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 effects. We hypothesized that TZB-30878 might have benefits from these dual effects as a medication for diarrhoea-predominant irritable bowel syndrome (d-IBS), and these studies were designed to confirm the pharmacological properties of TZB-30878 and its efficacy in an IBS-like animal model. The binding assays demonstrated that [3H]TZB-30878 selectively binds to human 5-HT1A and 5-HT3 receptors, with Ki values 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. Finally, 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 tandosiprone (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 [4-methyl-3H]-N-(2-(4-methoxyphenyl)-1-piperazinyl)(ethyl)-N-(2-pyridyl)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, anxieties and antimotility 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-HT₃ 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-HT₁₅ 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-HT₃ receptors.

1) Studies have been focused on the role of the serotonin system in gastrointestinal transit. In the present study, we evaluated the following pharmacological profiles of TZB-30878: 1) The binding affinities for human recombinant 5-HT₁₅A and 5-HT₃ receptors were determined using [³H]TZB-30878; 2) the functional activities in vitro were determined in the GTPγS binding assay for 5-HT₁₅A receptors and in the contraction-response assay of the isolated guinea pig ileum for 5-HT₃ receptors; 3) 5-HT₁₅A agonistic action in vivo was evaluated based on the 5-HT behavioral syndrome in rats; and 4) 5-HT₃ antagonist action in vivo was evaluated based on 5-HT-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[2,3-³H]propylamino)-8-hydroxy-1,2,3,4-tetrahydro-4-phthalalene (8-OH-[³H]DPAT), and GTPγS were obtained from Amersham Biosciences (Buckinghamshire, England). [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-10655 maleate salt were obtained from Sigma-Aldrich (St. Louis, MO). Ethyl carbamate (urethane) was obtained from Tokyo Chemical Industry (Tokyo, Japan).

Radioligand Binding Assays. Human 5-HT₁₅A 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, 10 mM MgSO₄, 0.5 mM EDTA, and 0.1% (w/v) ascorbic acid, pH 7.4) to prepare a human 5-HT₁₅A receptor membrane preparation.

In the present study, we evaluated the following pharmacological profiles of TZB-30878: 1) The binding affinities for human recombinant 5-HT₁₅A and 5-HT₃ receptors were determined using [³H]TZB-30878; 2) the functional activities in vitro were determined in the GTPγS binding assay for 5-HT₁₅A receptors and in the contraction-response assay of the isolated guinea pig ileum for 5-HT₃ receptors; 3) 5-HT₁₅A agonistic action in vivo was evaluated based on the 5-HT behavioral syndrome in rats; and 4) 5-HT₃ antagonist action in vivo was evaluated based on 5-HT-induced bradycardia (von Bezold-Jarisch reflex) in anesthetized rats.
Beer, 1997) with partial modification. Human 5-HT1A receptors were carried out using the method described previously (Stanton and 1000 nM), 20 μl of buffer B or the test drugs (final concentrations: 0.03–20 nM TZB-30878 was tested in more than 20 other receptor and trans-

porter assays (Cerep, Celle l’Evescaut, France).

TZB-30878. The figure shows one of three analyses.

Fig. 1. Saturation binding curve of [3H]TZB-30878 to human 5-HT1A receptors expressed in CHO cells (A) and 5-HT3 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-HT3). Nonspecific binding was detected in the presence of 10 μM 


gumasetron. In addition, 5-HT3 (100 pM) was added to all

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 5-HT1A or 20 nM 5-HT3. Nonspecific binding was detected in the presence of 10 μM 

TZB-30878. The figure shows one of three analyses.

Nonspecific binding was determined in the presence of 10 μM GTPγS. In addition, TZB-30878 was tested in more than 20 other receptor and trans-

porter assays (Cerep, Celle l’Evescaut, France).

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.

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 my-

enteric 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 [137 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 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 thermostrobe (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
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 isolated by cutting the duodenum into the right common carotid artery and vein to measure the blood pressure. Blood pressure was recorded immediately before and to administer 5-HT, respectively. The duodenum was isolated by cutting the duodenum into the right common carotid artery and vein to measure the blood pressure. Blood pressure was recorded immediately before and after the injection of the test drugs. All experiments were performed in accordance with the Animal Experiment Law of Japan and the Guide for the Care and Use of Laboratory Animals (Cerep, except for 5-HT1A and 5-HT3 receptors (in ASKA; pharmaceutical Co., Ltd.).

### Table 1

<table>
<thead>
<tr>
<th>Receptor or Transporter</th>
<th>Subtype</th>
<th>Membrane Source</th>
<th>Ligand</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>A1, A3, A6</td>
<td>Human recombinant</td>
<td>°H^3HDFCPX</td>
<td>&gt;1 × 10^-6</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>α1, α2, α3, α4</td>
<td>Rat cerebral cortex</td>
<td>°H^3HPrazosin</td>
<td>≈1 × 10^-6</td>
</tr>
<tr>
<td>Dopamine</td>
<td>D1, D2, D3, D4</td>
<td>Human recombinant</td>
<td>°H^3HRX21002</td>
<td>≈1 × 10^-6</td>
</tr>
<tr>
<td>GABA</td>
<td>Nonselective</td>
<td>Rat cerebral cortex</td>
<td>°H^3HGABA</td>
<td>&gt;1 × 10^-6</td>
</tr>
<tr>
<td>Histamine</td>
<td>H1, H2, H3</td>
<td>Human recombinant</td>
<td>°H^3HPyrilamine</td>
<td>≈1 × 10^-6</td>
</tr>
<tr>
<td>Muscarinic</td>
<td>M1, M2, M3, M4</td>
<td>Human recombinant</td>
<td>°H^3HFenfluramine</td>
<td>≈1 × 10^-6</td>
</tr>
<tr>
<td>Muscarinic</td>
<td>M1, M2, M3, M4</td>
<td>Human recombinant</td>
<td>°H^3HFenfluramine</td>
<td>≈1 × 10^-6</td>
</tr>
<tr>
<td>Neurokinine</td>
<td>NK2, NK3</td>
<td>Human recombinant</td>
<td>°H^3HSPS-6103</td>
<td>&gt;1 × 10^-6</td>
</tr>
<tr>
<td>Neuroepetide</td>
<td>Y1, Y2, Y3</td>
<td>KAN-TS cells</td>
<td>°H^3HSPS-6103</td>
<td>&gt;1 × 10^-6</td>
</tr>
<tr>
<td>Opioid</td>
<td>δ2, κ, ν</td>
<td>Guinea-pig cerebral cortex</td>
<td>°H^3HDADLE</td>
<td>&gt;1 × 10^-6</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT1A, 5-HT2B</td>
<td>Rat cerebral cortex</td>
<td>°H^3HDADLE</td>
<td>&gt;1 × 10^-6</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT2B</td>
<td>Rat cerebral cortex</td>
<td>°H^3HDADLE</td>
<td>&gt;1 × 10^-6</td>
</tr>
<tr>
<td>Noradrenaline transporter</td>
<td>Dopamine transporter</td>
<td>Human recombinant</td>
<td>°H^3Hnoradrenaline</td>
<td>&gt;1 × 10^-6</td>
</tr>
<tr>
<td>Dopamine transporter</td>
<td>D1, D2, D3, D4</td>
<td>Human recombinant</td>
<td>°H^3HSPS-6103</td>
<td>&gt;1 × 10^-6</td>
</tr>
</tbody>
</table>

**Data Analysis and Statistical Evaluation.** GraphPad Prism (version 4.00; GraphPad Software Inc., San Diego, CA) was used for data analyses in all assays. Specific binding of the radiolabeled ligand was calculated and a saturation binding curve was generated by plotting $P = X$, where $X$ was the bound radiolabeled ligand and $B = Y$, bound radiolabeled ligand on the horizontal and vertical axes, respectively. To calculate $K_d$ values using nonlinear regression. The equation (one-site binding) was as follows: $Y = B_{max} \times X / (K_d + X)$. 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 ($K_d$) was calculated by multiplying the following equation: $K_d = IC_{50}/(1 + L/K_d)$. The ligand concentration was expressed as a percent of the standard concentration of radiolabeled ligand added.

Specific binding of °H3[^3H]GTPyS 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 of the maximal response of °H3[^3H]GTPyS.

### Statistical Analysis

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 using the Wilcoxon test.
Results

In Vitro Properties

Radioligand Binding Assays. The binding assays of [3H]8-OH-DPAT 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 Kd values of TZB-30878 toward 5-HT1A receptors and 5-HT3 receptors were 6.77 ± 10\(^{-10}\) and 8.90 ± 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 Ki 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 Ki for TZB-30878 was approximately 10 times greater than that for tandospirone and approximately 3/10 of that for alosetron.

The receptor selectivity of TZB-30878 for the 5-HT1A and 5-HT3 receptors was 300 times or more for that for the other receptors or transporters (adenosines A1, A2A, and A3; adrenalinics alpha1, alpha2, and beta1; dopaminics D1 and D2; GABA: histamines H1 and H2; muscarinics M3, M4, and M5; neurokinins NK2 and NK3; neuropeptides Y and Y2; opioids delta, k, and mu; serotonins 5-HT1A, 5-HT1B, 5-HT3, 5-HT5A, 5-HT5B, and 5-HT2; 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\textsubscript{max} values. S-(−)-8-OH-DPAT and tandospirone also increased binding of [35S]GTP\(\gamma\)S in a concentration-dependent manner. E\textsubscript{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\textsubscript{max} value was comparable with that of R-(+)-8-OH-DPAT.

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.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Drug & \(E_{\text{max}}\) \% \\
\hline
R-(+)-8-OH-DPAT & 100 \\
S-(−)-8-OH-DPAT & 81.3 \\
Tandospirone & 66.7 \\
TZB-30878 & 99.2 \\
WAY-100635 & 7.2 \\
\hline
\end{tabular}
\caption{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.}
\end{table}
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-HT$_3$ receptors. The maximum inhibitory action of both drugs reached the vehicle level, which clearly indicated that TZB-30878 possesses 5-HT$_3$ 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-HT$_{1A}$ receptors to the reduction of stress-induced defecation by TZB-30878, the effect of WAY-100635, a 5-HT$_{1A}$ 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-HT$_{1A}$ agonism and an antihyperdefecation effect via 5-HT$_3$ 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-HT$_{1A}$ and 5-HT$_3$ 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-HT$_{1A}$ and 5-HT$_3$ receptors, as well as to specific ligands (tandospirone and alosetron). The binding inhibition constant ($K_i$ 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]GTPγS 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-

Fig. 6. The effects of TZB-30878 and alosetron on 5-HT-induced bradycardia in rats. Anesthetized rats were injected i.v. with 5-HT (300 µg/kg) to evoke a transient bradycardia. After recovery, vehicle or test drugs were injected intraduodenally, and 5-HT was readministered 30 min later. n = 6/group. Values represent mean (±S.D.).

Fig. 7. The effects of TZB-30878, tandospirone, and alosetron on stress-induced defecation in rats. The upper bodies of lightly anesthetized rats were wrapped with adhesive tape, and the animals were returned to the observation cages. The number of feces dropped on the tray was counted 1 h after the wrapping. Vehicle or test drugs were administered p.o. 1 h before the restraint. n = 8/group. Values represent means ± S.D. $$$, P < 0.001 versus normal (N), Wilcoxon test. ***, P < 0.01 versus vehicle (V), nonparametric Dunnett multiple comparison test. Normal group represents animals without wrapping.

Fig. 8. The influence of 5-HT1A antagonist WAY-100635 on the inhibitory effect of TZB-30878 in stress-induced defecation in rats. Vehicle or TZB-30878 was injected i.p. 20 min after s.c. injection with saline (containing diluted hydrochloric acid) or WAY-100635. Five minutes later, lightly anesthetized rats were wrapped for 1 h, and the number of feces was counted. n = 8/group. Values represent means ± S.D. $$$, P < 0.001 versus normal (N), Wilcoxon test. ***, P < 0.01; **, P < 0.05; #, P < 0.001 versus vehicle (V), nonparametric Dunnett multiple comparison test. Normal group represents animals without wrapping.
tration of TZB-30878 induced a 5-HT syndrome and hypo-
thermia, 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$_1$ (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
trimetropin, 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). Endog-
enous 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, 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$_3$
agonism.

Miyata et al. (1992) reported that diazepam reduced re-
straint 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 syn-
drome 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 anxi-
olytic 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 stim-
ulation of the enteric neurons inhibits excitatory postsynap-
tic potentials and acetylcholine release in the myenteric
plexus and smooth muscle contraction (Dietrich and Kilbin-
ger, 1996). If 5-HT$_{1A}$ receptors negatively modulate 5-HT
neuronal activity in the myenteric plexus, the effects of en-
dogenous 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 tan-
dospirone did not decrease restraint stress-induced defecation,
although it induced the 5-HT syndrome via intraperito-
neal administration. We have already confirmed that
intraperitoneal administration of tandospirone partially and
significantly reduced restraint stress-induced defecation by
approximately 40% (unpublished observations). The consist-
istency 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 $\alpha_2$-adrenoceptors (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 re-
ported that 1-PP and other $\alpha_2$-adrenoceptor antagonists in-
creased fecal excretion in rats (Croci and Bianchetti, 1992).
Such effects of 1-PP in increasing fecal excretion may coun-
teract 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 an-
agonism 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 ramosetron) 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 antag-
onists and is expected to be a next-generation therapeutic
agent for d-IBS.

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