Synthesis and SAR of N-Chlorophenyl Substituted Piperazinylethyl-aminomethylpyrazoles as 5-HT3A Inhibitors

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The 5-HT3A receptors are one of ligand-gated ion channels and are known to be involved in visceral pain, anxiety, or anticancer agent-induced nausea and vomiting. In present study, we designed novel skeletons based on the developed 5-HT3A receptor antagonists and evaluated their effects on 5-HT3A receptor channel currents (I5-HT3A) of a series of pyrazole derivatives having N-chlorophenylpiperazine functionality (6-9). We found that most of N-p-chlorophenyl substituted piperazinyl-pyrazole derivatives (7b, 7c, 7e and 7h) exhibited the high potency for the inhibition of I5-HT3A, whereas the compound without chloride (6) or with m-chlorophenyl group (a serious of 8 and 9) showed the low potency. These results indicate that p-chlorophenyl group is might play an important role for increasing the inhibitory potency on I5-HT3A.

Key Words: 5-HT3 receptor, 5-HT3A receptor channel activity, Novel 5-HT3 receptor channel current blockers, Chlorophenyl substituted piperazinylethylaminomethylpyrazoles

Introduction

Serotonin (5-Hydroxytryptamine, 5-HT) is an important neurotransmitter playing the diverse functions in intracellular communications. 5-HT receptors are classified into over the 8 different receptor subtypes according to structural or pharmacological differences.1 While most of serotonin receptors are GTP-binding protein coupled receptors, the 5-HT1 subtypes are the only ionotropic receptors.2 Activation of this receptor mediates fast depolarizing responses and is permeable for the monovalent and divalent cations. The 5-HT1 receptors consist of homomorphic pentamers.

5-HT3 receptors are mainly expressed in brain stem and spinal cord in central nervous system (CNS) and in intestine in peripheral nervous system (PNS).3 In CNS, 5-HT3A receptors are known for nausea and vomiting, whereas are known for visceral pain such as irritable bowel syndrome in PNS.3,5 In the early 1990s the introduction of 5-HT3 receptor antagonists into markets was perceived great success to attenuate side effects related with chemotherapy-induced emesis. It is well known that nausea and vomiting induced by chemotherapy of cancer patients result from activation of 5-HT3A receptors in the brain stem. Therefore, several 5-HT3 antagonists are currently available and show efficac in the control of the emesis induced by anticancer chemotherapy. For example, tropisetron, ondansetron, granisetron, dolasetron, and palonsetron are in markets and bemesetron (MDL-72222), and renzapride are underdeveloped state in phase II or Phase III. The palonosetron and indisetron were launched in 2003 and 2004, respectively.12,13,14 (Fig. 1).

Recent studies show that 5-HT3 receptor antagonist were also focused on other CNS related beneficial effects such as pain, anxiety, cognitive function, anti-inflammatory and immune modulatory properties.12

In present study, we designed novel skeletons based on known 5-HT3A antagonists for the development of novel 5-HT3A antagonists. Thus, we developed novel target molecule (6-9), called N-chlorophenyl substituted piperazinylethylaminomethylpyrazole, which is contained piperazine ring, chlorphenyl and pyrazole group (Fig. 2). Each group was reconstructed from chlorphenyl group of MDL-72222, piperazine moiety of lerisetron and MCI-225, and pyrazol moiety of indisetron (Fig. 1). Here, we first report novel syntheses of N-chlororoaryl substituted piperazinylethylaminomethyl pyrazole derivatives 6-9 (Fig. 2) and in vitro evaluations against 5-HT3A receptor channel activities at single cell level. We also mention the structure-activity relationships of the synthesized compounds.

Figure 1. Examples of recently developed 5-HT3A antagonists.
Results and Discussion

Chemistry. The core structure of the target compound was piperazinylethylaminomethylpyrazole which connected to pyrazole and piperazine through alkylamine chain (Fig. 2). The chlorophenyl group was introduced to each ring as substituents. The synthesis of target compound was outlined in Scheme 1. The desired compounds 6-9 were synthesized by reductive amination of pyrazole aldehyde (4) with corresponding arylpiperazylethylamine (5) in moderate yields. The pyrazole aldehydes having various R1 and R2 substituents were prepared by previously developed process in our group.15 Aryl piperazinylethylamine (5) was obtained by Gabriel amine synthetic method from the commercially available arylpiperazine. To introduce chlorophenyl substituent in core target molecule, p-chlorophenylpiperazine, m-chlorophenylpiperazine and p-chlorophenyl(phenyl)methylpiperazine were selected and reacted with several pyrazole aldehydes. In detail synthetic methods were noted as follow. Claisen-condensation of commercially available various ketones (1) with diethylketone gave 2,4-dioxo-4-alkyl-butyric acid ethyl esters (2). These β-ketoester compounds undergo cycloaddition reaction with phenylhydrazine or t-butylhydrazine, yielding a pyrazole 3-carboxy acid ethyl ester (3), which was then oxidized by DIBAL-H from ester to aldehyde (4). Gabriel-

![Scheme 1](image)

Scheme 1. Regent and condition: (a) NaOEt/EtOH, diethyloxalate, (b) R1NH2H2HCl, EtOH, (c) DIBAL-H, CH2Cl2, (d) NaBH(OAc)3, CH2Cl2.

![Scheme 2](image)

Scheme 2. Regent and condition: (a) NaBH(OAc)3, CH2Cl2.
Among the currents (tested on biotinylations on receptors. All of synthesized target compounds exhibited inhibitory effects on the target compounds. The data indicated that several compounds showed high inhibitory activities about 2 µM of IC\textsubscript{50} values of the compounds. As shown similar inhibitory activities on the receptors, compound 7\texttext{f, 8b (R\texttext{1} = R\texttext{2} = Ph)}, the compound 7\text{d} (2.4 µM), 7\text{f} (3.1 µM) having p-chlorophenyl as R\texttext{1} group showed lower IC\textsubscript{50} values than the compound 8a (31.0 µM), 8b (20.3 µM) and 9a (39.9 µM) having m-chlorophenyl or p-chlorophenyl(phe- nyl)methyl group. And also as compared with the compound in the pyrazole ring were showed moderate inhibitory potency. The synthetic pathway of selected compounds was presented in Scheme 1 and 2. The novel chlorophenyl substituted target compounds were synthesized and all compounds were fully characterized by spectroscopic analysis, and some of selected compounds were summarized in Table 1.

**Biological activity.** Next, all piperazinylethylpyrazole derivatives (6-9) were estimated for their in vitro effects on 5-HT\texttext{1A} receptor channel activities by examining of the inhibitory effects of the target compounds 6-9 on the 5-HT-mediated inward peak currents (I\texttext{SS}) in oocytes expressing wild-type mouse 5-HT\texttext{1A} receptors. All of synthesized target compounds exhibited inhibitions on I\texttext{SS}. The inhibitory potencies of each compound tested on I\texttext{SS} were estimated with IC\textsubscript{50} values (Table 1).

The data indicated that several compounds showed high inhibitory effects on I\texttext{SS} along with low IC\textsubscript{50} values. According to the structure activity relationships (SAR) studies of aromatic substituents (Ar) on piperazine, p-chlorophenyl group (a series of 7) tends to exhibit better activities than those of other functional groups such as phenyl group (6), m-chlorophenyl group (8), and p-chlorophenyl(phenyl)methyl substituted ones (9). Among the p-chlorophenylpiperazine substituted compounds, the compound 7b and 7h having one n-propyl group in pyrazole ring, were the most potent inhibitor that exhibited IC\textsubscript{50} values of 1.3 µM, which were comparable to the well-known 5-HT\texttext{1A} receptor antagonist MDL-72222 (Table 1). Compared with a series of the compound having same R\texttext{1} and R\texttext{2} substituents such as compound 7d, 8a, 9a (R\texttext{1} = t-butyl, R\texttext{2} = p-tolyl) between compound 7f, 8b (R\texttext{1} = R\texttext{2} = Ph), the compound 7d (2.4 µM), 7f (3.1 µM) having p-chlorophenyl as R\texttext{1} group showed lower IC\textsubscript{50} values than the compound 8a (31.0 µM), 8b (20.3 µM) and 9a (39.9 µM) having m-chlorophenyl or p-chlorophenyl(phenyl)methyl group. And also as compared with the compound having different substituents, compound 7b and 7h possessing n-propyl group in R\texttext{2} which showed lowest IC\textsubscript{50} value of 1.3 µM, these data revealed that linear alkyl-chained functionality on R\texttext{2} leads to show high inhibitory activity on I\texttext{SS}. As shown similar activities about 2 µM of IC\textsubscript{50} values of the compounds 7d (R\texttext{1} = i-butyl, R\texttext{2} = p-tolyl) and 7e (R\texttext{1} = Ph, R\texttext{2} = p-tolyl) having different R\texttext{1} substituents, R\texttext{2} substituent little affected the activity.

Compounds of structurally bulky R\texttext{2} substituent such as 4-cyclohexylphenyl (7k, IC\textsubscript{50} = 17.7 ± 2.2 µM), 4-piperidinylphenyl (7l, 6.4 ± 0.3 µM), and p-chlorophenyl (7a, 28.3 ± 5.0 µM) in the pyrazole ring were showed moderate inhibitory potency.

### Table 1. In vitro 5-HT\texttext{1A} receptor blocking effects of 5-(N-aryl)piperazinylethyl)aminomethylypyrazoles

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substituent</th>
<th>Ar</th>
<th>R\texttext{1}</th>
<th>R\texttext{2}</th>
<th>V\texttext{max}</th>
<th>IC\textsubscript{50} (µM)</th>
<th>nH</th>
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<tr>
<td>6</td>
<td>Ph</td>
<td>t-butyl</td>
<td>p-chloro-Ph</td>
<td>88.7 ± 5.8</td>
<td>41.9 ± 0.4</td>
<td>2.6 ± 0.8</td>
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<tr>
<td>7a</td>
<td>p-chloro-Ph</td>
<td>t-butyl</td>
<td>p-chloro-Ph</td>
<td>100.5 ± 4.3</td>
<td>28.3 ± 5.0</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>7b</td>
<td>p-chloro-Ph</td>
<td>t-butyl</td>
<td>n-propyl</td>
<td>97.3 ± 5.6</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>7c</td>
<td>p-chloro-Ph</td>
<td>t-butyl</td>
<td>i-butyl</td>
<td>102.8 ± 2.1</td>
<td>2.1 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td>p-chloro-Ph</td>
<td>t-butyl</td>
<td>p-tolyl</td>
<td>102.6 ± 6.5</td>
<td>2.4 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>7e</td>
<td>p-chloro-Ph</td>
<td>Ph</td>
<td>p-tolyl</td>
<td>95.4 ± 3.5</td>
<td>2.2 ± 0.2</td>
<td>1.8 ± 0.3</td>
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<tr>
<td>7f</td>
<td>p-chloro-Ph</td>
<td>Ph</td>
<td>Ph</td>
<td>101.3 ± 2.6</td>
<td>3.1 ± 0.3</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>7g</td>
<td>p-chloro-Ph</td>
<td>Ph</td>
<td>methyl</td>
<td>98.5 ± 1.2</td>
<td>2.5 ± 0.3</td>
<td>1.4 ± 0.1</td>
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<tr>
<td>7h</td>
<td>p-chloro-Ph</td>
<td>Ph</td>
<td>n-propyl</td>
<td>94.8 ± 4.8</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.3</td>
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<tr>
<td>7i</td>
<td>p-chloro-Ph</td>
<td>Ph</td>
<td>i-butyl</td>
<td>104.3 ± 3.5</td>
<td>3.3 ± 0.9</td>
<td>1.4 ± 0.4</td>
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<tr>
<td>7j</td>
<td>p-chloro-Ph</td>
<td>Ph</td>
<td>2-furyl</td>
<td>97.3 ± 2.0</td>
<td>4.3 ± 0.3</td>
<td>2.0 ± 0.2</td>
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<tr>
<td>7k</td>
<td>p-chloro-Ph</td>
<td>Ph</td>
<td>(4-cyclohexyl)Ph</td>
<td>95.0 ± 5.6</td>
<td>17.7 ± 2.2</td>
<td>1.9 ± 0.3</td>
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<tr>
<td>7l</td>
<td>p-chloro-Ph</td>
<td>Ph</td>
<td>(4-piperidinyl)Ph</td>
<td>95.9 ± 1.6</td>
<td>6.4 ± 0.3</td>
<td>1.9 ± 0.1</td>
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<tr>
<td>8a</td>
<td>m-chloro-Ph</td>
<td>t-butyl</td>
<td>p-tolyl</td>
<td>98.4 ± 5.5</td>
<td>31.0 ± 5.0</td>
<td>1.1 ± 0.2</td>
<td></td>
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<tr>
<td>8b</td>
<td>m-chloro-Ph</td>
<td>Ph</td>
<td>Ph</td>
<td>96.9 ± 4.3</td>
<td>20.3 ± 1.9</td>
<td>1.5 ± 0.2</td>
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<tr>
<td>8c</td>
<td>m-chloro-Ph</td>
<td>Ph</td>
<td>2-furyl</td>
<td>95.9 ± 2.8</td>
<td>9.4 ± 0.7</td>
<td>1.5 ± 0.2</td>
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<tr>
<td>8d</td>
<td>m-chloro-Ph</td>
<td>Ph</td>
<td>Ph</td>
<td>87.6 ± 3.6</td>
<td>21.8 ± 1.8</td>
<td>1.6 ± 0.2</td>
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<tr>
<td>9a</td>
<td>(p-CIPh)CHPh</td>
<td>t-butyl</td>
<td>p-tolyl</td>
<td>74.2 ± 1.5</td>
<td>39.9 ± 0.5</td>
<td>1.9 ± 0.4</td>
<td></td>
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<tr>
<td>9b</td>
<td>(p-CIPh)CHPh</td>
<td>Ph</td>
<td>p-tolyl</td>
<td>73.1 ± 3.1</td>
<td>8.2 ± 0.8</td>
<td>2.2 ± 0.4</td>
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<tr>
<td></td>
<td>MDL-72222</td>
<td></td>
<td></td>
<td>99.6 ± 17.7</td>
<td>0.77 ± 0.16</td>
<td>1.3 ± 0.27</td>
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</table>
than small substituents such as methyl, propyl and phenyl group. And also, m-CIPh- or (p-CIPh)CIPh-groups in piperazine exhibited low activity in the inhibition of 5-HT3. Therefore, these results indicate that the presence of chloro group on p-position of phenyl play important roles in exerting the 5-HT3 receptor current inhibitions. The Hill coefficients indicate the number of binding molecule with 5-HT3A receptor. As shown in Table 1, the modification of Ar, R1 and R2 substituent led to no significant change in Hill coefficient. The Hill coefficients of the compound 7f and 8a showed 1.1 nH and the compound 7j, 7k and 7l showed 2.0 nH, 1.9 nH and 1.9 nH, respectively. It seems that one molecule of compound 7f and 8a might bind to 5-HT3A receptors, whereas two molecules of the compounds 7k and 7l having comparatively large functionality such as cyclohexylphenyl or piperidinlyphenyl group, might bind to 5-HT3A receptor to exhibit their effects.

In summary, a new series of N-chlorophenyl substituted piperazinylethlamino pyrazoles were designed, synthesized and examined for the 5-HT3A, inhibitions potency and SAR. Among all compounds, 7b and 7h show the most potent for 5-HT3 receptor inhibition activity. The results of structure activity relationships (SAR) indicate that the chlorophenyl group is key role in biological activity against 5-HT3 receptor. The position of substituted in phenyl ring is particularly important, due to all p-chloro substituted compounds showed lower IC50 values. These findings suggest that novel chlorophenyl substituted pipersazinylethlamino pyrazole derivatives could be a lead compound of 5-HT3A inhibitor.

Experimental Section

Preparation of 3-formyl-1-(t-butyl)-5-(p-chlorophenyl)pyrazole (4a) General procedure. To a solution of ethoxyacetyl piperazine (4.34 mmol) in toluene (15 mL) was added slowly DIBAL-H (8.68 mL, 8.62 mmol) at -78 °C, and the solution was stirred for 30 min. The acetal methanol was added to the reaction mixture. The solution was filtered off by celite and extracted with ethyl acetate. The organic layer dried by MgSO4, filtered, and the solvent evaporated under vacuum condition and concentrated with under reduced pressure. The crude product was purified by flash column chromatography (hexane/ethyl acetate/methylene chloride = 3/1/1) to afford 4a (81%); 1H NMR (300 MHz, CDCl3) δ 7.35-7.47 (m, 4H), 7.18-7.33 (m, 9H), 6.15 (s, 1H), 3.93 (s, 2H), 3.17 (m, 2H), 2.97 (t, J = 6.0 Hz, 2H), 2.62-2.84 (m, 6H).

1-t-Butyl-3-[2-[4-(4-chlorophenyl) piperazin-1-yl]ethyl] aminomethyl pyrazole 7a. (68%); 1H NMR (300 MHz, CDCl3) δ 7.35-7.42 (m, 4H), 7.18-7.33 (m, 9H), 6.15 (s, 1H), 3.44 (s, 2H), 3.18 (m, 2H), 2.74 (t, J = 5.4 Hz, 4H), 2.63 (t, J = 5.8 Hz, 2H), 2.55 (m, 6H), 1.58-1.43 (m, 11H), 0.89 (t, J = 7.6 Hz, 3H).

1-t-Butyl-5-[4-(4-chlorophenyl)piperazin-1-yl]ethyl] aminomethyl pyrazole 7c. (28%); Mp = 133 ~ 134 °C; 1H NMR (300 MHz, CDCl3) δ 7.25-7.37 (m, 4H), 6.09 (s, 1H), 3.51 (s, 2H), 3.18 (m, 4H), 2.74 (t, J = 5.6 Hz, 2H), 2.62 (t, J = 5.6 Hz, 2H), 2.45-2.59 (m, 6H), 1.80 (m, 1H), 1.44 (s, 9H), 0.90 (d, J = 7.2 Hz, 6H).

1-t-Butyl-3-[2-[4-(4-chlorophenyl) piperazin-1-yl]ethyl] aminomethylpyrazole 7d. (81%); 1H NMR (300 MHz, CDCl3) δ 7.20-7.31 (m, 5H), 7.07-7.19 (m, 4H), 5.74 (s, 1H), 3.78 (s, 2H), 3.06 (m, 2H), 2.77 (m, 4H), 2.50-2.68 (m, 6H), 2.38 (s, 3H).

3-[2-[4-(Chlorophenyl)piperazin-1-yl]ethyl] aminomethyl-1,5-diphenylpyrazole 7f. (75%); 1H NMR (300 MHz, CDCl3) δ 7.11-7.36 (m, 14H), 6.03 (s, 1H), 3.97 (s, 2H), 3.16 (m, 4H), 2.94 (t, J = 6.0 Hz, 2H), 2.68 (m, 4H), 2.50 (m, 2H).

3-[2-[4-(Chlorophenyl)piperazin-1-yl]ethyl] aminomethyl-1-methyl-1-phenylpyrazole 7g. (51%); 1H NMR (300 MHz, CDCl3) δ 7.33-7.39 (m, 6H), 7.21-7.32 (m, 3H), 6.21 (s, 1H), 3.89 (s, 2H), 2.89 (m, 2H), 2.23-2.75 (m, 12H), 1.45 (m, 2H), 0.90 (t, J = 7.0 Hz, 3H).

5-iso-Butyl-3-[2-[4-(4-chlorophenyl)piperazin-1-yl]ethyl] amino-methyl-1-phenylpyrazole 7i. (53%); 1H NMR (300 MHz, CDCl3) δ 7.36-7.50 (m, 6H), 7.15-7.34 (m, 3H), 6.19 (s, 1H), 3.89 (s, 2H), 3.15 (m, 2H), 2.68 (m, 2H), 2.31-2.61 (m, 10H), 1.78 (m, 1H), 0.86 (d, J = 6.9 Hz, 6H).

3-[2-[4-(Chlorophenyl)piperazin-1-yl]ethyl] aminomethyl-1-(2-furyl)-1-phenylpyrazole 7j. (63%); 1H NMR (300 MHz, CDCl3) δ 7.32-7.49 (m, 6H), 7.18-7.21 (m, 4H), 6.65 (s, 2H), 5.73-5.88 (m, 2H), 4.39 (t, J = 5.6 Hz, 4H), 2.74 (t, J = 5.4 Hz, 4H), 2.62 (t, J = 5.8 Hz, 2H), 2.55 (m, 6H), 1.58-1.43 (m, 11H), 0.89 (t, J = 7.6 Hz, 3H).
3-[2-[4-(Chlorobenzhydryl)piperazin-1-yl]ethyl] aminomethyl-5-(4-methylphenyl)-1-phenylpyrazole 8a. (96%): 1H NMR (300 MHz, CDCl3) δ 7.32-7.56 (m, 6H), 6.13 (7, J = 7.8 Hz, 1H), 6.71-6.90 (m, 3H), 6.67 (s, 1H), 6.33 (m, 1H), 5.97 (m, 1H), 3.99 (s, 2H), 3.13 (m, 4H), 2.90 (m, 2H), 2.48-2.71 (m, 6H); 13C NMR (75 MHz, CDCl3) δ 152.3, 151.2, 144.4, 142.6, 140.1, 135.4, 134.9, 130.0, 129.1, 128.6, 125.9, 119.2, 115.6, 113.8, 111.2, 108.9, 106.1, 155.2, 52.9, 48.6, 46.5, 45.3.

1-tet-Butyl-3-[2-[4-(Chlorobenzhydryl)piperazin-1-yl]ethyl] aminomethyl-5-(4-methylphenyl)-1-phenylpyrazole 9a. (41%): 1H NMR (300 MHz, CDCl3) δ 7.32-7.40 (m, 4H), 7.18-7.31 (m, 5H), 7.13 (d, J = 5.8 Hz, 2H), 7.08 (d, J = 6.3 Hz, 2H), 6.15 (s, 1H), 4.18 (s, 1H), 4.06 (s, 2H), 2.72 (t, J = 5.6 Hz, 4H), 2.49 (m, 4H), 2.32-2.45 (m, 1H), 1.38 (s, 9H). 13C NMR (75 MHz, CDCl3) δ 141.9, 141.1, 138.5, 132.6, 130.4, 130.1, 129.1, 128.7, 128.6, 128.5, 128.4, 127.7, 127.8, 108.8, 61.5, 52.8, 51.6, 45.0, 43.5, 31.1, 31.1, 29.7, 21.3; FT-IR (KBr, cm-1) 3356 (-NH), 2924, 2814, 1450, 1010, 912, 806; HRMS (FAB) m/z calcd for C30H25ClN5 (M+H)+ 556.3207, Found 556.3207.

Preparation of Xenopus oocytes and microinjection. Xenopus laevis frogs were purchased from Xenopus I (Ann Arbor, MI, USA). Their care and handling were in accordance with the highest standards of institutional guidelines. For isolation of oocytes, frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester. Oocytes were surgically removed and separated by collagenase treatment followed by agitation two hours in a Ca2+-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units/mL penicillin and 100 µg/mL streptomycin. Stage V-VI oocytes were collected and stored in ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 µg/mL gentamicin. This oocyte-containing solution was maintained at 18°C with continuous gentle shaking and changed daily. Electrophysiological experiments with oocytes were performed with thin 5 ~ 6 days of their isolation. Chemicals were bath-applied. One day after harvest, 40 nL of cRNAs were injected into the animal or vegetal pole of each oocyte using a 10 µL VWR micro-dispenser (VWR Scientific, San Francisco, CA, USA) fitted with a tapered glass pipette tip that was 15 - 20 µm in diameter.

**In vitro transcription of 5-HT3A receptor cDNAs.** For generation of wild-type cRNAs, recombinant plasmids containing the wild-type 5-HT3A receptor cDNA were linearized by digestion with appropriate restriction enzymes. All cRNAs were prepared using T3 RNA polymerase and the mMessage mMACHINE transcription kit (Ambion, Austin, TX, USA). The final cDNA products were resuspended at 1 µg/µL with RNase-free water and stored at -80°C until use. The absence of degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining.

**Data recording.** A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings. The chamber was constructed by milling two concentric wells into the chamber bottom (diameter/height: upper well, 8.3 mm; lower well, 6.5 mm) and gluing plastic mesh (~ 0.4 mm grid diameter) onto the bottom of the upper well. The perfusion inlet (~ 1 mm in diameter) was formed through the wall of the lower well, and a suction tube was placed on the edge of the upper well. The oocyte was placed on the net that separated the upper and lower wells, with the net grids serving to keep the oocyte in place during the electrophysiological recordings. Oocytes were impaled with two microelectrodes filled with 3 M KCl (0.2 ~ 0.7 MΩ). Recordings were performed in ND96 solution. The electrophysiological experiments were performed at room temperature using an Oocyte Clamp (OC-725C; Warner Instruments, Hamden, CT, USA) and stimulation and data acquisition were controlled by pClamp 8 (Axon Instruments, Union City, CA, USA).

**Data analysis.** To obtain concentration-response curves of the effect of Compounds on Is,IH, the peak amplitudes at different concentrations of compounds were plotted and then fitted to the following Hill equation using the Origin software (OriginLab Corp, Northampton, MA, USA): Response = Emax - E0 [(C - IC50)^n]/1 + (IC50)^n, where Emax and E0 are maximal and minimal responses, respectively. [C] is concentration of compounds and nH is the Hill coefficient. IC50 is the concentration of compounds required to decrease the response by 50%. All values are presented as means ± S.E.M. The differences between means of control and treatment data were determined using the unpaired Student’s t-test or one-way ANOVA. A value of p < 0.05 was considered statistically significant.

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**References.**