Discovery and SAR of N-(1-((substituted piperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide derivatives: 5-Hydroxytryptamine receptor 4 agonist as a potent prokinetic agent

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A B S T R A C T
A series of novel benzamide derivatives, altering the 4-fluorophenylalkyl moiety in cisapride, were synthesized as 5-HT4 receptor agonists, and SAR of these analogs was examined on in vitro and in vivo prokinetic activities. These compounds were synthesized for high 5-HT4 receptor binding affinities and low hERG affinities. Several types of analogs were obtained and screened for 5-HT4 binding, hERG blocking, agonism, and gastric emptying assessment. Among the analogues, compound 23g showed promising results compared with the other analogs with respect to gastric emptying rates in rats. Therefore, we suggest that it may be a clinical candidate for the development of a potent prokinetic agent to treat GI disorders.

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1. Introduction
Serotonin (5-hydroxytryptamine, 5-HT) functions as both a hormone and a neurotransmitter, interacting with different receptors [1–4]. There exist several members of the serotonin receptor family, which all play important roles in various disorders. In particular, the role and distribution of 5-HT4 receptors within the gastrointestinal (GI) tract have been well-established in different species including humans, guinea pigs [5], mice [6], and rats [7]. Due to its potential roles in central and peripheral disorders, 5-HT4 has been an attractive target for the treatment of GI disorders such as irritable bowel syndrome, chronic constipation, gastroparesis, dyspepsia, and gastroesophageal reflux disease [8].

Dyspepsia is defined as pain or discomfort in the upper abdomen [9]. In particular, functional dyspepsia is associated with abnormalities in gastric or duodenal physiology, including abnormal sensitivity to gastric acid, delayed gastric emptying, impaired gastric accommodation, and visceral hypersensitivity [9–11]. Prokinetic agents such as 5-HT4 agonists are potential therapeutic agents to treat GI tract disorders including functional dyspepsia (Fig. 1) [12,13]. Of the representative prokinetic agents, cisapride (Propulsid™) was launched in 1988 and has shown potent effects in patients with GI disorders [14–16]. However, cisapride was withdrawn from the US market in 2000 as a result of adverse cardiovascular effects associated with QT prolongation due to the fact that it is a potent blocker of the hERG (human ether-a-go-go related gene) channel [17,18].

cis-4-Amino-5-chloro-2-methoxy-N-(3-methoxypiperidin-4-yl) benzamide (cis-norcisapride) is a metabolite of cisapride [19], and a pharmacophore that is a potential 5-HT4 receptor agonist and a potential 5-HT3 receptor antagonist [20]. Moreover, cis-norcisapride does not bind the hERG channel (IC50 > 10 μM). The portion of cisapride that binds to the 5-HT4 receptor can be divided into parts A and B (Fig. 2) [21]. Part A contains cis-norcisapride and part B contains linkers with functional groups. We expected, as shown in Fig. 2(c), that modifying part B into adequate structures may result in an enhanced binding affinity to the 5-HT4 receptor and a reduced inhibition of the hERG potassium ion channel.
2. Results and discussion

2.1. Chemistry

Our approach was to examine various linkers and functional groups in cisapride part B that show effective binding affinity and agonism to the 5-HT₄ receptor, but are not hERG blockers. Herein we describe the synthetic strategy and structure–activity relationships (SAR) of diverse benzamide derivatives as 5-HT₄ receptor agonists.

Initially, we synthesized compound 8, well-known as cis-norcisapride by following the route as depicted in Scheme 1. Further, we modified part B of the pharmacophore by incorporating three different types of moieties such as alkyl heteroaryl, alkyl amide, and alkyl piperidinyl groups into compound 8 as shown in Scheme 2. Compounds 13a–e containing alkyl heteroaryl moieties were synthesized as shown in Scheme 2A. 1-Bromo-3-chloropropane underwent SN₂ reaction with heteroaryl compounds like pyrrole, imidazole, triazole, and tetrazole to provide compounds 10a–e, which were coupled with compound 8 to give the desired products 13a–e. Subsequently, we synthesized the alkyl amide derivatives 17a–d using the strategy shown in Scheme 2B. Acryloyl chloride in the presence of triethylamine reacted with cyclic amines to provide the corresponding amides 16a–d, which...

**Scheme 1.** Reagents and conditions: (a) PhI(OAc)₂, KOH, MeOH, 0 °C for 30 min, then rt. 3 h, 71%; (b) NaH, MeI, DMF, 0 °C for 30 min, then rt. 4 h, 75%; (c) 5% H₂SO₄, rt., 78%; (d) NaBH₃CN, NH₄OAc, MeOH, 80 °C, 3 h, 35%; (e) TEA, EDC, HOBt, DMF, rt., 5 h, 83%; (f) KOH, 2-propanol, 0 °C for 15 min, then reflux for 6 h, 72%. Compound 1 and 6 are commercially available.

**Scheme 2A.** Reagents and conditions: (a) PhI(OAc)₂, KOH, MeOH, 0 °C for 30 min, then rt. 3 h, 71%; (b) NaH, MeI, DMF, 0 °C for 30 min, then rt. 4 h, 75%; (c) 5% H₂SO₄, rt., 78%; (d) NaBH₃CN, NH₄OAc, MeOH, 80 °C, 3 h, 35%; (e) TEA, EDC, HOBt, DMF, rt., 5 h, 83%; (f) KOH, 2-propanol, 0 °C for 15 min, then reflux for 6 h, 72%. Compound 1 and 6 are commercially available.

**Scheme 2B.** Acryloyl chloride in the presence of triethylamine reacted with cyclic amines to provide the corresponding amides 16a–d, which...
upon coupling with compound 8 in ethanol at room temperature furnished the desired compounds 17a–d.

In addition, compounds 23a–o containing alkyl piperidinyl groups were synthesized using the synthetic routes depicted in Scheme 2C. Substituted piperidinyl alcohols 18a–o were reacted with different acyl chlorides to afford the corresponding amides 19a–o. Subsequently, the free hydroxyl group of the amides was substituted with either mesylates 20a–i or halides 21j–o. Compounds 20a–i, 21j–k, and 21n–o were coupled with compound 8 to afford the products 23a–i, 23j–k, and 23n–o, respectively. Additionally, compounds 21l–m were converted to the sulfur analogues by Lawesson’s reagent to furnish the thioamides 22l–m, which on reaction with compound 8 provide the thio-substituted compounds 23l–m. All compounds were purified by column chromatography and characterized by 1H and 13C NMR. Detailed experimental procedures and characterization are provided in the experimental section.

Further, we also synthesized various salts of benzamide derivative 23g. As depicted in Scheme 3, compound 23g was reacted with several acids like hydrochloric acid, hydrobromic acid, citric acid, methanesulfonic acid, maleic acid, and L-tartaric acid in appropriate solvent to furnish the corresponding salts 24a–29a. These acid salts showed good solubility in distilled water. In addition, single enantiomers of 23g were prepared using the diastereomeric salt resolution method as described in Scheme 4 cis-norcisapride (8) was separated into the (+)-form and (–)-form using suitable optically active acids ((±)-DBTA and (–)-DBTA) [24], and then S82 reactions between 20g and a single enantiomer of cis-norcisapride were performed to obtain compound 11 and 12, i.e., the (+)-form and (–)-form of 23g, respectively.

2.2. Pharmacological evaluation
2.2.1. Structure–activity relationship (in vitro and in vivo efficacy, and hERG binding assay)

Structure–activity relationship (SAR) data based on 5-HT4 binding affinity (IC50), hERG binding affinity (IC50), and functional potency (pEC50) for the compounds (13a–23d) are shown in Table 1. Compounds containing alkyl heteroaryl functional groups (13a–e) showed relatively low binding affinities for the 5-HT4 receptor. Moreover, compounds 13b, 13c, and 13d weakly inhibited hERG. Compounds containing alkyl amide functional groups (17a, 17b, and 17d) showed lower binding affinities for the 5-HT4 receptor than that of cisapride. However, compound 17c, containing a benzyl group along with an alkylamide functional group, showed dramatically higher binding affinity to the 5-HT4 receptor than that of cisapride, however, it also strongly inhibited hERG.

In accordance with the SAR data for compounds 23a–d, consisting of amide derivatives of the alkyl piperidinyl group in part B, they have higher binding affinities for the 5-HT4 receptor than that of cisapride and lower affinities for hERG. The 4-piperidyl analog of compound 23a displayed a higher binding affinity and functional activity compared to cisapride.
potency value (pEC_{50}) for the 5-HT_4 receptor than that of 3-piperidyl derivative 23b. Furthermore, a comparison between 23a and 23c illustrated that the –CH_2– linker was better for 5-HT_4 binding than –CH_2CH_2– in these series. Interestingly, introducing carbamate protection to the piperidinyl nitrogen (23d) improved 5-HT_4 receptor binding affinity and did not affect hERG binding. Thus, the initial SAR results for compounds 13a–23d revealed that benzamide derivatives, which are composed of cis-norcisapride (part A) and piperidine analogs, have potential as long as the nitrogen does not act as a tertiary amine (part B).

Next, various N-substituted analogs of compound 23a were tested for hERG and 5-HT_4 receptor binding affinities as well as for gastric emptying rate (Table 2). Biological assays of compounds 23a and 23e–i indicated that the binding affinities for 5-HT_4 receptor increased gradually by lengthening or branching of the alkyl chain of the amide group. However, the gastric emptying results were not always relevant to homologation. The gastric emptying rates of compounds 23a and 23g were comparatively equal or superior to that of cisapride, whereas that of compound 23h was slightly less than that of cisapride. However, compounds 23g and 23h were found to have good binding affinities for the 5-HT_4 receptor and negligible inhibition of hERG (IC_{50} > 10 \mu M) relative to cisapride.

Compounds containing piperidyl carboxylates (23d, 23j, and 23k) had similar binding affinities to those of the compounds 23a and 23e–i, however, their hERG binding affinities were relatively higher and gastric emptying rates were not improved from that of compound 23g. Pharmacological evaluation results of thio-derivatives 23l and 23m were inferior to compound 23g in terms

Scheme 3. Diverse salt forms of compound 23g: (a) 2-propanol, from r.t. to 0 °C for 2 h; (b) 2-propanol, from r.t. to 0 °C for 2 h; (c) acetone, reflux for 1 h, cooled to 0 °C for 12 h; (d) 2-propanol, from r.t. to 0 °C for 2 h; (e) 2-propanol, from r.t. to 0 °C for 3 h; (f) acetone, r.t. for 2 h.

Scheme 4. Preparation of each single enantiomer of compound 23g by diastereomeric salt resolution.
of hERG binding. Although 23n of the piperidyl carboxamide derivatives showed the highest binding affinity (0.052 μM of 50% inhibition concentration) of all the compounds, it showed a very low gastric emptying rate (46%). On the other hand, 23o showed a high binding affinity (0.073 μM of 50% inhibition concentration) for the 5-HT4 receptor, however, also slightly inhibited hERG (7.551 μM of 50% inhibition concentration).

Thus, after screening all compounds for 5-HT4 and hERG binding affinities, agonism, and gastric emptying rates in rats, compound 23g was found to be the best pre-candidate for a novel prokinetic agent.

Fig. 3 shows that a 0.1 mg/kg oral dose of compound 23g minimally enhanced gastric emptying, whereas that of cisapride was 0.5 mg/kg. The mean gastric emptying rates were 46.2% and 46.9% in the rat model at 0.1 and 0.5 mg/kg doses of compound 23g, respectively. However, the gastric emptying rate was 44.5% in the same model at a 0.5 mg/kg dose of cisapride [21]. Consequently, compound 23g produced more potent gastric emptying stimulation in rats than that of cisapride.

In addition to the study as described above, the chiral (+)-form of 23g, 11 was more potent than the racemic mixture, in binding affinity to the 5-HT4 receptor and in vivo gastric emptying rate as shown in Table 3. The melting point and solubility in aqueous conditions of the different salts 24–29 are shown in Table 4. Some
salts of 23g, hydrochlorate 24, methansulfonate 27, and maleate 28, synthesized as shown in Scheme 3, were found to be stable and soluble in aqueous conditions (see Table 5).

### 2.2.2. Pharmacokinetic properties of 23g and 23h

Compounds 23g and 23h are illustrated in Table 5 for in vivo efficacy, functional potency, and rat pharmacokinetic (PK) tests. As shown in Table 5, compound 23g displayed gastric emptying results superior to those of compound 23h and cisapride in the rat model (normal model: 59.1% compared with 56.4% and 57.1%; cisplatin model: 58.6% compared with 56.6% and 57.2%). Furthermore, with respect to pharmacokinetic properties, 23g showed longer T1/2 and higher Cmax and AUCinf values than 23h in a rat PK study.

### Table 2
Pharmacological evaluation of N-substituted piperidinyl methoxypiperidine benzamide derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>P</th>
<th>5-HT6 IC50 (µM)</th>
<th>hERG (IC50, µM)</th>
<th>Gastric emptying rate % (rat iv 5 mg/kg, normal model)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisapride</td>
<td>–</td>
<td>0.483 &lt;1</td>
<td>57.1</td>
<td></td>
</tr>
<tr>
<td>23e</td>
<td></td>
<td>0.592 &gt;10</td>
<td>39.5</td>
<td></td>
</tr>
<tr>
<td>23a</td>
<td></td>
<td>0.329 &gt;10</td>
<td>57.8</td>
<td></td>
</tr>
<tr>
<td>23f</td>
<td></td>
<td>0.178 &gt;10</td>
<td>48.6</td>
<td></td>
</tr>
<tr>
<td>23g</td>
<td></td>
<td>0.134 &gt;10</td>
<td>59.1</td>
<td></td>
</tr>
<tr>
<td>23h</td>
<td></td>
<td>0.118 &gt;10</td>
<td>56.4</td>
<td></td>
</tr>
<tr>
<td>23i</td>
<td></td>
<td>0.078 10</td>
<td>54.3</td>
<td></td>
</tr>
<tr>
<td>23d</td>
<td></td>
<td>0.190 &gt;10</td>
<td>56.2</td>
<td></td>
</tr>
<tr>
<td>23j</td>
<td></td>
<td>0.377 4.580</td>
<td>55.9</td>
<td></td>
</tr>
<tr>
<td>23k</td>
<td></td>
<td>0.134 &lt;1</td>
<td>57.7</td>
<td></td>
</tr>
<tr>
<td>23l</td>
<td></td>
<td>0.593 2.862</td>
<td>61.0</td>
<td></td>
</tr>
<tr>
<td>23m</td>
<td></td>
<td>0.155 1.132</td>
<td>67.3</td>
<td></td>
</tr>
<tr>
<td>23n</td>
<td></td>
<td>0.052 &gt;10</td>
<td>46.0</td>
<td></td>
</tr>
<tr>
<td>23o</td>
<td></td>
<td>0.073 7.551</td>
<td>59.9</td>
<td></td>
</tr>
</tbody>
</table>
2.2.3. Off-target screening study of 23g
We examined compound 23g (1e2 mM) for binding affinity to other pharmacologically relevant receptors. Fig. 4 shows that compound 23g selectively bound to the 5-HT4 receptor, whereas it did not show even 50% inhibition of any other receptors. Therefore, it is expected that compound 23g is a potent prokinetic agent without concern for side effects arising from non-specificity.

3. Conclusion
In this study, we intended to find compounds that retained the prokinetic efficacies of cisapride but did not block the hERG channel. We designed novel benzamide derivatives of 5-HT4 receptor agonists and tested diverse compounds. We successfully found part B of a compound with negligible hERG binding and an enhanced gastric emptying rate. We determined that compound 23g was better than cisapride in terms of high 5-HT4 receptor binding, lower cardiac adverse effects due to lower hERG binding, and enhanced gastric emptying in various models. In particular, the chiral (+)-form of 23g was more potent than the racemic mixture with respect to binding affinity to the 5-HT4 receptor and in vivo gastric emptying rate. Thus, compound 23g or (+)-form of 23g could be a good candidate to treat GI disorders, particularly functional dyspepsia. We plan to investigate additional in vivo efficacy and toxicity for the single enantiomer of 23g.

4. Experimental section
4.1. Chemistry
Reagents and solvents were purchased from commercial suppliers (Sigma–Aldrich and TCI) used as provided, unless indicated otherwise. All other solvents used for reactions were analytical grade and used as provided. Column chromatography was carried out using Merck silica gel 60 (230–400 mesh). The melting points were determined on Mettler Toledo FP900 thermosystem. 1H NMR and 13C NMR spectra were recorded on Varian 400 MHz spectrophotometer by CDCl3 or DMSO-d6 and the chemical shifts are reported in δ (ppm) and are relative to the central peak of these solvents. Mass spectra were obtained with JMS-700 Mstation mass spectrometer (JEOL Ltd.).

4.1.1. 1-(3-Chloropropyl)-1H-1,2,4-triazole (10d)
1,2,4-Triazole sodium derivative (1 g, 10.983 mmol) was dissolved in N,N-dimethylformamide (10 mL) and cooled to 0 °C. Sodium hydride (60% dispersion in mineral oil, 570 mg, 14.25 mmol) was added to the reaction mixture and stirred for 20 min at 0 °C. 1-Bromo-3-chloropropane (2.08 g, 13.2 mmol) was added dropwise to the reaction mixture. The reaction mixture was stirred for 12 h at room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (hexane:ethyl acetate = 4:1) to obtain the target compound (600 mg, 64% yield).

Table 3
5-HT4 binding affinities and GE (%) rates of 23g and each single enantiomer of compound 23g.

<table>
<thead>
<tr>
<th>Compound</th>
<th>5-HT4 IC50 (μM)</th>
<th>Gastric emptying rate % (rat iv 5 mg/kg, normal model)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23g</td>
<td>0.134</td>
<td>59.1</td>
</tr>
<tr>
<td>11 (+)-isomer of 23g</td>
<td>0.068</td>
<td>0.617</td>
</tr>
<tr>
<td>12 (-)-isomer of 23g</td>
<td>&gt;10</td>
<td>56.5</td>
</tr>
</tbody>
</table>

Table 4
Melting point and solubility of several salts of 23g.

<table>
<thead>
<tr>
<th>Melting point (°C)</th>
<th>Solubility in D.W. (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>&gt;15</td>
</tr>
<tr>
<td>25</td>
<td>&lt;5</td>
</tr>
<tr>
<td>26</td>
<td>&gt;30</td>
</tr>
<tr>
<td>27</td>
<td>&gt;10</td>
</tr>
<tr>
<td>28</td>
<td>&gt;10</td>
</tr>
<tr>
<td>29</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

Table 5
Functional potency, hERG binding, in vivo efficacy and oral PK data of 23g and 23h in rats.

<table>
<thead>
<tr>
<th>Item/parameters</th>
<th>Functional potency pEC50</th>
<th>hERG (IC50, μM)</th>
<th>In vivo efficacy (rat gastric emptying rate (%))</th>
<th>Rat PK (oral, 5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>normal</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>cisapride</td>
<td>6.99</td>
<td>&lt;1</td>
<td>57.1</td>
<td>57.2</td>
</tr>
<tr>
<td>23g</td>
<td>7.34</td>
<td>&gt;10</td>
<td>59.1</td>
<td>58.6</td>
</tr>
<tr>
<td>23h</td>
<td>7.00</td>
<td>&gt;10</td>
<td>56.6</td>
<td>0.77</td>
</tr>
</tbody>
</table>
temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO$_4$ and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (800 mg, 60% yield).

$^1$H NMR(CDC$_3$): $\delta$ 8.21 (d, $J = 8.0$ Hz, 1H), 8.07 (s, 1H), 6.65 (s, 2H), 6.28 (s, 1H), 6.13 (s, 2H), 4.56 (s, 2H). $^1$C NMR (400 MHz, CDC$_3$): $\delta$ 152.35, 143.59, 46.03, 41.09, 31.80.

### 4.1.3. cis-4-Amino-5-chloro-N-[1-(3-(1H-pyrrol-1-yl)propyl)-3-chloro-2-methoxy-N-(3-methoxypiperidin-4-yl)benzamide (13a)

1-(2-Bromoethyl)-1H-pyrrole (717 mg, 4.12 mmol), potassium carbonate (660 mg, 4.775 mmol) and potassium iodide (106 mg, 0.64 mmol) were added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-[3-methoxypiperidin-4-yl]benzamide (1 g, 3.188 mmol) in N,N-dimethylformamide (20 mL) in order. The reaction mixture was heated to 90 °C for 15 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO$_4$ and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (430 mg, 33% yield).

$^1$H NMR(CDC$_3$): $\delta$ 8.21 (d, $J = 8$ Hz, 1H), 8.08 (s, 1H), 6.69 (s, 2H), 6.29 (s, 1H), 6.13 (s, 2H), 4.40 (s, 2H), 4.20-4.12 (m, 1H), 4.04 (t, $J = 7.2$ Hz, 2H), 3.87 (s, 3H), 3.45-3.37 (m, 4H), 3.02-2.93 (m, 1H), 2.85-2.73 (m, 3H), 2.34-2.18 (m, 2H), 1.93-1.78 (m, 2H). $^1$C NMR (400 MHz, CDC$_3$): $\delta$ 163.75, 157.57, 146.69, 132.96, 120.76, 112.56, 111.50, 108.20, 97.89, 76.66, 59.20, 57.17, 55.97, 53.82, 52.02, 47.98, 47.55, 27.78. HRMS (FAB): calcd for C$_{21}$H$_{29}$ClN$_4$O$_3$ [M+H]$^+$ 421.2001, found 421.2010.

4.1.4. cis-4-Amino-5-chloro-N-[1-(3-(1H-imidazol-1-yl)propyl)-3-methoxy-piperidin-4-yl]-2-methoxybenzamide (13c)

1-(3-Chloropropyl)-1H-imidazole (596 mg, 4.12 mol), potassium carbonate (660 mg, 4.775 mmol) and potassium iodide (106 mg, 0.64 mmol) were added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-[3-methoxy-piperidin-4-yl]benzamide (1 g, 3.188 mmol) in N,N-dimethylformamide (20 mL) in order. The reaction mixture was heated to 90 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO$_4$ and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (700 mg, 52% yield).

$^1$H NMR (400 MHz, CDC$_3$): $\delta$ 8.19 (d, $J = 8.4$ Hz, 1H), 8.06 (s, 1H), 7.49 (s, 1H), 7.03 (s, 1H), 6.92 (s, 1H), 6.28 (s, 1H), 4.49 (bs, 2H), 4.19-4.08 (m, 1H), 4.08-3.92 (m, 2H), 3.85 (s, 3H), 3.44-3.38 (m, 4H), 2.99-2.83 (m, 1H), 2.78-2.65 (m, 1H), 2.35-2.18 (m, 2H), 2.18-2.03 (m, 2H), 1.99-1.67 (m, 4H). $^1$C NMR (400 MHz, CDC$_3$): $\delta$ 163.75, 157.56, 146.76, 137.41, 132.92, 129.28, 112.45, 111.44, 97.85, 57.14, 55.96, 54.19, 53.74, 51.44, 48.15, 44.53, 28.14, 27.73. HRMS (FAB): calcd for C$_{21}$H$_{29}$ClN$_4$O$_3$ [M+H]$^+$ 422.1954, found 422.1955.

4.1.5. cis-4-Amino-5-chloro-N-[1-(3-(1,2,4-triazol-1-yl)propyl)-3-methoxy-piperidin-4-yl]-2-methoxybenzamide (13d)

1-(3-Chloropropyl)-1H-1,2,4-triazole (600 mg, 4.12 mol), potassium carbonate (660 mg, 4.775 mmol) and potassium iodide (106 mg, 0.64 mmol) were added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-[3-methoxy-piperidin-4-yl]benzamide (1 g, 3.188 mmol) in N,N-dimethylformamide (20 mL) in order. The reaction mixture was heated to 90 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO$_4$ and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (610 mg, 45% yield).

$^1$H NMR (400 MHz, CDC$_3$): $\delta$ 8.17 (d, $J = 8$ Hz, 1H), 8.11 (s, 1H), 8.0 (s, 1H), 7.91 (s, 1H), 6.27 (s, 1H), 4.48 (s, 2H), 4.28-4.06 (m, 3H), 3.84 (s, 3H), 3.43-3.37 (m, 4H), 2.99-2.83 (m, 1H), 2.77-2.64 (m, 1H), 2.34-1.97 (m, 6H), 1.90-1.65 (m, 2H). $^1$C NMR (400 MHz, CDC$_3$): $\delta$ 163.75, 157.55, 151.92, 146.76, 143.65, 132.91, 112.44, 111.43, 97.83, 57.16, 55.97, 53.99, 53.71, 51.26, 48.17, 47.11, 27.69. HRMS (FAB): calcd for C$_{21}$H$_{29}$ClN$_4$O$_3$ [M+H]$^+$ 423.1906, found 423.1911.

4.1.6. cis-4-Amino-5-chloro-N-[1-(3-(1H-tetrazol-1-yl)propyl)-3-methoxy-piperidin-4-yl]-2-methoxybenzamide (13e)

1-(3-Chloropropyl)-1H-tetrazole (604 mg, 4.12 mol), potassium carbonate (660 mg, 4.775 mmol) and potassium iodide (106 mg, 0.64 mmol) were added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-[3-methoxy-piperidin-4-yl]benzamide (1 g, 3.188 mmol) was dissolved in N,N-dimethylformamide (20 mL) in order. The reaction mixture was heated to 100 °C for
12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO₄ and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (795 mg, 59% yield).

¹H NMR (DMSO-d₆): 6.38 (s, 1H), 8.08 (d, J = 7.6 Hz, 1H), 7.72 (s, 1H), 6.49 (s, 1H), 5.99 (s, 2H), 4.47 (t, J = 6.8 Hz, 2H), 4.00 (bs, 1H), 3.85 (s, 3H), 3.39–3.28 (m, 5H), 2.75 (bs, 1H), 2.25 (t, J = 6.6 Hz, 2H), 2.22–2.03 (m, 2H), 2.00 (t, J = 6.6 Hz, 2H), 1.75–1.58 (m, 18H). ¹³C NMR (400 MHz, CDCl₃): δ 163.11, 157.85, 149.11, 144.57, 132.06, 110.38, 109.62, 98.09, 76.30, 56.54, 56.35, 54.25, 53.40, 50.73, 47.77, 46.17, 28.08, 26.81. HRMS (FAB): calcd for C₁₈H₂₆ClN₇O₃ [M+H]+ 543.2273, found 543.2279.

4.1.10. cis-4-Amino-5-chloro-N-[1-(3-(4-benzylpiperidin-1-yl)-3-oxopropyl)-3-methoxy(piperidin-4-yl)-2-methoxybenzamide (17c)

1-(4-Benzylpiperidin-1-yl)prop-2-ene-1-one (383 mg, 1.908 mmol) was slowly added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methoxy(piperidin-4-yl)-benzamide (500 mg, 1.59 mmol) in ethanol (5 mL). The reaction mixture was stirred for 12 h at room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO₄ and evaporated in vacuo. The residue was purified by column chromatography (chloroform:methanol = 20:1) to obtain the target compound (318 mg, 37% yield).

¹H NMR (400 MHz, CDCl₃): δ 8.19 (d, J = 8.4 Hz, 1H), 8.06 (s, 1H), 7.30–7.22 (m, 2H), 7.22–7.13 (m, 1H), 7.13–7.07 (m, 2H), 6.27 (s, 1H), 4.56 (d, J = 13.2 Hz, 1H), 4.43 (s, 2H), 4.21–4.12 (m, 1H), 3.87–3.76 (m, 4H), 3.38 (m, 4H), 3.09–2.99 (m, 1H), 2.99–2.83 (m, 1H), 2.59–2.40 (m, 5H), 2.31–2.17 (m, 2H), 1.91–1.62 (m, 5H), 1.20–1.02 (m, 2H). ¹³C NMR (400 MHz, CDCl₃): 170.00, 163.72, 157.55, 146.67, 139.91, 132.97, 129.08, 128.30, 126.06, 124.96, 112.59, 111.50, 97.88, 57.14, 55.94, 54.15, 53.91, 53.86, 53.47, 51.60, 47.87, 45.86, 42.93, 41.94, 38.25, 32.55, 31.76, 27.80. HRMS (FAB): calcd for C₂₉H₂₅ClN₅O₅ [M+H]+ 543.2733, found 543.2739.

4.1.11. cis-4-Amino-5-chloro-N-[1-(3-oxo-3-(morpholino-1-yl)propyl)-3-methoxy(piperidin-4-yl)-2-methoxybenzamide (17d)

1-Morpholinoprop-2-ene-1-one (269 mg, 1.908 mmol) was slowly added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methoxy(piperidin-4-yl)-benzamide (500 mg, 1.59 mmol) in ethanol (5 mL). The reaction mixture was stirred for 12 h at room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO₄ and evaporated in vacuo. The residue was purified by column chromatography (chloroform:methanol = 20:1) to obtain the target compound (292 mg, 40% yield).

¹H NMR (400 MHz, CDCl₃): δ 8.19 (d, J = 8.4 Hz, 1H), 8.06 (s, 1H), 6.28 (s, 1H), 4.44 (s, 2H), 4.20–4.12 (m, 1H), 3.85 (s, 3H), 3.68–3.56 (m, 4H), 3.47–3.39 (m, 6H), 3.09–2.97 (m, 1H), 2.84–2.67 (m, 3H), 2.54 (t, J = 7.6 Hz, 2H), 2.28–2.17 (m, 2H), 1.86–1.77 (m, 2H). ¹³C NMR (400 MHz, CDCl₃): 170.31, 163.71, 157.53, 146.67, 132.95, 112.54, 111.49, 97.85, 66.87, 66.61, 60.40, 57.17, 55.94, 53.93, 53.93, 51.67, 47.92, 45.94, 41.87, 30.78, 27.75. HRMS (FAB): calcd for C₂₉H₂₅ClN₅O₅ [M+H]+ 545.2065, found 545.2065.

4.1.12. 1-(4-(Hydroxymethyl)piperidin-1-yl)-2-methylprop-1-ene (19g)

Diisopropylethylamine(DIPEA) was slowly added to a stirred solution of piperidin-4-ylmethanol (1.3 g, 11.29 mmol) in methanol (20 mL) and the reaction mixture was stirred for 30 min at 0 °C. Isobutyl chloride (1.42 mL, 13.55 mmol) was added dropwise to the reaction mixture at the same temperature. The reaction mixture was cooled down to 0 °C and 1 h at room temperature. The reaction mixture was stirred for 2 h at 0 °C and 1 h at room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO₄ and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 2:1) to obtain the target compound (1.42 g, 76.8 mg).

¹H NMR (400 MHz, CDCl₃): 4.68–4.47 (m, 1H), 4.03–3.79 (m, 1H), 3.44 (d, J = 5.2 Hz, 2H), 2.83–2.64 (m, 2H), 2.62–2.37 (m, 2H), 1.90–1.62 (m, 3H), 1.19–0.97 (m, 8H).

4.1.13. 1-(Isobutylpiperidin-1-yl)-2-methylmethanesulfonate (20g)

1-(4-(Hydroxymethyl)piperidin-1-yl)-2-methylprop-1-ene
ethyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (19g) (1 g, 5.398 mmol) was dissolved in dichloromethane 20 mL and cooled to 0 °C. Triethylamine (1.51 mL, 10.796 mmol) was added to the reaction mixture and stirred for 30 min at 0 °C. Methanesulfonyl chloride (0.5 mL, 6.478 mmol) was added dropwise for 30 min and stirred for 3 h at the same temperature. The reaction mixture was stirred for 1 h at room temperature and extracted with dichloromethane and 1 M citric acid aqueous solution. The organic layer was dried with MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (n-hexane:EtOAc = 1:1) to obtain the target compound (1.21 g, 85% yield).

H NMR (400 MHz, CDCl3): δ 4.79 – 4.49 (m, 1H), 4.08 – 3.81 (m, 1H), 3.67 – 3.40 (m, 2H), 3.07 – 2.73 (m, 1H), 2.65 – 2.77 (m, 2H), 2.64 – 2.38 (m, 1H), 1.99 – 1.74 (m, 4H), 1.27 – 1.03 (m, 9H).

4.1.14. cis-4-Amino-5-chloro-N-(1-((1-propionylpiperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (23a) (1-Propionylpiperidin-4-yl)methyl methanesulfonate (20a) (381 mg, 1.53 mmol), potassium carbonate (246 mg, 1.78 mmol) and potassium iodide (42 mg, 0.25 mmol) were added to a solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methoxypiperidin-4-yl) benzamide (400 mg, 1.275 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 100 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetonitrile = 4:1) to obtain the target compound (115 mg, 19% yield).

H NMR (400 MHz, CDCl3): δ 8.18 (d, J = 8 Hz, 1H), 8.08 (s, 1H), 6.29 (s, 1H), 4.60 (d, J = 13.2 Hz, 1H), 4.41 (s, 2H), 4.25 – 4.16 (m, 1H), 3.96 – 3.78 (m, 4H), 3.42 (s, 4H), 3.04 – 2.83 (m, 2H), 2.78 – 2.61 (m, 1H), 2.54 (t, J = 13.2 Hz, 1H), 2.39 – 2.07 (m, 6H), 1.96 – 1.64 (m, 6H), 1.20 – 0.99 (m, 5H).

13C NMR (400 MHz, CDCl3): δ 172.17, 163.76, 157.55, 146.60, 133.01, 112.68, 111.53, 97.86, 64.29, 56.28, 56.00, 54.02, 52.22, 47.91, 45.57, 41.76, 34.01, 31.54, 31.34, 30.64, 30.52, 27.80, 9.63. HRMS (FAB): calcd for C23H35ClN4O4 [M+H]+ 467.2420, found 467.2425.

4.1.15. cis-4-Amino-5-chloro-N-(1-((1-propionylpiperidin-3-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (23b) (1-Propionylpiperidin-3-yl)methyl methanesulfonate (20b) (190 mg, 0.765 mmol), potassium carbonate (123 mg, 0.89 mmol) and potassium iodide (21 mg, 0.125 mmol) were added to a solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methoxypiperidin-4-yl) benzamide (200 mg, 0.638 mmol) in N,N-dimethylformamide (5 mL) in order. The reaction mixture was heated to 100 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetonitrile = 4:1) to obtain the target compound (115 mg, 19% yield).

H NMR (400 MHz, CDCl3): δ 8.18 (d, J = 7.6 Hz, 1H), 8.04 (s, 1H), 6.27 (s, 1H), 4.50 (s, 2H), 4.23 – 3.08 (m, 3H), 3.83 (s, 3H), 3.65 (s, 3H), 3.38 (s, 4H), 2.98 – 2.83 (m, 1H), 2.79 – 2.58 (m, 3H), 2.29 – 2.07 (m, 5H), 1.90 – 1.56 (m, 5H), 1.14 – 0.97 (m, 2H).

13C NMR (400 MHz, CDCl3): δ 163.80, 157.55, 155.98, 146.77, 132.85, 112.40, 111.37, 97.83, 76.67, 46.36, 56.72, 55.94, 54.00, 52.45, 52.19, 47.93, 43.94, 33.75, 30.72, 20.60, 27.75, 14.16. HRMS (FAB): calcd for C22H27ClIN4O4 [M+H]+ 469.2212, found 469.2215.

4.1.18. cis-4-Amino-5-chloro-N-1(1-((1-acetylpiperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (23e) (1-Acetylpirperidin-4-yl)methyl methanesulfonate (20e) (360 mg, 1.53 mmol), potassium carbonate (246 mg, 1.78 mmol) and potassium iodide (42 mg, 0.25 mmol) were added to a solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methoxypiperidin-4-yl) benzamide (400 mg, 1.275 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 90 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetonitrile = 4:1) to obtain the target compound (27 mg, 5% yield).

H NMR (400 MHz, CDCl3): δ 8.18 (d, J = 8 Hz, 1H), 8.07 (s, 1H), 6.29 (s, 1H), 4.57 (d, J = 13.2 Hz, 1H), 4.44 (s, 2H), 4.23 – 4.14 (m, 1H), 3.86 (s, 3H), 3.78 (d, J = 13.2 Hz, 1H), 3.41 (s, 4H), 3.07 – 2.82 (m, 2H), 2.75 – 2.59 (m, 1H), 2.53 (t, J = 13.2 Hz, 1H), 2.26 – 2.09 (m, 4H), 2.07 (s, 3H), 1.94 – 1.65 (m, 5H), 1.18 – 0.98 (m, 2H).

13C NMR (400 MHz, CDCl3): δ 168.83, 163.75, 157.55, 146.65, 132.97, 112.61, 111.48, 97.86, 64.27, 56.79, 55.98, 54.15, 52.20, 47.91, 46.55, 41.65, 41.61, 33.96, 31.44, 31.31, 30.56, 30.42, 27.83, 21.58. HRMS (FAB): calcd for C22H27ClIN4O4 [M+H]+ 453.2263, found 453.2267.
4.1.19. cis-4-Amino-5-chloro-N-(1-((1-butyrylpiperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (23f)

(1-Butyryl)piperidin-4-yl)methyl methanesulfonate (20f) (381 mg, 1.53 mmol), potassium carbonate (246 mg, 1.78 mmol) and potassium iodide (42 mg, 0.25 mmol) were added to a solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (400 mg, 1.275 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 100 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (115 mg, 19% yield).

4.1.19.1. cis-4-Amino-5-chloro-N-(1-((1-butyrylpiperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (23f)

(1-Butyryl)piperidin-4-yl)methyl methanesulfonate (20f) (381 mg, 1.53 mmol), potassium carbonate (246 mg, 1.78 mmol) and potassium iodide (42 mg, 0.25 mmol) were added to a solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (400 mg, 1.275 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 100 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (115 mg, 19% yield).

4.1.19.2. cis-4-Amino-5-chloro-N-(1-((1-butyrylpiperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (23f)

(1-Butyryl)piperidin-4-yl)methyl methanesulfonate (20f) (381 mg, 1.53 mmol), potassium carbonate (246 mg, 1.78 mmol) and potassium iodide (42 mg, 0.25 mmol) were added to a solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (400 mg, 1.275 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 90 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (115 mg, 19% yield).

4.1.20. cis-4-Amino-5-chloro-N-(1-((1-isobutylpiperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (23g)

(1-Isobutyl)piperidin-4-yl)methyl methanesulfonate (20g) (403 mg, 1.53 mmol), potassium carbonate (246 mg, 1.78 mmol) and potassium iodide (42 mg, 0.25 mmol) were added to a solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (400 mg, 1.275 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 100 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (428 mg, 70% yield).

4.1.20.1. cis-4-Amino-5-chloro-N-(1-((1-isobutylpiperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-methoxybenzamide (23g)

(1-Isobutyl)piperidin-4-yl)methyl methanesulfonate (20g) (403 mg, 1.53 mmol), potassium carbonate (246 mg, 1.78 mmol) and potassium iodide (42 mg, 0.25 mmol) were added to a solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (400 mg, 1.275 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 100 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (428 mg, 70% yield).

4.1.23. Ethyl 4-((cis-4-(4-amino-5-chloro-2-methoxybenzamido)-4-methyl)-3-methoxypiperidin-4-yl)methyl)propionitrile-1-carboxylate (23j)

Ethyl 4-(bromomethyl)piperidin-1-carboxylate (410 mg, 1.64 mmol), potassium carbonate (281 mg, 2.03 mmol) and potassium iodide (48 mg, 0.29 mmol) were added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (455 mg, 1.45 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 90 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 5:1) to obtain the target compound (222 mg, 32% yield).

4.1.23.1. Ethyl 4-((cis-4-(4-amino-5-chloro-2-methoxybenzamido)-4-methyl)-3-methoxypiperidin-4-yl)methyl)propionitrile-1-carboxylate (23j)

Ethyl 4-(bromomethyl)piperidin-1-carboxylate (410 mg, 1.64 mmol), potassium carbonate (281 mg, 2.03 mmol) and potassium iodide (48 mg, 0.29 mmol) were added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (455 mg, 1.45 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 90 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 5:1) to obtain the target compound (222 mg, 32% yield).

4.1.24. Propyl 4-((cis-4-(4-amino-5-chloro-2-methoxybenzamido)-3-methoxypiperidin-1-yl)methyl)piperidine-1-carboxylate (23k)

Propyl 4-((bromomethyl)piperidin-1-carboxylate (434 mg, 1.64 mmol), potassium carbonate (281 mg, 2.03 mmol) and potassium iodide (48 mg, 0.29 mmol) were added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (455 mg, 1.45 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 90 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (200 mg, 28% yield).

4.1.24.1. Propyl 4-((cis-4-(4-amino-5-chloro-2-methoxybenzamido)-3-methoxypiperidin-1-yl)methyl)piperidine-1-carboxylate (23k)

Propyl 4-((bromomethyl)piperidin-1-carboxylate (434 mg, 1.64 mmol), potassium carbonate (281 mg, 2.03 mmol) and potassium iodide (48 mg, 0.29 mmol) were added to a stirred solution of cis-4-amino-5-chloro-2-methoxy-N-(3-methylpiperidin-4-yl)benzamide (455 mg, 1.45 mmol) in N,N-dimethylformamide (10 mL) in order. The reaction mixture was heated to 90 °C for 12 h and then cooled to room temperature and extracted with ethyl acetate and water. The organic layer was dried over anhydrous MgSO4 and evaporated in vacuo. The residue was purified by column chromatography (chloroform:acetone = 4:1) to obtain the target compound (200 mg, 28% yield).
temperature and extracted with ethyl acetate and water. The
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cis (719 mg, 2.886 mmol), potassium carbonate (465 mg, 3.364 mmol)
4.1.26. 4-((cis-4-(4-Amino-5-chloro-2-methoxybenzamido)-3-
heated to 90°C and then cooled to room temperature and
extracted with ethyl acetate and water. The organic layer was
dried over anhydrous MgSO4 and evaporated in vacuo. The residue
was purified by column chromatography (chloroform:acetone = 3:1) to
obtain the target compound (310 mg, 27% yield).

1H NMR (400 MHz, CDCl3): δ 8.17 (d, J = 0.80 Hz, 1H), 8.02 (s, 1H),
6.27 (s, 1H), 4.56 (s, 2H), 4.21–4.08 (m, 1H), 3.81 (s, 3H), 3.60 (d,
J = 13.2 Hz, 2H), 3.37 (s, 4H), 2.98–2.83 (m, 1H), 2.76 (s, 6H),
2.73–2.56 (m, 4H), 2.50–2.31 (m, 1H), 2.22–2.01 (m, 4H), 1.90–1.55 (m,
5H), 1.09–1.02 (m, 2H). 13C NMR (400 MHz, CDCl3): δ 165.17,
163.82, 157.55, 146.89, 132.79, 112.16, 110.07, 87.18, 76.66, 64.44,
56.73, 55.92, 53.89, 52.20, 47.93, 46.97, 38.51, 34.01, 30.81, 30.71,
27.78. HRMS (FAB): calcd for C23H35ClN4O3S [M+H]+ 497.2348, found 497.2355.

4.1.26. 4-((cis-4-4-Amino-5-chloro-2-methoxybenzamido)-3-
heptadecane-1-thione (225m)

Lawesson's reagent (724 mg, 1.79 mmol) was added to a stirred
solution of 1-(4-(bromomethyl)piperidin-1-yl)-2-methylpropane-1-thione
(730 mg, 2.763 mmol), potassium carbonate (480 mg, 3.473 mmol)
and potassium iodide (74 mg, 0.446 mmol) were added to a stirred
solution of cis-4-amino-5-chloro-N-(3-methoxypiperidin-4-yl)benzamide (755 mg, 2.406 mmol) in N,N-
dimethylformamide (15 mL) in order. The reaction mixture was
heated to 90°C for 12 h and then cooled to room temperature and
extracted with ethyl acetate and water. The organic layer was
dried over anhydrous MgSO4 and evaporated in vacuo. The residue
was purified by column chromatography (chloroform:acetone = 4:1) to
obtain the target compound (380 mg, 33% yield).

1H NMR (400 MHz, CDCl3): δ 8.17 (d, J = 7.6 Hz, 1H), 8.03 (s, 1H),
6.28 (s, 1H), 5.64 (d, J = 12.4 Hz, 1H), 4.49 (s, 2H), 4.36 (d, J = 13.2 Hz,
1H), 4.21–4.08 (m, 1H), 3.83 (s, 3H), 3.38 (s, 4H), 3.23–3.03 (m, 2H),
3.01–2.78 (m, 2H), 2.72–2.57 (m, 1H), 2.26–1.68 (m, 9H), 1.34–1.03 (m,
5H). 13C NMR (400 MHz, CDCl3): δ 208.95, 208.50, 163.79,
157.56, 146.77, 132.83, 112.28, 111.35, 111.35, 107.78, 76.64, 63.76, 63.73,
56.80, 56.76, 55.98, 54.28, 54.05, 52.26, 51.95, 50.71, 50.67, 49.06,
49.04, 47.96, 36.42, 33.89, 31.96, 31.68, 30.21, 30.10, 27.77, 23.12,
23.08, 23.03, 23.01. HRMS (FAB): calcd for C23H35ClN4O5S [M+H]+
497.2348, found 497.2355.

4.1.28. cis-4-Amino-5-chloro-N-(1-((1-(2-methylpropanethioyl)
piperidin-4-yl)methyl)-3-methoxypiperidin-4-yl)-2-
heptadecane-1-thione (23m)

1-(4-(Bromomethyl)piperidin-1-yl)-2-methylpropane-1-thione
(730 mg, 2.763 mmol), potassium carbonate (480 mg, 3.473 mmol)
and potassium iodide (74 mg, 0.446 mmol) were added to a stirred
solution of cis-4-amino-5-chloro-N-(3-methoxypiperidin-4-yl)benzamide (722 mg, 2.3 mmol) in N,N-
dimethylformamide (15 mL) in order. The reaction mixture was
heated to 90°C for 12 h and then cooled to room temperature and
extracted with ethyl acetate and water. The organic layer was
dried over anhydrous MgSO4 and evaporated in vacuo. The residue
was purified by column chromatography (chloroform:acetone = 4:1) to
obtain the target compound (400 mg, 36% yield).

1H NMR (400 MHz, CDCl3): δ 8.18 (d, J = 8.0 Hz, 1H), 8.06 (s, 1H),
6.29 (s, 1H), 5.52 (d, J = 13.2 Hz, 1H), 4.44 (s, 2H), 4.25–4.13 (m, 2H),
3.86 (s, 3H), 3.40 (s, 4H), 3.17 (tj, J = 13.2 Hz, 1H), 3.04–2.82 (m, 4H),
2.75–2.63 (m, 1H), 2.31–2.10 (m, 4H), 2.04–1.73 (m, 6H), 1.35–1.11 (m,
5H). 13C NMR (400 MHz, CDCl3): δ 203.64, 203.60, 163.78,
157.54, 146.67, 132.91, 112.53, 111.45, 97.87, 76.73, 63.72, 56.83,
56.78, 55.99, 54.28, 54.06, 52.25, 51.98, 50.47, 49.00, 49.58,
47.94, 37.27, 37.25, 33.57, 31.69, 31.57, 30.19, 30.06, 27.72, 13.47.

4.2. Biological evaluation

4.2.1. 5-HT4 receptor binding assay

5-HT4 receptor binding assay were performed using membrane
preparations from Cos-7 cells expressing human 5-HT4. The mem-
brane protein preparation was conducted as described previously [25].
Briefly, cells were washed with phosphate buffered saline (PBS)
and centrifuged at 300 g for 5min. The resulting pellet was
suspended in ice-cold HEPES buffer (50 mM, pH 7.4), centrifuged at
40,000 g for 30 min at 4°C. The final pellet was resuspended in
HEPES buffer, and protein quantification assay. Competitive
(30–400 nM) of test compounds and 30 nM of [3H]-GR113808.
Non-specific binding was defined using 10 μM of 5-HT. The I$_{50}$
value (the concentration of test compound that inhibits the binding of the radioactive ligand by 50%) was determined by linear regression of the displacement curve.

4.2.2. hERG channel assay

hERG binding affinities of test compounds were obtained using Predictor™ hERG fluorescence polarization assay (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The IC_{50} values of compounds (10–10,000 nM) were calculated by eliminating maximum polarization value of 30 μM E-4031, a specific hERG blocker.

4.2.3. Functional potency evaluation; 5-HT4 receptor agonist activity on carbachol-induced contraction of rat esophageal thoracic muscularis mucosa (TMM) preparations

Rats were sacrificed by a blow on the head, and the most distal 1.2 cm of the esophagus was isolated. The esophageal segments were prepared as described previously [26]. Briefly, the external muscularis propria, containing the outer longitudinal and circular muscle layers of the esophagus, was carefully removed in order to isolate the smooth muscle of the tunica muscularis mucosa. The preparations were suspended longitudinally under an initial tension of approximately 0.5 g in modified Krebs–Henseleit solution at 37°C and saturated with 95% O2 and 5% CO2. The ionic composition of the Krebs–Henseleit solution (mM) was NaCl 118, KCl 4.75, CaCl2 2.5, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25 and glucose 10. This solution routinely contained indomethacin (3 μM) to prevent the relaxation effects of prostanoids, methysergide (1 μM) to block 5-HT1 and 5-HT3 receptor.

Tissues were left to equilibrate with Krebs–Henseleit solution for 60 min (with washing every 15 min) before starting the experiment. Responses were recorded isometrically through a force displacement transducer (FT03, GRASS technology, U.S.A.) coupled to a chart recorder (Labchart 5, AD Instruments, Australia).

The preparations were contracted by addition of a submaximal concentration of carbachol (3 μM) into the bathing solution. Upon establishing a stable contraction, accumulative concentration–effect curve for relaxation to 5-HT was constructed. After construction of the control curve, the tissue was washed with fresh modified Krebs–Henseleit solution and allowed to recover for 60 min before recontracting with carbachol. Potency relative to 5-HT was calculated from experiments in which two concentration–effect curves were constructed in the same preparation: the first to 5-HT itself and the second to a test compound.

4.2.4. Gastric emptying evaluation

Gastric emptying was measured according to the method [27] of with some modifications. Male Sprague–Dawley rats (220–250 g) were fasted for 18 h ad libitum access to water. (i) Normal rats were given 2 mL of semisoloid meals by gavages at 50 min after drug administration. Following 30 min animals were sacrificed by a blow on the head, and the most distal 1.2 cm of the esophagus was isolated. The esophageal segments were prepared as described previously [26]. Briefly, the external muscularis propria, containing the outer longitudinal and circular muscle layers of the esophagus, was carefully removed in order to isolate the smooth muscle of the tunica muscularis mucosa. The preparations were suspended longitudinally under a initial tension of approximately 0.5 g in modified Krebs–Henseleit solution at 37°C and saturated with 95% O2 and 5% CO2. The ionic composition of the Krebs–Henseleit solution (mM) was NaCl 118, KCl 4.75, CaCl2 2.5, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25 and glucose 10. This solution routinely contained indomethacin (3 μM) to prevent the relaxation effects of prostanoids, methysergide (1 μM) to block 5-HT1 and 5-HT3 receptor.

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4.2.5. Other receptor binding assay

Other receptor binding assays were performed by MDS Pharma Services, Taiwan Ltd., using compound 23g at a screening concentration of 1 or 2 μM. The radioligand binding affinities of 23g were determined at the following receptors: 5-HT receptors (5-HT1A, 5-HT1B, 5-HT2A, 5-HT3A, 5-HT5A receptors), non-5-HT receptors (Adrenergic α1, Adrenergic α2, Adrenergic β1, Cholecystokinin 1, Dopamine 2L, Dopamine 2S, Dopamine 3, Motilin, Muscarinic M2, Muscarinic M3, Opiate κ (OP2), Opiate μ (OP3), Somatostatin sst2, Tachykinin NK1, Tachykinin NK2).

4.3. Chiral HPLC analyses

4.3.1. Instruments

Analytical HPLC apparatus consisted on a Agilent G1311A quaternary pump, a G1319A autosampler, a G1316A column oven, a ChemStation Datasystem (Agilent).

4.3.2. HPLC operating condition

Analytical chromatographic separations were carried out on a Chiralpak IA column (250 mm × 4.6 mm I.D.) with a mobile phase consisting of heptane: isopropanol: ethanol: diethylamine in the ratio 80:10:10:0.1 (v/v/v/v) at a flow rate of 1.0 mL/min and maintaining the column temperature at 30°C. The injection volume was 10 μL and the detection wavelength was set at 220 nm.

4.3.3. Preparation of sample

Sample was prepared by dissolving of accurate weight of 25 mg in 50 mL of methanol (0.5 mg/mL).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.12.006.

References


