

Article

Synthesis and Protective Effect of New Ligustrazine-Benzoic Acid Derivatives against CoCl₂-Induced Neurotoxicity in Differentiated PC12 Cells

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Abstract: A series of novel ligustrazine-benzoic acid derivatives were synthesized and evaluated for their protective effect against cobalt chloride-induced neurotoxicity in differentiated PC12 cells. Combining hematoxylin and eosin staining, we found compound that (3,5,6-trimethylpyrazin-2-yl)methyl 3-methoxy-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (**4a**) displayed promising protective effect on the proliferation of the injured PC12 cells (EC₅₀ = 4.249 μM). Structure-activity relationships are briefly discussed.

Keywords: ligustrazine; neuroprotective effect; cobalt chloride; PC12 cell; HE staining

1. Introduction

Stroke is one of the most devastating diseases in China as well as worldwide. Most strokes (80%) are ischemic and the disease relates with both cerebrovascular system and cranial nerves [1]. Currently, treatments for ischemic stroke include the use of thrombolytic and neuroprotective agents;

thrombolytic drugs lyse blood clots to restore blood flow, and neuroprotective treatments prevent cell death during and after ischemia and reperfusion [2,3]. Despite the remarkable progress achieved in the therapy of stroke during the last two decades, there is no effective chemotherapy for cerebral ischemic stroke and neuroprotective agents that could attenuate lots of the clinical problems of ischemic stroke [1,4], so the development of new neuroprotective agents that inhibit neuronal damage induced by cerebral ischemia is of great significance [4].

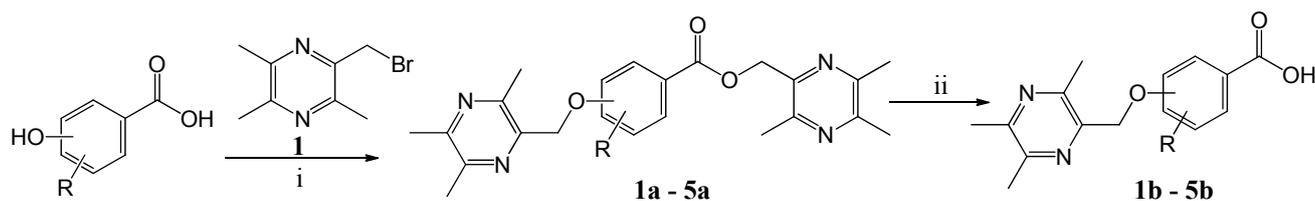
Ligustrazine (2,3,5,6-tetramethylpyrazine, TMP), one of the major effective components of the Chinese traditional medicinal herb *Ligusticum Chuanxiong Hort*, has been widely used for ischemic stroke therapy in China for many years. TMP has multiple mechanisms of action, including thrombolysis and neuroprotection, both of which were important for effectively protecting the brain tissue from ischemic and reperfusion damage in ischemic stroke [5–8]. A series of previous studies proved that ligustrazinyloxy-aromatic acid derivatives could inhibit platelet aggregation and protect damaged endothelial cell proliferation, and that they exhibited much higher activities than TMP [9–11]. Many of these aromatic acids, such as protocatechuic acid, vanillic acid, salicylic acid, the parent compounds of ligustrazine derivatives, also showed interesting neuroprotective activity [12–16]. To further improve TMP's neuroprotective effects, inspired by the biological characteristics of TMP and aromatic acids, we integrated the ligustrazine ring and hydroxybenzoic acid fragments into one molecule according to the combination principle in medicinal chemistry, constructing a series of new ligustrazine-benzoic acid derivatives.

PC 12 is a cell line derived from a rat adrenal medulla pheochromocytoma. The differentiated PC12 cells induced by nerve growth factor (NGF) have the typical characteristic of the neurons in form and function; therefore it is widely used as a model for *in vitro* neuron research [17,18]. Cobalt chloride (CoCl_2), a water-soluble compound, was used in this investigation because it is one of the best-known chemical inducer of PC12 injury; CoCl_2 -induced neurotoxicity in differentiated PC12 cells is commonly used to screen new candidates for the intervention of stroke [19,20]. Herein, the novel ligustrazine-benzoic acid derivatives' neuroprotective activities were evaluated on CoCl_2 -induced damage in differentiated PC12 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and hematoxylin and eosin (HE) staining. Structure-activity relationships (SARs) of these new compounds are also discussed.

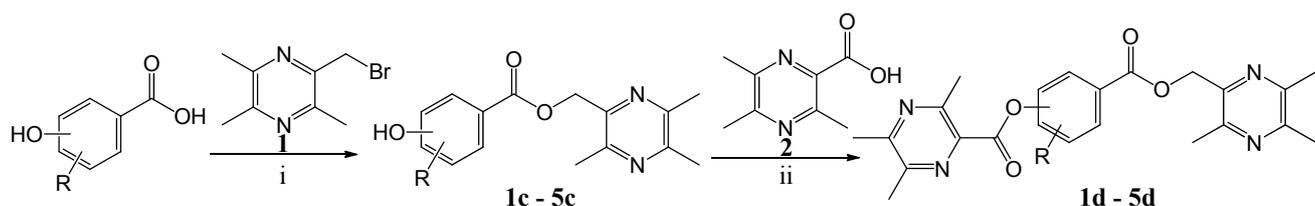
2. Results and Discussion

2.1. Chemistry

All the designed derivatives were synthesized via the routes outlined in Scheme 1 and Scheme 2. As the important intermediates, 2-(bromomethyl)-3,5,6-trimethylpyrazine (**1**) and 3,5,6-trimethylpyrazine-2-carboxylic acid (**2**) were prepared according to our previous study [21,22]. In Scheme 1, typical synthetic procedures for **1a–5a** involved the combination of bromo TMP and other hydroxybenzoic acids through the formation of ester and ether bonds under alkaline conditions, followed by hydrolysis of the corresponding ligustrazinyloxybenzoic acid esters **1a–5a** in 20% (w/v) KOH aqueous solution, to obtain compounds **1b–5b**.

Scheme 1. Synthetic routes to ligustrazine derivatives **1a–5a**, **1b–5b**.

Reagents and Conditions: (i) DMF, K_2CO_3 , 85 °C, 1.5 h; (ii) 20% KOH, 60 °C, 45 min.

Scheme 2. Synthetic routes to ligustrazine derivatives **1c–5c**, **1d–5d**.

Reagents and Conditions: (i) DMF, $NaHCO_3$, r.t., 12 h; (ii) CH_2Cl_2 , EDCI/DMAP, r.t., 16 h.

In Scheme 2, single ester combination of TMP with hydroxybenzoic acids **1c–5c** was achieved by controlling the reaction temperature and alkali catalyst strength. Compounds **1c–5c** were reacted with 3,5,6-trimethylpyrazine-2-carboxylic acid (**2**) catalyzed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) in CH_2Cl_2 , respectively; we thus successfully obtained compounds **1d–5d**. The structures of all the target compounds (Table 1) were confirmed by 1H -NMR, ^{13}C -NMR, MS and high resolution mass (HRMS). Of all the compounds, **1a–5a**, **2b**, **5b**, **1c–5c**, **1d–5d** were new compounds and **1b**, **3b**, **4b** had been reported previously [11,23], but none of the compounds' neuroprotective activities had been explored.

Table 1. The structures of ligustrazine-benzoic acid derivatives.

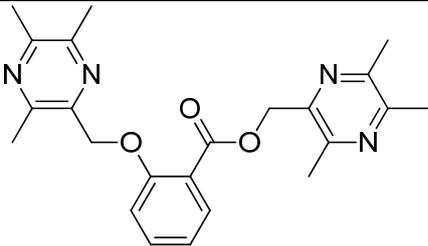
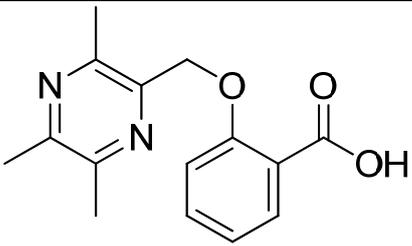
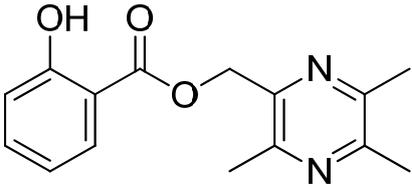
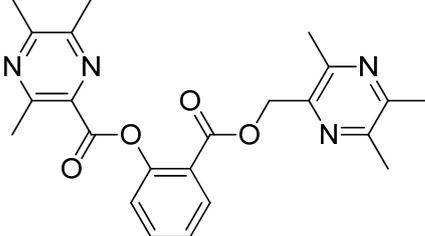
| Structure | Yield | Structure | Yield |
|---|-------|--|-------|
|  | 61.1% |  | 85.1% |
|  | 64.0% |  | 47.6% |

Table 1. Cont.

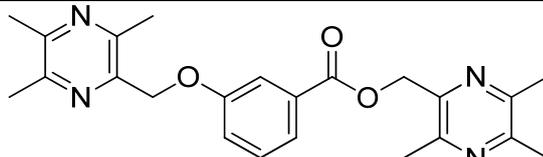
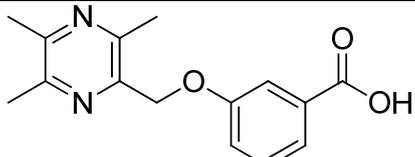
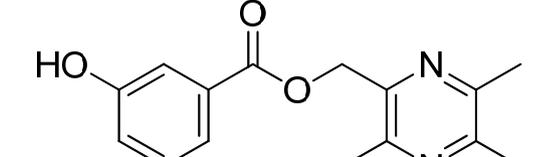
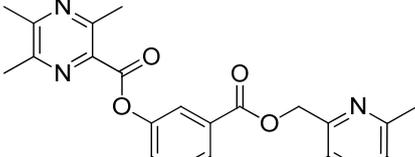
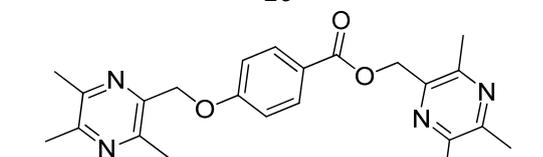
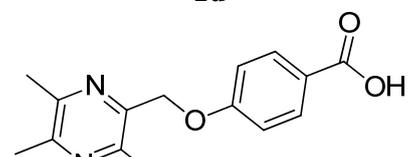
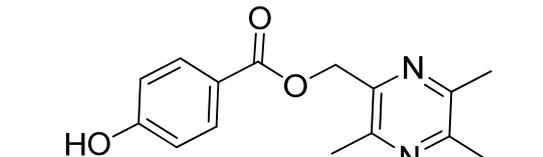
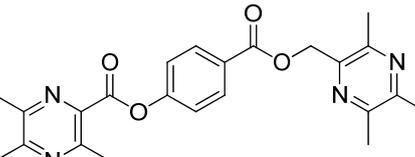
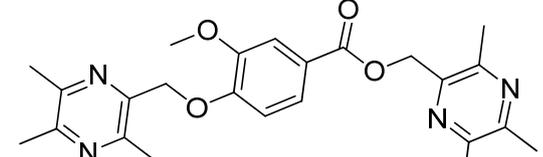
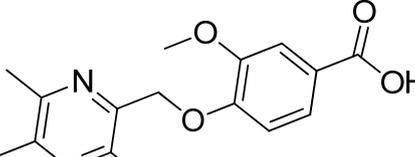
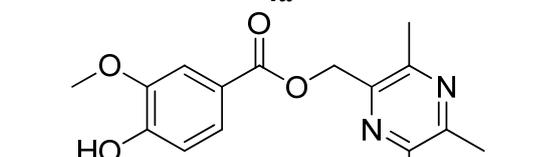
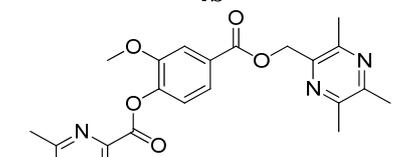
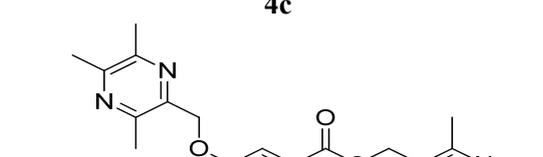
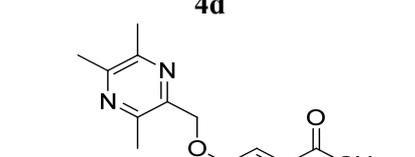
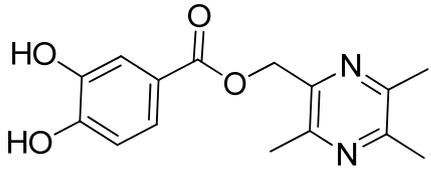
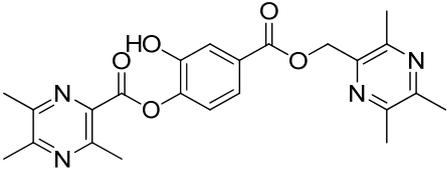
| Structure | Yield | Structure | Yield |
|---|-------|--|-------|
|  | 61.7% |  | 81.9% |
| 2a | | 2b | |
|  | 64.4% |  | 52.6% |
| 2c | | 2d | |
|  | 61.7% |  | 89.7% |
| 3a | | 3b | |
|  | 63.2% |  | 60.1% |
| 3c | | 3d | |
|  | 62.5% |  | 87.4% |
| 4a | | 4b | |
|  | 67.5% |  | 54.7% |
| 4c | | 4d | |
|  | 58.4% |  | 87.1% |
| 5a | | 5b | |

Table 1. Cont.

| Structure | Yield | Structure | Yield |
|---|-------|--|-------|
|  | 51.2% |  | 54.7% |
| 5c | | 5d | |

2.2. Biological Activities

2.2.1. Protective Effect on Injured Neuronal-like PC12 Cells

Using TMP as the positive control drug, all the synthesized compounds were tested for their protective effects on neuronal-like PC12 cells damaged by CoCl_2 . This revealed the proliferation rates (%) at different concentration and 50% effective concentrations (EC_{50}) for protecting damaged PC12 cells of the ligustrazine-benzoic acid derivatives in Table 2. The results showed that TMP and its derivatives presented protective effects on injured differentiated PC12 cells and most of the ligustrazine derivatives were more active (with lower EC_{50} values) than TMP ($\text{EC}_{50} = 64.459 \mu\text{M}$).

Table 2. The EC_{50} of the ligustrazine-benzoic acid derivatives for protecting damaged PC12 cells.

| Compound | Proliferation rate (%) | | | | | EC_{50} (μM) |
|----------|------------------------|------------------|------------------|-------------------|--------------------|------------------------------------|
| | 60 μM | 30 μM | 15 μM | 7.5 μM | 3.75 μM | |
| 1a | 61.63 | 58.87 | 27.69 | 18.97 | 4.58 | 27.359 |
| 2a | 16.27 | 25.49 | 27.48 | 33.71 | 15.65 | 40.715 |
| 3a | 81.60 | 77.58 | 64.50 | 32.08 | 30.07 | 11.467 |
| 4a | 126.60 | 117.10 | 89.76 | 52.98 | 31.12 | 4.249 |
| 5a | 47.14 | 36.44 | 36.01 | -7.22 | -5.94 | 45.029 |
| 1b | 5.50 | 32.37 | -1.76 | -8.25 | -16.00 | 90.345 |
| 2b | 16.91 | 27.96 | 6.99 | -22.43 | -22.76 | 95.484 |
| 3b | 27.30 | 46.91 | 33.84 | 11.21 | 6.44 | 38.493 |
| 4b | 6.29 | 10.84 | -2.28 | -10.40 | -10.73 | >100 |
| 5b | 27.14 | 0.47 | -0.08 | -3.71 | -5.67 | 85.723 |
| 1c | 21.88 | 21.67 | 19.32 | -1.87 | -17.07 | 73.801 |
| 2c | 55.78 | 90.74 | 96.07 | 72.74 | 58.09 | 5.828 |
| 3c | 57.72 | 18.75 | 9.20 | 22.27 | -1.86 | 43.909 |
| 4c | 9.18 | 12.54 | 14.28 | 12.04 | 7.31 | 66.230 |
| 5c | 15.68 | 27.93 | 27.45 | -7.01 | -11.72 | 68.265 |
| 1d | 50.28 | 41.08 | 22.15 | 13.55 | 2.86 | 37.398 |
| 2d | 29.07 | 44.19 | 37.79 | 36.63 | 1.163 | 33.245 |
| 3d | 46.79 | 40.13 | 41.79 | 36.13 | 33.47 | 22.210 |
| 4d | 15.27 | 62.41 | 51.74 | 30.06 | 1.16 | 29.683 |
| 5d | 11.50 | 37.43 | 23.97 | 14.19 | 11.90 | 46.678 |
| TMP | 14.71 | 12.11 | 11.76 | 10.60 | 9.44 | 64.459 |

The derivatives containing bis-ligustrazine substituents **1a–5a** and **2d–5d** exhibited high potency, with EC₅₀ values below 50 μM; among them **4a** was the most active compound, with an EC₅₀ value of 4.249 μM. We found that most of bis-ligustrazine derivatives' neuroprotective activities were better than that of the single-ligustrazine compounds. This result revealed the combinational effects of two ligustrazinyl pharmacophores. This structure-activity relationship analysis was in agreement with the previously studied protective effects of ligustrazine derivatives on damaged ECV-304 cells [24]. However, this principle did not work in the case of **2c** (EC₅₀ = 5.828 μM) on the injured PC12 cells model; this was possibly caused by the usage of different cell types.

In addition, it was observed that ether-joined derivatives' protective effects were better than those of the ester ones, as exemplified by comparison of the respective pairs of compounds such as **1a** > **1d**, **3a** > **3d**, **4a** > **4d**, **5a** > **5d**. Structure-activity relationship analysis among **1a–5a** revealed that compounds with substituents at the *para*-position of the benzyloxy moiety seemed to be more active than those with substituents at other positions in the same series, and approximately followed a tendency in activity 4-OH, 3-OCH₃ > 4-OH > 2-OH > 3-OH > 3, 4-OH. These findings may provide a new framework for the design of new ligustrazine derivatives as neuroprotective drugs for treating cerebral ischemic stroke.

Moreover, previous studies displayed that **3b** could inhibit platelet aggregation properties [11,23]; while the current study also showed that **3b** and its congener structures exhibited good neuroprotective activities. Based on the above evidence, we reason that ligustrazine-benzoic acid derivatives possess both thrombolytic and neuroprotective effects and may be more efficacious than either a thrombolytic or neuroprotective agent alone.

2.2.2. Effect of **4a** on CoCl₂-Induced Cell Injury

Under optical microscopy, we found that undifferentiated PC12 cells proliferated to form clone-like cell clusters without neural characteristics as shown in Figure 1-I-A; normal differentiated PC12 cells showed round cell bodies with fine dendritic networks, and the cell edges were intact and clear (Figure 1-I-B); moreover, the mean value expressed as percent of neurite-bearing cells in NGF treated cells was 51.7% (Figure 1-II). In contrast, incubation of cells with 200 mM of CoCl₂ for 12 h induced shrinkage of the cell bodies, disappearance of cell reticular formation, and disruption of the dendritic networks (Figure 1-I-C); the mean value of neurite-bearing cells (21.8%, Figure 1-II) showed a significant decrease. Pretreatment with **4a** dramatically alleviated morphological manifestations of cell damage and led to a pronounced increase (65.8%, Figure 1-II) in neurite-bearing cells compared to model cells (Figure 1-I-D).

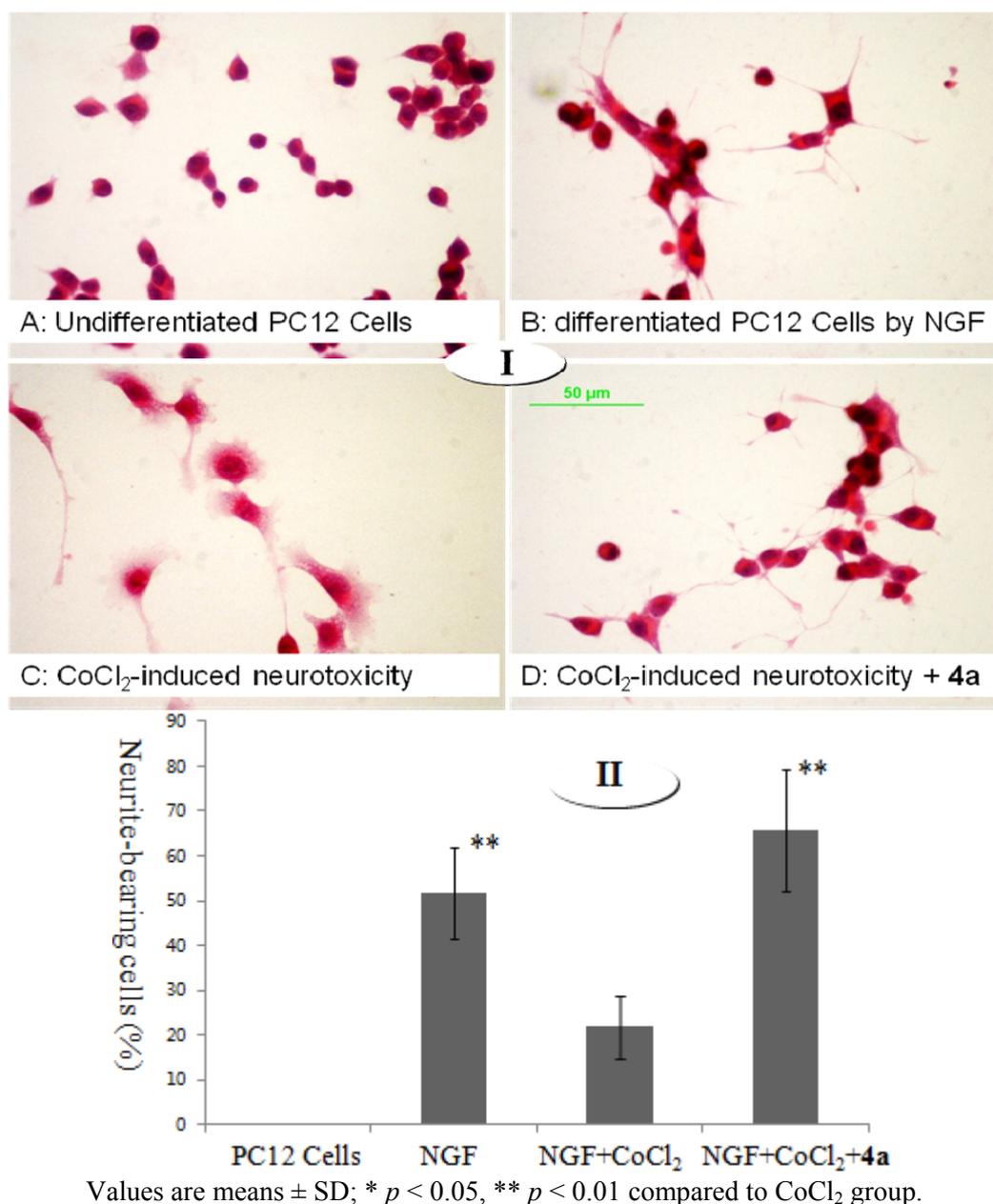
3. Experimental Section

3.1. Chemistry

Reactions were monitored by TLC using silica gel coated aluminum sheets (Qingdao Haiyang Chemical Co., Qingdao, China) and visualized in UV light (254 nm). ¹H-NMR and ¹³C-NMR assays were recorded on a Bruker AVANCE 500 NMR spectrometer (Fällanden, Switzerland) and chemical shifts are reported in δ (ppm). Mass spectra were recorded with an LC Autosampler Device: Standard

G1313A instrument (Agilent, New York, NY, USA). HRMS spectra were obtained using a Thermo Scientific™ LTQ Orbitrap XL hybrid FTMS instrument (Thermo Technologies, New York, NY, USA). Melting points (uncorrected) were measured on an X-5 micro melting point apparatus (Beijing, China). Flash column chromatography was performed using 200–300 mesh silica gel. The yields were calculated based on the last step reaction. All chemicals and solvents used were analytical or high-performance liquid chromatography grade.

Figure 1. Effect of **4a** on differentiated PC12 cell injury induced by CoCl_2 .



2-(Bromomethyl)-3,5,6-trimethylpyrazine (1). Compound **1** was prepared according to our previously reported method [21]. The crude product, with 70% purity, was not purified further as it caused a strong mucous membrane irritation.

3,5,6-Trimethylpyrazine-2-carboxylic acid (2). Compound **2** was prepared according to the method described by Wang *et al.* and Li *et al.*, with minor modifications [22,25]. To a solution of trimethylpyrazine (5.0 g, 36.8 mmol) in water (200 mL), aqueous potassium permanganate (KMnO₄) solution (8.6 g KMnO₄:150 mL water) was added dropwise at room temperature over about 60 min. Upon completion of the addition, the mixture was stirred at 50 °C for 12 h, then the warm reaction mixture was filtered and washed with hot water (300 mL, 90 °C). The filtrate and washing liquor were combined, cooled to 0–5 °C, and the pH adjusted to 2.0 with concentrated hydrochloric acid. Extraction was performed with ethyl acetate (200 mL × 3), and the organic phase was dried with anhydrous sodium sulfate. The solvent was removed by distillation under vacuum, and the residue was recrystallized from acetone to produce a light yellow solid (2.39 g, yield: 47.8%), m.p.: 162–163 °C [26].

3.1.1. General Procedure for the Preparation of Ligustrazine Derivatives **1a–5a** (Scheme 1)

Compound **1** (9.0 mmol) and the corresponding hydroxybenzoic acid (3.0 mmol) were dissolved in dry DMF, then K₂CO₃ (6.0 mmol) was added and the mixture was kept at 85 °C for 1.5 h under a nitrogen atmosphere. The warm reaction mixture was poured into ice-water and the crude product was extracted with ethyl acetate. After drying the organic layer over anhydrous Na₂SO₄ and evaporating the solvent under vacuum, the crude products were purified by flash chromatography and recrystallization from acetone.

(3,5,6-Trimethylpyrazin-2-yl)methyl 2-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (1a). White solid, m.p.: 76.2–76.9 °C. ¹H-NMR (CDCl₃) (ppm): 7.84 (m, 1H, Ar-H), 7.45 (m, 1H, Ar-H), 7.16 (m, 1H, Ar-H), 6.98 (m, 1H, Ar-H), 5.36 (d, *J* = 2 Hz, 2H, -CH₂), 5.22 (d, *J* = 2 Hz, 2H, -CH₂), 2.45–2.52 (m, 18H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 165.8, 158.0, 151.2, 151.2, 150.4, 149.4, 148.8, 148.3, 145.2, 144.9, 133.6, 132.0, 120.6, 120.1, 113.6, 70.7 (-CH₂), 65.8 (-CH₂), 21.7 (-CH₃), 21.7 (-CH₃), 21.4 (-CH₃), 21.3 (-CH₃), 20.6 (-CH₃), 20.4 (-CH₃). MS (ESI) *m/z*: 407.1 [M+H]⁺, HRMS (ESI) *m/z*: 407.20633 [M+H]⁺, calcd. for C₂₃H₂₇N₄O₃ 407.20049.

(3,5,6-Trimethylpyrazin-2-yl)methyl 3-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (2a). White solid, m.p.: 135.1–135.7 °C. ¹H-NMR (CDCl₃) (ppm): 7.70 (brs, 1H, Ar-H), 7.67 (m, 1H, Ar-H), 7.35 (m, 1H, Ar-H), 7.21 (m, 1H, Ar-H), 5.45 (s, 2H, -CH₂), 5.18 (s, 2H, -CH₂), 2.51–2.60 (m, 18H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 166.0, 158.6, 151.5, 151.5, 149.9, 149.4, 149.0, 148.8, 145.2, 144.8, 131.1, 129.5, 122.7, 120.2, 115.4, 70.0 (-CH₂), 66.0 (-CH₂), 21.7 (-CH₃), 21.7 (-CH₃), 21.5 (-CH₃), 21.4 (-CH₃), 20.6 (-CH₃), 20.6 (-CH₃). MS (ESI) *m/z*: 407.0 [M+H]⁺, HRMS (ESI) *m/z*: 407.20618 [M+H]⁺, calcd. for C₂₃H₂₇N₄O₃ 407.20049.

(3,5,6-Trimethylpyrazin-2-yl)methyl 4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (3a). White solid, m.p.: 70.2–71.0 °C. ¹H-NMR (CDCl₃) (ppm): 8.00 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.02 (d, *J* = 9.0 Hz, 2H, Ar-H), 5.42 (s, 2H, -CH₂), 5.21 (s, 2H, -CH₂), 2.52–2.59 (m, 18H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 165.9, 162.5, 151.6, 151.3, 150.0, 149.4, 149.0, 148.7, 145.1, 145.0, 131.8, 122.6, 114.5, 70.0 (-CH₂), 65.7 (-CH₂), 21.7 (-CH₃), 21.7 (-CH₃), 21.5 (-CH₃), 21.4 (-CH₃), 20.6 (-CH₃), 20.6 (-CH₃). MS (ESI) *m/z*: 407.0 [M+H]⁺, HRMS (ESI) *m/z*: 407.20627 [M+H]⁺, calcd. for C₂₃H₂₇N₄O₃ 407.20049.

(3,5,6-Trimethylpyrazin-2-yl)methyl 3-methoxy-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy] benzoate (**4a**). White solid, m.p.: 108.8–109.6 °C. ¹H-NMR (CDCl₃) (ppm): 7.64 (dd, *J* = 8.5, 1.5 Hz, 1H, Ar-H), 7.55 (d, *J* = 1.5 Hz, 1H, Ar-H), 7.06 (d, *J* = 8.5 Hz, 1H, Ar-H), 5.43 (s, 2H, -CH₂), 5.27 (s, 2H, -CH₂), 3.88 (s, 3H, -OCH₃), 2.52–2.62 (m, 18H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 166.0, 152.1, 151.3, 151.2, 150.0, 149.2, 149.2, 149.1, 148.7, 145.2, 145.2, 123.6, 122.8, 112.7, 112.6, 70.7 (-CH₂), 65.7 (-CH₂), 56.0 (-OCH₃), 21.6 (-CH₃), 21.6 (-CH₃), 21.5 (-CH₃), 21.4 (-CH₃), 20.6 (-CH₃), 20.6 (-CH₃). MS (ESI) *m/z*: 437.2 [M+H]⁺, HRMS (ESI) *m/z*: 437.21692 [M+H]⁺, calcd. for C₂₄H₂₉N₄O₄ 437.21106.

(3,5,6-Trimethylpyrazin-2-yl)methyl 3,4-bis[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoate (**5a**). White solid, m.p.: 132.9–133.7 °C. ¹H-NMR (CDCl₃) (ppm): 7.76 (brs, 1H, Ar-H), 7.69 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.05 (d, *J* = 8.5 Hz, 1H, Ar-H), 5.43 (s, 2H, -CH₂), 5.23 (s, 2H, -CH₂), 5.17 (s, 2H, -CH₂), 2.47–2.60 (m, 27H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 165.8, 152.8, 151.3, 151.3, 151.1, 150.1, 150.0, 149.3, 149.0, 148.6, 148.6, 148.1, 145.4, 145.1, 145.1, 124.5, 122.8, 115.4, 113.1, 71.1 (-CH₂), 70.8 (-CH₂), 65.8 (-CH₂), 21.7 (-CH₃), 21.7 (-CH₃), 21.6 (-CH₃), 21.5 (-CH₃), 21.3 (-CH₃), 21.3 (-CH₃), 20.6 (-CH₃), 20.6 (-CH₃), 20.5 (-CH₃). MS (ESI) *m/z*: 557.2 [M+H]⁺, HRMS (ESI) *m/z*: 557.28503 [M+H]⁺, calcd. for C₃₁H₃₇N₆O₄ 557.27980.

3.1.2. General Procedure for the Preparation of Ligustrazine Derivatives **1b–5b** (Scheme 1)

An aqueous solution of KOH (4.0 mmol) was added to a solution of ligustrazine derivatives **1a–5a** (2.0 mmol) in ethanol (30 mL). The mixture was stirred at 60 °C for 40 min (checked by TLC). Upon completion of the reaction, pH was adjusted to 3 with 10% hydrochloric acid, and then the solution was extracted with ethyl acetate (100 mL). The organic layer was washed with water (100 mL × 2), dried over anhydrous Na₂SO₄ and evaporated. The solids were dried under vacuum and purified by recrystallization from acetone to give pure target compounds.

2-[(3,5,6-Trimethylpyrazin-2-yl)methoxy]benzoic acid (**1b**). White solid, m.p.: 172.2–172.9 °C. ¹H-NMR (CDCl₃) (ppm): 8.10 (d, *J* = 7.5 Hz, 1H, Ar-H), 7.53 (t, *J* = 8.0 Hz, 1H, Ar-H), 7.16 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.12 (t, *J* = 7.5 Hz, 1H, Ar-H), 5.40 (s, 2H, -CH₂), 2.53 (brs, 6H, -CH₃), 2.52 (s, 3H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 166.2, 157.0, 151.6, 149.1, 146.9, 143.6, 134.4, 133.6, 122.6, 122.6, 113.6, 68.1 (-CH₂), 21.5 (-CH₃), 20.7 (-CH₃), 20.0 (-CH₃). MS (ESI) *m/z*: 273.0 [M+H]⁺, HRMS (ESI) *m/z*: 273.12250 [M+H]⁺, calcd. for C₁₅H₁₇N₂O₃ 273.11609.

3-[(3,5,6-Trimethylpyrazin-2-yl)methoxy]benzoic acid (**2b**). White solid, m.p.: 139.2–139.9 °C. ¹H-NMR (CDCl₃) (ppm): 7.79 (brs, 1H, Ar-H), 7.74 (m, 1H, Ar-H), 7.39 (m, 1H, Ar-H), 7.25 (m, 1H, Ar-H), 5.25 (s, 2H, -CH₂), 2.64 (s, 3H, -CH₃), 2.57 (brs, 6H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 170.7, 158.5, 151.5, 149.9, 149.0, 145.4, 131.2, 129.6, 123.1, 120.7, 115.6, 69.7 (-CH₂), 21.5 (-CH₃), 21.3 (-CH₃), 20.4 (-CH₃). MS (ESI) *m/z*: 273.0 [M+H]⁺, HRMS (ESI) *m/z*: 273.12234 [M+H]⁺, calcd. for C₁₅H₁₇N₂O₃ 273.11609.

4-[(3,5,6-Trimethylpyrazin-2-yl)methoxy]benzoic acid (**3b**). White solid, m.p.: 160.7–161.4 °C. ¹H-NMR (CDCl₃) (ppm): 8.07 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.07 (d, *J* = 8.5 Hz, 2H, Ar-H), 5.26 (s, 2H, -CH₂), 2.63 (s, 3H, -CH₃), 2.56 (s, 3H, -CH₃), 2.56 (s, 3H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 171.2,

162.9, 151.6, 150.0, 148.9, 145.1, 132.3, 122.4, 114.6, 69.9 ($-\underline{\text{C}}\text{H}_2$), 21.7 ($-\underline{\text{C}}\text{H}_3$), 21.4 ($-\underline{\text{C}}\text{H}_3$), 20.5 ($-\underline{\text{C}}\text{H}_3$). MS (ESI) m/z : 273.0 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 273.12234 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{15}\text{H}_{17}\text{N}_2\text{O}_3$ 273.11609.

3-Methoxy-4-[(3,5,6-trimethylpyrazin-2-yl)methoxy]benzoic acid (4b). White solid, m.p.: 190.1–190.9 °C. $^1\text{H-NMR}$ (CDCl_3) (ppm): 7.74 (dd, $J = 8.5, 1.5$ Hz, 1H, Ar-H), 7.61 (d, $J = 1.5$ Hz, 1H, Ar-H), 7.12 (d, $J = 8.5$ Hz, 1H, Ar-H), 5.31 (s, 2H, $-\underline{\text{C}}\text{H}_2$), 3.92 (s, 3H, $-\underline{\text{O}}\underline{\text{C}}\text{H}_3$), 2.65 (s, 3H, $-\underline{\text{C}}\text{H}_3$), 2.54 (s, 3H, $-\underline{\text{C}}\text{H}_3$), 2.55 (s, 3H, $-\underline{\text{C}}\text{H}_3$). $^{13}\text{C-NMR}$ (CDCl_3) (ppm): 171.2, 152.5, 151.5, 150.1, 149.2, 148.8, 145.1, 124.2, 122.5, 112.7, 112.6, 70.6 ($-\underline{\text{C}}\text{H}_2$), 56.0 ($-\underline{\text{O}}\underline{\text{C}}\text{H}_3$), 21.6 ($-\underline{\text{C}}\text{H}_3$), 21.3 ($-\underline{\text{C}}\text{H}_3$), 20.5 ($-\underline{\text{C}}\text{H}_3$). MS (ESI) m/z : 303.2 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 303.13272 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{16}\text{H}_{19}\text{N}_2\text{O}_4$ 303.12666.

3,4-bis[(3,5,6-Trimethylpyrazin-2-yl)methoxy]benzoic acid (5b). White solid, m.p.: 183.8–184.5 °C. $^1\text{H-NMR}$ (CDCl_3) (ppm): 7.76 (d, $J = 1.7$ Hz, 1H, Ar-H), 7.69 (dd, $J = 8.5, 1.7$ Hz, 1H, Ar-H), 7.13 (d, $J = 8.5$ Hz, 1H, Ar-H), 5.27 (s, 2H, $-\underline{\text{C}}\text{H}_2$), 5.24 (s, 2H, $-\underline{\text{C}}\text{H}_2$), 2.53–2.58 (m, 18H, $-\underline{\text{C}}\text{H}_3$). $^{13}\text{C-NMR}$ (CDCl_3) (ppm): 170.4, 153.1, 151.5, 151.4, 150.2, 150.2, 148.7, 148.6, 148.0, 145.4, 145.1, 124.9, 122.8, 115.7, 113.0, 70.9 ($-\underline{\text{C}}\text{H}_2$), 70.7 ($-\underline{\text{C}}\text{H}_2$), 21.6 ($-\underline{\text{C}}\text{H}_3$), 21.6 ($-\underline{\text{C}}\text{H}_3$), 21.3 ($-\underline{\text{C}}\text{H}_3$), 21.2 ($-\underline{\text{C}}\text{H}_3$), 20.5 ($-\underline{\text{C}}\text{H}_3$), 20.5 ($-\underline{\text{C}}\text{H}_3$). MS (ESI) m/z : 423.0 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 423.20093 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{23}\text{H}_{27}\text{N}_4\text{O}_4$ 423.19541.

3.1.3. General Procedure for the Preparation of Ligustrazine Derivatives **1c–5c** (Scheme 2)

Compound **1** (3.0 mmol) and hydroxybenzoic acid (3.0 mmol) were dissolved in dry DMF (25 mL), then NaHCO_3 (4.0 mmol) was added and the mixture was kept at room temperature for 12 h under nitrogen atmosphere. Then reaction mixture was poured into ice-water and the crude product was extracted with ethyl acetate. After drying the organic layer over anhydrous Na_2SO_4 and evaporating the solvent under vacuum, the crude products were purified by flash chromatography and recrystallization from acetone.

(3,5,6-Trimethylpyrazin-2-yl)methyl 2-hydroxybenzoate (1c). White solid, m.p.: 84.4–85.1 °C. $^1\text{H-NMR}$ (CDCl_3) (ppm): 10.66 (s, 1H, $-\underline{\text{O}}\underline{\text{H}}$), 7.85 (dd, $J = 8.0, 1.5$ Hz, 1H, Ar-H), 7.47 (td, $J = 8.0, 1.5$ Hz, 1H, Ar-H), 7.01 (d, $J = 8.0$ Hz, 1H, Ar-H), 6.87 (t, $J = 8.0$ Hz, 1H, Ar-H), 5.49 (s, 2H, $-\underline{\text{C}}\text{H}_2$), 2.61 (s, 3H, $-\underline{\text{C}}\text{H}_3$), 2.56 (s, 3H, $-\underline{\text{C}}\text{H}_3$), 2.54 (s, 3H, $-\underline{\text{C}}\text{H}_3$). $^{13}\text{C-NMR}$ (CDCl_3) (ppm): 169.5, 161.6, 151.7, 149.2, 149.1, 144.2, 135.9, 130.1, 119.2, 117.7, 112.3, 65.8 ($-\underline{\text{C}}\text{H}_2$), 21.7 ($-\underline{\text{C}}\text{H}_3$), 21.4 ($-\underline{\text{C}}\text{H}_3$), 20.6 ($-\underline{\text{C}}\text{H}_3$). MS (ESI) m/z : 273.2 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 273.12241 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{15}\text{H}_{17}\text{N}_2\text{O}_3$ 273.11609.

(3,5,6-Trimethylpyrazin-2-yl)methyl 3-hydroxybenzoate (2c). White solid, m.p.: 110.1–110.8 °C. $^1\text{H-NMR}$ (CDCl_3) (ppm): 7.53 (m, 1H, Ar-H), 7.46 (brs, 1H, Ar-H), 7.26 (m, 1H, Ar-H), 7.04 (m, 1H, Ar-H), 5.42 (s, 2H, $-\underline{\text{C}}\text{H}_2$), 2.59 (s, 3H, $-\underline{\text{C}}\text{H}_3$), 2.55 (s, 3H, $-\underline{\text{C}}\text{H}_3$), 2.53 (s, 3H, $-\underline{\text{C}}\text{H}_3$). $^{13}\text{C-NMR}$ (CDCl_3) (ppm): 166.2, 156.6, 151.7, 149.5, 149.2, 144.9, 129.7, 121.6, 121.6, 120.9, 116.3, 65.6 ($-\underline{\text{C}}\text{H}_2$), 21.4 ($-\underline{\text{C}}\text{H}_3$), 21.2 ($-\underline{\text{C}}\text{H}_3$), 20.3 ($-\underline{\text{C}}\text{H}_3$). MS (ESI) m/z : 273.0 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 273.12201 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{15}\text{H}_{17}\text{N}_2\text{O}_3$ 273.11609.

(3,5,6-Trimethylpyrazin-2-yl)methyl 4-hydroxybenzoate (**3c**). White solid, m.p.: 183.2–184.0 °C. ¹H-NMR (DMSO-*d*₆) (ppm): 10.39 (s, 1H, -OH), 7.81 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.84 (d, *J* = 8.5 Hz, 2H, Ar-H), 5.34 (s, 2H, -CH₂), 2.50 (s, 3H, -CH₃), 2.47 (s, 3H, -CH₃), 2.45 (s, 3H, -CH₃). ¹³C-NMR (DMSO-*d*₆) (ppm): 165.7, 162.6, 151.3, 149.2, 148.9, 145.4, 132.1, 120.3, 115.9, 65.3 (-CH₂), 21.7 (-CH₃), 21.5 (-CH₃), 20.6 (-CH₃). MS (ESI) *m/z*: 273.1 [M+H]⁺, HRMS (ESI) *m/z*: 273.12231 [M+H]⁺, calcd. for C₁₅H₁₇N₂O₃ 273.11609.

(3,5,6-Trimethylpyrazin-2-yl)methyl 4-hydroxy-3-methoxybenzoate (**4c**). White solid, m.p.: 147.8–148.4 °C. ¹H-NMR (CDCl₃) (ppm): 7.62 (dd, *J* = 8.4, 1.5 Hz, 1H, Ar-H), 7.54 (d, *J* = 1.5 Hz, 1H, Ar-H), 6.90 (d, *J* = 8.4 Hz, 1H, Ar-H), 5.41 (s, 2H, -CH₂), 3.91 (s, 3H, -OCH₃), 2.58 (s, 3H, -CH₃), 2.52 (s, 3H, -CH₃), 2.51 (s, 3H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 166.0, 151.3, 150.3, 149.3, 149.0, 146.2, 145.1, 124.4, 121.7, 114.1, 111.8, 65.6 (-CH₂), 56.1 (-OCH₃), 21.6 (-CH₃), 21.4 (-CH₃), 20.6 (-CH₃). MS (ESI) *m/z*: 303.1 [M+H]⁺, HRMS (ESI) *m/z*: 303.13297 [M+H]⁺, calcd. for C₁₆H₁₉N₂O₄ 303.12666.

(3,5,6-Trimethylpyrazin-2-yl)methyl 3,4-dihydroxybenzoate (**5c**). White solid, m.p.: 184.8–185.5 °C. ¹H-NMR (DMSO-*d*₆) (ppm): 9.86 (s, 1H, -OH), 9.42 (s, 1H, -OH), 7.35 (brs, 1H, Ar-H), 7.31 (m, 1H, Ar-H), 6.81 (d, *J* = 8.0 Hz, 1H, Ar-H), 5.32 (s, 2H, -CH₂), 2.50 (s, 3H, -CH₃), 2.46 (s, 3H, -CH₃), 2.43 (s, 3H, -CH₃). ¹³C-NMR (DMSO-*d*₆) (ppm): 165.8, 151.4, 151.1, 149.2, 148.9, 145.6, 145.4, 122.5, 120.6, 116.7, 115.9, 65.3 (-CH₂), 21.7 (-CH₃), 21.5 (-CH₃), 20.6 (-CH₃). MS (ESI) *m/z*: 289.1 [M+H]⁺, HRMS (ESI) *m/z*: 289.11725 [M+H]⁺, calcd. for C₁₅H₁₇N₂O₄ 289.11101.

3.1.4. General Procedure for the Preparation of Ligustrazine Derivatives **1d–5d** (Scheme 2)

To a solution of ligustrazine derivatives **1c–5c** (1.0 mmol) in CH₂Cl₂ (30 mL) were successively added compound **2** (1.2 mmol), EDCI (1.5 mmol), and DMAP (catalytic amount), and the mixture was stirred at room temperature for 16 h under nitrogen atmosphere. The organic layer was washed with brine and water, respectively. After drying the organic layer over anhydrous Na₂SO₄ and evaporating the solvent under vacuum, the crude products were purified by flash chromatography and the target compound was recrystallized from acetone.

2-[[3,5,6-Trimethylpyrazin-2-yl)methoxy]carbonyl]phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**1d**). White solid, m.p.: 89.4–90.1 °C. ¹H-NMR (CDCl₃) (ppm): 8.16 (m, 1H, Ar-H), 7.63 (m, 1H, Ar-H), 7.39 (m, 1H, Ar-H), 7.26 (m, 1H, Ar-H), 5.30 (s, 2H, -CH₂), 2.68 (s, 3H, -CH₃), 2.63 (brs, 6H, -CH₃), 2.45 (brs, 6H, -CH₃), 2.42 (s, 3H, -CH₃). ¹³C-NMR (CDCl₃) (ppm): 164.3, 164.1, 155.2, 153.0, 151.3, 151.0, 149.5, 149.5, 148.7, 144.2, 137.3, 134.2, 132.4, 126.4, 124.2, 123.2, 66.3 (-CH₂), 23.0 (-CH₃), 22.5 (-CH₃), 21.7 (-CH₃), 21.7 (-CH₃), 21.3 (-CH₃), 20.4 (-CH₃). MS (ESI) *m/z*: 421.0 [M+H]⁺, HRMS (ESI) *m/z*: 421.18494 [M+H]⁺, calcd. for C₂₃H₂₅N₄O₄ 421.17976.

3-[[3,5,6-Trimethylpyrazin-2-yl)methoxy]carbonyl]phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**2d**). White solid, m.p.: 135.1–135.7 °C. ¹H-NMR (CDCl₃) (ppm): 7.97 (m, 1H, Ar-H), 7.91 (brs, 1H, Ar-H), 7.50 (m, 1H, Ar-H), 7.45 (m, 1H, Ar-H), 5.44 (s, 2H, -CH₂), 2.81 (s, 3H, -CH₃), 2.61 (s, 3H, -CH₃), 2.61 (s, 3H, -CH₃), 2.58 (s, 3H, -CH₃), 2.51 (s, 3H, -CH₃), 2.50 (s, 3H, -CH₃). ¹³C-NMR (CDCl₃)

(ppm): 165.2, 164.2, 155.5, 152.7, 151.5, 150.8, 149.7, 149.3, 149.1, 144.6, 137.9, 131.4, 129.5, 127.5, 126.8, 123.3, 66.1 ($-\underline{\text{C}}\underline{\text{H}}_2$), 22.9 ($-\underline{\text{C}}\underline{\text{H}}_3$), 22.3 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.7 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.6 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.5 ($-\underline{\text{C}}\underline{\text{H}}_3$), 20.6 ($-\underline{\text{C}}\underline{\text{H}}_3$). MS (ESI) m/z : 421.0 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 421.18512 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{23}\text{H}_{25}\text{N}_4\text{O}_4$ 421.17976.

4-[[*(3,5,6-Trimethylpyrazin-2-yl)methoxy*]carbonyl]phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**3d**). White solid, m.p.: 89.5–90.2 °C. $^1\text{H-NMR}$ (CDCl_3) (ppm): 8.14 (d, $J = 8.5$ Hz, 2H, Ar-H), 7.32 (d, $J = 9.0$ Hz, 2H, Ar-H), 5.46 (s, 2H, $-\underline{\text{C}}\underline{\text{H}}_2$), 2.83 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.63 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.63 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.60 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.54 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.52 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$). $^{13}\text{C-NMR}$ (CDCl_3) (ppm): 165.4, 163.9, 155.7, 154.7, 152.8, 151.5, 149.7, 149.3, 149.1, 144.7, 137.8, 131.4, 127.6, 122.0, 66.0 ($-\underline{\text{C}}\underline{\text{H}}_2$), 22.9 ($-\underline{\text{C}}\underline{\text{H}}_3$), 22.4 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.7 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.7 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.5 ($-\underline{\text{C}}\underline{\text{H}}_3$), 20.6 ($-\underline{\text{C}}\underline{\text{H}}_3$). MS (ESI) m/z : 421.0 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 421.18521 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{23}\text{H}_{25}\text{N}_4\text{O}_4$ 421.17976.

2-Methoxy-4-[[*(3,5,6-trimethylpyrazin-2-yl)methoxy*]carbonyl]phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**4d**). White solid, m.p.: 113.2–113.8 °C. $^1\text{H-NMR}$ (CDCl_3) (ppm): 7.73 (m, 1H, Ar-H), 7.72 (brs, 1H, Ar-H), 7.26 (d, $J = 8.0$ Hz, 1H, Ar-H), 5.46 (s, 2H, $-\underline{\text{C}}\underline{\text{H}}_2$), 3.88 (s, 3H, $-\text{O}\underline{\text{C}}\underline{\text{H}}_3$), 2.84 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.64 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.62 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.61 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.54 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.53 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$). $^{13}\text{C-NMR}$ (CDCl_3) (ppm): 165.6, 163.4, 155.4, 152.7, 151.5, 151.1, 149.7, 149.3, 149.1, 144.7, 144.0, 138.0, 128.7, 123.0, 122.9, 113.6, 66.0 ($-\underline{\text{C}}\underline{\text{H}}_2$), 56.1 ($-\text{O}\underline{\text{C}}\underline{\text{H}}_3$), 22.8 ($-\underline{\text{C}}\underline{\text{H}}_3$), 22.4 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.7 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.7 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.5 ($-\underline{\text{C}}\underline{\text{H}}_3$), 20.6 ($-\underline{\text{C}}\underline{\text{H}}_3$). MS (ESI) m/z : 451.2 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 451.19550 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{24}\text{H}_{27}\text{N}_4\text{O}_5$ 451.19032.

2-Hydroxy-4-[[*(3,5,6-trimethylpyrazin-2-yl)methoxy*]carbonyl]phenyl 3,5,6-trimethylpyrazine-2-carboxylate (**5d**). White solid, m.p.: 182.2–182.9 °C. $^1\text{H-NMR}$ (CDCl_3) (ppm): 9.70 (brs, 1H, $-\text{OH}$), 7.88 (s, 1H, Ar-H), 7.83 (m, 1H, Ar-H), 7.01 (d, $J = 8.5$ Hz, 1H, Ar-H), 5.40 (s, 2H, $-\underline{\text{C}}\underline{\text{H}}_2$), 2.72 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.58 (brs, 6H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.53 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.51 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$), 2.50 (s, 3H, $-\underline{\text{C}}\underline{\text{H}}_3$). $^{13}\text{C-NMR}$ (CDCl_3) (ppm): 165.3, 163.2, 156.2, 153.2, 152.8, 151.3, 149.6, 149.3, 149.1, 145.1, 137.9, 137.2, 129.6, 125.1, 121.7, 117.4, 65.5 ($-\underline{\text{C}}\underline{\text{H}}_2$), 22.5 ($-\underline{\text{C}}\underline{\text{H}}_3$), 22.2 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.5 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.3 ($-\underline{\text{C}}\underline{\text{H}}_3$), 21.2 ($-\underline{\text{C}}\underline{\text{H}}_3$), 20.5 ($-\underline{\text{C}}\underline{\text{H}}_3$). MS (ESI) m/z : 437.2 $[\text{M}+\text{H}]^+$, HRMS (ESI) m/z : 437.18036 $[\text{M}+\text{H}]^+$, calcd. for $\text{C}_{23}\text{H}_{25}\text{N}_4\text{O}_5$ 437.17467.

3.2. Bio-Evaluation Methods

3.2.1. Protective Effect on Damaged Differentiated PC12 Cells [9,24–26]

PC12 cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum, 10% (v/v) horse serum and 100 U/mL penicillin-streptomycin (Thermo Technologies, New York, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2 . When cells achieved the desired density of >80% confluency original medium was removed and cells were cultured with the serum-free medium for 14 h. Then the cells were suspended in 1640 medium supplemented with 10% (v/v) fetal bovine serum, and seeded into poly-L-lysine-coated 96-well culture plates at 7×10^3 cells/well, differentiated by treated with 50 ng/mL NGF for 48 h. After these, the differentiated PC12 cells were pretreated with various concentrations (60, 30, 15, 7.5, 3.75 μM) of ligustrazine derivatives

for 36 h. All measurements were performed after the cells were induced by CoCl_2 (final concentration, 200 μM) for 12 h. Control differentiated cells were not treated with ligustrazine derivatives and CoCl_2 . CoCl_2 was dissolved in RPMI 1640 medium. ligustrazine derivatives were dissolved in DMSO. The final concentration of DMSO was less than 0.1% (v/v).

After MTT solution (20 μL , 5 mg/mL) was added to each well, the plate was incubated for a further 4 h at 37 °C. The supernatant was removed carefully by pipetting from wells without disturbing the attached cells and formazan crystals were solubilized by adding 200 μL of DMSO to each well and shaken for 15 min. The absorbance at 490 nm was measured with a BIORAD 550 spectrophotometer (Bio-rad, California, CA, USA). The proliferation rates of damaged PC12 cells were calculated by the formula $[\text{OD}_{490}(\text{Compd}) - \text{OD}_{490}(\text{CoCl}_2)] / [\text{OD}_{490}(\text{NGF}) - \text{OD}_{490}(\text{CoCl}_2)] \times 100\%$; the EC_{50} values were defined as the concentration of compounds that produced a 50% proliferation of surviving cells and calculated using the following equation: $-\text{pEC}_{50} = \log C_{\text{max}} - \log 2 \times (\sum P - 0.75 + 0.25P_{\text{max}} + 0.25P_{\text{min}})$, Where C_{max} = maximum concentration, $\sum P$ = sum of proliferation rates, P_{max} = maximum value of proliferation rate and P_{min} = minimum value of proliferation rate.

3.2.2. Observation of Morphologic Changes [26,27]

The PC12 cells culture procedure was similar to that described in section 3.2.1. After pretreatment with the serum-free medium for 14 h, cells were seeded at a concentration of 7×10^4 cell/mL in a volume of 0.8 mL on a poly-L-lysine-coated sterile cover slip in 6-well tissue culture plates, and differentiated by treating with NGF (50 ng/mL) for 48 h. Then the differentiated PC12 cells were pretreated with compound **4a** (60 μM) for 36 h prior to exposure to CoCl_2 . After induction by CoCl_2 for 12 h, culture medium was removed and the cells were fixed in 95% ethyl alcohol for 15 min. The cells were washed twice with PBS and stained with hematoxylin for 8 min. The stained nuclei were washed twice with diluted PBS, and consequently stained with eosin for 8 min. Next, H&E stained sections were dehydrated through gradient ethanol and cleared in xylene. The residual procedure was processed in accordance with the hematoxylin and eosin (HE) staining protocol. The cellular morphology and percentage of cells showing neurite outgrowth was determined by light microscopy (Nikon, Kobe, Japan). Cells with one or more neurites whose lengths were at least twice the diameter of the cell body were scored as differentiated cells. Cell differentiation rate was calculated as the number of differentiated cells/total cells. Neurite outgrowth was determined from at least three different regions of interest in three independent experiments. All data are expressed as mean \pm SD. Data analysis was carried out using SAS software, version 8.1 (SAS Institute, Cary, NC, USA). Statistically significant differences between the samples were evaluated by Student's t-test and $p < 0.05$ was considered significant.

4. Conclusions

In conclusion, a series of novel ligustrazine-benzoic acid derivatives was designed, synthesized and biologically evaluated for their protective effects on the damaged differentiated PC12 cell proliferation. The preliminary biological results have demonstrated that most of ligustrazine-benzoic acid derivatives exhibited good protective effects in comparison with TMP. Among the active compounds, **4a** is the most active congener, with EC_{50} 4.249 μM , which is much higher than that of

TMP, and representing a most promising lead for further investigation. Studies of the anti-experimental stroke effects and mechanism of **4a** in a rat middle cerebral artery occlusion (MCAO) stroke model are in progress and will be reported in the near future.

Supplementary Materials

Supplementary materials can be accessed at: <http://www.mdpi.com/1420-3049/18/10/13027/s1>.

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Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds **1a–5a**, **1b–5b**, **1c–5c**, **1d–5d** are available from the authors.

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