Design, Synthesis, and Neuroprotective Effects of Novel Cinnamamide-Piperidine and Piperazine Derivatives

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Pharmaceut Fronts 2023;5:e132–e140.

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Keywords
► stroke
► cinnamic acid
► derivatives
► hERG
► neuroprotective

Abstract
In our previous studies, Fenazinel has shown good neuroprotective effects; however, when Fenazinel entered phase 1 clinical trials, it was associated with certain side effects. This study aimed to explore novel neuroprotective agents with higher potency and lower toxicity. Evidence suggested that cinnamic acid and its analogs may serve as promising lead compounds for stroke treatment. In this study, a series of Fenazinel derivatives were first synthesized with potential neuroprotective effects with fragments including cinnamic acid and its analogs as key functional groups. The methyl thiazolyl tetrazolium assay was performed to assess the neuroprotective effects of the compounds in glutamate-induced neurotoxicity in SH-SY5Y cells. The hERG binding assay was conducted to assess drug-induced QT prolongation or other cardiotoxicity. The neuroprotective activity of the most potent compound in vivo was tested through the survival time of mice under the hypoxic condition and a middle cerebral artery occlusion model. Our data suggested that among those derivatives, compound 9d

1 These authors contributed equally to this work.

received October 28, 2022
accepted August 10, 2023
article published online September 12, 2023

ISSN 2628-5088.

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Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany
Introduction

Stroke ranks as the second most common cause of death and a leading cause of disability worldwide. It can be categorized into ischemic stroke and hemorrhagic stroke, with the former being more prevalent, accounting for approximately 80% of all stroke cases. Currently, tissue plasminogen activator remains the only Food and Drug Administration-approved drug for treating ischemic stroke. Edaravone, a free-radical scavenger, has been approved for use in the treatment of acute ischemic stroke in Japan and China. Therefore, the identification and validation of novel therapeutic strategies for stroke is of paramount importance.

In previous studies, we designed and synthesized a series of dicarbonylalkyl piperazine derivatives to explore their neuroprotective properties. Specifically, compound 1 (Fenazinel, Fig. 1, also named SIPI5052/5052) and compound 8 demonstrated promising neuroprotective effects both in vitro and in vivo. In 2006, Fenazinel entered phase 1 clinical trials in China as a novel neuroprotective agent. However, during clinical trials, Fenazinel’s administration was associated with certain side effects: elevated serum phosphocreatine kinase activity in two patients, and potential premature atrial contractions in another patient. As a result, we conducted a comprehensive evaluation of Fenazinel’s pharmacological profile to determine any significant off-target activity or metabolic disorders associated with the compound. Follow-up studies revealed that Fenazinel had mild activity in the hERG patch-clamp K⁺ channel binding assay, with an IC50 value of 8.64 μmol/L. Meanwhile, its major metabolite M1 (3) in the human body had a hERG IC50 value of 0.43 μmol/L, suggesting M1 might contribute to drug-induced QT prolongation or other cardiotoxicity. Given the increasing regulatory emphasis on drug-induced QT prolongation, we believe it is essential to mitigate the hERG activity of these compounds through structural modification.

In recent years, traditional Chinese medicine has gained extensive acceptance for treating nervous system diseases. Cinnamic acid (3-phenylprop-2-enoic acid) and its analogues (ferulic acid, sinapic acid, p-methoxycinnamic acid, etc.) are prolific in plants. These compounds display an array of pharmacological activities, including antioxidant properties, neuroprotection, antithrombotic effects, angiogenesis promotion, and vascular protective capacities. These attributes suggest that cinnamic acid and its analogues could serve as promising lead compounds for stroke treatment.

In this study, we sought to mitigate potential cardiac risks by replacing groups within the structures of Fenazinel or M1 with fragments from cinnamic acid and its analogues, thereby creating novel cinnamamide-piperidine and piperazine derivatives. We anticipated that the integration of these natural active ingredients with Fenazinel would generate a synergistic effect in neuroprotection, while simultaneously mitigating the risk of cardiotoxicity.

Results and Discussion

Chemistry

The synthesis of the analogues, designated as 9a-m, was obtained following the processes depicted in various schemes. As outlined in Scheme 1, different substituted benzyl/acetaldehyde 4a-l reacted with malonic acid via the Perkin reaction to afford intermediate 5a-t, and then coupled intermediate 7 with triethylamine in the presence of HBTO (O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyl-uronium hexafluorophosphate)
to produce compounds 8a–l. Finally, the target compound 9a–l was obtained by a salification reaction with hydrochloric acid. The analogues are listed in Table 1.

The synthesis of 9m is shown in Scheme 2. A nucleophilic substitution of tert-butyl piperazine-1-carboxylate with N-benzyl-2-chloroacetamide generated intermediate 10 and aqueous HCl-mediated deprotection afforded intermediate 11, which coupled with 4-methoxycinnamic acid provided compound 12. Ultimately, the target compound 9m was obtained by a salification reaction with hydrochloric acid.

### Biological Activity

To test the potential neuroprotective activities of these target compounds, a preliminary screening was performed investigating neuroprotection on impairment induced by glutamic acid deprivation in SH-SYSY cells, as evaluated by methyl thiazolyl tetrazolium (MTT) assay. The results are shown in Table 1. Six compounds (9a–b, 9i, 9k–m) showed slight neuroprotection capacity at two test concentrations (1 and 10 μmol/L), with cell survival rates ranging from 50.24 to 57.83%. Two compounds (9c and 9d) exhibited moderate to good neuroprotective effect at two levels of concentration in comparison with Fenazinel (9c: 60.09% and 57.41%; 9d: 56.53% and 59.65% viable rate at 1 and 10 μmol/L, respectively). The compound 9j exhibited weaker activity at the low concentration of 1 μmol/L, but showed better protective activity than the positive compound Fenazinel at the high concentration of 10 μmol/L.

The preliminary structure–activity relationship (SAR) showed the neuroprotective activities of the derivatives (9c and 9d) with two methoxyl groups on the benzene ring were better than those of mono-substituted (9a and 9h) and trisubstituted derivatives (9b). The length of the left carbon chain had no significant effect on the activity of the compound (9k and 9l). Meanwhile, the piperidine derivative (9m) and piperazine derivative (9a) had no significant difference in activity.

Evidence suggested that neuroprotective agents may be cytotoxic at high concentrations.22–25 To further evaluate the potency and toxicity of compounds, we selected the most potent compounds 9c and 9d to test their neuroprotection capacity in high concentrations (10, 20, 50, and 100 μmol/L), using Fenazinel as the positive control group. According to the results of the MTT assay (Fig. 2), different concentrations of all compounds’ solutions exhibited different degrees of damage toward SH-SYSY cells, and with the increase in concentration, the survival rate of SH-SYSY cells decreased gradually, showing a dose–effect relationship. Among them, compound 9d showed similar cell viability to Fenazinel at four test concentrations, which was worth further investigation as a novel neuroprotective agent.

Based on the above analysis, compound 9d was further evaluated in hERG binding assay and hypoxia tolerance model in mice (Table 2). Compound 9d showed weak inhibition in the hERG binding assay with an IC50 value of 24.61 μmol/L and almost threefold increased value compared with Fenazinel, which indicated that the possibility of compound 9d causing drug-induced QT prolongation was lower. Hypoxia tolerance assay in vivo showed that compound 9d could prolong the survival time of mice under the hypoxic condition at a dose of 20 mg/kg compared with the control group and was slightly weaker than the Fenazinel group. Therefore, it can be considered as a new lead compound for further development in specific tests for a potential neuroprotective agent.

To investigate the activity of 9d (also named SIPI7591/7591) in vivo, we tested its potential anti-ischemic stroke effects on a rat model of middle cerebral artery (MCA) occlusion (MCAO). Then MCAO rats were injected intraperitoneally with compound 7591 (5 mg/kg), Fenazinel/5052 (5 mg/kg), and
### Table 1 Preliminary in vitro neuroprotective activity of the targeted compounds of 9a–m

<table>
<thead>
<tr>
<th>Compd.</th>
<th>R</th>
<th>n</th>
<th>X</th>
<th>Glu deprivation test, survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 µmol/L</td>
</tr>
<tr>
<td>9a</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>54.45 ± 0.23</td>
</tr>
<tr>
<td>9b</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>51.32 ± 0.38</td>
</tr>
<tr>
<td>9c</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>60.09 ± 1.61</td>
</tr>
<tr>
<td>9d</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>56.53 ± 3.52</td>
</tr>
<tr>
<td>9e</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>57.19 ± 0.60</td>
</tr>
<tr>
<td>9f</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>46.37 ± 0.53</td>
</tr>
<tr>
<td>9g</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>56.04 ± 5.68</td>
</tr>
<tr>
<td>9h</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>48.06 ± 1.38</td>
</tr>
<tr>
<td>9i</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>55.98 ± 1.08</td>
</tr>
<tr>
<td>9j</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>50.69 ± 0.22</td>
</tr>
<tr>
<td>9k</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>54.47 ± 1.32</td>
</tr>
<tr>
<td>9l</td>
<td></td>
<td>1</td>
<td>CH</td>
<td>54.45 ± 2.47</td>
</tr>
<tr>
<td>9m</td>
<td></td>
<td>0</td>
<td>N</td>
<td>57.83 ± 1.15</td>
</tr>
<tr>
<td>Fenazine</td>
<td></td>
<td>–</td>
<td>–</td>
<td>56.27 ± 0.86</td>
</tr>
<tr>
<td>Vehicle (Damage model)</td>
<td></td>
<td></td>
<td></td>
<td>50.58 ± 1.13</td>
</tr>
</tbody>
</table>
Edaravone (5 mg/kg), respectively. The results (Fig. 3) showed that the positive control drug Fenazinel significantly reduced the area of cerebral infarction. Meanwhile, compound 9d exhibited a trend toward reducing the infarct area, although the difference was not statistically significant. While we did not observe significant neuroprotective effects of Edaravone groups in this experiment. Further, in vivo experiments on neuroprotective effects are underway.

**Conclusion**

Based on the SAR analysis of Fenazinel and its toxic metabolite M1, we designed and synthesized a series of novel cinnamamide-piperidine and piperazine derivatives. The results showed that most of these target compounds exhibited potent protective capacities against glutamate-induced cell damage in SH-SY5Y cells, with compound 9d being particularly effective. Furthermore, in subsequent experiments, compound 9d displayed weak hERG inhibitory activity, showing a prolonged lifetime of mice in the hypoxia tolerance model in vivo and exhibiting a trend toward reducing the infarct area in the MCAO model. These promising results suggest that compound 9d may be a valuable candidate for neuroprotection worthy of in-depth study. Additional mechanistic studies and pharmaceutical evaluations of compound 9d are currently underway and will be reported in due course.

**Table 2** In vitro and in vivo data for selected compounds

<table>
<thead>
<tr>
<th>Compd.</th>
<th>hERG IC50\textsuperscript{a} (μmol/L)</th>
<th>Hypoxia tolerance assay\textsuperscript{b}, survival time (second)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 mg/kg</td>
</tr>
<tr>
<td>9d</td>
<td>24.61</td>
<td>4,819.0 ± 1,041.2</td>
</tr>
<tr>
<td>Fenazinel</td>
<td>8.64</td>
<td>5,678.0 ± 1,465.6</td>
</tr>
<tr>
<td>Control (2% DMSO)</td>
<td>–</td>
<td>3,803.4 ± 780.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} hERG Patch clamp screen as described in Dubin et al.\textsuperscript{26} IC50 values represent the concentration to inhibit 50% of hERG current (IKr). Numbers represent IC50 values generated from 3-point concentration–response relationships in duplicate.

\textsuperscript{b} Hypoxia tolerance assay in mice as described in Wang et al.\textsuperscript{6}
Experimental Section

Reagents and Materials
Unless otherwise specified, all reagents and solvents were purchased from commercial sources (Bidepharm Inc.; Aladdin Inc.; Tansooe Inc., and Sigma-Aldrich) and used without further purification. All air-sensitive reactions were performed under an atmosphere of argon with magnetic stirring. 1H nuclear magnetic resonance (NMR) and 13C NMR spectra were collected on Bruker AVANCE III spectrometers in CDCl3, DMSO-d6, and d6-methanol. Chemical shifts were reported as values in parts per million (ppm). The reference resonance peaks were set at 7.26 ppm (CHCl3), 2.50 ppm (CD2OD) for 1H NMR spectra and at 77.23 ppm (CDCl3), 39.52 ppm (DMSO-d6), and 49.00 ppm (CD3OD) for 13C NMR spectra. Low-resolution mass spectra were determined on an Agilent triple quadrupole mass spectrometer with a 1,220 in pole mass spectrometer. All air-sensitive reactions were performed under an atmosphere of argon with magnetic stirring.

General Synthetic Procedure of Intermediate 5a–l
To a solution of substituted benzaldehyde/phenylacetaldheyde (5.0 mmol, 1.0 equiv.) and malonic acid (5.0 mmol, 1.0 equiv.) in pyridine (20 mL) was added piperidine (2 mL). The reaction mixture was stirred at 120°C for 5 hours, at which point TLC indicated that the reaction was complete. After cooling to room temperature, the reaction solution was poured into 50 mL 2N HCl solution to precipitate a white solid. After stirring for 1 hour, the solid was separated by Buchner funnel filtration and washed with water (10 mL x 3) to obtain the crude substitute cinnamic acid 5a–l.

General Synthetic Procedure of Intermediate 7
To a solution of 2-chloroacetyl chloride (36.8 g, 0.25 mol, 1.25 equiv.) and triethylamine (64.5 g, 0.4 mol, 2.0 equiv.) in acetonitrile (80 mL) was added benzylamine (21.43 g, 0.2 mol, 1.0 equiv.) dropwise. The reaction mixture was stirred at room temperature for 6 hours. After reaction completion shown by TLC, acetonitrile was evaporated and recrystallized with ethanol/H2O to afford the desired N-benzyl-2-chloroacetamide (18.4 g, yield: 51%).

To a solution of tert-butylpiperidin-4-ylicarbamate (20.0 g, 0.1 mol, 1.0 equiv.) in acetone (40 mL) was added N-benzyl-2-chloroacetamide (18.4 g, 0.1 mol, 1.0 equiv.), K2CO3 (27.6 g, 0.2 mol, 2.0 equiv.), and KI (0.83 g, 5.0 mmol, 0.025 equiv.). The reaction mixture was stirred at 40°C for 6 hours. The reaction solution was cooled to room temperature, filtered, and concentrated under reduced pressure to obtain intermediate 6 (32.5 g, yield: 93.6%).

To a solution of intermediate 6 (32.5 g, 0.09 mol) in ethyl acetate (80 mL) was added HCl at ethyl acetate solution to pH 3 to 4. After stirring at 21°C for 1 hour, the solid was separated by Buchner funnel filtration and washed with ethyl acetate (10 mL) to afford the desired intermediate 7 (27.9 g, yield: 93.1%).

General Synthetic Procedure of 9a–9l
A solution of substituted cinnamic acid 5a–5l (5.0 mmol, 1.0 equiv.) and HBTU (5.5 mmol, 1.1 equiv.) in N,N-dimethylformamide (50 mL) was stirred at room temperature for 1 hour. And then, to the mixture solution was added intermediate 7 (5.0 mmol, 1.0 equiv.) and triethylamine (15.0 mmol, 3.0 equiv.) dropwise. After stirring at room temperature for 6 hours, the reaction droplets were slowly added to iced-cold water (50 mL) to precipitate the white solid and then stirred for 1 hour. The solid was separated by Buchner funnel filtration and washed with diethyl ether (10 mL x 3). After drying, methanol recrystallization, and hydrochloric acid salt formation, the target compound 9a–9l was obtained.

General Synthetic Procedure of 9m
To a solution of 1-tert-butylcarbonyl piperazine (20.0 g, 0.1 mol, 1.0 equiv.) in acetone (100 mL) was added N-benzyl-2-chloroacetamide (18.4 g, 0.1 mol, 1.0 equiv.), K2CO3 (27.6 g, 0.2 mol, 2.0 equiv.), and KI (0.83 g, 5.0 mmol, 0.025 equiv.). The reaction mixture was stirred at 40°C for 6 hours. After completion, the reaction solution was cooled to room temperature, filtered, and concentrated under reduced pressure to obtain intermediate 10 (31.9 g, yield: 89.1%).

To a solution of intermediate 10 (31.9 g, 0.09 mol) in ethyl acetate (100 mL) was added HCl at ethyl acetate solution to pH 3 to 4. After stirring at 21°C for 1 hour, the solid was separated by Buchner funnel filtration and washed with ethyl acetate (10 mL) to afford the desired intermediate 11 (26.29 g, yield: 89.7%).

A solution of p-methoxycinnamic acid (5.0 mmol, 1.0 equiv.) and HBTU (5.5 mmol, 1.1 equiv.) in N,N-dimethylformamide (50 mL) was stirred at room temperature for 1 hour. Then, intermediate 7 (5.0 mmol, 1.0 equiv.) and triethylamine (TEA, 15.0 mmol, 3.0 equiv.) were added dropwise. After stirring at room temperature for 6 hours, the reaction droplets were slowly...
added to ice-cold water (50 mL) to precipitate the white solid and then continued stirring for 1 hour. The solid was separated by Buchner funnel filtration, washed with diethyl ether (10 mL x 3), and dried to obtain intermediate 12. After hydrochloric acid salt formation, the target compound 9m was obtained (1.02 g, 91.4%).

(E)-(N-(1-(2-benzylamino)-2-oxoethyl)piperidin-4-yl)-3-(4-methoxyphenyl)acrylamide hydrochloride (9a): yield: 85.5%, mp: 256.7–257.5°C. ESI-MS (m/z): calculated for [M + H]+ 408.2209; found 408.33. 1H NMR (400 MHz, DMSO-d6): δ 10.17 (s, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.50 (d, J = 12.0 Hz, 2H), 7.41 (s, 1H), 7.37–7.25 (m, 5H), 6.98 (d, J = 8.0 Hz, 2H), 6.53 (d, J = 16.0 Hz, 1H), 4.36 (d, J = 4.0 Hz, 2H), 4.15–4.00 (m, 3H), 3.79 (s, 3H), 3.51 (d, J = 12.0 Hz, 2H), 3.25–3.18 (m, 2H), 2.08–1.98 (m, 2H), 1.88–1.79 (m, 2H).

(E)-(N-(1-(2-benzylamino)-2-oxoethyl)piperidin-4-yl)-3-(3,4,5-trimethoxyphenyl)acrylamide hydrochloride (9b): yield: 85.8%, mp: 220.0–221.4°C. ESI-MS (m/z): calculated for [M + H]+ 468.2420; found 468.24. 1H NMR (400 MHz, DMSO-d6): δ 10.08 (s, 1H), 9.29 (t, J = 8.0 Hz, 1H), 8.35 (d, J = 8.0 Hz, 1H), 7.10 (s, 1H), 7.37–7.26 (m, 5H), 6.90 (d, J = 4.0 Hz, 2H), 6.61 (d, J = 16.0 Hz, 1H), 4.36 (d, J = 4.0 Hz, 2H), 4.06–3.99 (m, 3H), 3.81 (s, 6H), 3.68 (s, 3H), 3.52–3.47 (m, 2H), 3.25–3.11 (m, 2H), 1.99 (s, 2H), 1.86–1.78 (m, 2H).

(E)-(N-(1-(2-benzylamino)-2-oxoethyl)piperidin-4-yl)-3-(3,4-dimethoxyphenyl)acrylamide hydrochloride (9c): yield: 85.3%, mp: 253.8–254.9°C. ESI-MS (m/z): calculated for [M + H]+ 438.2315; found 438.27. 1H NMR (400 MHz, DMSO-d6): δ 10.06 (s, 1H), 9.23 (d, J = 8.0 Hz, 1H), 8.29 (d, J = 8.0 Hz, 1H), 7.38–7.25 (m, 6H), 7.13 (t, J = 8.0 Hz, 2H), 6.99 (d, J = 8.0 Hz, 1H), 6.53 (d, J = 12.0 Hz, 2H), 4.36 (d, J = 4.0 Hz, 2H), 4.12–3.88 (m, 1H), 3.78 (d, J = 4.0 Hz, 6H), 3.50 (d, J = 12.0 Hz, 2H), 3.20 (q, J = 12.0 Hz, 2H), 2.06–1.98 (m, 2H), 1.85–1.77 (m, 2H).

(E)-(N-(1-(2-benzylamino)-2-oxoethyl)piperidin-4-yl)-3-(2,5-dimethoxyphenyl)acrylamide hydrochloride (9d): yield: 79.1%, mp: 177.4–179.4°C. ESI-MS (m/z): calculated for [M + H]+ 408.2209; found 408.2. 1H NMR (400 MHz, DMSO-d6): δ 8.25 (t, J = 8.0 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H), 7.63 (d, J = 12.0 Hz, 1H), 7.50 (d, J = 4.0 Hz, 1H), 7.38–7.34 (m, 3H), 7.26–7.21 (m, 3H), 7.07 (d, J = 8.0 Hz, 1H), 6.98 (t, J = 8.0 Hz, 1H), 6.65 (d, J = 16.0 Hz, 1H), 4.30 (d, J = 4.0 Hz, 2H), 3.85–3.63 (m, 1H), 2.97 (s, 2H), 2.77 (d, J = 12.0 Hz, 2H), 2.17 (t, J = 8.0 Hz, 2H), 1.77 (d, J = 8.0 Hz, 2H), 1.54–1.45 (m, 2H).

(E)-(N-(1-(2-benzylamino)-2-oxoethyl)piperidin-4-yl)-3-(3,4,5-trimethoxyphenyl)acrylamide hydrochloride (9e): yield: 82.1%, mp: 190.3–192.7°C. ESI-MS (m/z): calculated for [M + H]+ 468.2420; found 468.2. 1H NMR (400 MHz, DMSO-d6): δ 8.25 (t, J = 8.0 Hz, 1H), 8.00 (d, J = 8.0 Hz, 1H), 7.50 (d, J = 16.0 Hz, 1H), 7.34–7.23 (m, 6H), 6.88 (d, J = 8.0 Hz, 1H), 6.57 (d, J = 16.0 Hz, 1H), 4.30 (d, J = 4.0 Hz, 2H), 3.83 (s, 3H), 3.80 (s, 3H), 3.75 (s, 3H), 3.67–3.60 (m, 1H), 2.97 (s, 2H), 2.77 (d, J = 12.0 Hz, 2H), 2.18 (t, J = 8.0 Hz, 2H), 1.77 (d, J = 8.0 Hz, 2H), 1.53–1.45 (m, 2H).

N-(1-(2-benzylamino)-2-oxoethyl)piperidin-4-yl)cinnamamide hydrochloride (9f): yield: 87.4%, mp: 148.4–149.5°C. ESI-MS (m/z): calculated for [M + H]+ 378.2103; found 378.2. 1H NMR (400 MHz, DMSO-d6): δ 8.50 (t, J = 8.0 Hz, 2H), 8.25 (t, J = 4.0 Hz, 1H), 8.10 (dd, J = 3.6 Hz, 4.4 Hz, 1H), 7.87–7.85 (m, 1H), 7.32 (t, J = 8.0 Hz, 2H), 7.26–7.22 (m, 6H), 6.29 (d, J = 8.0 Hz, 1H), 4.30 (t, J = 4.0 Hz, 2H), 3.48 (s, 1H), 2.97 (s, 2H), 2.72 (d, J = 4.0 Hz, 2H), 2.21 (t, J = 4.0 Hz, 2H), 1.80 (d, J = 8.0 Hz, 2H), 1.49–1.43 (m, 2H).

(E)-(N-(1-(2-benzylamino)-2-oxoethyl)piperidin-4-yl)-4-phenylbut-2-enamide hydrochloride (9g): yield: 75.3%.
Neuroprotection Assay against Glu-Induced Cell Damage in SH-SY5Y Cells

Cortical Neuron Culture
Postnatal 1-day-old Sprague-Dawley (SD) Suckling rats (Sipu-BiKAI, SCXK2008-0016) were decapitated under sterile conditions in an ultra-clean table, and bilateral cortices were collected in ice-cold D-Hanks solution (Boster) with a curved forceps. The meninges, blood vessels, and other tissues were carefully removed, and the tissue was cut to 1 mm³ by iris scissors. Then, an appropriate amount of trypsin (0.125%) was added and digested at 37°C for 20 minutes. The trypsin was discarded, and the whole culture solution (DMEM [Corning Cellgro] containing 10% serum was added. Except for the normal group and the model group, model group, drug treatment group, and positive control group (for primary screening compounds screening was expressed as mean ± standard deviation. For rigorous statistical comparisons require at least three independent batches of experiments.

Hypoxia Tolerance Assay
A total of 30 male ICR (Institute of Cancer Research) mice (Sipu-BiKAI, SCXK2008-0016), weighing 18 to 20 g, were divided into three groups: DMSO control group, Fenazinel, 9d in the dose of 20 mg/kg (10 animals per group). The injection volume was 0.1 mL/10 g. After the mice were injected with each sample through the tail vein, the mice in each group were placed in 250 mL grinding mouth bottles containing 5 g sodium lime (1 mouse per bottle), capped, and sealed. The respiratory arrest was taken as an indication of death, and the survival time of the mice was observed. The t-test was used for statistical analysis, and all data were expressed as mean ± standard deviation.

hERG Inhibitory Activity Assay
Cell Culture Preparation
CHO-hERG cells were cultured in 175 cm² culture flasks, and when the cell density had grown to 60 to 80%, the culture medium was removed, washed once with 7 mL phosphate-buffered saline, and then digested with 3 mL Detachin. After complete digestion, add 7 mL culture medium to neutralization, then centrifugation, suction up to the supernatant, and add 5 mL culture medium to resuspension, to ensure the cell density of 2–5 × 10⁶/mL.

Electrophysiological Recording Process
The single-cell high-impedance sealing and whole-cell patch clamp method were all performed automatically by the Qpatch instrument. The after-hyperpolarization mode was obtained, the cell was clamped at −80 mV, followed by a 50 milliseconds prepotential of −50 mV before a 5-second +40 mV depolarization stimulus, and then repolarized to −50 mV for 5 seconds. Then go back to −80 millivolts. This voltage stimulus was applied every 15 seconds and recorded for 2 minutes followed by 5 minutes of extracellular fluid recording and then the administration process was started. The compound concentration was 40, 13.33, 4.44, 1.48, 0.49, and 0.16 μmol/L, respectively, starting from the lowest tested concentration. Each test concentration was administered for 2.5 minutes, and after all, concentrations...
were administered consecutively, and the positive control compound 3 μmol/L Fenazinel was administered. At least three cells (n ≥ 3) were tested for each concentration.

**MCAO-Induced Cerebral Ischemia/Reperfusion Injury Model**

Pharmacological studies were performed by the Center for Pharmacological Evaluation and Research according to protocols approved by the Animal Care and Use Committee of the Shanghai Institute of Pharmaceutical Industry. All experiments are reported in compliance with the ARRIVE (Animal Research: Reporting in vivo Experiments) guidelines. Healthy male SD rats were divided into four groups, namely, model group, compound 7591 (5 mg/kg), Fenazinel/5052 (5 mg/kg), and Edaravone (5 mg/kg). The rats were anesthetized by intraperitoneal injection of 12% chloral hydrate (360 mg/kg) and fixed on the operating table supine. The nylon thread with a diameter of 0.26 mm and a length of 20 mm entered from the external carotid artery to the proximal end of the anterior cerebral artery. All blood flow sources of the MCA were blocked. Then, 1.5 hours later, the nylon thread was pulled out to re-flow the blood. The rats were intraperitoneally injected test samples and raised in separate cages.

The rats were sacrificed 24 hours after administration. Their brains were taken and on average cut into five slices. Then, pathological sections were placed in TTC solution and incubated at 37°C for 5 to 10 minutes for staining. The injected test samples and raised in separate cages.

The rats were sacrificed 24 hours after administration. Their brains were taken and on average cut into five slices. Then, pathological sections were placed in TTC solution and incubated at 37°C for 5 to 10 minutes for staining. The infarct area is not colored, and the normal brain tissue is stained red.

**Conflict of Interest**

None declared.

**Acknowledgments**

We gratefully acknowledge financial support from the National Science and Technology Major Project (Grant No. 2018ZX09711002-002-009), the National Natural Science Foundation of China (Grant No. 81703358), the Science and Technology Commission of Shanghai Municipality (Grant Nos. 17431903900, 18QB1404200, 21511908000, 22ZR1460300), and the Graduate Innovation Fund Project of China State Institute of Pharmaceutical Industry (Grant Nos. YJS2021013, YJS2021011).

**Reference**