Gastroprokinetic agent, mosapride inhibits 5-HT₃ receptor currents in NCB-20 cells

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INTRODUCTION

Mosapride is a gastroprokinetic agent that increases gastrointestinal (GI) motility by stimulating contraction of the GI tract [1,2]. Although not completely understood, the prokinetic mechanism of mosapride is mediated by agonist action on the 5-hydroxytryptamine (5-HT, serotonin) type 4 (5-HT₄) receptor in the enteric nervous system that facilitate cholinergic excitatory neurotransmission and thereby, increase propulsive motility [3]. However, several studies have shown that some of benzamide gastroprokinetics also inhibited fast transient depolarization to 5-HT mediated by 5-HT type 3 (5-HT₃) receptors in the myenteric neurons [4-6] and suggested the 5-HT₃ receptor antagonistic properties of these drugs.

5-HT₃ receptors belong to the Cys-loop receptor family of ligand-gated ion channels [7], are permeable to cations, and can induce a fast inward current in neurons [8]. 5-HT₃ receptors are expressed in neural tissues throughout the central and the peripheral nervous system [9,10], including enteric neurons in the GI tract [11,12], and have been proposed as a potential drug treatment target for various neurologic disorders [13-15]. The activation of 5-HT₃ receptors in the GI tract is known to be involved in the pharmacological effects of mosapride to treat GI disorders.
in the regulation of GI motility, visceral sensation, secretion, and certain variants of the HTR3 gene are associated with a number of functional GI diseases, such as irritable bowel syndrome (IBS), gastroesophageal reflux disease, and anorexia [13]. Besides the established role of 5-HT₃ receptor antagonists in anti-emetic drugs [16], a meta-analysis study by Zheng et al. [17] reported that 5-HT₃ receptor antagonists were effective treatments for non-constipated IBS or diarrhea-predominant IBS. The mixed action of mosapride, i.e., an agonist of the 5-HT₄ receptor and an antagonist of the 5-HT₃ receptor, could be essential to understanding whether it is beneficial or harmful in the treatment of GI motility disorders, especially diarrhea-predominant IBS. However, to our knowledge, there have been no reports evaluating the direct effects of mosapride on 5-HT₃ receptor ion channel function through the patch clamp technique. Accordingly, we tested the effects of mosapride on the 5-HT₃ receptor using whole-cell voltage clamp recording combined with a fast drug application technique.

METHODS

Materials

Mosapride citrate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Serotonin hydrochloride and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture reagents were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture

NCB-20 neuroblastoma cells were provided by Dr. Lovinger (National Institute on Alcohol Abuse and Alcoholism [NIAAA], Bethesda, MD, USA). The NCB-20 cells were maintained under previously described conditions [18]. Frozen cell stocks were maintained in liquid nitrogen and thawed as needed. The cells were grown in medium containing 89% Dulbecco’s Modified Eagle’s Medium, 10% fetal bovine serum, and 1% hypoxanthine aminopterin thymidine supplement and were cultured in an incubator at 37°C with 5% CO₂. The cells were seeded onto 35-mm culture dishes at least 2 days prior to recording. Culture medium was changed to extracellular solution 1–2 h before recording and the cells were transferred onto cover glasses and moved to the recording chamber.

Electrophysiology

Whole-cell patch-clamp recordings were performed at room temperature (25°C) on the stage of an inverted microscope (DM IRM; Leica, Wetzler, Germany). The cells were continuously perfused with an extracellular solution containing (in mM) 150 NaCl, 2.5 KCl, 2.5 CaCl₂, 0.1 MgCl₂, 10 N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid (HEPES), and 10 D-glucose (pH adjusted to 7.4 with NaOH and osmolality adjusted to 340 mOsm/kg with sucrose) at a rate of 2–3 ml/min. Patch pipettes were made from borosilicate glass capillaries (1B150F-4; World Precision Instruments, Sarasota, FL, USA) which were pulled with a horizontal micropipette puller (P-97; Sutter Instrument Co., Novato, CA, USA). The pipette tips had resistances of around 2 MΩ when filled with internal solution containing (in mM) 140 CsCl, 2 MgCl₂, 5 ethylene glycol bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid, and 10 HEPES (pH adjusted to 7.4 with CsOH, osmolality adjusted to 310 mOsm/kg with sucrose). Whole-cell currents were recorded using an Axopatch 200B amplifier and pClamp 9 software (Molecular Devices, Sunnyvale, CA, USA). 5-HT₃ receptor currents were recorded at a holding potential of −50 mV unless otherwise stated. The signals were filtered at 2 kHz, digitized at 10 kHz, and saved on a PC using DigiData 1322 and pClamp 9 software (Molecular Devices). The liquid junction potentials were zeroed before a gigaohm seal was formed. The capacitive currents were compensated with analog compensation, but leak subtraction was not used in this study.

Drug preparation and application

After a whole-cell configuration, the cells were lifted up and positioned in front of one side of two theta glass micropipette barrels, which were pulled from theta glass capillary tubes (Clark Borosilicate Theta; Warner Instruments, Hamden, CT, USA; 2 mm outer diameter, 1.4 mm inner diameter, 0.2 mm septum thickness) to an outer diameter of ~300 mm and continuously perfused with extracellular solution. The extracellular solution, which contained agonists with or without mosapride, flowed through a different side of the theta glass micropipette. The solutions were rapidly switched around the cell by the linear movement of the theta glass micropipette mounted on a piezoelectric translator (P-601 PiezoMove Z. Actuator; Physik Instrumente, Karlsruhe, Germany) driven by a Piezo Servo Controller (E-625, Physik Instrumente) and triggered by pClamp 9 software (Molecular Devices), which displaced the theta glass micropipette laterally in msec time resolution. Therefore, the cells could be exposed to the drug-containing extracellular solution for a defined period of time, then rapidly returned to the drug-free extracellular solution. The solution flow was driven by gravity from the reservoirs and solution switching was controlled by a perfusion valve control system (VC-8; Warner Instruments). The drugs were diluted with extracellular solution to the final concentration from stock solutions in distilled water (5-HT) or dimethyl sulfoxide (mosapride). The solutions were freshly prepared prior to each experiment.

Data analysis

The current peak amplitudes were measured using Clampfit.
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10 software (Molecular Devices), by determining the differences in currents before and during agonist application. Since peak amplitudes of the 5-HT₃ receptor currents induced by agonist application varied between cells, the data were normalized to the maximal values in each cell. The rise slopes of currents were also analyzed to evaluate 5-HT₃ receptor activation using the built-in statistical tools in Clampfit 10. Receptor desensitization was measured as the decay in 5-HT, receptor current during agonist application for 10 sec, then the currents decay slopes were analyzed.

The concentration-response data were fitted to a sigmoid curve, calculated by the four-parameter logistic equation using Prism 8 software (GraphPad Software, San Diego, CA, USA).

For the EC₅₀ of 5-HT:

\[ Y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + 10^{\log\text{EC}_{50} - X\text{HillSlope}}} \]  

where the bottom is the minimal response, the top is the maximal response by 5-HT, X is the logarithmic concentrations of 5-HT, and EC₅₀ is the 5-HT concentration that gives a half-maximal response.

For the IC₅₀ of mosapride:

\[ Y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + 10^{\log\text{IC}_{50} - X\text{HillSlope}}} \]  

where the bottom is the maximally inhibited response by mosapride, the top is the maximal response by 5-HT alone, X is the logarithmic concentrations of mosapride and IC₅₀ is the concentration of mosapride resulting in a half-maximal response.

Interaction kinetics between the drug and channel are described on the basis of a first-order blocking scheme [19]. The apparent rate constants for association (k₊) and dissociation (k₋) were calculated from the following equations:

\[ \tau_D = \frac{I}{(k_+\text{[D]} + k_-)} \]  
\[ K_d = \frac{k_-}{k_+} \]

where \( \tau_D \) is the drug-induced time constant, calculated from single exponential fits to the decay of currents by mosapride (for 5 sec) 3 sec after 5-HT application.

All averaged values are presented as means ± standard error of the mean. Statistical significance was determined by Student’s t-tests. p-values < 0.05 were considered statistically significant.

RESULTS

To study the direct effects of mosapride on the 5-HT₃ receptor induced currents, we examined the concentration-dependent effect of mosapride (0.3, 1, 3, 10, 30 μM) on the currents by 3 μM 5-HT, a concentration near the EC₅₀ of 5-HT₃ receptor currents reported in our previous study [18]. The application of 30 μM mosapride for 5 sec did not induce any current (Fig. 1A). The co-application of mosapride decreased peak amplitudes of 3 μM 5-HT-evoked currents in a concentration-dependent manner (Fig. 1A). The IC₅₀ of mosapride that resulted in peak amplitude reduction, calculated using equation (2) in the Materials and Methods,

![Fig. 1. Concentration-dependent inhibition of 5-hydroxytryptamine type 3 (5-HT₃) receptor currents by mosapride.](image)

(A) Representative current traces induced by 3 μM 5-HT co-applied with 0.3, 1, 3, 10, 30 μM mosapride. The open horizontal bar indicates the drug application period. Mosapride alone did not induce any current even at high concentration (indicated by arrowhead). (B) Averaged concentration-dependent inhibition of mosapride on the 5-HT₃ receptor current peak amplitude. The data were normalized values (○) to the peak amplitude induced by 3 μM 5-HT alone and a line was obtained from fitting these data to equation (2) in the Methods. (C) Averaged concentration-dependent block of the 5-HT₃ receptor current rise slope by mosapride. Mosapride concentration-dependently decreased the rise slopes of currents at low concentrations (0.3, 1, 3 μM). The data are expressed as means ± standard error of the mean.

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was 4.03 ± 0.04 μM with a Hill coefficient of –1.45 ± 0.13 (Fig. 1B, n = 10). Mosapride also decreased the current rise slopes, an indicator of activation of the 5-HT, receptor [20]. The inhibition of the rise slope was concentration dependent, seen at low concentrations of mosapride (0.3, 1, 3 μM) in the tested range (Fig. 1C). We then studied whether mosapride inhibited the 5-HT, receptor in a competitive manner. Fig. 2A presents the representative 5-HT, receptor currents by 1, 3, 10, 30 μM of 5-HT with or without co-application of mosapride (10 μM, near the IC50 based on this study). The co-application of mosapride inhibited the currents activated by 1–30 μM 5-HT (Fig. 2B). The E50 value of peak currents amplitudes by the 5-HT alone, calculated using equation (1), was 1.88 ± 0.10 μM with a Hill coefficient of 2.55 ± 0.12 (n = 10). The E50 value of the peak currents amplitudes by the co-application of 5-HT with 10 μM of mosapride was 4.03 ± 0.04 μM with a Hill coefficient of –1.45 ± 0.13 (n = 10). The E50 value of the peak currents amplitudes by the co-application of 5-HT with 10 μM of mosapride was 4.80 ± 0.32 μM with a Hill coefficient of 2.60 ± 0.26 (n = 8). The co-application of 10 μM mosapride increased the E50 (p < 0.001, unpaired t-test), however, the Hill coefficients were not significantly different statistically (p = 0.8532). The peak currents amplitudes induced by 30 μM of 5-HT did not show significant difference between 5-HT application alone and 5-HT co-applied with 10 μM mosapride (97.0 ± 2.8% and 95.4 ± 3.2% of the 10 μM 5-HT response, respectively; p = 0.7282, unpaired t-test). The data are expressed as means ± standard error of the mean.

In order to determine whether mosapride affected the closed state of the 5-HT, receptor, we compared the extent of 5-HT, receptor current blocking by pretreatment with 10 μM mosapride for 1 min prior to the co-application of 10 μM 5-HT with the co-application of 5-HT and mosapride without pretreatment. There were significant differences in the inhibition of peak currents when the co-application of 5-HT and mosapride after 1 min pretreatment (31.1 ± 4.1% of 5-HT alone) compared to co-application without pretreatment (85.0 ± 2.9%) (Fig. 3B, n = 10, p < 0.001, paired t-test). These results suggest that mosapride probably bound to the closed state and blocked channel opening of the 5-HT, receptors. To address the effects of mosapride on the 5-HT, receptor in detail, i.e., whether it binds to the closed or open state of ion channels, we next tested the effects of mosapride during the application of 3 μM 5-HT. Different doses (1, 3, 10, 30 μM) of mosapride were co-treated 3 sec after 5-HT application for 5 sec, then 5-HT, receptors were reactivated upon termination of the mosapride application. The currents activated by 3 μM 5-HT were decreased following the subsequent application of mosapride in a concentration-dependent manner (Fig. 4). These results suggest that mosapride bound to the open state of 5-HT, receptors, then accelerated the decay of 5-HT, receptor currents by increasing desensitization, i.e., an open-channel blocking mechanism. The rate of current decay by mosapride (for 5 sec) 3 sec after the 5-HT application was fitted to a single exponential function and the time constants (τp) were taken to estimate the drug-open-channel interaction kinetics. The relationship between τp and concentration of mosapride was described by equation (3) in Materials and Methods. The slope of this linear function yielded an association rate constant (k+1 = 0.02 μM–1 sec–1) and the intercept at the y axis gave a dissociation rate constant (k–1 = 0.27 sec–1, Fig. 4B, n = 9). From equation (4), the apparent Kd (k–1 / k+1) was 12.5

Fig. 2. Competitive inhibition of mosapride on the 5-hydroxytryptamine type 3 (5-HT,) receptor currents. (A) Representative traces of 5-HT, receptor currents induced by 1, 3, 10, 30 μM 5-HT in the presence (black traces) or absence of 10 μM of mosapride (gray traces), near IC50 seen in Fig. 1B. The open horizontal bars indicate the drug application period. (B) Averaged concentration-response curve of 5-HT, receptor currents in the presence ( ● ) or absence (○ ) of mosapride. The data were normalized to the peak amplitude induced by 10 μM 5-HT and lines were obtained by fitting these data to equation (1) in the Methods. The E50 of 5-HT was increased from 1.88 ± 0.10 μM (n = 10) to 4.80 ± 0.32 μM (n = 8) by mosapride (p < 0.001, unpaired t-test) without Hill coefficient changes. There were no significant differences in the peak amplitudes induced by 30 μM 5-HT alone and the co-application of mosapride with 30 μM 5-HT (p = 0.7282, unpaired t-test). The data are expressed as means ± standard error of the mean.
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\[ \mu \text{M} \] and it was much higher than the IC₅₀ of 4.03 \( \mu \text{M} \) shown in Fig. 1B.

To examine if mosapride altered the desensitization of the 5-HT₃ receptor, a long duration (10 sec) of high concentration 5-HT (10 \( \mu \text{M} \)), with or without mosapride, was applied to induce more profound desensitization following channel activation, then the current decay slopes were analyzed. Fig. 5A shows the superimposed current traces induced by 5-HT 10 \( \mu \text{M} \) for 10 sec with or without mosapride (10 \( \mu \text{M} \)). The decay slope of currents during the 5-HT application were increased by the co-application of mosapride (0.12 ± 0.02 pA/ms for 5-HT alone and 0.15 ± 0.03 pA/ms for mosapride co-application; Fig. 5B, n = 10, p < 0.01, paired t-test). These results suggest that mosapride accelerated 5-HT₃ receptor desensitization. The effects of mosapride on the 5-HT₃ receptor desensitization recovery time course were studied using two pulses of 5-HT application in variable inter-pulse intervals (1, 5, 10, 30, 60 sec), then the peak amplitudes of the second pulse were normalized to the first peak amplitude (paired-pulse ratio) and plotted against the inter-pulse intervals. Fig. 5 shows the representative superimposed current traces evoked by two pulses of 10 \( \mu \text{M} \) 5-HT for 5 sec in the inter-pulse intervals of 1, 5, 10, 30, 60 sec with (5D) or without (5C) 10 \( \mu \text{M} \) mosapride. A single exponential function was fitted to the 5-HT₃ receptor desensitization recovery time course with a time constant of 3.85 ± 0.47 sec (n = 9) for 5-HT alone and 3.99 ± 0.44 sec (n = 9) for 5-HT and mosapride co-application (Fig. 5E, p = 0.5588, unpaired t-test). These results suggest that mosapride did not change the 5-HT₃ receptor desensitization recovery time course.

DISCUSSION

In the present study, we examined the direct inhibitory action of mosapride, a gastroprokinetic drug, on 5-HT₃ receptors naturally expressed on NCB-20 cells. Our results suggest that mosapride predominantly inhibited 5-HT₃ receptor function by blocking channel opening, because mosapride reduced the peak current amplitudes accompanied by an attenuation in the current rise slopes, an indicator of the activation of 5-HT₃ receptor...
On the 5-HT₃ receptors suggests the possibility that mosapride bound to closed-state 5-HT₃ receptors also, and thereby, blocked the subsequent ion channel opening. To investigate the binding of mosapride with the 5-HT₃ receptors during channel open state, mosapride was co-applied 3 sec after 5-HT application. At this time, the inward currents still remained, thus a large proportion of the 5-HT₃ receptors probably stayed in an open state. The application of mosapride used in this protocol inhibited 5-HT₃ receptor currents in a concentration-dependent manner based on time constants analysis. These results suggest that mosapride also bound to the open-state of 5-HT₃ receptor, then accelerated channel desensitization. Taken together, these results suggest that mosapride acted on both the closed and open-state 5-HT₃ receptors, then inhibited the 5-HT₃ receptors. Also, mosapride bound to the agonist binding sites of the 5-HT₃ receptor, both in the closed and open states, thus, competitively blocking the channel open or accelerating channel desensitization. In the same experiment, the calculated IC₅₀ values of mosapride on 5-HT₃ receptor currents (Fig. 4B) were three times larger than the IC₅₀ values shown in Fig. 1B. The larger IC₅₀ values for the open state of the 5-HT₃ receptor suggest that mosapride had a higher affinity for this receptor in the closed state, thus, mosapride showed a strong inhibition in this state. This interpretation is supported by our findings that 1) the concentration-response curve of 5-HT on 5-HT₃ receptor currents was shifted to the right, without a change in the maximal effect produced by mosapride (Fig. 2); and 2) its inhibitory effect on the 5-HT₃ receptor was profoundly augmented by pretreatment with mosapride (Fig. 3). These are typical characteristics of channel blockers that competitively bind to the agonist binding sites of receptors, then block channel opening in ligand-gated ion channels [19]. Mosapride also altered the desensitization properties of 5-HT₃ receptor channels, i.e., the slopes for current decay produced by the application of 5-HT for longer times (10 sec) were increased by mosapride but did not change the desensitization recovery time course (Fig. 4). In many ligand-gated ion channels, desensitization is correlated with the receptor activation, i.e., if more channels are opened, they will be more strongly desensitized, and thus the rate of desensitization is increased in an agonist concentration dependently [20]. Therefore, the acceleration of receptor desensitization by mosapride in our experiment was possibly by secondary effects following the primary inhibition of opening. However, a direct effect of mosapride on the 5-HT₃ receptor desensitization itself could not be excluded because some drugs have been known to modulate 5-HT₃ receptor desensitization process directly [21,22]. Thus, further studies clarifying the effect of mosapride on receptor desensitization process are necessary. In our study, mosapride inhibited the 5-HT₃ receptor currents in a voltage-independent manner without a reversal potential change and there was no use-dependency in the inhibition (Supplementary Figs. 1 and 2). These results suggest that mosapride preferentially bound to the 5-HT₃ receptor in a closed state and inhibited ion channel opening, because the volt-

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**Fig. 5. Effect of mosapride on the desensitization of 5-hydroxytryptamine type 3 (5-HT₃) receptor and the time course of desensitization recovery.** (A) Representative 5-HT₃ receptor currents induced by 10 µM of 5-HT for 10 sec with (black trace) or without (gray trace) 10 µM mosapride. The open horizontal bars indicate the drug application period. (B) Averaged bar graph shows the effects of mosapride on the current decay slope after the long application of 5-HT to test the effect of mosapride on 5-HT₃ receptor desensitization (n = 10, *p < 0.01, paired t-test). (C, D) Superimposed sample current traces evoked by two pulses of 10 µM 5-HT for 5 sec in the inter-pulse intervals of 1, 5, 10, 30, 60 sec with (D) or without (C) 10 µM mosapride. Gray arrowheads indicate the first application and the black arrowheads indicate the second application of 5-HT. (E) Averaged data of the paired-pulse ratio (the second peak amplitudes / the first peak amplitude) plotted against the inter-pulse intervals. A single exponential function was fitted to the data and compared to the time constants of 5-HT alone (∗, n = 9) and the co-application 5-HT and mosapride (●, n = 9). Mosapride did not change the time course of desensitization recovery of 5-HT₃ receptor (p = 0.5588, unpaired t-test). The data are expressed as means ± standard error of the mean.
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The 5-HT₃ receptors modulate the release of various neurotransmitters from neurons, such as dopamine, GABA and glutamate, and are involved in a complex neuronal function in the nervous system. In the peripheral nervous system, especially in the GI system, 5-HT₃ receptors were widely expressed in the afferent neurons of the submucosal plexus and are known to be involved in GI functions, such as visceral perception, secretion, and motility. The activation of 5-HT₃ receptors in the extrinsic primary afferent neurons in the submucosal plexus has been reported to trigger a reflex inducing nausea and emesis. Thus, 5-HT₃ receptor antagonists exert their anti-emetic effect by blocking these receptors and are widely used in the treatment of chemotherapeutic agent-induced nausea. 5-HT₃ receptors are also known to mediate a small subset of fast excitatory postsynaptic potential of enteric neurons. This can contribute to regulation of the propulsive motility of the gut, and if blocked, generates constipation, a common adverse effect of 5-HT₃ receptor antagonists for using anti-emetic purposes. Collectively, 5-HT₃ receptor inhibition blocks visceral perception and propulsive motility and these actions can be exploited for the treatment of GI disorders, such as IBS. IBS is a complex disorder characterized by abdominal pain or discomfort associated with disordered defecation, either constipation, diarrhea, or mixed. Hyper-sensitivity of visceral perception in the GI tracts is known to be a pathophysiological mechanism of IBS. Therefore, blocking visceral perception mediated by 5-HT₃ receptors in the submucosal extrinsic primary afferent neuron could be an important strategy for the treatment of IBS. Even the 5-HT₃ receptor antagonists are major gastrokinetic drugs for the treatment of IBS and the additional 5-HT₃ receptor blocking could be very useful for the treatment of certain forms of IBS, like diarrhea-potentiate IBS. Ramostron, cilansetron, ondansetron, and alosetron have been reported to be selective 5-HT₃ receptor antagonists, effective for treating diarrhea-potentiate IBD with rare serious adverse reactions. Even though a receptor binding assay reported that mosapride showed a weak affinity for 5-HT₃ receptors, mosapride exerted its prokinetic effect in the GI tract mainly through selective 5-HT₃ receptor agonist activity, and thus was effective in improving overall symptoms in patients with GI disorders, including chronic gastritis, gastro-esophageal reflux diseases, and functional dyspepsia. Our patch clamp study showing that mosapride directly inhibited 5-HT₃ receptor function expressed in NCB-20 cells provides evidence that mosapride has substantial 5-HT₃ receptor antagonist activity, as well as 5-HT₃ receptor agonist activity, and could contribute to the above mentioned pharmacological actions of mosapride. Moreover, this combined effect on the 5-HT₃ receptor, 5-HT₃ receptor antagonist activity as well as 5-HT₃ receptor agonist activity, suggests that the clinical use of mosapride could be extended to the treatment of IBS, especially diarrhea-prominent types.

In summary, we showed that mosapride directly inhibited 5-HT₃ receptor, receptor-induced currents in a competitive manner by binding to both the closed and open states of the receptor, but preferentially to the closed state. In addition to the known pharmacologic activity of mosapride, selective 5-HT₃ receptor agonist activity, the additional 5-HT₃ receptor antagonist activity could also contribute to the therapeutic effects of mosapride for the treatment of functional GI disorders.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including two figures can be found with this article online at http://pdf.medrang.co.kr/paper/pdf/Kjpp/Kjpp2019-23-05-s001.pdf.

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