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# Effects of TAK-480, a Novel Tachykinin $NK_2$–Receptor Antagonist, on Visceral Hypersensitivity in Rabbits and Ricinoleic Acid–Induced Defecation in Guinea Pigs

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**Abstract.** TAK-480, 4-(difluoromethoxy)-*N*-((1*R*,2*S*)-2-(((3*aR*,4*R*,9*bR*)-4-(methoxymethyl)-2,3,3*a*,4,5,9*b*-hexahydro-1*H*-pyrrolo[3,2-*c*]quinolin-1-yl)carbonyl)cyclohexyl)benzamide, is a novel tachykinin $NK_2$–receptor antagonist. In this study, we investigated its antagonistic activity and efficacy in animal models of visceral hypersensitivity and stimulated bowel function which have been implicated to underlie the symptoms in irritable bowel syndrome (IBS). TAK-480 showed potent binding affinity for human $NK_2$ receptors with a marked species difference and a 10,000-fold selectivity versus $NK_1$ and $NK_3$ receptors. TAK-480 dose-dependently antagonized colonic contractions induced by administration of the $NK_2$ receptor–selective agonist beta-$Ala^8$-NKA(4-10) ($\beta$A-NKA) in anesthetized rabbits. In a rabbit model of intracolonic zymosan-induced visceral hypersensitivity, TAK-480 markedly inhibited the visceromotor response to colorectal distension, in contrast to the moderate inhibition by the serotonin 5-$HT_3$–receptor antagonist alosetron. In addition, TAK-480 suppressed ricinoleic acid–induced defecation without affecting spontaneous defecation in guinea pigs, whereas alosetron suppressed both. Furthermore, TAK-480 inhibited smooth muscle contractions produced by natural tachykinins (substance P, neurokinin A, and neurokinin B) as well as $\beta$A-NKA in an isolated human colon. In conclusion, the novel $NK_2$-receptor antagonist TAK-480 improved visceral hypersensitivity and accelerated defecation without causing constipation in experimental animals. Furthermore, the potent functional blockade of $NK_2$ receptors in human colon might suggest the potential effectiveness of TAK-480 in IBS patients.

***Keywords***: $NK_2$-receptor antagonist, TAK-480, irritable bowel syndrome, visceral hypersensitivity, stimulated defecation

## Introduction

Irritable bowel syndrome (IBS) is a chronic functional disorder of the gut associated with altered bowel habits (constipation, diarrhea, or alternation of these) and abdominal pain/discomfort in the absence of demonstrable organic changes explaining these symptoms (1). Lowered visceral pain/discomfort thresholds and enhanced colonic motor responses to colorectal stimulation have been well documented, confirming the presence of visceral hypersensitivity and exaggerated colonic motility in the pathology of IBS (2 – 4). The prevalence rate of IBS has been reported as 10% – 20% in developed countries; although it is not fatal, refractory symptoms can severely compromise the quality of life (5, 6). IBS is commonly classified into three subtypes according to the bowel pattern: diarrhea-predominant (D-IBS), constipation-predominant, and mixed pattern (1). The serotonin 5-$HT_3$–receptor antagonist alosetron is currently available as a pharmacological medication in the US to treat

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D-IBS in women (7). However, the restricted dispensation of alosetron prescriptions because of its serious adverse effects and its relatively modest therapeutic benefits has discouraged the widespread use of this agent for managing IBS (7). Thus, it has become essential to develop and launch a novel product with more potent efficacy and better tolerability for patients with IBS.

Remarkable efforts and progress made in understanding gastrointestinal (GI) physiology and pathophysiology has lead to the establishment of tachykinins, which are key mediators of GI functions (8 – 10). Tachykinins are associated with a family of neuropeptides that includes substance P (SP), neurokinin A (NKA), and neurokinin B (NKB), which can modulate communication between neurons and effector cells by acting through their preferential receptors $NK_1$, $NK_2$, and $NK_3$, respectively (11). Of these three receptors, $NK_2$ is mainly distributed in the human GI tract and is known to be primarily responsible for inducing colonic contractions mediated by smooth muscle cells (12, 13). In addition to its dominant contribution to gut motility, neuronal excitation via the $NK_2$ receptor has been pharmacologically identified in sensory dorsal root ganglion (DRG) neurons (14); it was shown to be involved in visceral nociception and colonic hypersensitivity associated with colitis and psychological stress in animal studies (15, 16). The peptidic $NK_2$-receptor antagonist nepadutant has been reported to inhibit colonic motility changes induced by intravenous (i.v.) infusion of NKA without affecting normal motility in healthy volunteers (17). It is interesting that in the same study, nepadutant also ameliorated IBS-like symptoms induced by NKA, such as abdominal pain and discomfort. Given that hypersensitivity and hypermotility of the gut are potentially associated with IBS symptoms, these findings provide an attractive therapeutic rationale for developing $NK_2$-receptor antagonists for the treatment of IBS.

TAK-480, 4-(difluoromethoxy)-*N*-((1*R*,2*S*)-2-(((3*aR*,4*R*,9*bR*)-4-(methoxymethyl)-2,3,3*a*,4,5,9*b*-hexahydro-1*H*-pyrrolo[3,2-*c*]quinolin-1-yl)carbonyl)cyclohexyl) benzamide, is a novel tachykinin $NK_2$–receptor antagonist, highly selective to the human $NK_2$ receptor, thereby implicating its prospective $NK_2$-receptor antagonism in clinical settings (Fig. 1). In this study, we attempted to investigate the effect of this compound on visceral hypersensitivity and enhanced colonic function, commonly recognized as IBS characteristics, in order to investigate the potential application of TAK-480 for the treatment of this disease. Our study clearly demonstrated that TAK-480 possesses a unique property showing species-related differences in the binding affinity for the $NK_2$ receptor (higher affinity for human, rabbit, and guinea pig in nM order and lower affinity for rat in $\mu$M order) in accordance with heterogeneity of the receptor (18). Therefore, we investigated the efficacy of TAK-480 in non-rodent animal models partially-modified from original ones: a rabbit model of zymosan-induced visceral hypersensitivity and a guinea-pig model of ricinoleic acid–induced defecation, which partially mimic common characteristics of IBS (19, 20). In addition, we examined $NK_2$-receptor antagonism in isolated human colon specimens to further predict the clinical efficacy of TAK-480.

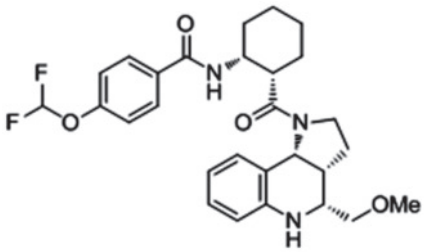


**Fig. 1.** Chemical structure of TAK-480.

## Materials and Methods

*Animals*

Experiments were conducted with male New Zealand White/Kbl rabbits (1.0 – 1.5 kg; Kitayama Rabesu, Ina) and male Hartley guinea pigs (360 – 400 g; SLC Japan, Hamamatsu). Animals were housed under standard controlled environmental conditions with a 12-h light/dark cycle and free access to water and food. All procedures were approved by the Takeda Pharmaceutical Company's Experimental Animal Care and Use Committee.

*Human colon tissue*

Human colon samples were obtained from 11 patients with colon cancer (age, 33 – 87 years; female:male, 10:1) with informed consent implemented in relation to the Declaration of Helsinki. Non-cancerous specimens were surgically dissected and immediately immersed in ice-cold Krebs solution (120.7 mM NaCl, 15.5 mM $NaHCO_3$, 5.9 mM KCl, 1.2 mM $NaH_2PO_4$, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 11.5 mM glucose). The specimens were transferred to the laboratory of Takeda Pharmaceutical Company. All procedures were approved by the ethical committees of Saiseikai-Nakatsu Hospital (Osaka) and Takeda Pharmaceutical Company (Kanagawa).

*Drugs*

TAK-480 and alosetron hydrochloride were synthesized at Takeda Pharmaceutical Company. For some experiments, alosetron hydrochloride was additionally purchased from Bosche Scientific (New Brunswick, NJ,

USA). For i.v. administration, TAK-480 was dissolved in a solution containing equivalent volumes of dimethylacetamide (Wako, Osaka) and polyethylene glycol 400 (Wako), whereas alosetron was dissolved in saline. Compound solutions were administered in a volume of 0.05 mL/kg. For intraperitoneal (i.p.) administration, both compounds were suspended in 0.5% methylcellulose solution (Shin-Etsu Chemical Co., Ltd., Tokyo) and administered as volumes of 2 mL/kg.

*Tachykinin-receptor binding assay*

For the $NK_1$-receptor binding assay, human IM9 cell membranes, a lymphoblastoma cell line that expresses $NK_1$ receptors, was used as the source of $NK_1$ receptors and [$^{125}$I]Bolton-Hunter–labeled Lys$^3$ substance P (GE Healthcare Japan, Tokyo) was used as the $NK_1$-receptor ligand. In brief, various concentrations of test compounds and radiolabeled ligand were incubated with IM9 membranes in a final volume of 0.2 mL buffer [50 mM Tris-HCl (pH 7.4) containing 0.5% DMSO, 2.3 mM $MnCl_2$, 0.02% BSA, 1.5 $\mu$g/mL chymostatin, 30 $\mu$g/mL bacitracin, and 30 $\mu$g/mL APMSF] at room temperature for 30 min. The reactions were terminated by rapid filtration through a GF/C filter plate (PerkinElmer Japan, Yokohama) presoaked with 0.3% polyethyleneimine, following by ten washes with a washing buffer [50 mM Tris-HCl (pH 7.4) containing 0.02% BSA]. Filters were dried and soaked in 20 $\mu$L/well of MicroScint$^{TM}$-0 (PerkinElmer Japan) and receptor-bound radioactivity was then measured using a TopCount liquid scintillation counter (PerkinElmer Japan).

For the $NK_2$-receptor binding assay, various concentrations of test compounds and [$^{125}$I]NKA (GE Healthcare Japan) were incubated with the cell membranes of CHO cells expressing human, rabbit, or rat $NK_2$ receptors or with intestinal membranes obtained from guinea pigs or mice in a final volume of 0.2 mL reaction buffer at room temperature for 30 min. The tissue membranes from guinea pigs and mice were prepared as follows: Male mice (20 – 30 g, C57BL/6N; CLEA Japan, Inc., Tokyo) and male Hartley guinea pigs (6-week-old, SLC Japan) were euthanized by dry ice and the intestine removed rapidly. The tissues were washed several times in ice-cold suspension buffer [50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 40 $\mu$g/mL bacitracin, 2 $\mu$g/mL chymostatin, and 40 $\mu$g/mL APMSF]. Then, the tissues were weighed, cut into thin pieces, and homogenized with a polytron sonicator in ice-cold suspension buffer. The homogenates were centrifuged at 2,000 rpm (Roter: R17A, Hitachi himac CR21G; Hitachi Koki Co., Ltd., Tokyo) for 10 min at 4°C. The supernatants were collected, suspended in suspension buffer, and centrifuged at 30,000 rpm (Roter: 50.2 Ti, Beckman Coulter Optima L-100XP; Beckman Coulter, Inc., Brea, CA, USA) for 20 min at 4°C. The pellets were collected and each washed twice with incubation buffer [50 mM Tris-HCl (pH 7.4), 300 mM KCl, 10 mM EDTA, and 50 mM Tris-HCl buffer (pH 7.4)]. The pellets were suspended in assay buffer [50 mM Tris-HCl (pH 7.4), 3 mM $MnCl_2$, 0.02% BSA, 40 $\mu$g/mL bacitracin, and 40 $\mu$g/mL APMSF] to give a concentration of 4 mg of protein per mL. Protein was determined with a protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard.

For the $NK_3$-receptor binding assay, various concentrations of test compounds and [$^{125}$I]His, MePhe$^7$-NKB (PerkinElmer Japan) were incubated with CHO cell membranes expressing human $NK_3$ receptors in a final volume of 0.2 mL reaction buffer at room temperature for 30 min.

Cell membrane sources and concentrations of radiolabeled ligands used in the binding affinity assays for each tachykinin receptor are summarized in Table 1. Ligand concentrations corresponded approximately to $K_d$ values in order to obtain comparable responses over the experiments with different cell sources. For determination of non-specific binding, corresponding non-labeled ligands were employed (2 $\mu$M of SP, 1 $\mu$M of NKA, and 4 $\mu$M of NKB; Peptide Institute, Osaka).

*Proximal colonic contraction in anesthetized rabbits: in vivo $NK_2$-receptor antagonism*

Rabbits were starved for 48 h before the experiments. Under urethane (1.5 g/kg, i.p.) anesthesia, a surgical airway was established with a tracheal cannula, and a polyethylene catheter was inserted into a jugular vein for drug administration. For detection of intracolonic pressure, a balloon catheter (2-cm long) made from a latex condom was inserted via a midline incision into the proximal colon and secured in place with sutures. The other end of the balloon catheter was connected to a pressure transducer and baseline balloon pressure was set to approximately 5 mmHg. During the experiment, the body temperature was kept constant at 37°C using a heat pad (Terumo, Tokyo).

At least 1 h after surgery, transient colonic contractions were produced every 15 min by i.v. administration of beta-Ala$^8$-NKA(4-10) ($\beta$A-NKA, 0.1 nmol/kg; Bachem AG, Bubendorf, Switzerland) at a volume of 0.1 mL/kg. After reproducible contractions were confirmed twice (baseline response), vehicle or single dose of TAK-480 was administered i.v. 5 min before the next $\beta$A-NKA challenge, and its inhibitory effect was evaluated. Colonic contractions were sampled using a pressure transducer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) connected to a personal computer with Chart5

**Table 1.** Membrane sources and radiolabeled ligands employed for in vitro binding affinity assays for tachykinin receptors

| Tachykinin receptor | Species | Membrane | | Radiolabeled ligand | |
|---|---|---|---|---|---|
| | | source | conc. ($\mu$g/well) | ligand | conc. (pM) |
| $NK_2$ | human | recombinant | 2 | [$^{125}$I]neurokinin A | 500 |
| | rabbit | recombinant | 1 | | 500 |
| | guinea pig | tissue | 50 | | 1000 |
| | rat | recombinant | 5 | | 100 |
| | mouse | tissue | 40 | | 500 |
| $NK_1$ | human | IM9 | 25 | [$^{125}$I]Bolton-Hunter–labeled Lys$^3$ substance P | 140 |
| $NK_3$ | human | recombinant | 2 | [$^{125}$I]His, MePhe$^7$-neurokinin B | 200 |

software (ADInstruments, Sydney, Australia) and displayed as an increase in balloon pressure normalized to the baseline response before vehicle or TAK-480 treatment.

*Colorectal distension (CRD) and hypersensitivity in rabbits*

As described previously (19), all surgical procedures were performed under pentobarbital sodium anesthesia. The lower left abdominal musculature (abdominal external oblique muscle) was exposed, and a force transducer was attached for monitoring abdominal muscle contractions, that is, the visceromotor response (VMR) to CRD. The transducer was secured in place with sutures, and the other end was subcutaneously tunneled out to the back of the head. Incisions were sutured and penicillin (Meiji Co., Ltd., Tokyo) was prophylactically administered to avoid infection.

Following a recovery period from surgery (more than 3 days), the effect of TAK-480 and alosetron on VMR to CRD was examined. The animals were starved for 48 h but were provided free access to water before the experiment. As reported previously (19), VMR to CRD in rabbits is blunted in the physiologic state; therefore, to enhance VMR, zymosan suspension (25 mg/mL, 4 mL, 24 mL/h; MP Biomedicals, Inc., Solon, OH, USA) was intracolonically administered following 5% *N*-acetyl-L-cysteine solution (4 mL, 24 mL/min; Wako) through a silicone catheter (o.d., 3 mm; i.d., 2 mm) rectally inserted up to 8-cm proximally from the anal sphincter. During the treatment, animals were placed in a clear syringe chamber (i.d., 13 – 15 cm, length, 45 – 60 cm; Osaka Riko, Osaka).

One hour after zymosan treatment, a latex balloon (length, 6 cm) was rectally inserted 2 cm beyond the anal verge and securely taped to the tail. To acclimatize the animals to the testing procedure, they were subjected to constant-pressure balloon inflation at 30 mmHg for 10 min. Following a 10-min recovery period from acclimatization, the number of VMR observed during CRD (35 mmHg, 10 min) was counted as a pre-treatment value. A post-treatment value was similarly determined 10 min after i.v. drug administration via auricular vein. The inhibition rate for each compound was calculated by comparing values before and after the drug treatment as follows: inhibition rate (%) = 1 − post-treatment value / pre-treatment value. Abdominal muscle contractions were recorded via a force transducer, and these contractions were amplified and monitored using Chart5 software (ADInstruments). Balloon pressure was constantly regulated using a barostat pressure controlling system (Biotex, Kyoto).

*Ricinoleic acid–induced defecation in guinea pigs*

One day before the experiment, each guinea pig was housed separately in individual cages with free access to food and water to acclimatize them to the experimental environment. On the day of the experiment, ricinoleic acid (Tokyo Chemical Industry Co., Ltd., Tokyo) was orally administered at a volume of 10 mL/kg 2 h after food deprivation, and the number of fecal pellets was counted at 2, 4, and 6 h after ricinoleic acid challenge. Equivalent volume of water was orally administered in the untreated group (normal) instead of ricinoleic acid. In ricinoleic acid–treated groups, vehicle, TAK-480 (1, 3, or 10 mg/kg), or alosetron (1, 3, or 10 mg/kg) was administered i.p. 10 min before the challenge.

*Spontaneous defecation in guinea pigs*

Each guinea pig was housed individually 1 week before the experiment. After i.p. drug administration (vehicle, TAK-480 at 10 mg/kg, or alosetron at 10 mg/kg),

the number of fecal pellets excreted during the testing period (6 h) was counted. During the experiment, animals were provided free access to food and water.

*In vitro colonic contraction*

Longitudinal (rabbit) and circular (human) smooth muscle strips of the proximal colon were prepared after removing the mucosa with fine forceps. Specimens were placed in glass organ baths filled with 10 mL of Krebs solution bubbled with 95% $O_2$ / 5% $CO_2$ at 35°C. Baseline muscle tension was maintained at 1 g to allow isometric recording. The signal was sampled via an electric recorder (MLT-870, ADInstruments), amplified (MLT-119, ADInstruments), and monitored on a personal computer with Chart5 software (ADInstruments).

Agonist-induced colonic contractions were induced by the addition of $\beta$A-NKA (Bachem AG) to the organ bath. First, concentration-dependent colonic contractions were confirmed with cumulative application of $\beta$A-NKA in the range of 0.001 – 1 $\mu$M (pre-treatment challenge). After a subsequent wash-out, the effects of vehicle (DMSO) or TAK-480 (0.001 – 1 $\mu$M) on cumulative $\beta$A-NKA challenge (0.001 – 10 $\mu$M) were examined. The drugs were applied 15 and 60 min before beginning the cumulative $\beta$A-NKA challenge in rabbit and human colon, respectively. Colonic contractions were quantified as the increase in mechanical muscle tension from baseline and normalized as a percentage of the cumulative response with pre-treatment challenge. The organ bath was washed out three times after each $\beta$A-NKA challenge. Colon strips were single-use for each concentration of TAK-480 with the cumulative $\beta$A-NKA challenge.

In another experiment, SP (1 $\mu$M, Peptide Institute), NKA (0.1 $\mu$M, Peptide Institute), or NKB (1 $\mu$M, Peptide Institute) was applied to the organ bath to investigate the inhibitory effect of TAK-480 on natural tachykinin-induced colonic contractions. Submaximal ligand concentrations were employed according to the previous report (13). After confirming the reproducibility of the contractions with natural tachykinin and subsequent wash-out, vehicle (DMSO) was applied 15 min before the next tachykinin challenge. Subsequently, the inhibitory effect of TAK-480 (0.01 – 1 $\mu$M) was evaluated similarly to vehicle evaluation. The organ bath was washed-out three times after every tachykinin challenge. Colonic contractions were normalized as a percentage of vehicle-treated contraction.

*Data analysis*

Data are expressed as the mean ± S.E.M. Results were analyzed using the statistical analysis software SAS preclinical package version 5 (SAS Institute, Inc., Cary, NC, USA). Student's *t*-test or Williams' test were conducted as required and $P < 0.05$ and $P < 0.025$, respectively, were considered statistically significant. For evaluation of in vitro $NK_2$-receptor affinity and in vivo antagonistic activity, $IC_{50}$ and $ID_{50}$ values were calculated by a 2-parameter logistic regression and the SAS preclinical package version 5, respectively. For in vitro Magnus experiments, the averaged data from the same donor, containing 2 – 3 strips was used as n = 1. The $pA_2$ values of TAK-480 in human and rabbit colon strips were obtained using Schild plots. X-intercepts were calculated as $pA_2$ values from the linear regression of mean values of the log (CR − 1) versus the negative logarithm of TAK-480 concentration (CR: concentration ratio).

## Results

*In vitro binding affinity of TAK-480 for tachykinin receptors*

The binding affinity of TAK-480 to tachykinin receptors was investigated in receptor-expressing cells or tissue membranes. TAK-480 inhibited the specific binding of [$^{125}$I]NKA to the human $NK_2$ receptor in a concentration-dependent manner with an $IC_{50}$ value of 0.95 nM (Fig. 2A). On the other hand, It did not interact with the

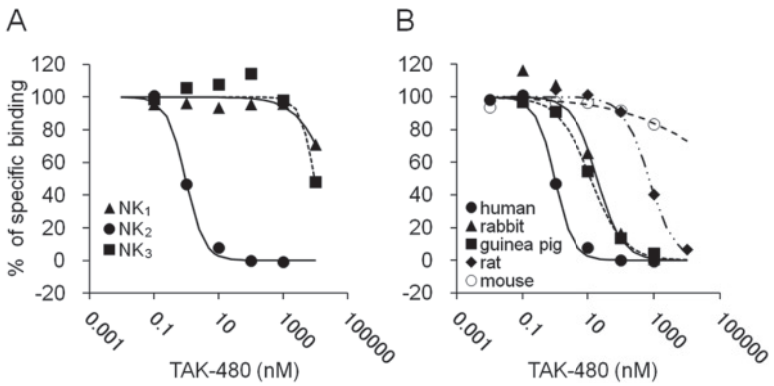


**Fig. 2.** In vitro binding affinity of TAK-480 to tachykinin receptors. Data are expressed as the mean of specific binding of radiolabeled ligand to tachykinin receptors. A: TAK-480 inhibited [$^{125}$I]neurokinin A (NKA) binding to human $NK_2$ receptors in a concentration-dependent manner, but did not affect [$^{125}$I]Bolton-Hunter–labeled Lys$^3$ substance P and [$^{125}$I]His, MePhe$^7$-NKB binding to human $NK_1$ and $NK_3$ receptors, respectively. B: TAK-480 showed marked species differences in binding affinity for $NK_2$ receptors (higher affinity for human, rabbit, and guinea pig and lower affinity for rodents).

human $NK_1$ and $NK_3$ receptors that bind [$^{125}$I]Bolton-Hunter–labeled Lys$^3$ substance P and [$^{125}$I]His, MePhe$^7$-NKB, respectively ($IC_{50}$ value > 10 $\mu$M). Interestingly, TAK-480 recognized rabbit, guinea pig, and rat $NK_2$ receptors with $IC_{50}$ values of 20, 12, and 720 nM, respectively, showing species-specific differences in binding affinity to $NK_2$ receptors (Fig. 2B). Even at 10 $\mu$M, TAK-480 did not interact with the mouse $NK_2$ receptor.

*In vivo $NK_2$-receptor antagonism of TAK-480*

We attempted to confirm the in vivo $NK_2$-receptor antagonism of TAK-480 using colonic contraction in rabbits as a pharmacodynamic marker. Under urethane anesthesia, phasic colonic contractions were produced by i.v. administration of the selective $NK_2$-receptor agonist $\beta$A-NKA (Fig. 3A). As shown in Fig. 3B, i.v. treatment with TAK-480 in doses ranging from 0.01 to 1 mg/kg suppressed $\beta$A-NKA-induced colonic contractions in a dose-dependent manner. Almost complete inhibition was observed at a dose of 1 mg/kg. $ID_{50}$ value was calculated as 0.12 mg/kg. Vehicle treatment did not alter the magnitude of colonic contractions compared with that of baseline contractions.

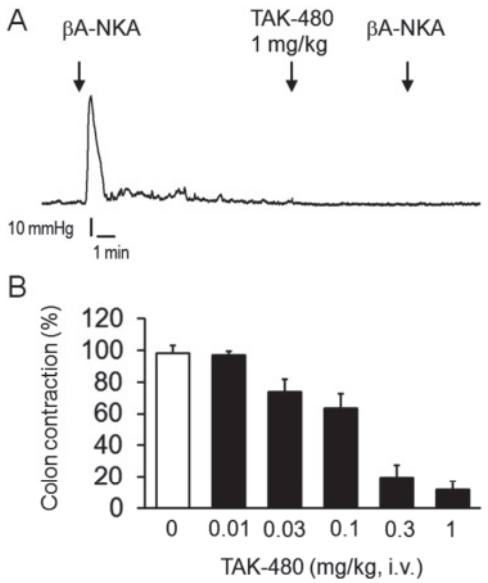


**Fig. 3.** Proximal colonic contraction produced by i.v. administration of $NK_2$-receptor agonist $\beta$A-NKA in urethane-anesthetized rabbits. A: Representative chart of $\beta$A-NKA-induced colonic contraction. B: TAK-480 inhibited $\beta$A-NKA-induced colonic contractions in a dose-dependent manner. Data are presented as the mean ± S.E.M. of colonic contractions normalized by the baseline response before each treatment (n = 5).

*Effect of TAK-480 on VMR to CRD in rabbits: comparison with alosetron*

The analgesic effect of TAK-480 on VMR to CRD was compared with that of alosetron in a rabbit model of intracolonic zymosan-induced visceral hypersensitivity. Figure 4A displays the dose-dependent inhibitory effect of TAK-480 on VMR, which resulted in significant analgesia at i.v. doses of 0.1 and 0.3 mg/kg. The inhibition rates to VMR were 35% and 46% at 0.1 and 0.3 mg/kg, respectively. Meanwhile, alosetron also showed a tendency to inhibit VMR, but this inhibition was modest and not significant at any dose (at most 17% inhibition at 1 mg/kg, i.v.; Fig. 4B). Vehicle treatment produced a slight exacerbation of VMR in both experiments regardless of the vehicle form (5% – 10% aggravation, Fig. 4: A and B).

*Effect of TAK-480 on ricinoleic acid–induced and spontaneous defecation in guinea pigs: comparison with alosetron*

The inhibitory effect of TAK-480 on exaggerated bowel function was evaluated by counting the fecal pellet output prompted by oral administration of ricinoleic acid in guinea pigs. Figure 5, A and C, summarizes the cumulative number of fecal pellets at 2, 4, and 6 h after peroral administration of ricinoleic acid in TAK-480- and alosetron-treated groups, respectively. Compared with the untreated normal group, ricinoleic acid produced a robust increase in defecation at all time points after the acid challenge in the vehicle-treated group. During the testing period, i.p. pre-treatment with TAK-480 and alosetron

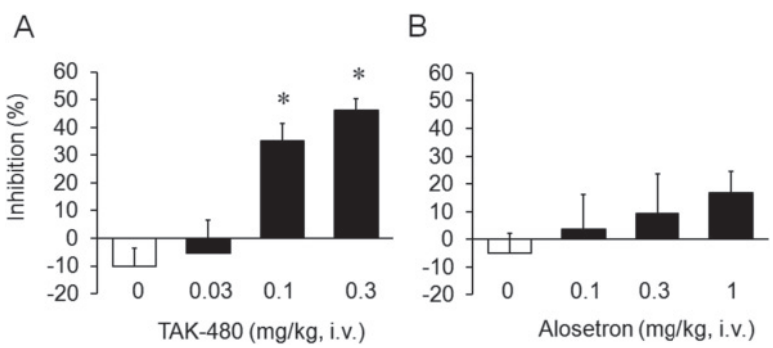


**Fig. 4.** Inhibitory effect of TAK-480 and alosetron on visceromotor response (VMR) induced by colorectal distension (CRD) in a rabbit model of zymosan-induced visceral hypersensitivity. Intravenous (i.v.) administration of TAK-480 significantly inhibited VMR in a dose-dependent manner (A) in contrast to less inhibition by alosetron (B). All data are expressed as the mean ± S.E.M. of inhibition rate (n = 6). *$P \leq 0.025$ vs. vehicle-treated group (Williams' test).

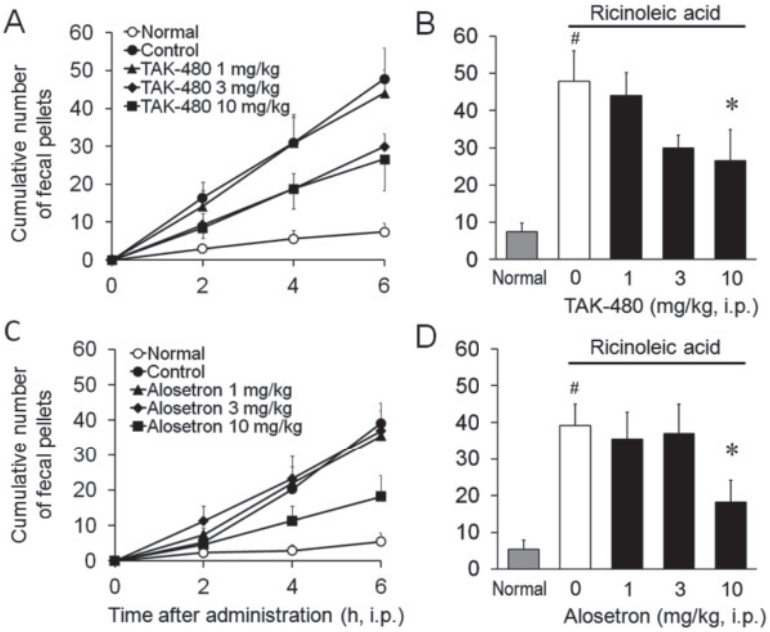


**Fig. 5.** Inhibitory effect of TAK-480 and alosetron on prompted defecation produced by oral administration of ricinoleic acid in guinea pigs. Pre-treatment with both drugs prevented a time-dependent increase in fecal pellets over the testing period (A: TAK-480, C: alosetron) and significantly suppressed cumulative defecation for 6 h after the acid challenge (B: TAK-480, D: alosetron). All data are expressed as the mean ± S.E.M. of the number of fecal pellets (n = 10). $^{\#}P \leq 0.05$ vs. normal group (Student's *t*-test). $^{*}P \leq 0.025$ vs. vehicle-treated group (Williams' test).

suppressed prompted defecation at 3 and 10 mg/kg and 10 mg/kg, respectively. A significant suppressive effect of both drugs on defecation enhanced by ricinoleic acid was demonstrated from the analysis at the 6 h time-point (Fig. 5: B and D).

In another experiment, the effect of each drug on spontaneous defecation was investigated for 6 h after i.p. drug administration in guinea pigs (Fig. 6). Alosetron at an effective dose for ricinoleic acid–induced defecation (10 mg/kg) significantly suppressed spontaneous fecal excretion compared to vehicle treatment. In comparison, TAK-480 (10 mg/kg) did not affect spontaneous defecation.

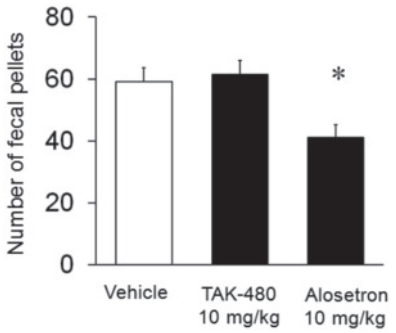


**Fig. 6.** Effect of TAK-480 and alosetron on spontaneous defecation in guinea pigs. Feces accumulated 6 h after drug treatment were counted. Alosetron, but not TAK-480, significantly suppressed spontaneous defecation at a pharmacologically effective dose in ricinoleic acid–induced defecation. All data are expressed as the mean ± S.E.M. of the number of fecal pellets (n = 10). $^{*}P \leq 0.05$ vs. vehicle-treated group (Student's *t*-test).

*Effect of TAK-480 on smooth muscle contraction of isolated colon*

To predict the efficacy of TAK-480 in human studies, the inhibitory effect of TAK-480 on $\beta$A-NKA-induced smooth muscle contraction was evaluated using colon strips isolated from humans and rabbits. $\beta$A-NKA positively produced smooth muscle contractions in response to ligand concentration, producing a concentration–response sigmoid curve in both species (Fig. 7A, human; Fig. 7B, rabbit). Compared with vehicle treatment, TAK-480 induced a right shift of the sigmoid curve in a concentration-dependent manner ranging from 0.001 to 1 $\mu$M. Based on the Schild plot analysis, the $pA_2$ values of TAK-480 were calculated as 8.9 and 8.2 in human and rabbit colons, respectively (Fig. 7C).

In addition, since the $NK_2$ receptor mainly mediates natural tachykinin-related gut motility in humans, the effect of TAK-480 on natural tachykinin-induced smooth muscle contraction was examined in an isolated human colon preparation. Submaximal concentrations of SP (1 $\mu$M), NKA (0.1 $\mu$M), and NKB (1 $\mu$M) were used to induce colonic contractions. As shown in Fig. 8, TAK-480 inhibited colonic contractions in a concentration-dependent fashion, which was reproduced using all three natural tachykinins. A complete blockade of colonic contraction was observed at a concentration of 1 $\mu$M.

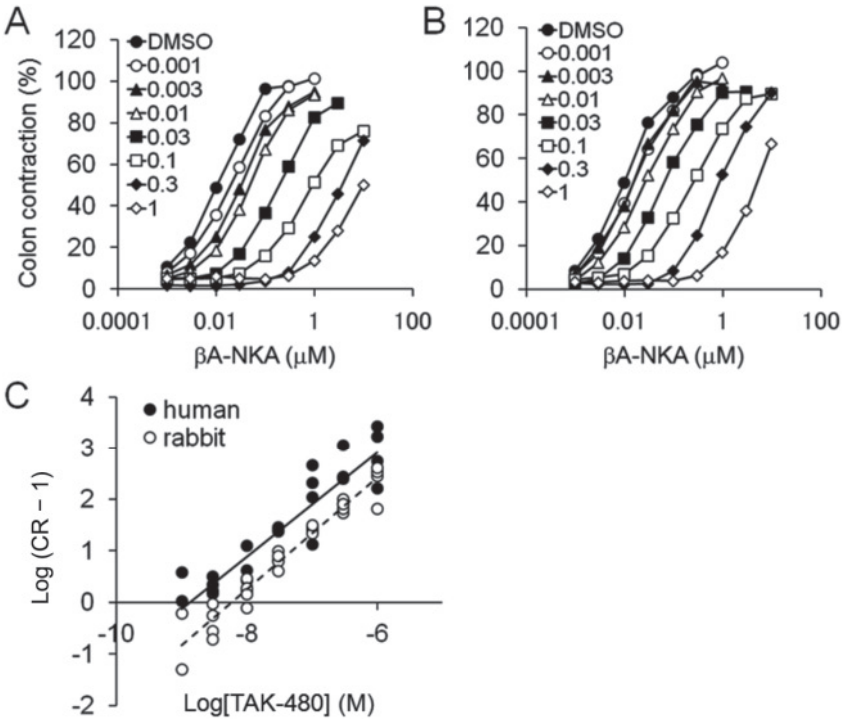


**Fig. 7.** Inhibitory effect of TAK-480 on colonic contractions induced by selective $NK_2$-receptor agonist $\beta$A-NKA in isolated human and rabbit preparations. An agonist response curve was obtained at each concentration of TAK-480. Note that the curves shifted to the right with an increase in compound concentration in both species (A: human, B: rabbit). All data are expressed as the mean of colonic contraction normalized to the maximum response with 1 $\mu$M $\beta$A-NKA application in DMSO (n = 4). The corresponding Schild plot was used for $pA_2$ calculations (C).

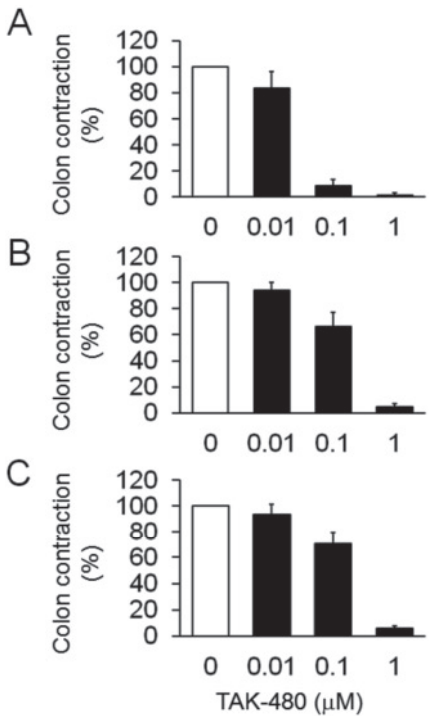


**Fig. 8.** Inhibitory effect of TAK-480 on natural tachykinin-induced circular smooth muscle contractions in the human colon. Substance P (SP)- (A), neurokinin A (NKA)- (B), and neurokinin B (NKB)- (C) induced colonic contractions were monitored. TAK-480 blocked all natural tachykinin-induced colonic contractions in a concentration-dependent manner. Data are expressed as the mean ± S.E.M. of colonic contractions normalized to the response of the vehicle-treated group (n = 7).

## Discussion

TAK-480 is a novel $NK_2$-receptor antagonist. Its unique chemical structure is completely different from that of other non-peptidic $NK_2$-receptor antagonists (21, 22). The remarkable complexity of the pharmacological characteristics of the tachykinin receptor has been documented based on past developments of selective tachykinin antagonists (10). One of the typical properties of the receptor is marked species-related variation in their affinity for synthesized non-peptide tachykinin receptor antagonists (18). Maggi et al. reported the heterogeneity of the $NK_2$ receptor in rabbits, guinea pigs, and human colonic smooth muscles and concluded that the $NK_2$ receptor in mammalian smooth muscle can be divided into two categories: classical $NK_2A$ (human, rabbit, and guinea pig) and non-classical $NK_2B$ (rat and hamster). Consistent with this heterogeneity, TAK-480 exhibited typical and marked species differences in its affinity for the $NK_2$ receptor, showing lower binding affinity for the rodent $NK_2$ receptor ($\mu$M order) in contrast to higher binding affinity for human, rabbit, and guinea-pig $NK_2$ receptors (nM order). Therefore, we employed rabbit and guinea-pig models for evaluating the efficacy of TAK-480. It would be reasonable to employ these species instead of rodents for the appropriate evaluation of human-active $NK_2$-receptor antagonists. In another property, although TAK-480 was orally inactive in rabbits and guinea pigs due to its poor stability in oxidative metabo-

lism (data not shown), it was stable in human oxidative metabolism, implying the oral activity of the compound in a clinical setting. Thus, in the present study, we conducted preclinical efficacy evaluation of TAK-480 via i.v. or i.p. routes instead of per oral (p.o.). Initially we attempted to investigate the in vivo $NK_2$-receptor antagonistic activity of TAK-480. For this purpose, we employed a rabbit model of agonist-induced colonic contractions which we validated previously as an in vivo screening assay for $NK_2$-receptor antagonists (23). In this model, TAK-480 inhibited $\beta$A-NKA-induced colonic contractions in a dose-dependent manner. This result confirmed the distinct $NK_2$-receptor antagonism of TAK-480 in vivo, permitting further in vivo studies in animal models, which partially mimic IBS pathology.

To investigate the effect of TAK-480 on colonic hypersensitivity, we used a rabbit model of CRD based on the pharmacologically validated procedure reported by Okano et al. (19). We have previously demonstrated the inhibitory effect of a series of $NK_2$-receptor antagonists on VMR to CRD in this model, correlating to their corresponding in vivo $NK_2$-receptor antagonistic activities (23). In the present study, instead of intracolonic acetic acid treatment employed in the previous report, zymosan, a protein–carbohydrate cell wall derivative of the yeast *Saccharomyces cerevisiae*, was administered intracolonically to induce visceral hypersensitivity (24, 25). In this model, TAK-480 dose-dependently and significantly inhibited VMR to CRD in accordance with its in vivo $NK_2$-receptor antagonism, which was confirmed in an agonist-induced colonic contraction model. This finding suggests the important contribution of the $NK_2$ receptor in the rabbit model of zymosan-induced visceral hypersensitivity, consistent with data from several rodent models of visceral hypersensitivity (15, 16, 26). In addition, the maximum inhibition rate of TAK-480 to VMR was comparable to that of the $\mu$-opioid receptor partial agonist buprenorphine and $NK_1$-receptor antagonist TAK-637, as demonstrated in our previous reports (approximately, up to 50% inhibition; ref. 19). These findings support the strong analgesic potency of TAK-480 as being possibly equivalent to that of conventional analgesics for the treatment of abdominal pain. On the other hand, the inhibitory effect of alosetron on VMR to CRD was moderate compared with that of TAK-480. Indeed, 5-$HT_3$ receptors are expressed on postsynaptic neurons in the spinal cord and are involved in neurotransmission from visceral afferents, thereby providing a therapeutic rationale for using 5-$HT_3$-receptor antagonists for abdominal pain (7). In addition, alosetron increased pain thresholds to balloon distension in IBS patients; however, the increase in pain thresholds was associated with an increase in colonic compliance, which could contribute to anti-nociceptive effects of alosetron in IBS patients (27). In the present study, we employed a barostat system for balloon distension and kept the intensity of colonic stimulation constant to evaluate the direct effect of drugs on colonic perception itself, excluding the influence on colonic compliance. Therefore, inhibitory effect of TAK-480 and alosetron on VMR could be attributed to their modulation of colonic perception. In addition to experimental procedures, neuronal excitation via the $NK_2$ receptor in sensory DRG neurons could also support the direct effect of TAK-480 on colonic perception (14). In this report, selective $NK_2$ agonist, $\beta$A-NKA, enhanced $Ca^{2+}$ currents in DRG neurons, and the enhancement was reversed by the $NK_2$-receptor antagonist MEN 10,376, suggesting a possible site of action by TAK-480 in primary colonic perception. Although there are no preclinical models that fully resemble IBS characteristics, these results suggest that TAK-480 might be more effective in treating the associated abdominal pain in patients with IBS by modulating colonic perception more directly.

To evaluate the efficacy of TAK-480 on enhanced colonic function, we investigated the inhibitory effect on ricinoleic acid–induced defecation in guinea pigs. Generally, castor oil–induced defecation is a well-established model to study the in vivo evaluation of the anti-motility/secretary effect of drugs in rodents (20). In our preliminary study, however, no enhanced defecation was observed in guinea pigs after p.o. treatment with castor oil (data not shown). Therefore, we used ricinoleic acid, which is considered to be an active substance hydrolyzed from castor oil by intestinal lipase. Ricinoleic acid has been demonstrated to potentiate the contractive responses to prostaglandin $E_2$ and acetylcholine in the ileum of guinea pigs (28). Although stool consistency was not altered demonstrably, ricinoleic acid significantly increased fecal pellet output as observed with castor oil in rodents. In this model, TAK-480 significantly and dose-dependently suppressed prompted defecation, suggesting an important role of the $NK_2$ receptor in enhanced defecation following mucosal stimulation by ricinoleic acid. In support of our finding, major involvement of the $NK_2$ receptor has been demonstrated in a rat model of castor oil–induced defecation in which endogenous tachykinins could account for enhanced gut motility and secretion (29, 30). Another finding of this study was that the maximum potency of TAK-480 was equivalent to that of alosetron. Given that alosetron has been demonstrated to be a strong anti-diarrheal agent in clinical and preclinical studies, TAK-480 could be effective in controlling enhanced bowel function which is observed in D-IBS (31 – 33).

Alosetron has been shown to be effective in the treatment of D-IBS symptoms in women, particularly in

managing bowel urgency and abdominal pain. However, it is currently available only on restricted use due to constipation, sometimes severe, being its most common side effect (31, 32, 34). Therefore, a novel, effective, and non-constipating approach is strongly desirable for treating D-IBS. In the current study, alosetron, but not TAK-480, significantly suppressed spontaneous defecation in guinea pigs at a pharmacologically effective dose in ricinoleic acid–induced defecation, providing experimental insight into the cause of severe constipation reported in IBS patients treated with alosetron. This result could be easily conceivable given that 5-HT is abundantly expressed in the GI tract, particularly in enterochromaffin cells, and is involved in gut motility, and secreted as an endocrine substance (7). Unlike alosetron, TAK-480 suppressed prompted defecation without affecting spontaneous defecation, suggesting that TAK-480 could be effective in altered bowel habits in D-IBS patients without the possible risk of severe constipation.

In drug development, it is important to predict the efficacy of drug candidates in human studies from preclinical data obtained using experimental animals. For this purpose, in vitro $NK_2$ antagonism was compared between human and rabbit colons using isolated colon strips. In both species preparations, TAK-480 inhibited agonist-induced smooth muscle contraction in a concentration-dependent manner, shifting the ligand response curve to the right with an increase in compound concentration. It was particularly encouraging that Schild plots revealed more potent $pA_2$ values in the human colon than that of the rabbit. These values provide interspecies compensation, predicting stronger efficacy of TAK-480 in the clinical setting compared with that found in experimental animal models. In a separate experiment using human colon strips, we investigated the inhibitory effect of TAK-480 on natural tachykinin-induced colonic contractions. Interestingly, all three natural tachykinin-induced colonic contractions were suppressed by TAK-480 in a concentration-dependent manner. Considering that TAK-480 showed a 10,000-fold higher selectivity to the human $NK_2$ receptor over the $NK_1$ and $NK_3$ receptors in vitro, the inhibitory effect of TAK-480 on colonic contractions is attributed to its ability to selectively block the $NK_2$ receptor. In support of this result, we recently demonstrated the predominant contribution of the $NK_2$ receptor to colonic contractions that were induced by the natural tachykinins SP, NKA, and NKB in the human colon as well as $NK_2$ expression in the smooth muscle layer and myenteric plexus (13). Given that natural tachykinins produce spasmogenic contractions in human colonic circular smooth muscles and are implicated in abnormal gut motility in certain pathophysiological conditions (35, 36), potent blockade of the $NK_2$ receptor, an integrator of natural tachykinin-related gut motility, could support the therapeutic rationale for using TAK-480 for the treatment of altered bowel habits in IBS patients.

In conclusion, the novel, potent, and highly selective $NK_2$-receptor antagonist, TAK-480, ameliorated intracolonic zymosan-induced visceral hypersensitivity and ricinoleic acid–induced defecation without suppressing spontaneous defecation at effective doses in animal models. Moreover, in vitro Magnus experiments using the human colon may predict stronger efficacy of TAK-480 in the clinical setting and imply its potential effectiveness to ameliorate abdominal pain and impaired colonic function in IBS patients without causing severe constipation.

## References


1 Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology. 2006;130:1480–1491.

2 Ritchie J. Pain from distension of the pelvic colon by inflating a balloon in the irritable colon syndrome. Gut. 1973;14:125–132.

3 Sagami Y, Shimada Y, Tayama J, Nomura T, Satake M, Endo Y, et al. Effect of a corticotropin releasing hormone receptor antagonist on colonic sensory and motor function in patients with irritable bowel syndrome. Gut. 2004;53:958–964.

4 Fukudo S, Kanazawa M, Kano M, Sagami Y, Endo Y, Utsumi A, et al. Exaggerated motility of the descending colon with repetitive distention of the sigmoid colon in patients with irritable bowel syndrome. J Gastroenterol. 2002;37 Suppl 14:145–150.

5 Hillila MT, Farkkila MA. Prevalence of irritable bowel syndrome according to different diagnostic criteria in a non-selected adult population. Aliment Pharmacol Ther. 2004;20:339–345.

6 Boyce PM, Koloski NA, Talley NJ. Irritable bowel syndrome according to varying diagnostic criteria: are the new Rome II criteria unnecessarily restrictive for research and practice? Am J Gastroenterol. 2000;95:3176–3183.

7 Crowell MD. Role of serotonin in the pathophysiology of the irritable bowel syndrome. Br J Pharmacol. 2004;141:1285–1293.

8 Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part I. Expression, release and motor function. Pharmacol Ther. 1997;73:173–217.

9 Lecci A, Capriati A, Maggi CA. Tachykinin $NK_2$ receptor antagonists for the treatment of irritable bowel syndrome. Br J Pharmacol. 2004;141:1249–1263.

10 Shimizu Y, Matsuyama H, Shiina T, Takewaki T, Furness JB. Tachykinins and their functions in the gastrointestinal tract. Cell Mol Life Sci. 2008;65:295–311.

11 Maggi CA. The mammalian tachykinin receptors. Gen Pharmacol. 1995;26:911–944.

12 Lecci A, Capriati A, Altamura M, Maggi CA. Tachykinins and tachykinin receptors in the gut, with special reference to $NK_2$ receptors in human. Auton Neurosci. 2006;126–127:232–249.

13 Nakamura A, Tanaka T, Imanishi A, Kawamoto M, Toyoda M, Mizojiri G, et al. Bi-directional regulation of human colonic smooth muscle contractility by tachykinin $NK_2$ receptors. J Pharmacol Sci. 2011;117:106–115.

14 Sculptoreanu A, de Groat WC. Protein kinase C is involved in

neurokinin receptor modulation of N- and L-type $Ca^{2+}$ channels in dorsal root ganglia neurons of the adult rat. J Neurophysiol. 2003;90:21–31.
15 Toulouse M, Coelho AM, Fioramonti J, Lecci A, Maggi C, Buéno L. Role of tachykinin $NK_2$ receptors in normal and altered rectal sensitivity in rats. Br J Pharmacol. 2000;129:193–199.
16 Kamp EH, Beck DR, Gebhart GF. Combinations of neurokinin receptor antagonists reduce visceral hyperalgesia. J Pharmacol Exp Ther. 2001;299:105–113.
17 Lordal M, Navalesi G, Theodorsson E, Maggi CA, Hellstrom PM. A novel tachykinin $NK_2$ receptor antagonist prevents motility-stimulating effects of neurokinin A in small intestine. Br J Pharmacol. 2001;134:215–223.
18 Maggi CA, Giuliani S, Patacchini R, Quartara L, Rovero P, Renzetti AR, et al. Heterogeneity of tachykinin NK-2 receptors in rabbit, guinea-pig and human smooth muscles. Neuropeptides. 1992;23:181–186.
19 Okano S, Ikeura Y, Inatomi N. Effects of tachykinin $NK_1$ receptor antagonists on the viscerosensory response caused by colorectal distention in rabbits. J Pharmacol Exp Ther. 2002; 300:925–931.
20 Awouters F, Megens A, Verlinden M, Schuurkes J, Niemegeers C, Janssen PA. Loperamide. Survey of studies on mechanism of its antidiarrheal activity. Dig Dis Sci. 1993;38:977–995.
21 Emonds-Alt X, Vilain P, Goulaouic P, Proietto V, Van Broeck D, Advenier C, et al. A potent and selective non-peptide antagonist of the neurokinin A ($NK_2$) receptor. Life Sci. 1992;50:PL101–PL106.
22 Cialdai C, Tramontana M, Patacchini R, Lecci A, Catalani C, Catalioto RM, et al. MEN15596, a novel nonpeptide tachykinin $NK_2$ receptor antagonist. Eur J Pharmacol. 2006;549:140–148.
23 Tanaka T, Matsumoto-Okano S, Inatomi N, Fujioka Y, Kamiguchi H, Yamaguchi M, et al. Establishment and validation of a rabbit model for in vivo pharmacodynamic screening of tachykinin $NK_2$ antagonists. J Pharmacol Sci. 2012;118:487–495.
24 Jones RC 3rd, Otsuka E, Wagstrom E, Jensen CS, Price MP, Gebhart GF. Short-term sensitization of colon mechanoreceptors is associated with long-term hypersensitivity to colon distention in the mouse. Gastroenterology. 2007;133:184–194.
25 Shinoda M, Feng B, Gebhart GF. Peripheral and central $P_2X$ receptor contributions to colon mechanosensitivity and hypersensitivity in the mouse. Gastroenterology. 2009;137:2096–2104.
26 McLean PG, Picard C, Garcia-Villar R, Moré J, Fioramonti J, Buéno L. Effects of nematode infection on sensitivity to intestinal distension: role of tachykinin $NK_2$ receptors. Eur J Pharmacol. 1997;337:279–282.
27 Delvaux M, Louvel D, Mamet JP, Campos-Oriola R, Frexinos J. Effect of alosetron on responses to colonic distension in patients with irritable bowel syndrome. Aliment Pharmacol Ther. 1998; 12:849–855.
28 Capasso F, Mascolo N, Autore G, Duraccio MR. Influence of ricinoleic acid on the contractions elicited by PGE2 on the guinea-pig ileum. J Pharm Pharmacol. 1984;36:64–65.
29 Croci T, Landi M, Emonds-Alt X, Le Fur G, Maffrand JP, Manara L. Role of tachykinins in castor oil diarrhoea in rats. Br J Pharmacol. 1997;121:375–380.
30 Emonds-Alt X, Advenier C, Cognon C, Croci T, Daoui S, Ducoux JP, et al. Biochemical and pharmacological activities of SR 144190, a new potent non-peptide tachykinin $NK_2$ receptor antagonist. Neuropeptides. 1997;31:449–458.
31 Camilleri M, Chey WY, Mayer EA, Northcutt AR, Heath A, Dukes GE, et al. A randomized controlled clinical trial of the serotonin type 3 receptor antagonist alosetron in women with diarrhea-predominant irritable bowel syndrome. Arch Intern Med. 2001;161:1733–1740.
32 Lembo T, Wright RA, Bagby B, Decker C, Gordon S, Jhingran P, et al; Lotronex Investigator Team. Alosetron controls bowel urgency and provides global symptom improvement in women with diarrhea-predominant irritable bowel syndrome. Am J Gastroenterol. 2001;96:2662–2670.
33 Hirata T, Keto Y, Nakata M, Takeuchi A, Funatsu T, Akuzawa S, et al. Effects of serotonin 5-$HT_3$ receptor antagonists on stress-induced colonic hyperalgesia and diarrhoea in rats: a comparative study with opioid receptor agonists, a muscarinic receptor antagonist and a synthetic polymer. Neurogastroenterol Motil. 2008;20:557–565.
34 Chang L, Drossman DA. Rome foundation endpoints and outcomes conference 2009: optimizing clinical trials in FGID. Am J Gastroenterol. 2010;105:722–730.
35 Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part II. Roles in neural excitation, secretion and inflammation. Pharmacol Ther. 1997;73:219–263.
36 Al-Saffar A, Hellström PM. Contractile responses to natural tachykinins and selective tachykinin analogs in normal and inflamed ileal and colonic muscle. Scand J Gastroenterol. 2001; 36:485–493.