Pharmacological Properties of 3-Amino-5,6,7,8-tetrahydro-2-{4-[4-(quinolin-2-yl)piperazin-1-yl]butyl}quinazolin-4(3H)-one (TZB-30878), a Novel Therapeutic Agent for Diarrhea-Predominant Irritable Bowel Syndrome (IBS) and Its Effects on an Experimental IBS Model

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ABSTRACT

3-Amino-5,6,7,8-tetrahydro-2-{4-[4-(quinolin-2-yl)piperazin-1-yl]butyl}quinazolin-4(3H)-one (TZB-30878) is a novel compound with both 5-hydroxytryptamine (5-HT)1A agonism and 5-HT3 antagonism properties. The binding assays demonstrated that [3H]TZB-30878 selectively binds to human 5-HT1A and 5-HT3 receptors, with dissociation constants (Kd) of 0.68 ± 0.03 and 8.90 ± 1.73 nM, respectively. Systemic administration of TZB-30878 inhibited 5-HT-induced bradycardia in a dose-dependent manner in rats. In behavioral assays TZB-30878 produced signs of 5-HT syndrome in rats. These results suggest that TZB-30878 has dual effects as a 5-HT1A receptor agonist and a 5-HT3 receptor antagonist. Furthermore, we evaluated the effects of TZB-30878 on wrap restraint stress-induced defecation in an IBS-like model in rats. TZB-30878 (1–10 mg/kg p.o.) normalized stress-induced defecation in a dose-dependent manner, whereas the 5-HT1A agonist tandospirone (30 and 100 mg/kg p.o.) and the 5-HT3 antagonist alosetron (1–10 mg/kg p.o.) did not show such effects. Furthermore, this efficacy of TZB-30878 was partly antagonized by a 5-HT1A antagonist, [O-methyl-3H]-N-(2-(4-methylphenyl)-1-piperazinyl)ethyl)-N-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY-100635). These results suggest that 5-HT1A receptor agonism and 5-HT3 receptor antagonism contribute to the efficacy of TZB-30878 in the IBS-like model. The efficacy of TZB-30878 supports the concept that the presence of both actions, namely 5-HT1A receptor agonism and 5-HT3 receptor antagonism, could be an important mechanism in the treatment of d-IBS.

Irritable bowel syndrome (IBS) is one of the most common functional disorders of the gastrointestinal tract, with major symptoms such as abdominal discomfort or pain accompanied by constipation or diarrhea (Talley et al., 1991; Drossman et al., 1993). The syndrome has been considered to be associated with psychological stress, abnormal gut motility, and abdominal visceral hyperalgesia (Whitehead and Crowell, 1991; Camilleri and Choi, 1997). Indeed, anxiolytics and antimitoty agents have been used for the treatment of this disorder (Friedman, 1991; Talley et al., 1991). However, the existing medications have not been adequately effective for patients with IBS.

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter found in both the brain and gut and is probably involved in brain-gut interactions in normal volunteers and possibly to a greater degree in patients with IBS (Kim and...
Camilleri, 2000; Talley, 2001). Azapirones, such as buspirone and tandospirone, have been used as a class of anxiolytics that are less sedative and more anxioselective than the benzodiazepines (Robinson, 1991; Sasa, 1997). The mechanism of their anxiolytic action is considered to be an agonistic action for one of the 5-HT receptor subtypes, the 5-HT$_{1A}$ receptors (Traber and Glaser, 1987; Sasa, 1997). In clinical trials, the improvement rating with tandospirone was higher than that with the placebo in patients with IBS (Kimura et al., 1992). This indicates the possible involvement of the 5-HT$_{1A}$ receptors in the treatment of IBS.

Clinical studies demonstrated accelerated transit through the small bowel or colon in some patients with diarrhea-predominant IBS (d-IBS) (Cann et al., 1983; Vassallo et al., 1992). It has been suggested that gastrointestinal transit may mediate the development of symptoms in IBS. Previous studies have focused on the role of the serotonin system in intestinal motor function, particularly with the 5-HT$_3$ receptor. Studies have focused on 5-HT$_1A$ receptor agonism and 5-HT$_3$ receptor antagonist, granisetron, has been shown to reduce postprandial rectal motor activity in IBS patients (Prior and Read, 1993). In addition, recent studies have demonstrated the effectiveness of a 5-HT$_3$ receptor antagonist, alosetron, in the treatment of IBS. We considered that the development of a unique compound could be valuable for the treatment of IBS. TZB-30878 is rapidly absorbed after oral administration, permeates the blood-brain barrier, and is eliminated via hepatic metabolism.

In the present study, we evaluated the following pharmacological profiles of TZB-30878: 1) The binding affinities for human recombinant 5-HT$_{1A}$ and 5-HT$_3$ receptors were determined using [3H]TZB-30878; 2) the functional activities in vitro were determined in the GTP$_\gamma$S binding assay for 5-HT$_{1A}$ receptors and in the contraction-response assay of the isolated guinea pig ileum for 5-HT$_3$ receptors; 3) 5-HT$_{1A}$ agonistic action in vivo was evaluated based on the 5-HT behavioral syndrome in rats; and 4) 5-HT$_3$ antagonistic action in vivo was evaluated based on 5-HT$_3$-induced bradycardia (von Bezold-Jarisch reflex) in anesthetized rats. The main aim of this study was to evaluate the effects of TZB-30878 on wrap restraint stress-induced defecation in rats, which is believed to be a pathological model for IBS (Williams et al., 1988). More specifically, we examined the value of having both actions for the treatment of IBS.

### Materials and Methods

#### Animals

Male Sprague-Dawley rats (Charles River Laboratories Japan, Inc., Yokohama, Japan), weighing 170 to 230 g, and female Hartley guinea pigs (Japan SLC, Inc., Shizuoka, Japan), weighing 280 to 350 g, were used. Animals were maintained under controlled environmental conditions with constant temperature (22°C) and a 12-h light/dark cycle (lighted from 8:00 AM to 8:00 PM) and were allowed free access to feed and water. All studies were conducted after approval by the Animal Research Committee of ASKA Pharmaceutical Co., Ltd., based on the criteria described in the Rules for the Care and Use of Laboratory Animals.

#### Drugs

All drugs were prepared fresh at each use. The drugs were administered at a dose volume of 2 ml/kg (i.p., s.c., or intraduodenal) or 5 ml/kg (p.o.). TZB-30878, tandospirone, and alosetron were prepared by ASKA Pharmaceutical Co., Ltd. (Kawasaki, Japan). [3H]TZB-30878, 2-[(N,N-di(2-naphthalenyl)-8-hydroxy-1,2,3,4-tetrahydro- 

#### Radioligand Binding Assays

Human 5-HT$_{1A}$ receptor (cloned human serotonin receptor subtype 1A produced in CHO cells; PerkinElmer Life and Analytical Sciences) was thawed on ice and diluted with buffer A (50 mM Tris-HCl, 0 mM MgSO$_4$, 0.5 mM EDTA, and 0.1% (w/v) ascorbic acid, pH 7.4) to prepare a human 5-HT$_{1A}$ receptor membrane preparation. The following procedures were performed in the three experiments. Twenty microliters of buffer A, 20 μl of [3H]TZB-30878 solution (final concentrations: 0.01–10 nM), and 500 μl of the human 5-HT$_{1A}$ receptor membrane preparation (including 1 U) were placed in tubes and mixed to prepare reaction mixtures in triplicate (nonspecific binding was determined in the presence of 10 μM TZB-30878). The reaction mixtures were incubated at 27°C for 1 h. The incubation was terminated by filtering the reaction mixtures through GF/C filters soaked in 0.3% polyethyleneimine and 50 mM Tris-HCl (pH 7.4) using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). The filters were washed twice with 5 ml of cold buffer C (50 mM Tris-HCl, pH 7.4). Radioactivity retained on the filters was counted with a liquid scintillation counter (LSC-5100; Aloka Co., Ltd., Tokyo, Japan). The assay procedure for the competitive analysis was essentially the same as described above. Assays were carried out after saturation binding of 8-OH-[3H]DPAT (final concentrations: 0.01–10 nM) to obtain the binding affinity (Kd value) of 8-OH-[3H]DPAT. Twenty microliters of buffer A or the test drugs (final concentrations: 0.03–1000 nM), 20 μl of 8-OH-[3H]DPAT solution (the final concentration was close to the Kd value), and 500 μl of the human 5-HT$_{1A}$ receptor membrane preparation (including 1 U) were placed in tubes and mixed to prepare reaction mixtures in triplicate. Nonspecific binding was determined in the presence of 10 μM 8-OH-DPAT.

Human 5-HT$_3$ receptor (human 5-HT$_3$ serotonin receptor, a membrane preparation of HEK293 cells expressing human 5-HT$_3$ receptor genes; PerkinElmer Life and Analytical Sciences) was thawed on ice and diluted with buffer B (50 mM Tris-HCl, 5 mM MgCl$_2$, and 1 mM EDTA, pH 7.5) to prepare the human 5-HT$_3$ receptor membrane preparation. The following procedures were performed in the three experiments. Twenty microliters of buffer B, 20 μl of [3H]TZB-30878 solutions (final concentrations: 0.03–20 nM), and 500 μl of the human 5-HT$_3$ receptor membrane preparation (including 1 U) were placed in tubes and mixed to prepare reaction mixtures in triplicate. Nonspecific binding was determined in the presence of 10 μM 8-OH-DPAT. Radioactivity retained on the filters was counted with a liquid scintillation counter. The assay procedure for the competitive analysis was essentially the same as that described above. Assays were carried out after saturation binding of [3H]BRL-43694 (granisetron) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). 5-HT creatinine sulfate complex, 2-methyl-5-HT maleate salt, 8-OH-DPAT, and WAY-100655 maleate salt were obtained from Sigma-Aldrich (St. Louis, MO). Ethyl carbamate (urethane) was obtained from Tokyo Chemical Industry (Tokyo, Japan).
Beer, 1997) with partial modification. Human 5-HT1A receptor bindings were carried out using the method described previously (Stanton and Hartley guinea pigs were sacrificed, and their temperatures measured before and after administration was designated as the index of hypothermia.

The surgical procedures and mean heart rate recordings were performed as follows. Animals were anesthetized with urethane at a concentration of 1 g recorded using an isotonic force transducer (TD-112S; Nihon Kohden, Tokyo, Japan). Experiments were started after stable contractions induced by 10 μM 2-methyl-5-HT were obtained at least three times. The vehicle (dimethyl sulfoxide) or the test drugs were added to the organ bath, and the preparations were exposed to the vehicle or test drugs for 20 min. Then 2-methyl-5-HT (10 μM) was added to the organ bath, and the contractions were recorded.

5-HT1A Receptor-Mediated Behavior and Hypothermia in Rats. Rats were acclimated to the test environment for 2 weeks before testing and conditioned to the test procedures during this period. On the day of the experiment, rats were acclimated to the test cage for 1 h. TZB-30878 or vehicle (saline containing diluted hydrochloric acid) was injected i.p., and then 5-HT1A receptor-mediated behavior (flat body posture and lower lip retraction) was measured in the test cage. Behavioral responses were measured at 5, 10, 20, and 30 min after administration using a 0 to 3 scale as described previously in the literature (Foreman et al., 1993). The maximum score obtained from each animal during the observation period was designated as the individual score. The rectal temperature was recorded before and at 30 min after administration of the test drugs using a thermistor probe (MGAIII-219; Nihon Kohden) that was inserted into the rectum, 3 cm from the anal orifice. The difference between the temperatures measured before and after administration was designated as the index of hypothermia.

5-HT-Induced Bradycardia (von Bezold-Jarisch Reflex) in Rats. The surgical procedures and mean heart rate recordings were performed as follows. Animals were anesthetized with urethane at a

**Fig. 1.** Saturation binding curve of [3H]TZB-30878 to human 5-HT1A receptors expressed in CHO cells (A) and 5-HT2 receptors expressed in HEK293 cells (B). Each receptor membrane preparation was incubated with increasing concentrations of [3H]TZB-30878 (0.01–10 nM 5-HT1A or 20 nM 5-HT2). Nonspecific binding was detected in the presence of 10 μM TZB-30878. The figure shows one of three analyses.

**Fig. 2.** Substitution curve of 8-OH-[3H]DPAT by TZB-30878 and tandospirone to human 5-HT1A receptors expressed in CHO cells (A) or [3H]BRL-43694 by TZB-30878 and alosetron to 5-HT3 receptors expressed in HEK293 (B). Each receptor membrane preparation was incubated with various concentrations of the drugs. Nonspecific binding was detected in the presence of 10 μM 8-OH-DPAT (5-HT1A) or granisetron (5-HT3). The figure shows one of three analyses.

**Fig. 3.** Inhibition of 5-HT3 Receptor-Mediated Contraction in Guinea Pig Ileum. Hartley guinea pigs were sacrificed, and their ileums were isolated. Longitudinal muscle strips including the myenteric plexus were prepared from a 1-cm-long segment of the ileum (Butler et al., 1990). The ileum preparations were suspended in an organ bath containing Tyrode’s solution [154 mM NaCl, 3 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 12 mM NaHCO3, 0.4 mM NaH2PO4, and 6 mM d-(-)-glucose], warmed to 37°C and aerated with a mixture of 5% CO2 and 95% O2. Isometric contractions under a loading tension of 1 g were recorded using an isotonic force transducer (TD-112S; Nihon Kohden, Tokyo, Japan). Experiments were started after stable contractions induced by 10 μM 2-methyl-5-HT were obtained at least three times. The vehicle (dimethyl sulfoxide) or the test drugs were added to the organ bath, and the preparations were exposed to the vehicle or test drugs for 20 min. Then 2-methyl-5-HT (10 μM) was added to the organ bath, and the contractions were recorded.

**5-HT1A** Agonist-Induced [35S]GTPγS Binding Assays. Assays were carried out using the method described previously (Stanton and Beer, 1997) with partial modification. Human 5-HT1A receptor (cloned human serotonin receptor subtype 1A produced in CHO cells; PerkinElmer Life and Analytical Science) was thawed on ice and diluted with incubation buffer (20 mM HEPES, 3 mM MgCl2, and 120 mM NaCl, pH 7.4). Membranes were incubated with GDP (20 μM) and the test drugs for 20 min at 30°C in a volume of 900 μl and then placed on ice for 15 min. [35S]GTPγS (100 μM) was added to all tubes to yield a final volume of 1 ml, and the tubes were further incubated for 30 min at 30°C. Incubation was terminated by filtering the mixtures through GF/B filters using a Brandel cell harvester. The filters were washed twice with 5 ml of cold wash buffer (20 mM HEPES and 3 mM MgCl2, pH 7.4 at 4°C). Radioactivity retained on the filters was counted by a liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM GTPγS.

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5-HT-Induced Bradycardia (von Bezold-Jarisch Reflex) in Rats. The surgical procedures and mean heart rate recordings were performed as follows. Animals were anesthetized with urethane at a
dose of 1.25 g/kg i.p., and then polyethylene cannulas were inserted into the right common carotid artery and vein to measure the blood pressure and to administer 5-HT, respectively. The duodenum was incised, decorticated 2 to 3 cm from the stomach and cannulated for intraduodenal administration of the test drugs. Blood pressure was monitored using a pressure amplifier (AP-601G; Nikon Kohden), and the mean heart rate was recorded by a tachometer (AT-601G; Nikon Kohden) triggered by blood pressure pulsation. 5-HT was injected i.v. at 300 μg/kg to evoke a transient bradycardia [von Bezold-Jarisch (BJ) reflex]. After recovery to normal blood pressure and heart rate, the test drugs and vehicle (0.5% Tween 80) were administered into the duodenum. Thirty minutes later, 5-HT was readministered i.v., and the bradycardia was assessed.

Wrap Restraint Stress-Induced Defecation in Rats. The method described by Williams et al. (1988) was used with slight modifications. Rats were acclimated to observation metal mesh cages placed on a tray from the day before the experiment. After acclimation, the rats were lightly anesthetized with ether, and their fore-shoulder, upper forelimbs, and thoracic trunks were wrapped with adhesive tape (2.5 × 50 cm). Immediately after wrapping, the rats were returned to the observation cages. The number of feces was counted at 1 h after the wrapping. Test drugs and vehicle (0.5% Tween 80) were administered p.o. 1 h before the restraint. In the antagonism assays, TZB-30878 or vehicle (saline containing diluted hydrochloric acid) was injected i.p. 20 min after s.c. treatment with the vehicle (saline) or a selective 5-HT1A receptor antagonist, WAY-100635. Five minutes later, the animals were wrapped for 1 h, and the number of feces was counted.

Data Analysis and Statistical Evaluation. GraphPad Prism (version 4.00; GraphPad Software Inc., San Diego, CA) was used for data analysis in all assays. Specific binding of the radiolabeled ligand was calculated and a saturation binding curve was generated by plotting $P = X$, unbound radiolabeled ligand) and $B = Y$, bound radiolabeled ligand) on the horizontal and vertical axes, respectively, to calculate $K_r$ values using nonlinear regression. The equation (one-site binding) was as follows: $Y = B_{max} \times X/K_r + X$. A substitution curve was generated by plotting the test drug concentrations and binding inhibition rates on the horizontal and vertical axes, respectively, to calculate the concentration that corresponded to a 50% binding inhibition rate ($IC_{50}$). The binding inhibition constant ($K_r$) value of the test drugs was calculated according to the following equation: $E = IC_{50}(1 + L/K_r)$. To determine the concentration of radiolabeled ligand added.

Specific binding of $[^{35}S]GTP\gamma S$ was calculated; the results were then presented as percent increases in binding from the basal value, and dose-response curves were plotted and analyzed. The maximal stimulation ($E_{max}$) achieved for each drug was expressed as percent the maximal response of $R(+)\-8\-OH-DPAT$.

Statistical analysis was performed using SAS (PreClinical Package version 5.0; SAS Institute, Tokyo, Japan). The dose response for the relative potency of the drugs was analyzed by analysis of variance followed by a nonparametric or parametric Dunnett multiple comparison test with the limit of significance level at $P < 0.05$. Results between the two groups were compared with the Wilcoxon test.
Results

Radioligand Binding Assays. The binding assays of [35S]GTP for TZB-30878 to human 5-HT1A and 5-HT3 receptors were performed three times each. A stable saturation curve (Fig. 1) was obtained from the assays, which indicates that TZB-30878 is a compound that possesses binding affinity to human 5-HT1A and 5-HT3 receptors. The K_i values of TZB-30878 toward 5-HT1A and 5-HT3 receptors were 6.771 × 10^{-10} ± 0.3292 × 10^{-10} and 8.903 × 10^{-9} ± 1.728 × 10^{-9} M, respectively.

Next, saturation binding analyses were conducted three times for each of the ligands, [3H]8-OH-DPAT, and [3H]BRL-43694. By using 5-HT1A and 5-HT3 receptors, the K_i values for TZB-30878 were compared with those for the 5-HT1A receptor agonist, tandospirone, and the 5-HT3 receptor antagonist, alosetron. TZB-30878 inhibited the binding of 8-OH-DPAT and BRL-43694 to these receptors in a concentration-dependent manner (Fig. 2). These results and the previously described results for the binding affinity indicated that TZB-30878 binds specifically to both human 5-HT1A and 5-HT3 receptors. Tandospirone and alosetron also inhibited binding of each radioligand to the receptors in a concentration-dependent manner. At the receptor binding level, the K_i for TZB-30878 was approximately 10 times greater than that for tandospirone and approximately \( \frac{1}{10} \) of that for alosetron.

The receptor selectivity of TZB-30878 for the 5-HT1A and 5-HT3 receptors was 300 times or more higher than that for histamines H1 and H2; muscarinics M1, M2, and M3; neurokinins NK1, NK2, and NK3; kinins NK2 and NK3; neuropeptides Y1 and Y2; opioids A1, A2, and A3; adrenelines D1 and D2; GABA; histamines H1 and H2; muscarinics M1, M2, and M3; neurokinins NK2 and NK3; neuropeptides Y1 and Y2; opioids \( \delta \), \( \kappa \), and \( \mu \); serotonin 5-HT1A, 5-HT1B, 5-HT3A, 5-HT3A, 5-HT3B, and 5-HT2C; norepinephrine transporter and dopamine transporter (Table 1).

5-HT1A Agonist-Induced [35S]GTP\(_{\gamma}S\) Binding Assays. The functional activity of TZB-30878 on the 5-HT1A receptor was compared with that of other 5-HT1A receptor agonists and antagonist (Fig. 3; Table 2). A 5-HT1A receptor agonist, R-(+)-8-OH-DPAT, increased binding of [35S]GTP\(_{\gamma}S\) to the receptor in a concentration-dependent manner. Generally, R-(+)-8-OH-DPAT is known to be a full agonist, and we have preliminarily confirmed that R-(+)-8-OH-DPAT and 5-HT exhibited comparable \( E_{\text{max}} \) values. S-(−)-8-OH-DPAT and tandospirone also increased binding of [35S]GTP\(_{\gamma}S\) in a concentration-dependent manner. \( E_{\text{max}} \) values were 81.3% for S-(−)-8-OH-DPAT and 66.7% for tandospirone compared with that for R-(+)-8-OH-DPAT. This result indicated that S-(−)-8-OH-DPAT and tandospirone were partial agonists. The 5-HT1A receptor antagonist, WAY-100635, did not affect the binding of [35S]GTP\(_{\gamma}S\). TZB-30878 increased the binding of [35S]GTP\(_{\gamma}S\) in a concentration-dependent manner, and the \( E_{\text{max}} \) value was comparable with that of R-(+)-8-OH-DPAT.

Inhibition of 5-HT3 Receptor-Mediated Contractions in Guinea Pig Ileum. The functional activity of TZB-30878 on the 5-HT3 receptor was compared with that of alosetron (Fig. 4). TZB-30878 alone did not affect longitudinal muscle strips with the myenteric plexus (data not shown). TZB-30878 suppressed the contractions caused by the 5-HT3 receptor agonist, 2-methyl-5-HT, in a concentration-dependent manner and nearly fully suppressed the contractions at 1 \( \mu \)M. This indicated that TZB-30878 possesses functional activity as a 5-HT3 receptor antagonist. In addition, the 50% suppression concentration (IC\(_{50}\)) of TZB-30878 was 0.014 \( \mu \)M. Alosetron showed similar contraction-suppressive effects with an IC\(_{50}\) of 0.017 \( \mu \)M.

In Vivo Properties

5-HT1A Receptor-Mediated Behavior and Hypothermia in Rats. TZB-30878 and tandospirone induced increases in flat body posture and lower lip retraction scores in rats (Figs. 5, A and B). TZB-30878 produced a dose-related effect on flat body posture and lower lip retraction scores. TZB-30878 and tandospirone induced statistically significant behavioral changes at 10 mg/kg or at 3 mg/kg or higher, respectively. In addition, TZB-30878 and tandospirone also decreased rectal temperature (Fig. 5C), which attained statistical significance at 3 mg/kg or higher. It was clarified that TZB-30878 stimulates the 5-HT1A receptor in vivo.

<table>
<thead>
<tr>
<th>Drug</th>
<th>( \text{E}_{\text{max}} ) %</th>
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<tr>
<td>R-(+)-8-OH-DPAT</td>
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<tr>
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</tr>
<tr>
<td>Tandospirone</td>
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<td>TZB-30878</td>
<td>99.2</td>
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<tr>
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TABLE 2
Ligand properties of [35S]GTP\(_{\gamma}S\) binding in human 5-HT1A receptors
Results are expressed as a percentage of the maximal response of R-(+)-8-OH-DPAT.

Fig. 3. The effects of several 5-HT1A ligands on [35S]GTP\(_{\gamma}S\) binding to human 5-HT1A receptors expressed in CHO cells. Specific [35S]GTP\(_{\gamma}S\) binding was measured in the presence of various concentrations of the test drug. Non-specific binding was detected in the presence of 10 \( \mu \)M GTP-S. Results are expressed as a percentage of the maximal response of R-(+)-8-OH-DPAT.
5-HT-Induced Bradycardia (BJ Reflex) in Rats. Intraduodenal administration of TZB-30878 (0.01–3 mg/kg) or alosetron (0.001–0.3 mg/kg) inhibited bradycardia induced by 5-HT (Fig. 6) in a dose-dependent manner. The 50% inhibitory doses were 0.152 and 0.00578 mg/kg, respectively. The BJ reflex is known to be mediated by 5-HT3 receptors. The maximum inhibitory action of both drugs reached the vehicle level, which clearly indicated that TZB-30878 possesses 5-HT3 receptor antagonistic action in vivo.

Animal Model

Wrap Restraint Stress-Induced Defecation in Rats. Wrap restraint stress caused a significant increase in defecation (Fig. 7). Stress-induced defecation was reduced by prior administration of TZB-30878 in a dose-dependent manner. The dose of 1 mg/kg exhibited no effects whereas both the 3 and 10 mg/kg doses significantly reduced stress-induced defecation. In addition, TZB-30878 strongly suppressed stress-induced defecation and completely normalized the number of defecations. These results indicated the effectiveness of oral administration of TZB-30878 for stress-induced defecation. Prior administration of alosetron (3 and 10 mg/kg) also significantly reduced stress-induced defecation. However, alosetron did not normalize the number of defecations, and the maximum effect plateaued. On the other hand, tandospirone did not suppressed stress-induced defecation at doses up to 100 mg/kg. The data on individual defecations showed a mixture of both suppressive and increasing trends.

To clarify the contribution of 5-HT1A receptors to the reduction of stress-induced defecation by TZB-30878, the effect of WAY-100635, a 5-HT1A receptor antagonist, was investigated (Fig. 8). After i.p. administration, TZB-30878 suppressed stress-induced defecation in a dose-dependent manner, which became statistically significant at 1 and 3 mg/kg. When WAY-100635 was administered before administration of TZB-30878, the suppressive effect of TZB-30878 was significantly reduced. However, the antagonistic effect of WAY-100635 did not completely eliminate the suppressive effect of TZB-30878 on stress-induced defecation.

Discussion

IBS is associated with psychological stress and alterations of gut motor function. We synthesized a novel compound, TZB-30878, which has dual pharmacological activities: it is expected to have an antistress effect via 5-HT1A agonism and an antihyperdefecation effect via 5-HT3 antagonism. The purpose of this study was to determine the effects of TZB-30878 in an IBS-like animal model. The present assays provide initial information that shows the importance and the usefulness of having both actions for the treatment of IBS.

The binding assays demonstrated that [3H]TZB-30878 bound to human 5-HT1A and 5-HT3 receptors with a high affinity in a reversible and saturable manner. In addition, a competitive analysis revealed that TZB-30878 selectively inhibited binding of well known ligands to human 5-HT1A and 5-HT3 receptors, as well as to specific ligands (tandospirone and alosetron). The binding inhibition constant (Kd value) of
TZB-30878 was 10 times greater than that of tandospirone and 1/10 that of alosetron. Furthermore, the results of the competitive analysis of the rat hippocampus (5-HT₁A) and entorhinal cortex (5-HT₃) showed similar affinities of TZB-30878 to human and rat receptors (data not shown). In addition, as TZB-30878 did not show affinity to any other typical receptors, its selectivity to 5-HT₁A and 5-HT₃ receptors was confirmed.

The relative efficacy of 5-HT₁A receptor agonists can be assessed using CHO cell membrane preparations of the human 5-HT₁A receptor with G protein subunits under controlled conditions. Under the conditions of our assay system we were able to detect partial agonists with an efficacy as low as approximately 80% of that of a full 5-HT₁A receptor agonist, R-(+)-8-OH-DPAT (as in the case of tandospirone), and did not detect the effects of a 5-HT₁A receptor antagonist, WAY-100635. As shown in the present study, TZB-30878 increased [³⁵S]GTP·S binding to 99.2% of the magnitude of R-(+)-8-OH-DPAT, indicating that TZB-30878 is a full agonist to 5-HT₁A receptors. It was suggested that TZB-30878 exhibited functional activity toward the G protein-coupled 5-HT₁A receptor. In another in vitro experiment, TZB-30878 inhibited 2-methyl-5-HT-evoked, 5-HT₃-mediated contractions in a dose-dependent manner but did not inhibit acetylcholine-evoked contractions (unpublished observations). These data suggest that TZB-30878 possesses dual functional activity mediated by 5-HT₁A and 5-HT₃ receptors.

Systemic administration of 5-HT₁A receptor agonists produces a behavioral syndrome including flat body posture and lower lip retraction (Lucki, 1992). This "5-HT syndrome" seems to be mediated through stimulation of the 5-HT₁A receptors in the central nervous system (CNS). In addition, 5-HT₁A receptor stimulation induces a hypothermic response (O'Connell et al., 1992). Therefore, an in vivo pharmacological study was conducted to explore the effects of TZB-30878 on both the 5-HT syndrome and hypothermia. The adminis-
tration of TZB-30878 induced a 5-HT syndrome and hypothermia, as with tandospirone. These results suggest that TZB-30878 stimulates 5-HT$_{1A}$ receptors in vivo, especially in the CNS.

The transient bradycardia induced by i.v. administration of 5-HT (von Bezold-Jarisch reflex) is the consequence of a reflex response of the vagus nerve after activation of sensory afferent fibers located mainly in the right ventricle (Paintal, 1973). The bradycardia induced by 5-HT was successfully inhibited by 5-HT$_3$ receptor antagonists (Fozard and Host, 1982). As a result, this inhibition is regarded as a useful index to assess in vivo 5-HT$_3$ receptor blocking activity. In the present experiments, TZB-30878 and alosetron inhibited the BJ reflex in a dose-dependent manner, which suggests that TZB-30878 is a 5-HT$_3$ antagonist that acts in vivo.

Williams et al. (1988) reported that wrap restraint stress caused intestinal signs in rats similar to IBS in humans without the formation of ulcers. The efficacy of anxiolytic agents, such as diazepam, and antimotility agents, such as trimethoprim, for IBS has been evaluated in this model (Yamamoto et al., 1998). Thus, the wrap restraint-induced stress model is useful for assessing the effects of therapeutic agents for IBS. It has been reported that immobilization stress results in an increase in plasma 5-HT concentrations in rats, which indicates the possibility that 5-HT acts as a mediator of gut dysfunction caused by stress (Sharma and Dey, 1981). Most of the endogenous 5-HT is considered to be contained within the gastrointestinal tract, particularly within the enterochromaffin cells of the gut mucosa, and 5-HT-containing neurons have been identified in the enteric nervous system of several species (Costa et al., 1982). Endogenous 5-HT may mediate restraint stress-induced acceleration in bowel function through the 5-HT$_3$ receptor (Miyata et al., 1992). In the present study, TZB-30878 reduced restraint stress-induced defecation as did alosetron. It is thought that TZB-30878 reduced the defecation at least partially through 5-HT$_3$ antagonism at the doses used in this assay. It is interesting that, although alosetron only partially inhibited restraint stress-induced defecation, as did alosetron, it is thought that TZB-30878 reduced the defecation at least partially through 5-HT$_3$ antagonism at the doses used in this assay. It is interesting that, although alosetron only partially inhibited restraint stress-induced defecation, TZB-30878 completely normalized this sign. Moreover, the effect of TZB-30878 was partly inhibited by a 5-HT$_{1A}$ antagonist, WAY-100635. These results indicate that stimulation of the 5-HT$_{1A}$ receptors by TZB-30878 reduces restraint stress-induced defecation. Taken together, these findings indicate that the suppression of restraint stress-induced defecation by TZB-30878 was the result of the contribution of both 5-HT$_{1A}$ agonism and 5-HT$_3$ antagonism.

Miyata et al. (1992) reported that diazepam reduced restraint stress-induced defecation and suggested a potential anxiolytic action in this model. 5-HT$_{1A}$ receptor agonists have been reported to show an anxiolytic effect similar to that of benzodiazepines (Traber and Glaser, 1987; Gordon and Hen, 2004). Because TZB-30878 produced the 5-HT syndrome and permeated the blood-brain barrier, an anxiolytic effect via the CNS may contribute in part to the action of TZB-30878 in this IBS-like model. Furthermore, it has been reported that 5-HT$_3$ receptor antagonists also have an anxiolytic effect in animal models (Filip, 1992; Roychoudhury and Kulkarni, 1997). If TZB-30878 possesses an anxiolytic effect, we should consider it in relation to the effects of 5-HT$_3$ receptors on the CNS.

On the other hand, because 5-HT$_{1A}$ receptors are localized in the enteric nerves (Galligan et al., 1988; Galligan, 1992), the 5-HT$_{1A}$ receptors in the myenteric plexus are likely to contribute to the reduction of the restraint stress-induced defecation. It has been reported that 5-HT$_{1A}$ receptor stimulation of the enteric neurons inhibits excitatory postsynaptic potentials and acetylcholine release in the myenteric plexus and smooth muscle contraction (Dietrich and Kilbinger, 1996). If 5-HT$_{1A}$ receptors negatively modulate 5-HT neuronal activity in the myenteric plexus, the effects of endogenous 5-HT through 5-HT$_3$ receptor stimulation may be reduced by TZB-30878. Further studies are needed to assess effects on the peripheral 5-HT$_{1A}$ receptors.

In contrast with TZB-30878, oral administration of tandospirone did not decrease restraint stress-induced defecation, although it induced the 5-HT syndrome via intraperitoneal administration. We have already confirmed that intraperitoneal administration of tandospirone partially and significantly reduced restraint stress-induced defecation by approximately 40% (unpublished observations). The inconsistency with the results for tandospirone may be due to its major metabolite, 1-(2-pyrimidinyl) piperazine (1-PP). After oral administration tandospirone is rapidly metabolized to 1-PP, and 1-PP has low affinity to the 5-HT$_{1A}$ receptors but has high affinity to the $\alpha_2$-adrenoeceptors (Miller et al., 1992). Because TZB-30878 does not possess the 1-PP structure, the effects of TZB-30878 are unrelated to 1-PP. It has been reported that 1-PP and other $\alpha_2$-adrenoeceptor antagonists increased fecal excretion in rats (Croci and Bianchetti, 1992). Such effects of 1-PP in increasing fecal excretion may counteract the effects of 5-HT$_{1A}$ receptor agonists in reducing restraint-stress induced defecation. Therefore, we have to consider the oral bioavailability when evaluating the action of tandospirone.

The most important finding in this study was that the inhibitory effects of TZB-30878 on restraint stress-induced defecation may be mediated not only by 5-HT$_3$ receptor antagonism but also by 5-HT$_{1A}$ receptor agonism. For 5-HT$_3$ receptor antagonism, the activity of TZB-30878 was weak compared with that of alosetron both in vitro and in vivo, despite the fact that TZB-30878 had more powerful efficacy than alosetron in the IBS-like model. From another point of view, the 5-HT$_3$ receptor works concomitantly with 5-HT$_{1A}$ receptor agonism in this model. A new concept suggested by our results is that the dual actions, 5-HT$_{1A}$ agonism and 5-HT$_3$ antagonism, can be expected to have a benefit for the treatment of IBS, and TZB-30878 is the first compound to fit this concept. Currently, other 5-HT$_3$ receptor antagonists (i.e., cilansetron and ramelsetron) for the treatment of d-IBS are under development. TZB-30878 could achieve an efficacy level higher than that for the existing 5-HT$_3$ receptor antagonists and is expected to be a next-generation therapeutic agent for d-IBS.


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