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ORIGINAL RESEARCH

Design, synthesis and neuroprotective activities of novel cinnamide derivatives containing benzylpiperazine moiety

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Abstract

A new series of cinnamide derivatives **6a–l** were synthesized by the reaction of acyl chlorides with various substituted benzylpiperazines. The structures were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS. The potential neuroprotective activities of cinnamide analogs were evaluated in differentiated rat pheochromocytoma cells (PC12 cells) and in mice subjected to acute cerebral ischemia. Among the series, **6a**, **6b**, and **6c**, featuring a 1,3-benzodioxole moiety, showed potent neuroprotection both in vivo and in vitro. The three compounds were selected and further studied to determine their mechanism of action. MTT assay, Hoechst 33342/PI double staining, and high content screening (HCS) revealed that pretreatment of the cells with **6a**, **6b**, and **6c** has significantly decreased the extent of cell apoptosis in a dose-dependent manner. The results of western blot analysis demonstrated these compounds suppressed apoptosis of glutamate-induced PC12 cells via caspase-3 pathway. These compounds can be lead compounds for further discovery of neuroprotective agents for treating cerebral ischemic stroke.

Keywords Cinnamide · Neuroprotection · Caspase-3 · Cerebral ischemia · Apoptosis

Introduction

Stroke is a leading cause of death and long-lasting disability in developed countries (Maki et al. 2013). In China, stroke is the second cause for mortality in all diseases. Particularly, ischemic strokes account for 60–80% of these strokes (Jia et al. 2011; Liu et al. 2007). With the rapid increase of aging population in China, stroke has become a critical cause for the health of the old, and stroke is becoming a major burden facing the government and health providers (Wang et al. 2011; Liu et al. 2007).

So far, therapeutic options remain limited. The medications commonly used for stroke can be divided into four groups: thrombolytic agents, antiplatelet agents, anticoagulants, and neuroprotective agents (Yousef et al. 2004). Neuroprotection is widely recognized to be a potential strategy for the treatment of ischemic stroke (Beresford et al. 2003), which may make the brain more resistant to damage from stroke, and considerable efforts have been devoted to the development of neuroprotective agents. However, little of them have reached routine clinical use because of their limited efficacy or unfavorable risk-benefit ratio in clinical trials (de Keyser et al. 1999). There is currently no treatment that has been conclusively proven by controlled clinical trials to be beneficial, except for thrombolytic treatment with recombinant tissue plasminogen activator for highly selected patients (Alexandrov et al. 2004). Nonetheless, ischemia/reperfusion after thrombolysis therapy is a big problem, which restrained clinical application of thrombolytic drugs (Warach and Latour 2004). Therefore, it is necessary to search for safer and more effective neuroprotective agents.

Current status of treating ischemic stroke promoted a great deal of interest in the area of neuroprotective agents. Previous work reported by our group suggested that cinnamide scaffold often affords neuroprotective compounds (Wu et al. 2008). Especially the compound **NY-308**, which has the (*E*)-*p*-methoxycinnamoyl moiety, exhibited excellent neuroprotection in vitro PC12 cells and in vivo rat focal

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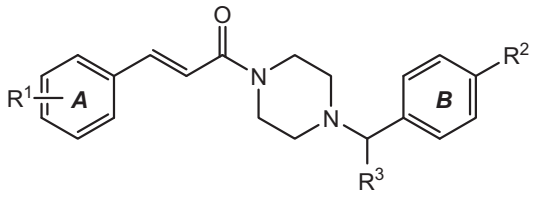
cerebral ischemic animal model (Wu et al. 2012). Structurally, the (*E*)-*p*-methoxycinnamoyl moiety in **NY-308** was believed to play a very important role in its activity. Therefore it became a pattern for target compounds mentioned in this paper. New compounds retained α,β-unsaturated carbonyl and basic center piperazine. Furthermore, we replaced diphenylmethylpiperazine of **NY-308** with benzylpiperazine and changed substituent groups of benzene rings to study of the substituent variability influence on the biological activity (Fig. 1).

Based on the excellent neuroprotective activity, **6a**, **6b**, and **6c** were chosen for further study of action mechanism. The elucidation of the molecular mechanism involved in this effect might provide novel insight into the process of stroke and other neurodegeneration and suggest the potential utility of cinnamide analogs for protection against neurological insults. Therefore, in vitro neuroprotective activities in the cell injury induced by glutamate, apoptosis assays, and the inhibition of caspase-3 were evaluated. Herein, the synthesis and preliminary biological evaluation of the novel cinnamide derivatives were reported.

Materials and methods

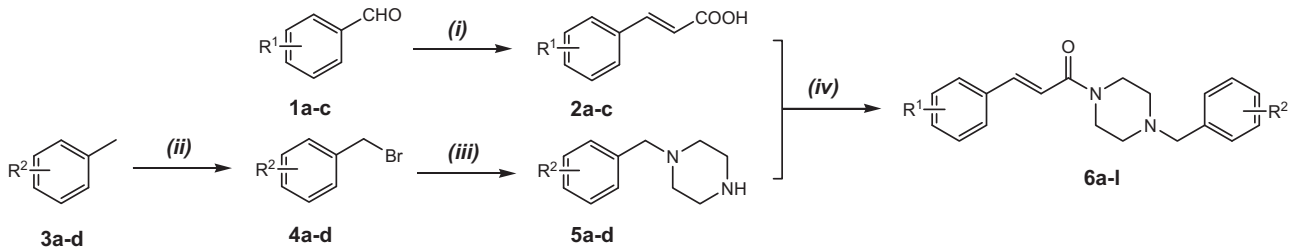
Chemistry

The title compounds were synthesized as outlined in the Scheme 1. Substituted benzaldehydes **1a–c** was coupled with malonic acid via a Knoevenagel reaction to give (*E*)-substitutedacrylic acids **2a–c** (Tanaka et al. 1983). The key intermediates benzyl bromides **4a–d**, were prepared by free-radical bromination of commercially available substituted toluenes **3a–d** with *N*-bromosuccinimide (NBS) (Sahin et al. 2008; Šterk et al. 2012). Potassium carbonate catalyzed coupling of **4a–d** with *N*-Boc-piperazine afforded the compounds **5a–d** in excellent yield (Long et al. 2010). The target compounds **6a–l** were finally obtained by acylation of various substituted benzylpiperazines with the corresponding acyl chlorides under mild conditions.



NY-308 (R^1 = 4-OMe; R^2 = 4-F; R^3 = 4-fluorophenyl)

Fig. 1 Structure of novel cinnamide deravatives



Scheme 1 Synthesis of cinnamide derivatives (**6a–l**). Reagents and conditions: (i) $CH_2(COOH)_2$, pyridine, piperidine, reflux, 2 h; (ii) NBS, AIBN, CCl_4, *h.v.*, reflux, 1 h; (iii) *N*-Boc-piperazine, CH_2Cl_2, K_2CO_3, reflux, 10 h; CH_2Cl_2, TFA, r.t., 2 h; (iv) $SOCl_2$, CH_2Cl_2, r.t., 6 h; Et_3N, CH_2Cl_2, CH_3COCH_3, r.t., 12 h

Biological activity

Bilateral common carotid artery occlusion

Kunming mice of both sexes were randomly divided into groups (10 mice per group). The test agents used in this study were **6a–l**. These compounds above were dissolved in aqueous 0.5% sodium carboxy methyl cellulose (CMCNa) solution before use and administered intraperitoneally (i.p.). Nimodipine was i.p. treated with 80 mg/kg as a positive control. The control group received normal saline (NS) in the same volume as other groups. All groups received drugs twice a day for 3 days. Sixty minutes after the last administration, all mice were anesthetized by ether. All groups underwent the operation for common carotid artery and vagus nerves ligation. Then the survival time of mice were recorded (Zhang et al. 2006; Yanpallewar et al. 2004).

Cell viability assay

PC12 cells obtained from New Drug Research Center of China Pharmaceutical University (Nanjing, China) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented 10% FBS (heat-inactivated), 5% HS (heat-inactivated), 100 IU/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator at 37 °C and 5% CO_2. PC12 cells were seeded in 96-well plates at a density of 1×10^5/mL. Prior to the treatments, PC12 cells were rinsed by DMEM for three times (5 min each), then covered with serum-free DMEM in which Glu (10 mmol/L) or Glu

(10 mmol/L) with compounds (0.1, 1.0, and 10 μmol/L) or Edaravone (90 μmol/L) were added. After incubation in a CO_2 incubator for 24 h, PC12 cells were subsequently tested by MTT assay (Mosmann 1983; Park et al. 2009).

Tetrazolium (10 μL/well; 5 mg/mL of stock in PBS) salt was added 4 h prior to completion of incubation periods. Thereafter, the reaction mixture was carefully taken out and 150 μL of dimethyl sulfoxide (DMSO) solution was added to each well to achieve solubilization of the formazan crystals formed in viable cells. The absorbance was read at 490 nm on an ELISA plate reader, with DMSO as a blank. All assays were performed in triplicate and repeated thrice. The degree of protection induced by the test compound was calculated using the following equation: protection (%) = ((C–B)/(A–B)) × 100, where A is the viability of control cultures, B is the viability of the cultures treated with Glu, and C is the viability of the cultures treated with Glu and the test compound.

Assessment with Hoechst 33342 and propidium iodide (PI) double staining

To further investigate the protective effects, Hoechst 33342 and PI double fluorescent staining was assayed (Wang et al. 2010; Ma et al. 2012). The PC12 cells were cultured in 96-well plates at 1×10^4 cells/well for 24 h. After the indicated treatment, the cells were incubated with 10 μg/mL Hoechst 33342 for 15 min, washed twice with PBS, incubated with 50 μg/mL PI working solution for an additional 15 min in the dark, and analyzed by high content screening (HCS) (Thermo Scientific, USA) within 24 h. The apoptotic nuclei were counted in at least 200 cells from nine randomly chosen fields in each treatment and are expressed as percentages of the total numbers of counted nuclei.

Western blot analysis

At the end of treatments, cells were collected with ice-cold radioimmunoprecipitation (RIPA) buffer (Beyotime, China). Then samples were homogenized and centrifuged at 13,000×*g* for 20 min at 4 °C to remove insoluble material. Protein concentration was determined using BCA Protein Assay kit (Beyotime, China) and the absorbance was measured using a microplate reader with a wavelength of 562 nm.

For western blot analysis (Li et al. 2014), equal amounts of protein (30 μg) were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes. These membranes were incubated with 5% (w/v) non-fat milk powder in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 h to block nonspecific binding sites. The membranes were then incubated overnight at 4 °C with the primary antibodies against caspase-3 (1:1000) (Cell Signaling Technology, USA) and actin (1:1000) (Beyotime, China). After washing with TBST, the membranes were incubated for 2 h at 30 °C with a 1:2000 dilution of horseradish peroxidase-conjugated antirabbit or antimouse IgG. After rewashing with TBST, the bands were developed by using a Immobilon Wester Chemiluminescent HRP Substrate kit (Millipore, USA) for 5 min, the protein bands were visualized with Tanon 5200 (Tanon Company Ltd., China).

Results and discussion

Chemistry

General

All chemicals used for the synthesis were of reagent grade and procured from Sigma-Aldrich, Shanghai, China. Melting points were determined on an electrothermal digital apparatus model WRR-401 (Shanghai, China) without correction. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR were recorded on a Bruker ACF-300 MHz instrument (Bruker) with $CDCl_3$ as the solvent and tetramethylsilane as an internal standard (chemical shifts are expressed as δ values, J in hertz). High resolution mass spectra (HRMS) were recorded on a MALDI Micro MX instrument (Waters). All the reactions were monitored by thin-layer chromatography (TLC) using precoated silica gel 60 F_{254} (Merck). The crude product was purified by column chromatography.

General procedures for the synthesis of (E)-substitutedacrylic acids (2a–c)

A mixture of corresponding substituted benzaldehydes **1** (50 mmol), malonic acid (75 mmol), and piperidine (1 mL) in 35 mL pyridine was refluxed for 2 h. The solvent was evaporated under reduced pressure. The residue was added to a mixture of 35 mL HCl and 70 g ice. The solid product was filtered and recrystallized in ethanol to obtain corresponding (*E*)-substitutedacrylic acids **2**.

General procedures for the synthesis of substituted benzyl bromides (4a–d)

A mixture of corresponding substituted toluenes **3** (70 mmol), *N*-bromosuccinimide (NBS, 70 mmol), and azobisisobutyronitrile (AIBN, 7 mmol) in 108 mL carbon tetrachloride was refluxed under irradiation conditions for 1 h. 108 mL cyclohexane was added to the mixture and the reaction was stirred for 10 min. After removal of precipitate by filtration, the filtrate was concentrated and recrystallized in ethanol to obtain corresponding substituted benzyl bromides **4**.

General procedures for the synthesis of substituted N-benzylpiperazines (5a–d)

To a stirring solution of *N*-Boc-piperazine (2 mmol) in CH_2Cl_2 (20 mL) was sequentially added substituted benzyl bromides **4** (1.6 mmol) and K_2CO_3 (7.2 mmol). The mixture was heated to reflux. After 10 h, the mixture was diluted with EtOAc and poured onto water. The organic layer was washed once with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude oil was taken up in CH_2Cl_2/TFA (1:1 v/v, 6 mL total). After the mixture was stirred for 2 h, the crude was concentrated in vacuo to obtain substituted *N*-benzylpiperazines **5** and used without purification.

General procedures for the synthesis of substituted cinnamoyl benzylpiperazines (6a–l)

To a stirred solution of corresponding (*E*)-substitutedacrylic acids **2** (4 mmol) in 15 mL dichloromethane were added thionyl chloride (2 mL) and piperadine (1 drop). The reaction mixture was stirred for 6 h at room temperature. After completion, the solvent was removed under reduced pressure. The residue was dissolved in acetone (15 mL) and reacted with the solution of corresponding *N*-(substitutedbenzyl)piperazines **5** (6 mmol) and triethylamine (5 mL) in dichloromethane (30 mL). After being stirred for 12 h at room temperature, the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using ethyl acetate/hexane (2:1 v/v) as eluent to afford target compounds.

(E)-1-(4-(4-bromobenzyl)piperazin-1-yl)-3-(benzo[d][1,3]dioxol-5-yl)prop-2-en-1-one (6a) Yellow solid; mp 124.6–126.5 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.56$ (1H, d, $J = 15.0$ Hz, Ar–CH=), 6.78 (7H, m, Ar–H), 6.66 (1H, d, $J = 15.0$ Hz, CO–CH=), 5.98 (2H, s, O–CH_2–O), 3.65 (4H, m, piperazine ring-H), 3.49 (2H, s, ArCH_2), 2.47 (4H, bs, piperazine ring-H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.5$ (C, C=O), 149.0 (C, Ar–C), 148.2 (C, Ar–C), 142.6 (CH, Ar–CH=), 136.7 (C, Ar–C), 131.5 (CH, Ar–C), 130.7 (C, Ar–C), 129.7 (C, Ar–C), 123.7 (CH, Ar–C), 121.1 (CH, Ar–C), 115.0 (CH, CO–CH=), 108.5 (CH, Ar–C), 106.3 (CH, Ar–C), 101.4 (CH_2, O–CH_2–O), 62.1 (CH_2, ArCH_2), 53.2 (CH_2, piperazine ring-C), 52.8 (CH_2, piperazine ring-C), 45.7 (CH_2, piperazine ring-C), 42.1 (CH_2, piperazine ring-C); HRESIMS *m/z* (pos): 429.0817 $C_{21}H_{22}BrN_2O_3$ (calcd. 429.0814).

(E)-1-(4-(4-chlorobenzyl)piperazin-1-yl)-3-(benzo[d][1,3]dioxol-5-yl)prop-2-en-1-one (6b) White solid; mp 125.8–128.4 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.57$ (1H, d, $J = 15.5$ Hz, Ar–CH=), 6.79 (7H, m, Ar–H), 6.66 (1H, d, $J = 15.5$ Hz, CO–CH=), 5.99 (2H, s, O–CH_2–O), 3.65 (4H, m, piperazine ring-H), 3.50 (2H, s, ArCH_2), 2.46 (4H, bs, piperazine ring-H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.5$ (C, C=O), 149.0 (C, Ar–C), 148.2 (C, Ar–C), 142.6 (CH, Ar–CH=), 136.2 (C, Ar–C), 133.1 (CH, Ar–C), 130.3 (C, Ar–C), 129.7 (C, Ar–C), 128.5 (CH, Ar–C), 123.7 (CH, Ar–C), 115.0 (CH, CO–CH=), 108.5 (CH, Ar–C), 106.3 (CH, Ar–C), 101.4 (CH_2, O–CH_2–O), 62.0 (CH_2, ArCH_2), 53.2 (CH_2, piperazine ring-C), 52.8 (CH_2, piperazine ring-C), 45.8 (CH_2, piperazine ring-C), 42.1 (CH_2, piperazine ring-C); HRESIMS *m/z* (pos): 385.1322 $C_{21}H_{22}ClN_2O_3$ (calcd. 385.1319).

(E)-1-(4-(4-ethoxybenzyl)piperazin-1-yl)-3-(benzo[d][1,3]dioxol-5-yl)prop-2-en-1-one (6c) Yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.56$ (1H, d, $J = 15.5$ Hz, Ar–CH=), 6.78 (7H, m, Ar–H), 6.66 (1H, d, $J = 15.5$ Hz, CO–CH=), 5.98 (2H, s, O–CH_2–O), 4.01 (2H, q, $J = 7.0$ Hz, CH_2, OCH_2CH_3), 3.64 (4H, m, piperazine ring-H), 3.49 (2H, s, ArCH_2), 2.47 (4H, bs, piperazine ring-H), 1.38 (3H, t, $J = 7.0$ Hz, CH_3, OCH_2CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.5$ (C, C=O), 158.3 (C, Ar–C), 148.9 (C, Ar–C), 148.1 (C, Ar–C), 142.4 (CH, Ar–CH=), 130.2 (CH, Ar–C), 129.7 (C, Ar–C), 129.2 (C, Ar–C), 123.6 (CH, Ar–C), 115.0 (CH, CO–CH=), 114.2 (CH, Ar–C), 108.4 (CH, Ar–C), 106.3 (CH, Ar–C), 101.3 (CH_2, O–CH_2–O), 63.3 (CH_2, ArCH_2), 62.1 (CH_2, OCH_2CH_3), 53.0 (CH_2, piperazine ring-C), 52.5 (CH_2, piperazine ring-C), 45.7 (CH_2, piperazine ring-C), 42.1 (CH_2, piperazine ring-C), 14.7 (CH_3, OCH_2CH_3); HRESIMS *m/z* (pos): 395.1974 $C_{23}H_{27}N_2O_4$ (calcd. 395.1971).

(E)-1-(4-(2-ethoxybenzyl)piperazin-1-yl)-3-(benzo[d][1,3]dioxol-5-yl)prop-2-en-1-one (6d) Yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.56$ (1H, d, $J = 15.5$ Hz, Ar–CH=), 6.78 (7H, m, Ar–H), 6.67 (1H, d, $J = 15.5$ Hz, CO–CH=), 5.98 (2H, s, O–CH_2–O), 4.02 (2H, q, $J = 7.0$ Hz, CH_2, OCH_2CH_3), 3.75 (4H, m, piperazine ring-H), 3.65 (2H, s, ArCH_2), 2.56 (4H, bs, piperazine ring-H), 1.40 (3H, t, $J = 7.0$ Hz, CH_3, OCH_2CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.4$ (C, C=O), 157.2 (C, Ar–C), 148.9 (C, Ar–C), 148.1 (C, Ar–C), 142.3 (CH, Ar–CH=), 130.6 (CH, Ar–C), 129.7 (C, Ar–C), 129.1 (C, Ar–C), 128.2 (CH, Ar–C), 123.6 (CH, Ar–C), 120.2 (CH, Ar–C), 115.1 (CH, CO–CH=), 111.5 (CH, Ar–C), 108.4 (CH, Ar–C), 106.3 (CH, Ar–C), 101.3 (CH_2, O–CH_2–O), 63.6 (CH_2, OCH_2CH_3), 55.7 (CH_2, ArCH_2), 53.1 (CH_2, piperazine ring-C), 52.6 (CH_2, piperazine ring-C), 45.8 (CH_2, piperazine ring-C), 42.2 (CH_2, piperazine ring-C), 14.9 (CH_3, OCH_2CH_3); HRESIMS *m/z* (pos): 395.1975 $C_{23}H_{27}N_2O_4$ (calcd. 395.1971).

(E)-1-(4-(4-ethoxybenzyl)piperazin-1-yl)-3-(4-bromophenyl) prop-2-en-1-one (6e) Yellow solid; mp 118.9–119.4 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.57$ (1 H, d, $J = 15.5$ Hz, Ar–CH=), 6.85 (8H, m, Ar–H), 6.82 (1H, d, $J = 15.5$ Hz, CO–CH=), 4.01 (2H, q, $J = 7.0$ Hz, CH_2, OCH_2CH_3), 3.63 (4H, m, piperazine ring-H), 3.48 (2H, s, $ArCH_2$), 2.47 (4H, bs, piperazine ring-H), 1.40 (3H, t, $J = 7.0$ Hz, CH_3, OCH_2CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.0$ (C, C=O), 158.3 (C, Ar–C), 141.3 (CH, Ar–CH=), 134.2 (C, Ar–C), 132.0 (CH, Ar–C), 130.3 (C, Ar–C), 129.1 (CH, Ar–C), 128.3 (CH, Ar–C), 123.6 (C, Ar-C), 117.8 (CH, CO–CH=), 114.3 (CH, Ar–C), 63.4 (CH_2, OCH_2CH_3), 62.2 (CH_2, $ArCH_2$), 53.1 (CH_2, piperazine ring-C), 52.6 (CH_2, piperazine ring-C), 45.8 (CH_2, piperazine ring-C), 42.2 (CH_2, piperazine ring-C), 14.8 (CH_3, OCH_2CH_3); HRE-SIMS *m/z* (pos): 429.1182 $C_{22}H_{26}BrN_2O_2$ (calcd. 429.1178).

(E)-1-(4-(2-ethoxybenzyl)piperazin-1-yl)-3-(4-bromophenyl) prop-2-en-1-one (6f) White solid; mp 80.8–82.5 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.57$ (1H, d, $J = 15.5$ Hz, Ar–CH=), 6.86 (8H, m, Ar–H), 6.82 (1H, d, $J = 15.5$ Hz, CO–CH=), 4.02 (2H, q, $J = 7.0$ Hz, CH_2, OCH_2CH_3), 3.75 (4H, m, piperazine ring-H), 3.68 (2H, s, $ArCH_2$), 2.57 (4H, bs, piperazine ring-H), 1.40 (3H, t, $J = 7.0$ Hz, CH_3, OCH_2CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.0$ (C, C=O), 157.3 (C, Ar–C), 141.2 (CH, Ar–CH=), 134.3 (C, Ar–C), 132.0 (CH, Ar–C), 131.9 (C, Ar–C), 130.7 (CH, Ar–C), 129.1 (CH, Ar–C), 128.4 (CH, Ar–C), 123.6 (C, Ar–C), 120.3 (CH, Ar–C), 117.9 (CH, CO–CH=), 111.6 (CH, Ar–C), 63.7 (CH_2, OCH_2CH_3), 55.8 (CH_2, $ArCH_2$) 53.2 (CH_2, piperazine ring-C), 52.6 (CH_2, piperazine ring-C), 45.9 (CH_2, piperazine ring-C), 42.3 (CH_2, piperazine ring-C), 14.9 (CH_3, OCH_2CH_3); HRESIMS *m/z* (pos): 429.1182 $C_{22}H_{26}BrN_2O_2$ (calcd. 429.1178).

(E)-1-(4-(4-chlorobenzyl)piperazin-1-yl)-3-(4-bromophenyl) prop-2-en-1-one (6g) White solid; mp 138.0–138.7 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.58$ (1H, d, $J = 15.5$ Hz, Ar–CH=), 7.28 (8H, m, Ar–H), 6.82 (1H, d, $J = 15.5$ Hz, CO–CH=), 3.65 (4H, m, piperazine ring-H) 3.51 (2H, s, $ArCH_2$), 2.47 (4H, bs, piperazine ring-H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.0$ (C, C=O), 141.4 (CH, Ar–CH=), 134.2 (C, Ar–C), 133.0 (C, Ar–C), 132.0 (CH, Ar–C), 131.4 (C, Ar–C), 130.2 (CH, Ar–C), 129.2 (CH, Ar–C), 128.5 (CH, Ar–C), 123.6 (C, Ar–C), 117.8 (CH, CO–CH=), 62.0 (CH_2, $ArCH_2$), 53.2 (CH_2, piperazine ring-C), 52.6 (CH_2, piperazine ring-C), 45.7 (CH_2, piperazine ring-C), 42.1 (CH_2, piperazine ring-C); HRESIMS *m/z* (pos): 419.0530 $C_{20}H_{21}BrClN_2O$ (calcd. 419.0526).

(E)-1-(4-(4-bromobenzyl)piperazin-1-yl)-3-(4-bromophenyl) prop-2-en-1-one (6h) White solid; mp 136.8–138.3 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.58$ (1H, d, $J = 15.5$ Hz, Ar–CH=), 7.22 (8H, m, Ar–H), 6.82 (1H, d, $J = 15.5$ Hz, CO–CH=) 3.64 (4H, m, piperazine ring-H), 3.49 (2H, s, $ArCH_2$), 2.47 (4H, bs, piperazine ring-H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.1$ (C, C=O), 141.5 (CH, Ar–CH=), 134.2 (C, Ar–C), 132.0 (CH, Ar–C), 131.5 (C, Ar–C), 130.7 (C, Ar–C), 129.6 (CH, Ar–C), 129.3 (CH, Ar–C), 123.7 (C, Ar–C), 121.2 (CH, Ar–C), 117.7 (CH, CO–CH=), 62.0 (CH_2, $ArCH_2$), 53.2 (CH_2, piperazine ring-C), 52.7 (CH_2, piperazine ring-C), 45.8 (CH_2, piperazine ring-C), 42.2 (CH_2, piperazine ring-C); HRESIMS *m/z* (pos): 463.0025 $C_{20}H_{21}Br_2N_2O$ (calcd. 463.0021).

(E)-1-(4-(4-ethoxybenzyl)piperazin-1-yl)-3-(4-chlorophenyl) prop-2-en-1-one (6i) White solid; mp 128.8–129.3 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.59$ (1H, d, $J = 15.5$ Hz, Ar–CH=), 6.85 (8H, m, Ar–H), 6.81 (1H, d, $J = 15.5$ Hz, CO–CH=), 4.01 (2H, q, $J = 7.0$ Hz, CH_2, OCH_2CH_3), 3.64 (4H, m, piperazine ring-H), 3.49 (2H, s, $ArCH_2$), 2.47 (4H, bs, piperazine ring-H), 1.40 (3H, t, $J = 7.0$ Hz, CH_3, OCH_2CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.0$ (C, C=O), 158.2 (C, Ar–C), 141.1 (CH, Ar–CH=), 135.2 (C, Ar–C), 133.8 (C, Ar–C), 130.2 (CH, Ar–C), 129.3 (CH, Ar–C), 128.9 (CH, Ar–C), 128.8 (C, Ar–C), 117.7 (CH, CO–CH=), 114.3 (CH, Ar–C), 63.4 (CH_2, OCH_2CH_3), 62.2 (CH_2, $ArCH_2$), 53.2 (CH_2, piperazine ring-C), 52.6 (CH_2, piperazine ring-C), 45.9 (CH_2, piperazine ring-C), 42.2 (CH_2, piperazine ring-C), 14.9 (CH_3, OCH_2CH_3); HRE-SIMS *m/z* (pos): 385.1687 $C_{22}H_{26}ClN_2O_2$ (calcd. 385.1683).

(E)-1-(4-(2-ethoxybenzyl)piperazin-1-yl)-3-(4-chlorophenyl) prop-2-en-1-one (6j) Brown solid; mp 70.0–72.8 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.59$ (1H, d, $J = 15.0$ Hz, Ar–CH=), 6.86 (8H, m, Ar–H), 6.81 (1H, d, $J = 15.5$ Hz, CO–CH=), 4.02 (2H, q, $J = 7.0$ Hz, CH_2, OCH_2CH_3), 3.77 (4H, m, piperazine ring-H), 3.68 (2H, s, $ArCH_2$), 2.59 (4H, bs, piperazine ring-H), 1.40 (3H, t, $J = 7.0$ Hz, CH_3, OCH_2CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.0$ (C, C=O), 157.3 (C, Ar–C), 141.3 (CH, Ar–CH=), 135.4 (C, Ar–C), 133.8 (C, Ar–C), 130.9 (CH, Ar–C), 129.0 (CH, Ar–C), 128.9 (C, Ar–C), 128.6 (CH, Ar–C), 120.3 (CH, Ar–C), 117.7 (CH, CO–CH=), 111.6 (CH, Ar–C), 63.7 (CH_2, OCH_2CH_3), 55.6 (CH_2, $ArCH_2$), 53.0 (CH_2, piperazine ring-C), 52.4 (CH_2, piperazine ring-C), 45.7 (CH_2, piperazine ring-C), 42.1 (CH_2, piperazine ring-C), 14.9 (CH_3, OCH_2CH_3); HRESIMS *m/z* (pos): 385.1686 $C_{22}H_{26}ClN_2O_2$ (calcd. 385.1683).

(E)-1-(4-(4-chlorobenzyl)piperazin-1-yl)-3-(4-chlorophenyl) prop-2-en-1-one (6k) White solid; mp 139.8–141.3 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 7.59$ (1H, d, $J = 15.5$ Hz, Ar–CH=), 7.28 (8H, m, Ar–H), 6.81 (1H, d, $J = 15.0$ Hz,

Table 1 Effects of title compounds on survival time of mice subjected to bilateral common carotid artery ligation

Compd	Survival time (min)					
	200 mg/Kg	100 mg/Kg	50 mg/Kg	25 mg/Kg	12.5 mg/Kg	6.25 mg/Kg
6a	16.38 ± 1.79*	14.92 ± 2.58*	5.97 ± 1.35*	5.47 ± 1.26*	3.90 ± 1.29*	2.73 ± 1.66
6b	16.22 ± 2.49*	11.85 ± 2.34*	5.96 ± 1.15*	6.37 ± 1.32*	5.34 ± 1.78*	4.31 ± 1.70*
6c	15.88 ± 2.01*	16.47 ± 1.95*	6.92 ± 1.22*	5.83 ± 1.54*	4.01 ± 0.44*	3.90 ± 1.51*
6d	10.04 ± 2.27*	5.14 ± 0.30*	2.51 ± 1.02	NE	NE	NE
6e	10.54 ± 2.80*	4.28 ± 1.12*	2.31 ± 1.28	NE	NE	NE
6f	6.46 ± 1.03*	5.14 ± 1.30*	1.17 ± 0.30	NE	NE	NE
6g	6.56 ± 1.43*	5.06 ± 2.18*	3.00 ± 1.19	NE	NE	NE
6h	12.31 ± 1.69*	6.18 ± 2.22*	8.54 ± 2.45*	2.08 ± 0.67	NE	NE
6i	7.58 ± 1.33*	11.03 ± 1.74*	6.16 ± 1.81*	4.27 ± 1.93*	3.06 ± 1.27	1.14 ± 0.30
6j	8.84 ± 2.03*	3.19 ± 0.48*	1.02 ± 0.44	NE	NE	NE
6k	6.90 ± 2.62*	4.14 ± 1.07*	2.68 ± 1.44	NE	NE	NE
6l	2.94 ± 1.81	4.37 ± 0.88*	2.26 ± 1.37	NE	NE	NE
NS	2.04 ± 0.61					
Nimodipine	3.52 ± 1.04*,[a]					

Values are in mean ± SEM; number of animals in each group = 10

NE not evaluated

*$p < 0.05$ versus NS

[a]Nimodipine 80 mg/Kg

CO–CH=), 3.64 (4H, m, piperazine ring-H), 3.50 (2H, s, $ArCH_2$), 2.47 (4H, bs, piperazine ring-H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.1$ (C, C=O), 141.4 (CH, Ar–CH=), 135.4 (C, Ar–C), 133.8 (C, Ar–C), 130.4 (C, Ar–C), 129.2 (CH, Ar–C), 129.0 (CH, Ar–C), 128.9 (CH, Ar–C), 128.5 (CH, Ar–C) 117.6 (CH, CO–CH=), 62.0 (CH_2, $ArCH_2$), 53.2 (CH_2, piperazine ring-C), 52.7 (CH_2, piperazine ring-C), 45.8 (CH_2, piperazine ring-C), 42.1 (CH_2, piperazine ring-C); HRESIMS *m/z* (pos): 375.1034 $C_{20}H_{21}Cl_2N_2O$ (calcd. 375.1031).

(E)-1-(4-(4-bromobenzyl)piperazin-1-yl)-3-(4-chlorophenyl) prop-2-en-1-one (6l) White solid; mp 151.7–153.1 °C; ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 7.59$ (1H, d, $J = 15.0$ Hz, Ar–CH=), 7.21 (8H, m, Ar–H), 6.81 (1H, d, $J = 15.5$ Hz, CO–CH=), 3.65 (4H, m, piperazine ring-H), 3.49 (2H, s, $ArCH_2$), 2.47 (4H, bs, piperazine ring-H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.1$ (C, C=O), 141.4 (CH, Ar–CH=), 136.6 (C, Ar–C), 135.4 (C, Ar–C), 133.8 (C, Ar–C), 131.5 (C, Ar–C), 130.7 (CH, Ar–C), 129.0 (CH, Ar–C), 128.9 (CH, Ar–C), 121.2 (CH, Ar–C), 117.6 (CH, CO–CH=), 62.1 (CH_2, $ArCH_2$), 53.2 (CH_2, piperazine ring-C), 52.7 (CH_2, piperazine ring-C), 45.8 (CH_2, piperazine ring-C), 42.2 (CH_2, piperazine ring-C); HRESIMS *m/z* (pos): 419.0530 $C_{20}H_{21}BrClN_2O$ (calcd. 419.0526).

Biological evaluation

Firstly, the target compounds were preliminary screened in mice underwent bilateral common carotid artery occlusion to study effects of compounds on acute cerebral ischemia. As shown in Table 1, most of the target compounds were active in mice i.p. at different doses, among which compounds **6a**, **6b**, **6c**, and **6i** at all doses significantly prolonged the survival time of mice subjected to acute cerebral ischemia and showed potent neuroprotective effects. Compound **6h** showed activities at high and median dose. Compounds **6d**, **6e**, **6f**, **6g**, **6j**, and 6**k** only presented protection at high dose while showed no effect at low or median dose.

In order to study the potential neuroprotective activity of the title compounds, another screening was performed investigating neuroprotection on impairment induced by glutamine (Glu) in PC12 cells, as evaluated by MTT assay. We damaged the PC12 cells with 10 mM L-glutamate and assessed the protective actions of twelve cinnamide derivatives. We found that most of the new compounds exhibited moderate to good protection against Glu-induced cell damage (Table 2).

As shown in Table 2, **6a**, **6b**, **6c**, **6d**, **6e**, **6f**, and **6i** showed good neuroprotective activity in glutamate-induced PC12 cells at all doses tested (protection >20%). It was found that the cumulative addition of the compounds **6a**, **6b** and **6c** (0.1–10 μM) caused concentration-dependent neuroprotective effects with the maximal effect observed at 10 μM. Compounds **6f** and **6i** showed a pattern of increased protection with increasing concentrations (1.0–10 and 0.1–1.0 μM, respectively) in terms of cell protection. Compounds **6d**, **6e**, and **6l** were observed to have the highest protection at the lowest concentrations of 0.1 μM (cell protection: 61.02, 71.82, and 34.02%, respectively). Compounds **6f**, **6g**, **6h**, and **6k** were observed to have the

Table 2 Neuroprotective effects of all target compounds against glutamate-induced neurotoxicity in PC12 cells

Compound	R^1	R^3	R^2	Cell viability (%)		
				0.1 μM	1.0 μM	10 μM
6a	R^1-phenyl = 1,3-benzodioxole	H	4-Br	32.06	45.34	63.56
6b	R^1-phenyl = 1,3-benzodioxole	H	4-Cl	32.84	48.02	72.16
6c	R^1-phenyl = 1,3-benzodioxole	H	4-OEt	37.64	52.99	85.40
6d	R^1-phenyl = 1,3-benzodioxole	H	2-OEt	61.02	49.95	35.51
6e	4-Br	H	4-OEt	71.82	62.12	61.17
6f	4-Br	H	2-OEt	23.61	25.26	45.01
6g	4-Br	H	4-Cl	8.41	9.46	52.64
6h	4-Br	H	4-Br	7.87	8.70	45.61
6i	4-Cl	H	4-OEt	33.68	42.54	35.71
6j	4-Cl	H	2-OEt	14.62	17.09	19.44
6k	4-Cl	H	4-Cl	7.92	15.19	22.92
6l	4-Cl	H	4-Br	34.02	18.54	14.83
NY-308				71.01	49.66	77.75
Edaravone				33.01[a]		

[a]Edaravone-90 μM

Table 3 Effect of some cinnamide on apoptosis of PC12 cells induced by glutamate

Compd	Rate of apoptotic cells (%)		
	0.1 μM	1.0 μM	10 μM
Control	19.27 ± 2.00		
Glutamate	43.97 ± 2.69[a]		
6a	42.82 ± 4.53	32.11 ± 2.85[b]	25.42 ± 2.73[b]
6b	30.04 ± 4.11[b]	24.42 ± 3.30[b]	17.34 ± 2.24[b]
6c	43.45 ± 4.00	36.44 ± 1.53[b]	31.95 ± 2.19[b]

[a]Significantly different from control group ($p < 0.05$)

[b]Significantly different from glutamate group ($p < 0.05$)

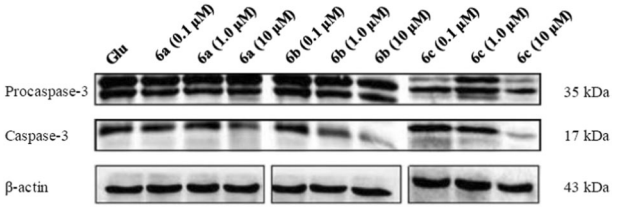


Fig. 2 Caspase-3 activity in PC12 cells pretreated with cinnamide derivatives

highest protection at the highest concentrations of 10 μM (cell protection: 45.01, 52.64, 45.61, and 22.92%, respectively).

Derivatives were substituted with different functional groups to study of the substituent variability influence on the biological activity and find new compounds with better neuroprotective activity. The electronic nature of the substituent group attached to the aromatic ring *B* led to a significant variation in neuroprotective activity. From the data shown above, it is clear that the presence of electron donating group at 4th position on aromatic ring *B* enhanced the activity when compared to electron withdrawing group (cell protection: **6c** > **6b**, **6c** > **6a**, **6e** > **6g**, **6e** > **6h**). On the other hand, for the different isoelectronic halogens, the lipophilic chloro substituent at the 4-position of *B* ring was more potent than the corresponding bromo-substituted derivatives (cell protection: **6g** > **6h**, **6b** > **6a**).

It was worthwhile to note that the introduction of 1,3-benzodioxole exerted more neuroprotective effect, such as **6a**, **6b**, **6c**, and **6d**, which indicated that larger hydrophobic groups might be favorable for increased neuroprotection.

For further investigation, apoptosis assays and western blot analysis were performed to determine their mechanism of action, at three concentration of 0.1, 1.0, and 10 μM, against glutamate-induced cell death. According to the in vitro and in vivo biological activities, the compounds **6a**, **6b**, and **6c** were selected and further studied.

Apoptosis after cerebral ischemia is one of the major pathways that leads to the process of cell death (Meng et al. 2011). Therefore, we investigated the anti-apoptotic effects of the compounds **6a**, **6b** and **6c** by Hoechst 33342/PI double staining assay using high content screening system. Quantitative analysis of the stained cells indicated that pretreatment of the cells with **6a**, **6b**, or **6c** has significantly decreased the extent of cell apoptosis in a dose-dependent manner. As shown in Table 3, after glutamate insult for 24 h, 43.97% of cultured cells showed typical characteristics of apoptosis. However, under pretreatments of **6a**, **6b**, and **6c** at three doses (0.1, 1.0, and 10 μM respectively), the apoptotic percentages were significantly reduced (Table 3). The above results suggest that **6a**, **6b**, and **6c** are capable of rescuing PC12 cells from glutamate-induced apoptotic death.

Another important finding, which confirmed the protective effect of these derivatives, was the data obtained from western blot analysis of procaspase-3 and caspase-3. As shown in Fig. 2, glutamate induced the appearance of cleaved active caspase-3, showing the involvement of caspase-3 in glutamate-induced cell death in PC12 neurons. In the cells pretreated with **6b** at three doses (0.1, 1.0, and 10 μM), bands of cleaved (active) caspase-3 were weaker compared to glutamate-treated cells, which illustrated that **6b** significantly inhibited the expression of caspase-3 induced by glutamate in a dose-dependent manner. The procaspase-3 level was not depressed significantly by

compounds **6a**, **6b**, and **6c**, while, **6a** and **6c** significantly inhibited the expression of caspase-3 at high doses. The above results demonstrated these compounds inhibited apoptosis of glutamate-induced PC12 cells via caspase-3 pathway.

Conclusion

In summary, twelve novel cinnamide analogs (**6a–l**) were designed and synthesized. Most target compounds showed neuroprotective activity both in vivo and in vitro, among which **6a**, **6b**, and **6c**, featuring a 1,3-benzodioxole moiety, exhibited the significant potency. Further studies showed that analogs **6a**, **6b**, and **6c** efficiently inhibited glutamate induced cell apoptosis via caspase-3 pathway. These findings may provide insight for our future design of cinnamide analogs with optimal neuroprotective activities. Cinnamide compounds might be useful as agents in the treatment of stroke and other neurological insults.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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