Serotonin (5-HT)₃-Receptor Antagonism of 4,5,6,7-Tetrahydrobenzimidazole Derivatives against 5-HT-Induced Bradycardia in Anesthetized Rats

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ABSTRACT—We investigated the mode of the 5-HT₃-receptor antagonism of 4,5,6,7-tetrahydrobenzimidazole derivatives, YM060, YM114 (KAE-393), YM-26103-2 and YM-26308-2, against 5-HT-induced transient bradycardia in anesthetized rats. Results were compared with those of ondansetron and granisetron. YM060 (0.03–0.1 µg/kg, i.v.), YM114 (0.03–0.3 µg/kg, i.v.), YM-26103-2 (0.01–0.03 µg/kg, i.v.), YM-26308-2 (0.01–0.03 µg/kg, i.v.) and granisetron (0.3–3 µg/kg, i.v.) displaced the 5-HT dose-response curve to the right, with apparent DR₂ values of 0.068, 0.068, 0.019, 0.011 and 0.69 µg/kg, i.v., respectively. Higher doses of these compounds inhibited 5-HT-induced bradycardia with a reduced maximal response. In contrast, ondansetron displaced the 5-HT dose-response curve to the right without affecting the maximal response. Judged by the apparent DR₂ values, YM060, YM114, YM-26103-2 and YM-26308-2 were approximately 13, 13, 50 and 79 times more potent than ondansetron, respectively, whereas granisetron was equipotent to ondansetron. Single i.v. doses of YM060 and granisetron inhibited 5-HT-induced bradycardia significantly longer than ondansetron. Moreover, inhibitory effects of p.o. doses of YM060 (3 µg/kg), YM114 (80 µg/kg), YM-26103-2 (12 µg/kg), YM-26308-2 (5 µg/kg) and granisetron (250 µg/kg) on the von Bezold-Jarisch reflex lasted for 3–6 hr, whereas ondansetron (700 µg/kg, p.o.) antagonized 5-HT₃ receptors for only 1 hr. In isolated guinea pig colon, the inhibitory effect of YM-compounds on 5-HT-induced contraction persisted significantly longer than those of ondansetron and granisetron after washout of the bath containing compounds. These results suggest that YM-compounds are highly potent 5-HT₃-receptor antagonists. Furthermore, non-competitive 5-HT₃-receptor antagonism of YM-compounds against the von Bezold-Jarisch reflex at higher doses may be reflected in their slow dissociation from the 5-HT₃ receptor, and that of granisetron may be reflected in its slow metabolism in anesthetized rats.

Keywords: 4,5,6,7-Tetrahydrobenzimidazole derivative, 5-HT₃-receptor antagonism, von Bezold-Jarisch reflex

Serotonin (5-HT) receptors have been classified into at least four subtypes: 5-HT₁, 5-HT₂, 5-HT₃ and 5-HT₄ (1). Among these, research into 5-HT₃ receptors, which are located in both the central and peripheral nervous systems (2–4), has advanced remarkably. This receptor has been implicated in anxiety (5, 6), schizophrenia (7), stress-induced gastrointestinal disorders (8) and vomiting responses to cancer chemotherapeutic agents (9, 10).

A number of 5-HT₃-receptor antagonists have been developed in the last decade: among these, ondansetron and granisetron are already available for clinical use. Recent reports (11, 12) have shown a novel 4,5,6,7-tetrahydrobenzimidazole derivative, YM060, to be a more potent and selective 5-HT₃-receptor antagonist than either of these two agents.

The mode of 5-HT₃-receptor antagonism of these compounds has been investigated in vitro using the ileum and colon of guinea pigs (11, 13, 14), vagus nerve of rats and rabbits (13–15) and the nodose ganglion of rabbits (16). Investigation of their mode of action in vivo systems, however, has not been performed. In the present study, we assessed the mode of the 5-HT₃-receptor antagonism...
of novel 4,5,6,7-tetrahydrobenzimidazole derivatives (Fig. 1) by measuring their ability to block 5-HT-induced bradycardia (the von Bezold-Jarisch reflex) in anesthetized rats, and the results were compared with those of ondansetron and granisetron. Moreover, we evaluated the dissociation of these derivatives from the 5-HT3 receptor using isolated guinea pig colon.

MATERIALS AND METHODS

The von Bezold-Jarisch reflex in anesthetized rats

Male Wistar rats (250-350 g) were used. Food and water were available ad libitum. In the oral administration tests, they were fasted for 24 hr before the experiments, but were allowed free access to water. The rats were anesthetized with urethane (1.25 g/kg, i.p.). A polyethylene tube was inserted into the trachea for artificial ventilation (60 respirations per min; tidal volume, 3.0 ml). The left common carotid artery was cannulated with a polyethylene catheter connected to a pressure transducer (MPU-0.5; Nihon Kohden, Tokyo). The pressure signal was amplified with a carrier amplifier (AP-621G, Nihon Kohden), and the heart rate was measured with a cardiotachometer (AT-600G, Nihon Kohden) triggered by the blood pressure pulse. The blood pressure and heart rate were monitored continuously and recorded (RMP-6008, Nihon Kohden). For intravenous drug administration, a catheter was inserted into the left femoral vein.

A dose-response curve for 5-HT-induced bradycardia was constructed by increasing doses of 5-HT (5–1000 µg/kg, i.v.) every 15 min. Because preliminary experiments showed the dose-response curve for 5-HT to be reproducible in the same animal (Fig. 2), test compounds were injected i.v. 10 min before constructing the second dose-response curve for 5-HT to investigate the mode of the 5-HT3-receptor antagonism. Only one dose of test compound was examined per animal. Apparent DR2 values for the test compounds were calculated to estimate their 5-HT3-receptor blocking activity.

In another series of experiments, the duration of 5-HT3-receptor antagonism after i.v. administration of test compounds was evaluated. A submaximal dose of 5-HT (30 µg/kg, i.v.) was used to examine the inhibitory effect of test compounds. After the control response to 5-HT was obtained, the inhibitory effects of single i.v. doses of YM060 (0.3 µg/kg), granisetron (10 µg/kg) and ondansetron (30 µg/kg) on the 5-HT-induced bradycardia was measured at 15- to 30-min intervals after dosing.

The duration of 5-HT3-receptor antagonism after p.o. administration of test compounds was evaluated. Thirty minutes after dosing, the rats were anesthetized and prepared surgically as described above. The inhibitory effects of single p.o. doses (approximating the ID80 value at 1 hr after dosing) of test compounds on the 5-HT (30 µg/kg, i.v.)-induced von Bezold-Jarisch reflex were measured at 1 hr intervals after dosing. Methylcellulose solution (MC, 0.5% w/v) was administered to the vehicle control group.

Evaluation of the antagonist dissociation in the guinea pig colon

Male Hartley guinea pigs (570-850 g) were used. Food and water were available ad libitum. The guinea pigs were killed by cervical dislocation. The distal portion of the colon (approximately 2-cm-long) was used. Tissues were mounted in organ baths containing 10 ml Krebs-bicarbonate solution maintained at 37°C and aerated with a mixture of 95% O2 and 5% CO2. The composition of Krebs-bicarbonate solution was: 118.4 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 11.1 mM dextrose, 25.0 mM NaHCO3 and 1.2 mM KH2PO4. Tissues were attached to isometric force-displacement transducers (SB-1T, Nihon Kohden) connected to a recorder (MC 6621; Graphtec, Tokyo) through carrier amplifiers (AP-621G, Nihon Kohden). Each tissue was placed under optimum resting force and allowed to equilibrate for approximately 30 min before exposure to test compounds. Isometric contraction under a loading tension of 1 g was recorded.

In preliminary experiments, 5-HT (10^{-7}–3 \times 10^{-5} M)
produced concentration-dependent contraction in the guinea pig colon (data not shown). A submaximal concentration of 5-HT (10^{-5} M) was used to evaluate the inhibitory effects of test compounds. After the control responses to 5-HT were obtained, tissues were incubated with high concentrations (30 times the IC_{50} value) of test compounds for 15 min. After washout of the bath containing test compounds, the 5-HT-induced contraction was measured every 15 min to determine the dissociation of test compounds from the 5-HT_{3} receptor in the guinea pig colon.

**Statistical analyses**

All values are expressed as the means±S.E.M. or as the means with 95% confidence limits. Probit analysis was used to obtain the ID_{50} and ID_{80} values. The dose-ratio was obtained from the ratio of ED_{50} values for 5-HT in the presence and absence of an antagonist. Apparent DR_{2} values (doses of test compounds produced a dose-ratio of 2 for 5-HT) and slopes of the curves were calculated (17). Statistical significance of values for the duration of action after i.v. dosing and washout was determined by analysis of variance at each time, and differences between treatment groups were compared by the Sheffe-S method, Mann-Whitney U-test and Newman-Keuls multiple range test. Student’s t-test was used to evaluate the duration of action after p.o. dosing between the test compound and control groups. Probabilities of <5% (P < 0.05) were considered significant.

**Drugs**

YM060 {[((R)-5-[(1-methyl-3-indolyl)carbonyl]-4,5,6,7-tetrahydro-1H-benzimidazole hydrochloride], YM114 (KAEC-393) {[((R)-5-[(2,3-dihydro-1-indolyl)carbonyl]-4,5,6,7-tetrahydro-1H-benzimidazole hydrochloride], YM-26103-2 {[((R)-5-[(1-methyl-3-indoliziny)carbonyl]-4,5,6,7-tetrahydro-1H-benzimidazole hydrochloride], YM-26308-2 {[((R)-5-[(3-methyl-1-indoliziny)carbonyl]-4,5,6,7-tetrahydro-1H-benzimidazole hydrochloride], ondansetron and granisetron were prepared by Yamanouchi Pharmaceutical Co., Ltd. 5-HT creatinine sulfate was purchased from E. Merck (Darmstadt, FRG). All drug doses are expressed as the free base. Drugs were dissolved in physiological saline for i.v. administration and were suspended in 0.5% MC solution for p.o. administration.

**RESULTS**

**Bradycardic response to 5-HT**

The basal heart rate in anesthetized rats was 400.3±36.6 beats/min (n=4). Bolus i.v. injection of 5-HT (5–80 µg/kg) evoked a dose-dependent and transient bradycardia. The second series of 5-HT injections in each rat produced almost the same bradycardic responses as the first, indicating the dose-response curve for 5-HT to be reproducible (Fig. 2).

**5-HT_{3}-receptor antagonism after i.v. administration in anesthetized rats**

YM060, YM114, YM-26103-2 and YM-26308-2 produced parallel shifts to the right of the 5-HT dose-response curves without affecting the maximal response to 5-HT at i.v. doses of 0.03–0.1, 0.03–0.3, 0.01–0.03 and 0.01–0.03 µg/kg, respectively. However, higher i.v. doses of YM060 (0.3 µg/kg), YM114 (1 µg/kg), YM-26103-2 (0.1 µg/kg) and YM-26308-2 (0.1 µg/kg) inhibited 5-HT-induced falls in heart rate with reduced maximal responses (Fig. 3). Similarly, lower doses of granisetron (0.3–3 µg/kg, i.v.) produced parallel shifts to the right of the dose-response curve without affecting maximal responses to 5-HT, while a higher dose of granisetron (10 µg/kg, i.v.) reduced maximal responses to 5-HT (Fig. 4). Ondansetron, unlike the YM-compounds and granisetron, produced parallel shifts to the right of the 5-HT curve with the same maximal response up to 100 µg/kg, i.v. (Fig. 5).

5-HT_{3}-receptor blocking activities of the test drugs were evaluated using apparent DR_{2} values, calculated using the dose-ratio obtained from the parallel-shifted dose-response curves. The apparent DR_{2} values are listed in Table 1. Judged by these values, YM060, YM114, YM-26103-2 and YM-26308-2 were approximately 13, 13, 50 and 79 times more potent than ondansetron, respectively, whereas granisetron and ondansetron were equipotent.
Fig. 3. Inhibitory effects of (a) YM060, (b) YM114, (c) YM-26103-2 and (d) YM-26308-2 on 5-HT-induced bradycardia in anesthetized rats. YM060 (○: 0 μg/kg, ○: 0.03 μg/kg, △: 0.1 μg/kg, □: 0.3 μg/kg), YM114 (●: 0 μg/kg, ○: 0.1 μg/kg, △: 0.3 μg/kg, □: 1 μg/kg), YM-26103-2 (●: 0 μg/kg, ○: 0.03 μg/kg, △: 0.1 μg/kg) and YM-26308-2 (●: 0 μg/kg, ○: 0.01 μg/kg, △: 0.03 μg/kg, □: 0.1 μg/kg) were injected i.v. 10 min before 5-HT (5–1000 μg/kg, i.v.). Values are the means ± S.E.M. from 4 to 6 animals.

Fig. 4. Inhibitory effect of granisetron on 5-HT-induced bradycardia in anesthetized rats. Granisetron (●: 0 μg/kg, ○: 0.3 μg/kg, △: 1 μg/kg, □: 3 μg/kg, ▲: 10 μg/kg) was injected i.v. 10 min before 5-HT (5–1000 μg/kg, i.v.). Values are the means ± S.E.M. from 4 to 8 animals.

Fig. 5. Inhibitory effect of ondansetron on 5-HT-induced bradycardia in anesthetized rats. Ondansetron (●: 0 μg/kg, ○: 1 μg/kg, △: 3 μg/kg, □: 10 μg/kg, ▲: 30 μg/kg, ▼: 100 μg/kg) was injected i.v. 10 min before 5-HT (5–1000 μg/kg, i.v.). Values are the means ± S.E.M. from 3 to 4 animals.
Duration of action after i.v. administration in anesthetized rats

To evaluate the duration of action of YM060, granisetron and ondansetron, their inhibitory effect on 5-HT ($30 \mu g/kg$, i.v.)-induced bradycardia was examined in anesthetized rats after higher doses of YM060 ($0.3 \mu g/kg$, i.v.), granisetron ($10 \mu g/kg$, i.v.) and ondansetron ($30 \mu g/kg$, i.v.) were injected. After i.v. injection, YM060 significantly inhibited the von Bezold-Jarisch reflex for a longer period than ondansetron, whereas the duration of action of granisetron was as long as that of YM060 (Fig. 6).

Duration of action after p.o. administration in anesthetized rats

To evaluate the duration of action, inhibitory effects of single p.o. doses of test compounds on the 5-HT ($30 \mu g/kg$, i.v.)-induced von Bezold-Jarisch reflex were measured at 1-hr intervals. Antagonist doses were determined from dose-response curves as approximate ID$_{80}$ values at 1 hr after dosing. Doses of YM060, YM114, YM-26103-2, YM-26308-2, granisetron and ondansetron were 3, 80, 12, 5, 250 and 700 $\mu g/kg$, p.o., respectively. As shown in Fig. 7, 5-HT$_3$-receptor antagonism by the YM-compounds and granisetron was sustained for 3 to 6 hr significantly, compared with the control group. In contrast, the blocking activity of ondansetron disappeared by 2 hr after p.o. administration.

Evaluation of the antagonist dissociation in the guinea pig colon

To evaluate the antagonist dissociation from 5-HT$_3$ receptors, inhibitory effects of test compounds on 5-HT ($10^{-5}$ M)-induced contraction in isolated guinea pig colon were examined after washout of the bath containing compounds. A submaximal concentration of 5-HT ($10^{-5}$ M) evoked 6.9±0.4 (n=24) g contraction in the guinea pig colon. YM060 ($10^{-6}$ M), YM114 ($10^{-6}$ M), YM-26103-2 ($3 \times 10^{-6}$ M), YM-26308-2 ($3 \times 10^{-6}$ M), granisetron ($3 \times 10^{-6}$ M) and ondansetron ($3 \times 10^{-5}$ M) prevented 5-HT-induced contraction with the degrees of inhibition measured.

### Table 1. Estimates of the apparent DR$_2$ values of antagonists on the bradycardic response to 5-HT in anesthetized rats

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>DR$_2$ ($\mu g/kg$, i.v.) [95% CL]</th>
<th>Relative potency (Ondansetron = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM060</td>
<td>0.068 [0.48–0.11]</td>
<td>13</td>
</tr>
<tr>
<td>YM114</td>
<td>0.068 [0.002–0.14]</td>
<td>13</td>
</tr>
<tr>
<td>YM-26103-2</td>
<td>0.019 [0.012–0.053]</td>
<td>50</td>
</tr>
<tr>
<td>YM-26308-2</td>
<td>0.011 [0.0081–0.014]</td>
<td>79</td>
</tr>
<tr>
<td>Ondansetron</td>
<td>0.87 [0.28–1.74]</td>
<td>1</td>
</tr>
<tr>
<td>Granisetron</td>
<td>0.69 [0.40–1.05]</td>
<td>1</td>
</tr>
</tbody>
</table>

Apparent DR$_2$ values are the means with 95% confidence limits.
of 84.8±4.5, 84.8±3.4, 89.8±3.0, 84.6±2.6, 82.8±1.4
and 83.0±4.701o, respectively, immediately after 15-min
incubation. 5-HT3-receptor blocking activity of all YM
compounds significantly persisted longer than that of
ondansetron and granisetron after washout of the bath
containing the compounds (Fig. 8).

**DISCUSSION**

In the present study, we investigated the mode of the 5-
HT3-receptor antagonism of YM060, YM114, YM-26103-
2, YM-26308-2, granisetron and ondansetron on the von
Bezold-Jarisch reflex in anesthetized rats. All YM-com-
ounds have an asymmetric center (Fig. 1), resulting in R-
and S-forms. As 5-HT3 receptors in the isolated guinea
pig colon have a stereochemical preference for the R-
form of YM-compounds (11, 18), we used only the R-
forms of these compounds in the present system.

Like YM060, the other YM-compounds have the ind-
dolyl or indolizinyl moiety as the aromatic component.
Although the position of the nitrogen atom varies among
these moieties, their 5-HT3-receptor blocking activities
are maintained. Based on the apparent DR2 values, the
rank order of potency was YM-26308-2>YM-26103-
2>YM060=YM114>granisetron>ondansetron.

Lower doses of all compounds tested caused a parallel
displacement of the 5-HT dose-response curve to the
right, consistent with simple competitive antagonist be-
behavior. At higher doses, ondansetron still produced a
parallel shift to the right without affecting the maximal
responses to 5-HT, whereas the YM-compounds and
granisetron reduced the maximal responses to 5-HT.
Three possibilities may explain these differences in the
mode of action between YM-compounds or granisetron
and ondansetron. First, the duration of the 5-HT3-recep-
tor blocking action of YM-compounds and granisetron,
unlike that of ondansetron, may be quite long due to their
slow metabolism or dissociation from the 5-HT3 receptor.
In the present study, the dissociation of all YM-com-
ounds from 5-HT3 receptors in the isolated guinea pig
colon was significantly slower than that of ondansetron.
Furthermore, the 5-HT3-receptor blocking activity of i.v.
or p.o. doses of YM060 was longer than that of ondan-
setron in anesthetized rats. Taken together, long-acting
properties of YM-compounds may be reflected in their
slow dissociation from 5-HT3 receptors. On the other
hand, the dissociation of granisetron from 5-HT3 recep-
tors in the guinea pig colon was as fast as that of ondan-
setron, whereas i.v. or p.o. doses of granisetron inhibited
5-HT-induced bradycardia significantly longer than ondan-
setron in anesthetized rats. The long-acting property of
granisetron, unlike that of YM-compounds, may be
reflected in its slow metabolism in anesthetized rats.

Ohen et al. (19) also reported that the duration of action
of granisetron was longer than that of ondansetron after
i.v. injection, and it was consistent with our observation.
Secondly, these 5-HT3-receptor antagonists may have
non-specific antagonistic activities at other receptors.
However, this possibility is unlikely because 5-HT-in-
duced transient bradycardia is mediated only through 5-
HT3 receptors, which are located on afferent vagus
nerves, and neither ketanserin, a 5-HT2-receptor an-
tagont (our observation, data not shown), nor methyser-
gide, a 5-HT, and 5-HT2-receptor antagonist (20), affect-
ed the 5-HT-evoked bradycardia. Furthermore, previous
reports have shown that neither YM-compounds (includ-
ing YM060) nor granisetron acts on other amine receptors
(11, 18), and they have no local anesthetic effect at the
doses used in the present in vivo study (data not shown).
Taken together, YM-compounds and granisetron are sug-
gested to be highly selective 5-HT3-receptor antagonists.
Finally, the pseudo-non-competitive antagonism of the
high affinity antagonist may be observed against the par-
tial agonist-induced response, although its mode of recep-
tor antagonism is a competitive against the full agonist.
In the present in vivo study, however, 5-HT was regarded
as a full agonist for 5-HT3 receptors. Moreover, the 5-
HT3-receptor blocking potency of granisetron was equipo-
tent to that of ondansetron. These results suggest that the
last possibility can not explain the differences in the mode
of the 5-HT3-receptor antagonism between YM-com-
ounds or granisetron and ondansetron in anesthetized
rats.

In isolated guinea pig ileum (14, 21) and colon (11, 18), YM-compounds, granisetron and ondansetron behave as competitive 5-HT₃-receptor antagonists. In isolated rabbit nodose ganglion, ondansetron behaves as a competitive 5-HT₃-receptor antagonist (14), whereas granisetron causes non-competitive or irreversible (16). Furthermore, in isolated rabbit vagus nerve, ondansetron behaves as a competitive 5-HT₃-receptor antagonist (14), whereas granisetron causes non-competitive antagonism (22). These results offer a new dimension for discussion on the criteria for the classification of 5-HT₃-receptor subtypes or a species difference in the 5-HT₃ receptor. Since the von Bezold-Jarisch reflex was mediated through vagus nerves in anesthetized rats, the behavior of 5-HT₃-receptor antagonists in the present study may be different from that in guinea pig ileum, colon and rabbit nodose ganglion. Although there are differences between an in vivo study and an in vitro study, the mode of action of 5-HT₃-receptor antagonists in the present in vivo study (rat vagus nerve) was consistent with that in the isolated rabbit vagus nerve. On the other hand, the dissociation of granisetron from 5-HT₃ receptors in the guinea pig colon was as fast as that of ondansetron. In rat vagus nerves, however, the dissociation of granisetron from 5-HT₃ receptors may be slower than that of ondansetron because of the difference in 5-HT₃-receptor subtypes between rat vagus nerve and guinea pig colon. It may be reflected in the long duration of action after i.v. doses of granisetron in anesthetized rats. To make it clear, however, further investigations have to be carried out.

In conclusion, YM060, YM114, YM-26103-2, YM-26308-2 and granisetron at lower doses behaved like competitive 5-HT₃-receptor antagonists against the von Bezold-Jarisch reflex in anesthetized rats, whereas at higher doses, their 5-HT₃-receptor antagonism was inconsistent with a competitive interaction. In contrast, ondansetron behaved like a competitive antagonist up to 100 μg/kg, i.v. This difference may be reflected in their slow metabolism or slow dissociation from 5-HT₃ receptors.

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