dose equivalent to the ED₅₀ values estimated for restraint-stress models. These findings therefore suggest the predominant involvement of M₁ receptors in stress-related altered bowel disorders. Darifenacin also inhibited 5-HT₃- and PGE₂-induced secretory diarrhea as potently as YM905, but was not potent against castor oil-induced diarrhea. This result may stem from a shorter duration of darifenacin action, since the time required to reach the onset of diarrhea after induction was much longer in castor oil-induced diarrhea than in 5-HT₃- and PGE₂-induced diarrhea. Moreover, darifenacin reversed morphine-induced ferret constipation as potently as YM905. Thus, darifenacin displayed a pharmacologic profile similar to that of YM905. Taken together, YM905 would suppress stress-mediated bowel dysfunction, secretory diarrhea and spastic constipation by the blockage of intestinal smooth muscle M₁ receptors, a mechanism identical to that of darifenacin action.

Propantheline, on the other hand, an antimuscarinic drug that has been used in clinical treatment of disorders due to smooth muscle dysfunction including IBS, was efficacious for all of the models used in this study, but the potencies were considerably weak. This is probably due to poor oral activity, since propantheline is a quaternary amine derivative, while YM904 and darifenacin are members of the tertiary amine-containing group.

A number of drugs in the antimuscarinic class are currently used for the control of IBS. However, their anticholinergic side effects such as tachycardia and dry mouth have presented serious problems that require discontinuation of prolonged drug therapy for IBS. In this respect, YM905 offers pharmacological advantages, since YM905 not only has little effect on the cardiac M₃ receptors that mediate the negative inotropic response, but also has recently been shown to selectively inhibit the smooth muscle M₁ receptors, as compared with the salivary gland M₁ receptors that mediate salivary secretory functions, as reported previously (12). Although further studies are required to fully understand the mechanism of YM905 action, YM905 may promise pharmacological advantages with fewer side effects in comparison to the conventional antimuscarinic drugs.

In summary, YM905 potently inhibited restraint stress-induced bowel dysfunction, as well as 5-HT₃-, PGE₂- and castor oil-induced secretory diarrhea. YM905 also potently suppressed morphine-induced spastic constipation. These effects of YM905 are possibly due to the blockage of muscarinic M₁ receptors. Consequently, these findings suggest that YM905 would be a useful drug for preventing bowel disorders such as IBS.

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