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Coumarin derivatives protect against ischemic brain injury in rats

Mingna Sun [a,1], Jinfeng Hu [a,1], Xiuyun Song [a], Donghui Wu [a], Linglei Kong [a], Yupeng Sun [a], Dongmei Wang [a], Yan Wang [a,**], Naihong Chen [a,***], Gang Liu [a,b,c,*]

[a] State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, 2 Nanwei Rd., Xicheng Dist., Beijing 100050, PR China
[b] Tsinghua-Peking Center for Life Sciences, School of Medicine, Tsinghua University, Haidian Dist., Beijing 100084, PR China
[c] Department of Pharmacology and Pharmaceutical Sciences, School of Medicine, Tsinghua University, Haidian Dist., Beijing 100084, PR China

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ABSTRACT

Neuroprotection strategies are of great importance in the treatment of ischemic brain injury. Screening of our in-stock coumarin derivatives identified compound **1** as exhibiting neuroprotective activity. Subsequently, a structural optimization was carried out, which led to the discovery of the potent compound **20**. This compound significantly attenuated the damage in a cell line derived from a pheochromocytoma of the rat adrenal medulla induced by oxygen–glucose deprivation *in vitro*. Furthermore, compound **20** exhibited clear neuroprotection in middle cerebral artery occlusion rats by reducing infarct size and brain-water content, improving neurological function, and suppressing neuronal loss and neuropathological changes in the cortex and hippocampus. Pharmacokinetic evaluation indicated that compound **20** could penetrate the blood–brain barrier of rats.

1. Introduction

Ischemic brain injury is considered one of the leading causes of death and adult disability in many countries because of its high mortality rate. Ischemic stroke, which results from occlusion of an artery in the brain, accounts for more than 80% of all strokes. A large body of evidence suggests that a transient or permanent reduction of cerebral blood flow often initiates brain ischemia and usually leads to neuronal cell death in the central core and penumbra. Transient ischemia was found to produce large amounts of free radicals and neurotoxicity in the penumbral cortex during reperfusion [1–3]. In particular, oxidative stress is a major factor in cerebral ischemic/reperfusion (I/R) damage because the brain consumes a large quantity of oxygen. The reactive oxygen and nitrogen species can lead to lipid peroxidation, protein oxidation and DNA damage in brain I/R injury [4]. Moreover, oxidative damage caused by reactive oxygen radicals has complex interactions with excitotoxicity, apoptosis and inflammation by triggering various critical cellular signal transduction pathways [5].

Antioxidant strategies have led to many results in numerous experimental stroke studies and clinical therapy (Fig. 1). Ebselen is a selenium compound that possesses glutathione peroxidase-like activity [6]. NXY-059, a nitrone-derived free radical trapping agent, protects against I/R injury by preventing the decline of mitochondrial functions, blocking cytochrome c efflux and subsequent caspase activation [7–9]. Tirilazad also possesses free-radical-scavenging activity [10,11]. Edaravone inhibits lipid peroxidation by scavenging free radicals, such as superoxide anion radical ($O_2^{-}\cdot$), nitric oxide radical ($NO\cdot$) and peroxynitrite anion ($ONOO^{-}$), and has been used in the treatment of cerebral infarctions [12–17]. Hydroxyl substituted coumarins are widely distributed in plants. Recently, it has been reported that many coumarin derivatives exhibit a neuroprotective effect by their antioxidant and anti-inflammatory activities, and the structure–activity relationships (SARs) of coumarin derivatives have been extensively investigated [18]. For example, esculetin shows neuroprotective effects on cerebral I/R injury in mice and exerts anti-apoptotic activity by upregulating the expression of B cell lymphoma/leukemia-2 (Bcl-2) and downregulating the expression of Bcl-2-associated X protein (Bax) [19].

In previous studies, we developed a series of 3-piperazine-substituted coumarin derivatives and discovered compound **1**,

* Corresponding author. Department of Pharmacology and Pharmaceutical Sciences, School of Medicine, Tsinghua University, Haidian Dist., Beijing 100084, PR China. Tel./fax: +86 10 63167165.
** Corresponding author. Tel./fax: +86 10 63165238.
*** Corresponding author. Tel./fax: +86 10 63165177.
E-mail addresses: wangyan@imm.ac.cn (Y. Wang), chennh@imm.ac.cn (N. Chen), gangliu27@tsinghua.edu.cn, gliu@imm.ac.cn, gangliu27@gmail.com (G. Liu).
[1] These authors contributed equally to this work.

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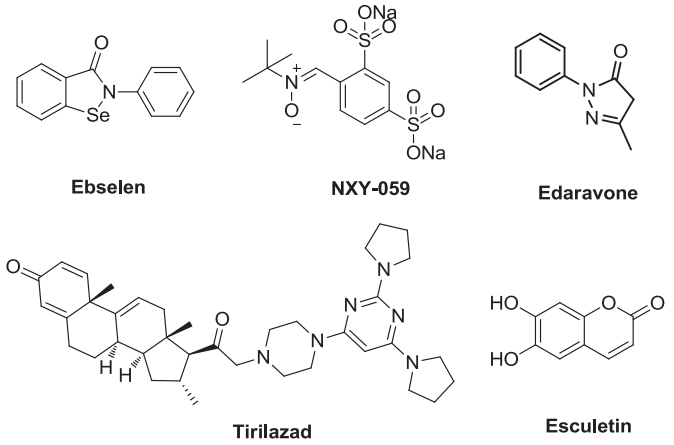


Fig. 1. Chemical structures of literature neuroprotective agents.

which exhibits anti-inflammatory activity as an antagonist of chemokine-like factor 1 (Fig. 2) [20]. Considering the potential antioxidant activity of the analogs with phenolic hydroxyl groups such as compound **1**, we screened 37 coumarin derivatives (see supplementary content, Table S1) in our in-stock library at a single dose through the assay of PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla) damaged by oxygen–glucose deprivation (OGD). A preliminary review of the data and structures suggested that appropriate substituents on the C5- and C7-positions (e.g., hydroxyls) and the C3-position (e.g., compounds **1**–**4**, Fig. 2) of coumarin might contribute to the neuroprotective activity of these compounds. This paper reports our optimization of compound **1** and the corresponding biological evaluation of neuroprotective activity *in vitro* and *in vivo*. With the aim of improving the neuroprotective activity, the optimization focused on the substituents of the piperazinyl group (compounds **6**–**8**), the linker between the piperazinyl group and the coumarin scaffold (compounds **11** and **17**) and the selective modification of the C5- and C7-hydroxyl groups (compounds **20**, **21**, **33**, **35** and **37**).

2. Results and discussion

2.1. Chemistry

Compounds **6**–**8** were prepared from the intermediate **5** through reaction with various acid anhydrides, isocyanates and isothiocyanates (Scheme 1). Compound **11**, with an ethylene linker between the piperazinyl group and the coumarin core, was prepared from phloroglucinol via a Pechmann reaction with **9** using PBr_3 as a catalyst, and then nucleophilic substitution with *N*-methylpiperazine in the presence of K_2CO_3 (Scheme 2A) [21]. The alkyl carbonyl compounds **17a** and **17b** were also designed and synthesized (Scheme 2B). Starting material **12** reacted with ethyl bromoacetate/propionate in the presence of sodium ethoxide (EtONa) to give the intermediates **14a** and **14b**, which subsequently reacted with phloroglucinol to be transformed into intermediates **15a** and **15b**, respectively. After hydrolysis and condensation with *N*-methylpiperazine catalyzed by benzotriazole-1-yloxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), the target compounds **17a** and **17b** were obtained, respectively [22,23].

To synthesize the target compounds **20** and **21**, the Pechmann reaction of **18** with **19** catalyzed by boron trifluoride diethyl ether ($BF_3 \cdot Et_2O$) and direct methylation of compound **1** by dimethyl sulfate (Me_2SO_4) were carried out (Scheme 3A). The former route resulted in compounds **20** and **21** with an equal amount of each, as analyzed by liquid chromatography–mass spectrometry (LC–MS) and thin-layer chromatography detection under ultraviolet 254 nm wavelength radiation, and it was too difficult to separate them by silica gel column chromatography. Interestingly, the direct methylation of compound **1** produced compound **22** but no C5-/C7-hydroxyl methylated products. This observation let us successfully develop the third synthetic route (Scheme 3B). Compound **1** was protected by *p*-toluenesulfonyl chloride (TsCl) in pyridine to give intermediate **23**. The anticipated compound **20** was prepared from **23** through five steps, involving benzylation of the *N*-methylpiperazine by benzyl bromide (BnBr), selective expulsion of the tosyl group by tetrabutylammonium fluoride (TBAF) to form the C5-hydroxyl group, methylation of the C5-hydroxyl group, removal of the protective tosyl group of the C7-hydroxyl group and debenzylation of *N*-methylpiperazine. The anticipated compound **21** was also prepared from **23** through five steps, involving selective expulsion of the tosyl group by TBAF to form the C5-hydroxyl group, benzylation of the *N*-methylpiperazine and the C5-hydroxyl group by BnBr, removal of the protective tosyl group of the C7-hydroxyl group, methylation of the C7-hydroxyl group and debenzylation of *N*-methylpiperazine.

Intermediates **32a**–**v** were prepared from the key intermediate **28** via a Mitsunobu reaction with appropriate alcohols in the presence of diethyl azodicarboxylate (DEAD) and triphenylphosphine (PPh_3). Removal of the protective tosyl group catalyzed by TBAF afforded the target compounds **33a**–**v** (Scheme 4). After removing the *tert*-butoxycarbonyl group of **32u** and **32v** by CF_3COOH in dichloromethane (DCM), the intermediates **34a** and

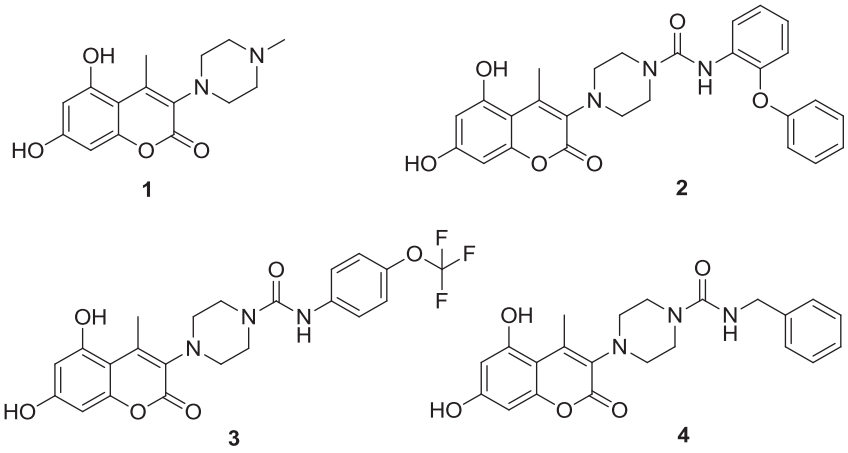
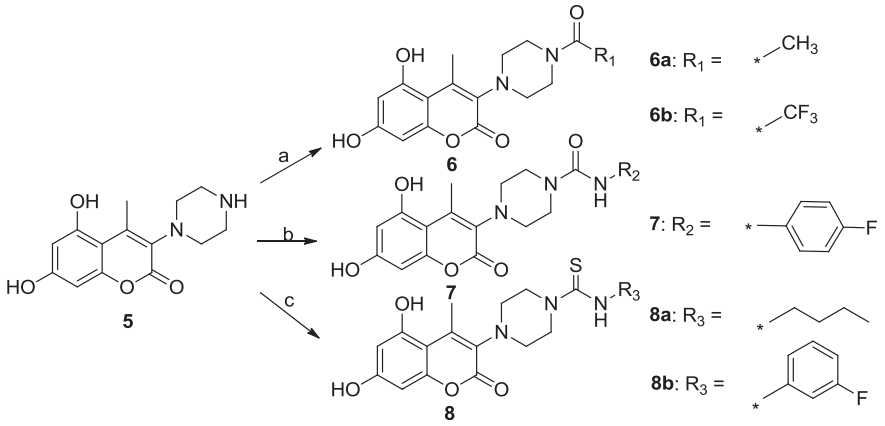


Fig. 2. Chemical structures of some in-stock coumarins with neuroprotective activity.



Scheme 1. Synthesis of coumarin analogs **6**–**8**. Reagents and conditions: (a) $(R_1CO)_2O$, triethylamine (TEA), tetrahydrofuran (THF), rt; (b) R_2NCO, THF/dimethylformamide (DMF), 50 °C; (c) R_3NCS, THF/DMF, 50 °C.

34b were obtained, respectively, which further afforded the target compounds **35a** and **35b** by removing the tosyl group. Treatment of intermediate **34a** with methanesulfonyl chloride and TsCl produced intermediates **36a** and **36b**, respectively, which were subsequently deprotected to achieve the anticipated sulfamide products **37a** and **37b** (Scheme 5).

2.2. Neuroprotective activity in vitro

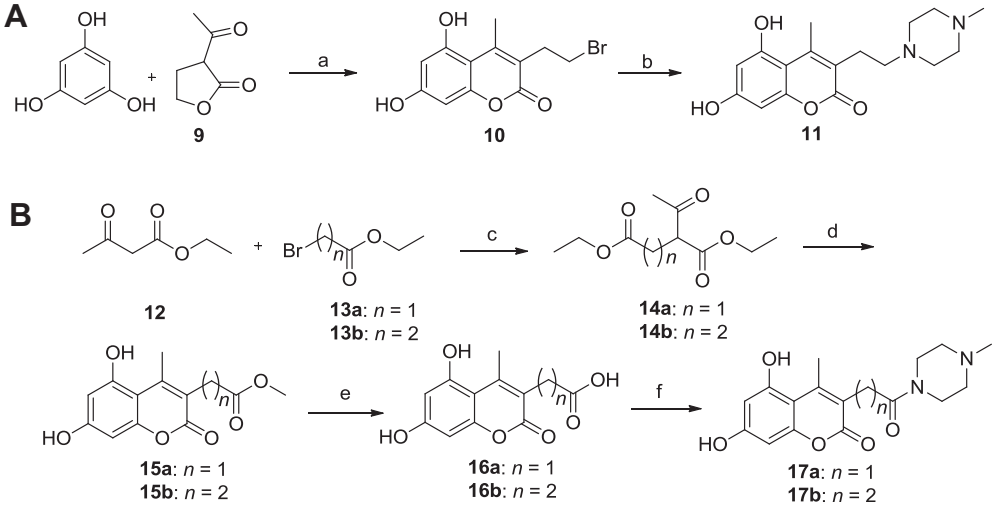
All synthesized compounds were evaluated for neuroprotective activity on damaged PC12 cells caused by OGD and proliferative effects on normal PC12 cells at a single dose of 10 μM.

The initial screening of the stock coumarins (Table S1) showed that nitrogen heterocycles at the C3-position could elicit neuroprotective activity and that free hydroxyl groups at the C5- or C7-position were helpful for the activity. While none of the newly synthesized compounds with modified piperazinyl groups (**6a**, **6b**, **7**, **8a** and **8b**) improved the neuroprotective activity. Those compounds (**11**, **17a** and **17b**) whose molecular flexibility was increased by adding carbon atoms between the coumarin nucleus and the piperazinyl group were also inactive. Selective methylation of the C5- or C7-hydroxyl group of compound **1** resulted in different potency. Selective methylation of the C5-hydroxyl group of compound **1** generated significant neuroprotective activity (**20**). However, selective methylation of the C7-hydroxyl group of compound **1** produced inactivity (**22**). (Table 1).

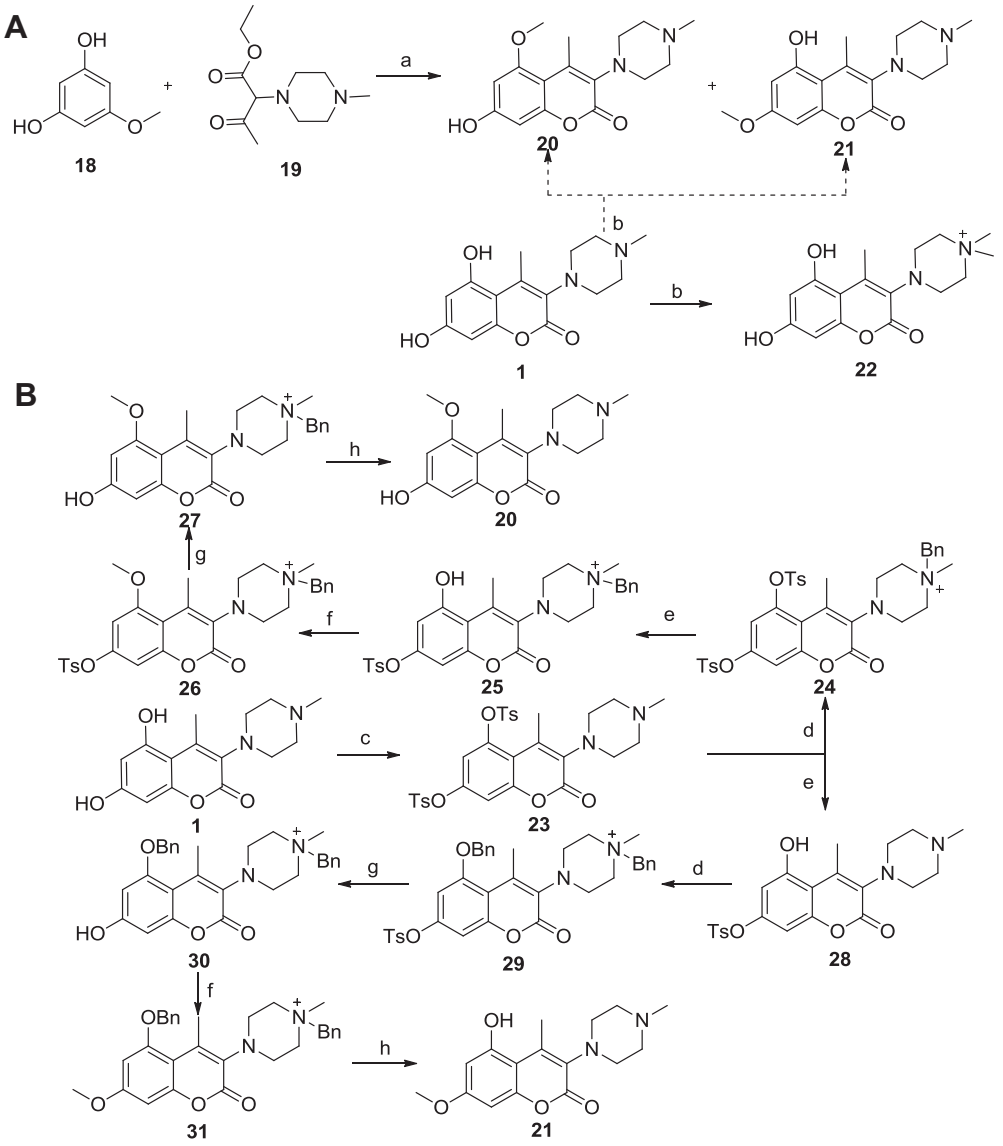
Further modification of compound **1** included elongation of the carbon chain length on the C5-position, introduction of other atoms into the carbon chain of the C5-position, and introduction of cyclic alkyls, aryls and heterocycles into the C5-position directly or with an interval of one or two carbons; unfortunately, none of the above methods resulted in satisfactory compounds. Investigation of rat pheochromocytoma PC12 cell viability indicated that many compounds (**33a**, **33b**, **33d**, **33e**, **33g**, **33h**, **33j**–**l**, **33n**, **33p**, **33q**, **33s**–**v** and **37b**) were cell proliferative activators for an unknown reason. Two compounds (**33c** and **33m**) exhibited significant cytotoxicity. The remaining compounds (**33f**, **33i**, **33o**, **33r**, **35a**, **35b** and **37a**) did not exhibit obvious protection in the tested assays (Table 2). All of the above syntheses and analysis proved that compound **20** was a potent new agent that effectively protected against damage of rat pheochromocytoma PC12 cells *in vitro*.

2.3. Neuroprotective activity in vivo

Being the most neuroprotective agent *in vitro* and without cell proliferative effect, compound **20** was selected to treat the middle cerebral artery occlusion (MCAO) rats to investigate the *in vivo* neuroprotective activity.



Scheme 2. Synthesis of coumarin analogs **11** and **17**. Reagents and conditions: (a) PBr_3, benzene, reflux; (b) 1-methylpiperazine, K_2CO_3, KI, butanone, reflux; (c) EtONa, ethanol (EtOH), rt → reflux; (d) phloroglucinol, HCl/methanol (MeOH), rt; (e) K_2CO_3, $EtOH/H_2O$, reflux; (f) 1-methylpiperazine, BOP, TEA, THF, rt.



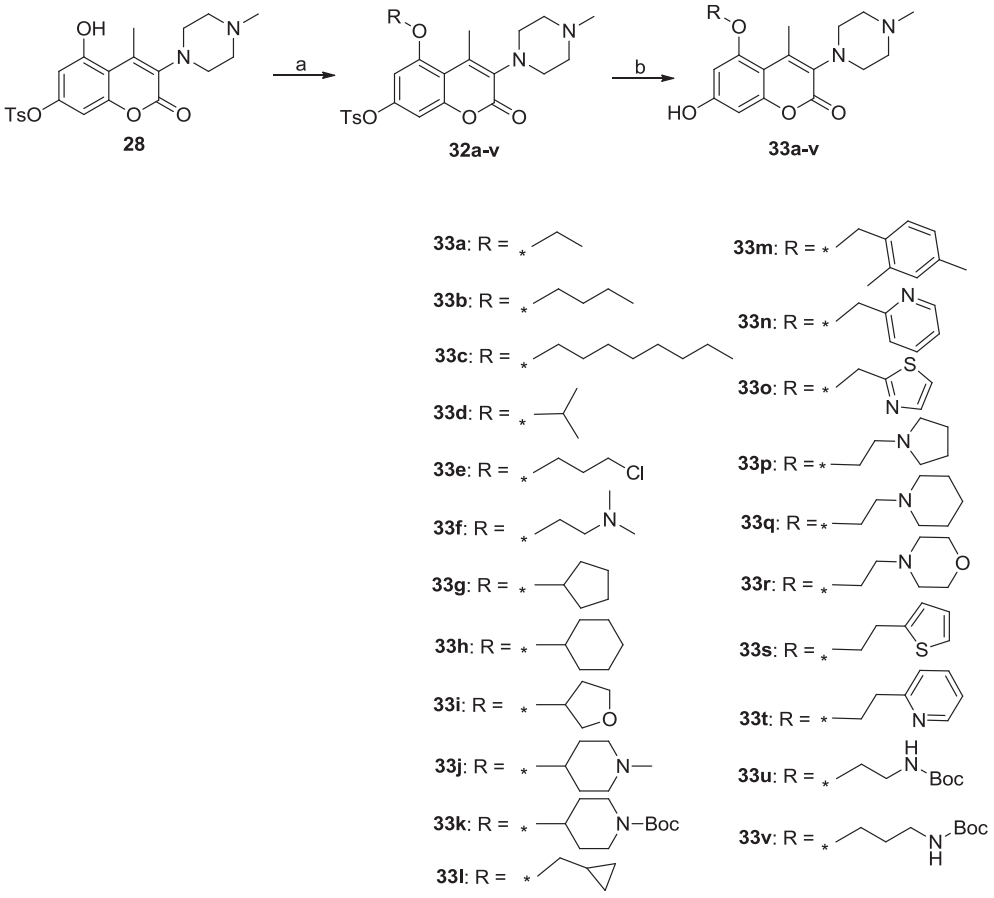
Scheme 3. Synthesis of coumarin analogs **20** and **21**. Reagents and conditions: (a) $BF_3{\cdot}Et_2O$, EtOH, reflux; (b) Me_2SO_4, K_2CO_3, acetone, rt; (c) TsCl, pyridine, rt; (d) BnBr, K_2CO_3, acetone, rt; (e) TBAF, THF, 0 °C; (f) Me_2SO_4, K_2CO_3, acetone, reflux; (g) TBAF, THF, rt; (h) Pd/C, $HCOONH_4$, THF, reflux.

*2.3.1. Protection by compound **20** against rat ischemic brain injury*

Injection of compound **20** using ethanol as cosolvent improved neurological function and reduced infarct size of rats at 24 h after reperfusion in a dose-dependent manner (Fig. 3). The neurological score of animals treated with compound **20** at doses of 1.5, 3.0 and 6.0 mg/kg was significantly lower than that of the vehicle control group (2.44 ± 0.81, 2.00 ± 0.47 and 1.84 ± 0.80, respectively, vs 3.00 ± 0.87, Fig. 3C). Compound **20** at doses of 3.0 and 6.0 mg/kg significantly reduced the percentage of the infarct area within the ipsilateral hemispheres compared with the vehicle control group (20.36 ± 7.9% and 18.89 ± 5.83%, respectively). The same result was observed for Edaravone at 6.0 mg/kg (Fig. 3A and B), with which the significant reduction of brain-water content also occurred (Fig. 3D).

*2.3.2. Effect of compound **20** on neuronal loss in the cortex and hippocampus*

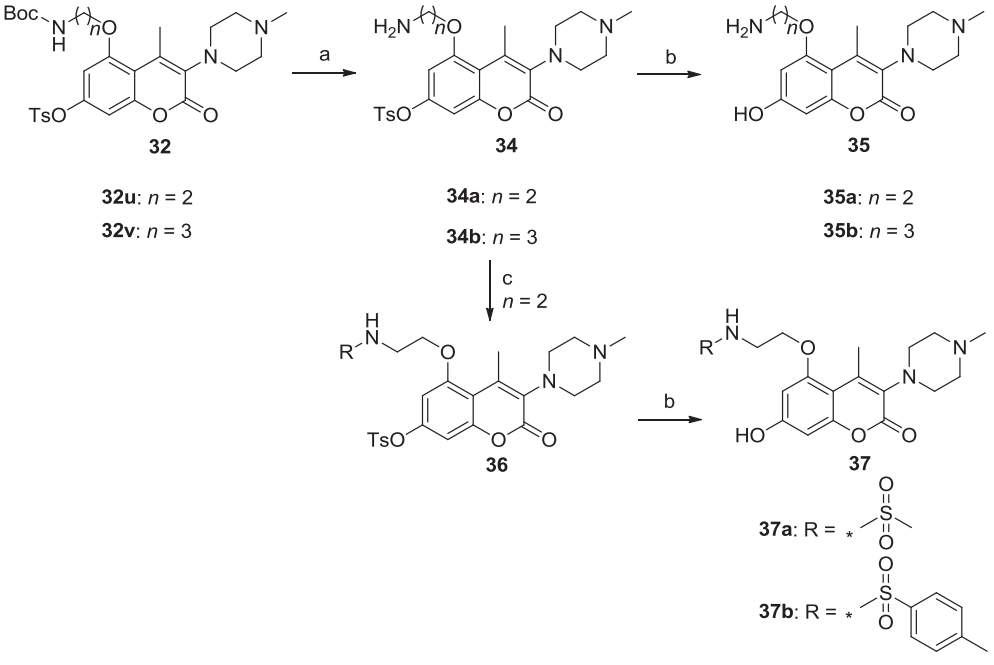
Typical neuropathological changes were observed in the cortex and the CA1 area of the hippocampus after MCAO. Fig. 4(A and C) shows representative photographs of Nissl staining from the hippocampus and cerebral cortex. The results of Nissl staining showed that neurons were clear and moderately sized with normal microstructure in untreated rats (Fig. 4a1 and c1). In contrast, the brain regions in MCAO rats were observed with significant neuronal shrinkage and dark staining (Fig. 4a2 and c2). These pathological changes were prominently suppressed in rats treated with compound **20** in a dose-dependent manner in the hippocampus and cortex (Fig. 4a3, a4, a5 and c3, c4, c5). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), which is based on the specific binding of terminal deoxynucleotidyl transferase to the 3′-OH ends of fragmented DNA, has become the most widely used *in situ* test for the study of apoptosis. Fig. 4(B and D) shows the results of TUNEL staining; the number of TUNEL-positive cells increased significantly in the hippocampus (Fig. 4b2) and cortex (Fig. 4d2) of MCAO rats showing apoptosis. A remarkable decrease in TUNEL-positive cells was observed in **20**-treated rat groups (Fig. 4b3, b4, b5 and d3, d4, d5). An ultrastructural study also provided strong evidence of neuronal damage (Fig. 5B). A high-electron-concentration neuronal nucleus was observed in MCAO rats, and the morphology was improved in **20**-treated groups (Fig. 5C, D and E).



Scheme 4. Synthesis of target compounds **33**. Reagents and conditions: (a) ROH, PPh_3, DEAD, THF, rt; (b) TBAF, THF, rt.

*2.3.3. Effect of compound **20** on the cell apoptosis-related proteins*

To explore the mechanism of protection against neuronal loss of compound **20** in MCAO rats, the cell apoptosis-related proteins were investigated. It is well known that apoptosis is actively regulated by several factors, including the decrease of antiapoptotic proteins such as Bcl-2, the increase of proapoptotic proteins such as Bax [24] and the activation of caspase-3, which is a major executioner of apoptotic signals [25,26]. The results (Fig. 6) showed that Bcl-2 expression



Scheme 5. Synthesis of target compounds **35** and **37**. Reagents and conditions: (a) CF_3COOH, DCM, 40 °C; (b) TBAF, THF, 50 °C; (c) RSO_2Cl, TEA, DCM, rt.

Table 1
Neuroprotection of coumarin derivatives at a single dose (10 μM) in damaged rat pheochromocytoma PC12 cells.

Compound	Proliferation[a]	OGD
Control[b]	100.0 ± 3.4	100.0 ± 5.9
Model[c]	–	44.2 ± 0.7##
1	107.4 ± 1.2	52.7 ± 3.7*
6a	98.1 ± 1.7	48.4 ± 0.5*
6b	97.4 ± 1.5	49.3 ± 1.9*
7	104.1 ± 3.2	46.3 ± 0.8
8a	103.3 ± 4.0	48.9 ± 1.2*
8b	101.1 ± 1.8	48.8 ± 0.9*
11	102.3 ± 1.4	45.9 ± 0.9
17a	97.7 ± 2.1	46.7 ± 2.1
17b	97.5 ± 3.7	46.1 ± 0.4
20	100.7 ± 5.4	66.0 ± 2.8**
21	99.8 ± 3.2	46.1 ± 0.4

*$p < 0.05$ vs model group.
**$p < 0.01$ vs model group.
##$p < 0.01$ vs control group ($n = 3$).
[a] Cell proliferation was performed in a full-culture medium and 10 μM tested compounds.
[b] A full-culture medium was used for the control group.
[c] OGD assay: the cells were treated with low-glucose medium and 5 mM sodium dithionite for 24 h.

decreased significantly compared with the control in both the cortex and the hippocampus, while compound **20** promoted the expression of Bcl-2. Remarkable increases in Bax and cleaved caspase-3 were also observed in MCAO rats; however, compound **20** inhibited the expression of Bax and cleaved caspase-3. These results indicate that compound **20** exhibits anti-ischemia activity by blocking the apoptosis pathway. Further studies on the antiapoptotic effect and its mechanism will be reported in detail in another journal.

Table 2
Neuroprotection of 5-hydroxyl group derivatives of compound **1** at a single dose (10 μM) in damaged rat pheochromocytoma PC12 cells.

Compound	Proliferation[a]	OGD
Control[b]	100.0 ± 3.4	100.0 ± 2.5
Model[c]	–	44.2 ± 0.7##
20	100.7 ± 5.4	66.0 ± 2.8**
33a	167.1 ± 2.9**	33.9 ± 5.7
33b	197.2 ± 7.7**	46.2 ± 3.9
33c	59.4 ± 3.0**	15.6 ± 1.2**
33d	119.1 ± 3.4**	51.8 ± 1.6
33e	196.9 ± 8.9**	36.3 ± 4.9
33f	103.9 ± 5.3	61.5 ± 4.8**
33g	181.1 ± 4.3**	33.8 ± 6.3
33h	176.8 ± 0.9**	36.1 ± 4.7
33i	106.9 ± 3.2	52.0 ± 2.0
33j	138.7 ± 7.8**	19.9 ± 3.7**
33k	137.3 ± 7.8**	51.5 ± 2.8
33l	184.4 ± 6.0**	37.7 ± 3.3
33m	51.6 ± 2.8**	27.3 ± 2.2*
33n	115.5 ± 4.5**	44.2 ± 5.9
33o	108.1 ± 4.9*	54.7 ± 2.8*
33p	134.7 ± 5.6**	48.2 ± 2.8
33q	144.1 ± 5.6**	24.9 ± 4.2*
33r	107.5 ± 2.3	56.9 ± 1.1*
33s	177.9 ± 8.4**	39.1 ± 4.7
33t	152.1 ± 2.4**	48.2 ± 1.5
33u	131.8 ± 4.8**	23.4 ± 2.6*
33v	153.3 ± 2.8**	15.8 ± 0.3**
35a	103.6 ± 5.5	49.2 ± 1.2
35b	107.4 ± 4.4	40.5 ± 4.2
37a	103.0 ± 2.9	40.3 ± 0.6
37b	140.1 ± 3.8**	45.1 ± 3.3

*$p < 0.05$ vs model group.
**$p < 0.01$ vs model group.
##$p < 0.01$ vs control group ($n = 3$).
[a] Cell proliferation was performed in a full-culture medium and 10 μM tested compounds.
[b] A full-culture medium was used for the control group.
[c] OGD assay: the cells were treated with low-glucose medium and 5 mM sodium dithionite for 24 h.

2.4. Pharmacokinetic evaluation of compound **20**

To obtain information on the *in vivo* behavior of compound **20**, its pharmacokinetic properties were studied. After intravenous administration of compound **20** to male Sprague Dawley rats (6 mg/kg) ($n = 4$), plasma and cortex samples were collected at intervals of 0, 2, 3.5, 3.75, 7.5, 15, 30 and 60 min. Samples were analyzed by LC–MS. The plasma and cortex concentration–time profiles of compound **20** are presented in Fig. 7. All of the pharmacokinetic parameters are listed in Table 3. The results showed that the C_{max} values were 13.309 and 0.122 mg/L in plasma and in the cortex at 2 min, respectively. The concentration of compound **20** in the plasma or the cortex was very low at 60 min. These results indicated that compound **20** was capable of crossing the blood–brain barrier (BBB) of Sprague Dawley rats.

3. Conclusions

In summary, 36 new coumarin derivatives were designed and synthesized in this study. A SARs study of these compounds was carried out, and a potent neuroprotective compound was discovered. Compound **20** significantly attenuated the PC12 cell damage induced by OGD *in vitro*, and exhibited clear neuroprotection in MCAO rats by reducing infarct size and brain-water content, improving neurological function, and suppressing neuronal loss and neuropathological changes in the cortex and hippocampus. Pharmacokinetic evaluation indicated that compound **20** was able to penetrate the BBB of rats. Mechanistic studies indicated that compound **20** acted against the apoptosis of nerve cells induced by oxygen and glucose deprivation/reoxygenation.

4. Experimental protocols

4.1. Chemistry

All materials used were of commercial grade without purification unless otherwise specified. All nuclear magnetic resonance (NMR) experiments were carried out on a Varian Mercury 300 or 400 or 500 or 600 MHz spectrometer or a Bruker Avance 400 MHz spectrometer using dimethylsulfoxide-d_6 (DMSO-d_6), methanol-d_4 and acetone-d_6 as the solvent. Chemical shifts were reported in ppm (δ) relative to the solvent, and coupling constants (J) were reported in Hz. Melting points were determined without correction with a Yanaco micromelting point apparatus. HPLC-MS analysis was performed on a Thermo Finnigan LCQ Advantage mass spectrometer equipped with an Agilent pump, an Agilent detector, an Agilent liquid handler, and a fluent splitter. The column used was a Kromasil C18 column (4.6 μm, 4.6 mm × 50 mm) from DIKMA for analysis. The eluent was a mixture of acetonitrile and water containing 0.05% HCOOH with a linear gradient from 5:95 (v/v) to 95:5 (v/v) of acetonitrile–H_2O within 5 min at a 1.0 mL/min flow rate for analysis. The UV detection was carried out at a UV wavelength of 254 nm. The 5% of the eluent was split into the MS system. Mass spectra were recorded in either positive or negative ion mode using electrospray ionization (ESI). High resolution LC-MS (HRMS) was carried out by Agilent LC/MSD TOF using a column of Agilent ZORBAX SB–C18 (rapid resolution, 3.5 μm, 2.1 mm × 30 mm) at a flow of 0.40 mL/min. The solvent is methanol/water = 75:25 (v/v) containing 5 mmol/L ammonium formate. The ion source is electrospray ionization (ESI). Flash column chromatography was performed with silica gel 60 (200–300 mesh) from Qingdao Haiyang

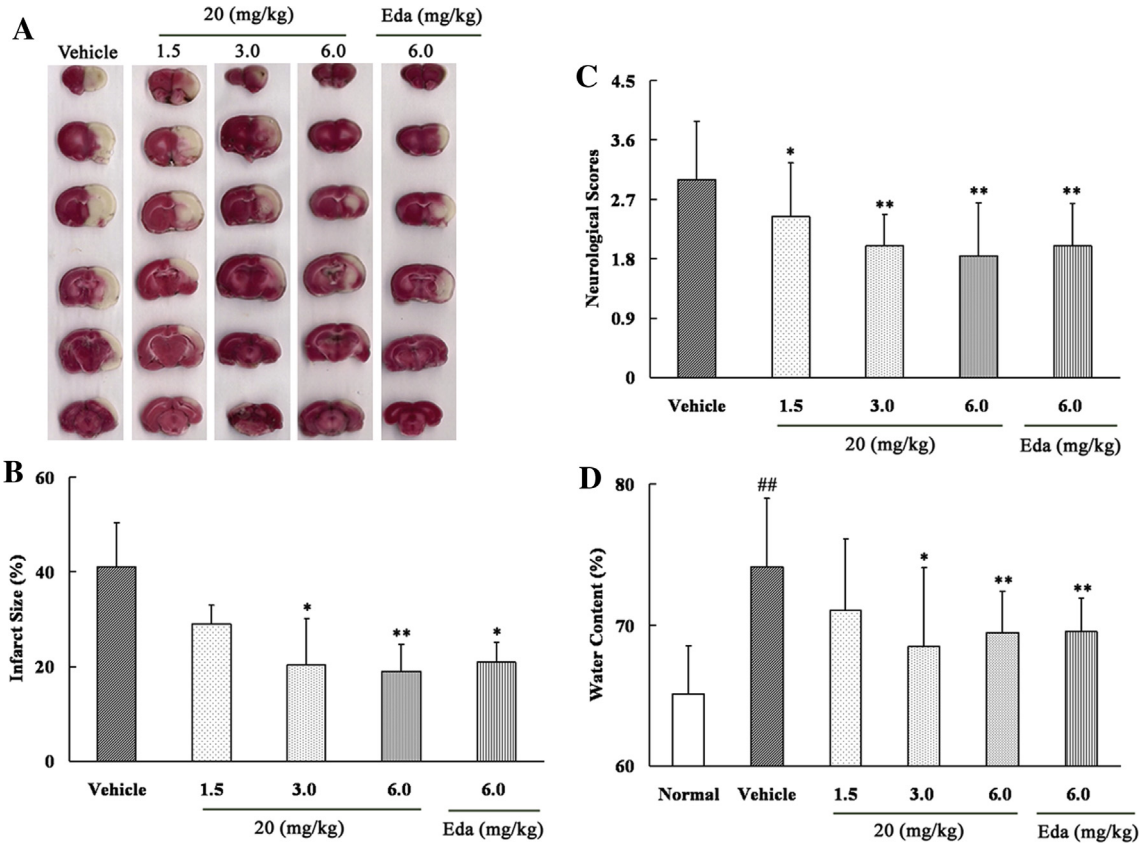


Fig. 3. Compound **20** effectively protected against rat ischemic brain injury. (A and B) Brain infarct size, (C) Neurological score and (D) brain-water content at 24 h after ischemia. After the rats underwent MCAO and were treated with compound **20**, edaravone or saline, they were scored by Longa's 5-point scale, and then the rat brains were stained with TTC. Finally, the brain-water content was determined. Data are expressed as the mean ± SD: (*) $p < 0.05$ vs vehicle group; (**) $p < 0.01$ vs vehicle group; (##) $p < 0.01$ vs normal group ($n = 12$).

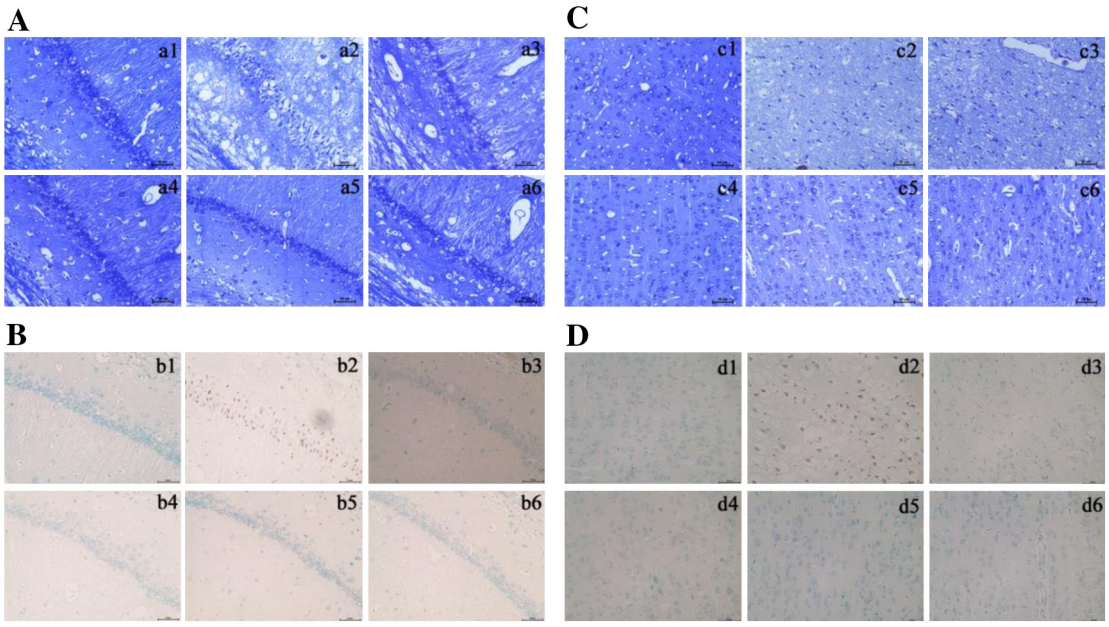


Fig. 4. Compound **20** decreased the neuronal loss in both the cerebral cortex and the hippocampus. After the rats underwent MCAO and were treated with compound **20**, Edaravone or saline, the rat brains were perfused and fixed with 4% buffered paraformaldehyde. Neuronal loss was observed by Nissl staining and TUNEL staining. (A) Hippocampus by Nissl staining; (B) hippocampus by TUNEL staining; (C) cerebral cortex by Nissl staining; (D) cerebral cortex by TUNEL staining. Parts a1, b1, c1 and d1 were from the untreated group. Parts a2, b2, c2 and d2 were from the vehicle control group. Parts a3, b3, c3 and d3 were from the 1.5 mg/kg **20** group. Parts a4, b4, c4 and d4 were from the 3.0 mg/kg **20** group. Parts a5, b5, c5 and d5 were from the 6.0 mg/kg **20** group. Parts a6, b6, c6 and d6 were from the 6.0 mg/kg Edaravone group (magnification 200×).

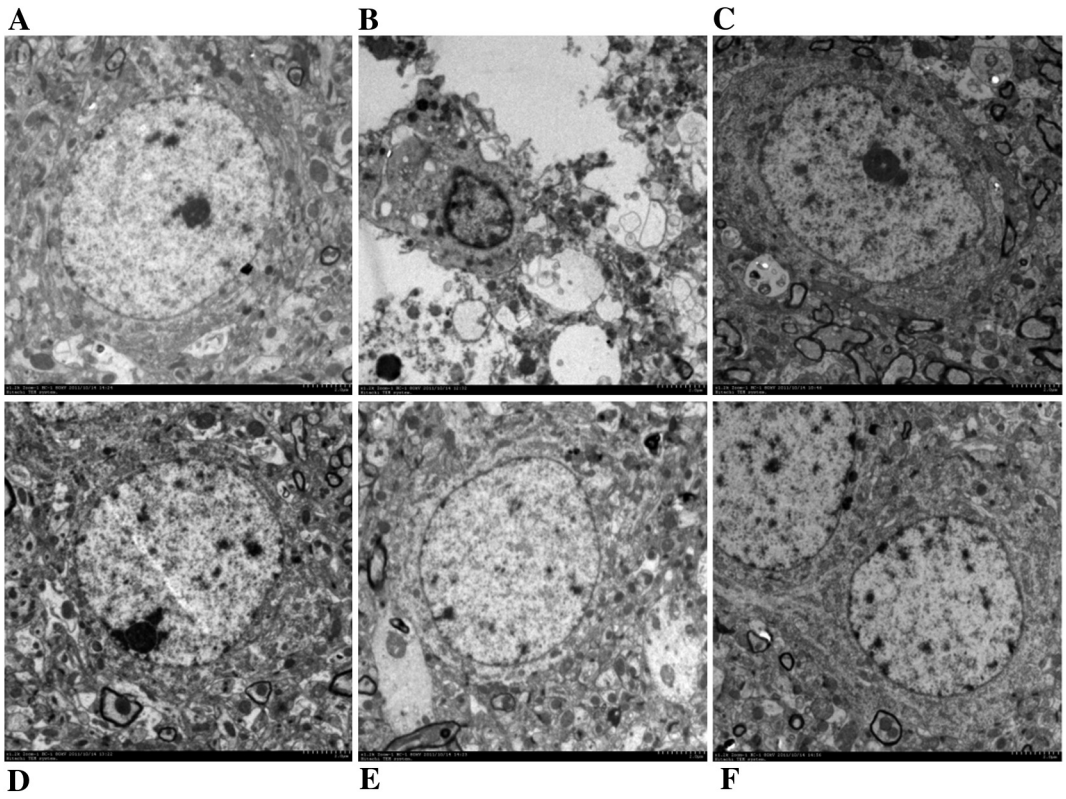


Fig. 5. The ultrastructural neuropathological changes were significantly suppressed by compound **20**, as observed by electron microscopy. After the rats underwent MCAO and were treated with compound **20**, Edaravone or saline, the rat brains were perfused, fixed and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and the ultrastructure was observed using electron microscopy. (A) Untreated group; (B) vehicle control group; (C) compound **20** (1.5 mg/kg)-treated group; (D) compound **20** (3.0 mg/kg)-treated group; (E) compound **20** (6.0 mg/kg)-treated group; (F) Edaravone (6.0 mg/kg)-treated group (magnification 12000×).

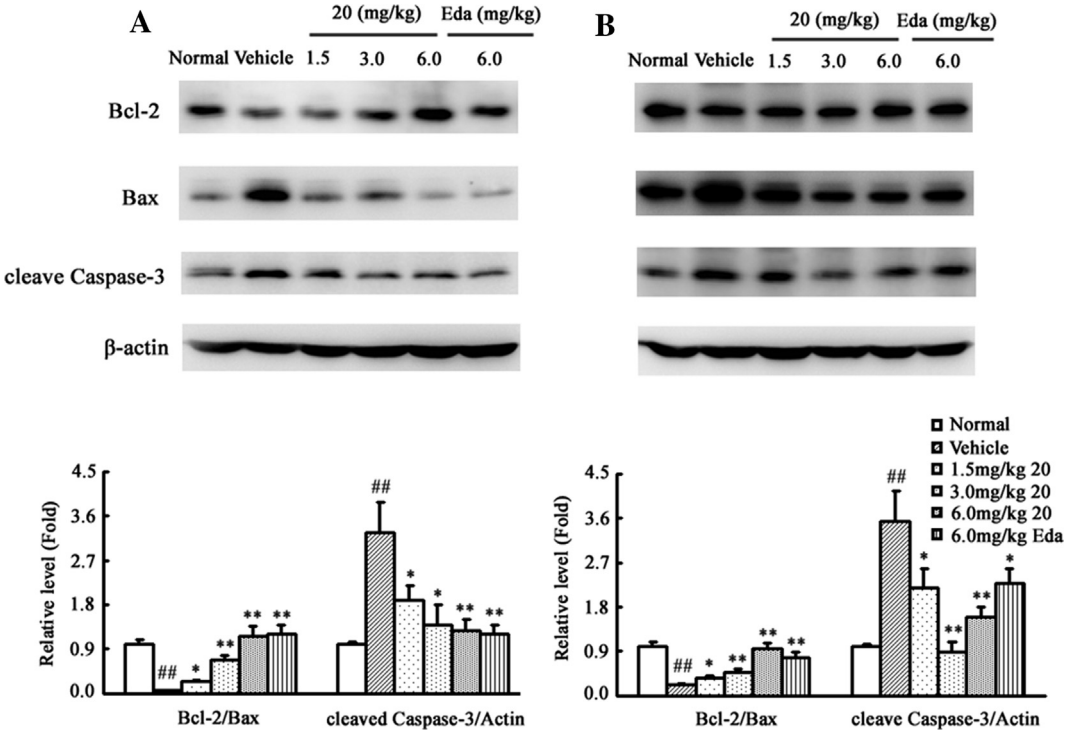


Fig. 6. Effect of compound **20** on Bcl-2, Bax and cleaved caspase-3 expression in the cerebral cortex and hippocampus. After the rats underwent MCAO and were treated with compound **20**, Edaravone or saline, the cerebral cortex and hippocampus were separated. The expression of Bcl-2, Bax and cleaved caspase-3 was detected by Western blot analysis. (A) Hippocampus; (B) Cerebral cortex. Quantitative analysis of the ratio of Bcl-2/Bax, the expression of cleaved caspase-3 by Science Lab 2005 Image Gauge software. ($n = 4$), (*) $p < 0.05$ vs vehicle group; (**) $p < 0.01$ vs vehicle group; (##) $p < 0.01$ vs normal group).

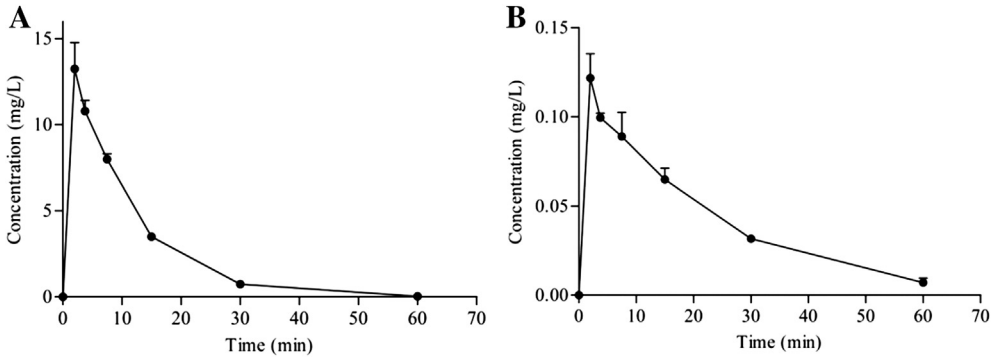


Fig. 7. Mean plasma (A) and mean cortex (B) concentration–time curves after a single intravenous dose of compound **20** (6 mg/kg, $n = 4$).

Chemical Factory. The tested compounds were purified until the purity was ≥95%, detected by HPLC under UV 254 nm wavelength, NMR, melting point, and HRMS.

*4.1.1. General procedure for the synthesis of compounds **6a** and **6b***

The key intermediate **5** was prepared according to the literature [20]. To a solution of **5** (0.1 mmol) in 5 mL of dry THF was added various acid anhydrides (0.1 mmol). The reaction mixture was stirred at room temperature for 30 min and then got clear. After the reaction was completed monitored by LC-MS analysis, the solvent was evaporated in vacuo. The final products were characterized after purification by silica gel column chromatography.

*4.1.1.1. 3-(4-Acetylpiperazin-1-yl)-5,7-dihydroxy-4-methyl-2H-chromen-2-one (**6a**).* This compound was obtained as green-yellow powder in 85% yield. mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (s, 1H), 10.18 (s, 1H), 6.25 (d, J = 1.6 Hz, 1H), 6.13 (d, J = 1.6 Hz, 1H), 3.8–2.6 (m, 8H), 2.65 (s, 3H), 2.01 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.10, 160.17, 157.95, 157.88, 154.50, 149.92, 128.11, 102.20, 99.30, 94.09, 49.43, 48.98, 46.44, 41.51, 21.22, 16.95. HRMS calcd for $C_{16}H_{19}N_2O_5$ ($M + H^+$) 319.1294; found 319.1289.

*4.1.1.2. 5,7-Dihydroxy-4-methyl-3-(4-(2,2,2-trifluoroacetyl)piperazin-1-yl)-2H-chromen-2-one (**6b**).* This compound was obtained as pale-yellow powder in 88% yield. mp 274–275 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.46 (s, 1H), 10.22 (s, 1H), 6.26 (d, J = 2.1 Hz, 1H), 6.13 (d, J = 2.1 Hz, 1H), 4.23–3.57 (m, 4H), 3.15–2.80 (m, 4H), 2.66 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.3, 158.0, 157.9, 154.5, 150.3, 127.6, 117.5, 115.2, 102.1, 99.4, 94.1, 49.441, 48.7, 46.2, 43.7, 17.00. HRMS calcd for $C_{16}H_{16}F_3N_2O_5$ ($M + H^+$) 373.1011; found 373.1012.

*4.1.2. Synthesis of 4-(5,7-dihydroxy-4-methyl-2-oxo-2H-chromen-3-yl)-N-(4-fluorophenyl)piperazine-1-carboxamide (**7**)*

To a solution of **5** (0.1 mmol) in dry THF 5 mL was added 1-fluoro-4-isocyanatobenzene (0.12 mmol). The reaction mixture was stirred at 50 °C. After the reaction was completed, the solvent was evaporated in vacuo. The final product **7** was obtained as white powder after purification by silica gel column chromatography eluting with DCM–CH_3OH (20:1, v/v). Yield 51%; mp 210–213 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.27 (s, 2H), 8.58 (s, 1H), 7.48 (dd, J = 8.7 Hz, 4.8 Hz, 2H), 7.07 (dd, J = 8.7 Hz, 4.8 Hz, 2H), 6.27 (d, J = 1.5 Hz, 1H), 6.13 (d, J = 2.1 Hz, 1H), 4.09–2.80 (m, 8H), 2.69 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.2, 158.0, 157.9, 155.0, 154.5, 150.0, 136.9, 136.9, 128.2, 121.3, 121.2, 114.9, 114.6, 102.3, 99.3, 94.1, 49.1, 44.5, 17.0. HRMS calcd for $C_{21}H_{21}FN_3O_5$ ($M + H^+$) 414.1465; found. 414.1457.

Table 3
Obtained pharmacokinetic parameters of compound **20** after a single intravenous dose (2×10^{-5} mol/kg) in the rat plasma and cortex.

	Parameters[a]					
	C_{max} (mg/L)	t_{max} (min)	$t_{1/2}$ (min)	AUC (min mg/L)	V_d (L)	CL (L/min)
In plasma	13.309	2.0	6.514	172.147	0.084	0.009
In cortex	0.122	2.0	15.526	2.544	12.273	0.558

[a] C_{max}: maximum measured concentration; t_{max}: time to reach the maximum measured concentration; $t_{1/2}$: half-life of the terminal slope of the concentration–time curve; AUC: area under the concentration–time curve; V_d: volume of distribution; CL: clearance.

*4.1.3. General procedure for the synthesis of compounds **8a** and **8b***

To a solution of **5** (0.1 mmol) in 5 mL of dry THF was added isothiocyanate (0.12 mmol). The reaction mixture was stirred at 50 °C. After the reaction was completed, the solvent was evaporated in vacuo. The final product was obtained after purification by silica gel column chromatography.

*4.1.3.1. N-butyl-4-(5,7-dihydroxy-4-methyl-2-oxo-2H-chromen-3-yl) piperazine-1-carbothioamide (**8a**).* This compound was obtained as off-white powder in 48% yield; mp 245–248 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.42 (s, 1H), 10.19 (s, 1H) 7.64 (t, J = 5.1 Hz, 1H), 6.25 (d, J = 2.4 Hz, 1H), 6.12 (d, J = 2.4 Hz, 1H), 4.5–2.5 (m, 8H), 3.52–3.46 (m, 2H), 2.66 (s, 3H), 1.56–1.47 (m, 2H),1.34–1.10 (m, 2H), 0.88 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.22, 161.80, 160.68, 158.14, 155.26, 151.14, 126.51, 102.06, 99.87, 92.93, 48.82, 47.98, 45.37, 30.57, 19.62, 17.02, 14.18. HRMS calcd for $C_{19}H_{26}N_3O_4S$ ($M + H^+$) 392.1644; found. 392.1632.

*4.1.3.2. 4-(5,7-Dihydroxy-4-methyl-2-oxo-2H-chromen-3-yl)-N-(3-fluorophenyl)piperazine-1-carbothioamide (**8b**).* This compounds was obtained as pale-yellow powder in 38% yield; mp 282–284 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 10.21 (s, 1H), 9.45 (s, 1H), 7.35–7.24 (m, 2H), 7.16 (d, J = 8.0 Hz, 1H), 6.91 (t, J = 7.8 Hz, 1H), 6.28 (d, J = 2.1 Hz, 1H), 6.14 (d, J = 2.1 Hz, 1H), 2.70 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.39, 162.09 (d, J = 240 Hz, 1C), 161.07, 159.08, 158.48, 155.10, 150.96, 143.42 (d, J = 11 Hz, 1C), 129.84 (d, J = 10 Hz, 1C), 127.93, 120.75 (d, J = 2 Hz, 1C), 111.88 (d, J = 24 Hz, 1C), 110.90 (d, J = 20 Hz, 1C), 102.67, 99.94, 94.33, 49.49, 49.37, 17.53. HRMS calcd for $C_{21}H_{20}FN_3O_4S$ ($M + H^+$) 430.12161; found. 430.12199.

*4.1.4. Synthesis of 5,7-dihydroxy-4-methyl-3-(2-(4-methylpiperazin-1-yl)ethyl)-2H-chromen-2-one (**11**)*

To a stirred solution of pholoroglucinol (2 mmol) in benzene (5 mL), **9** (2 mmol) and PBr_3 (6 mmol) were added at room temperature. The reaction mixture refluxed for 1 h and some precipitation was formed. Analyzed by LC-MS the solid was demonstrated

as the target intermediate **10** (460 mg, in 77% yield). mp > 252 °C decomposed. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.53 (s, 1H), 10.25 (s, 1H), 9.45 (s, 1H), 6.28 (d, J = 2.4 Hz, 1H), 6.15 (d, J = 2.4 Hz, 1H), 3.53 (t, J = 7.5 Hz, 2H), 3.04 (t, J = 7.5 Hz, 2H), 2.55 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.6, 160.5, 157.8, 154.9, 151.2, 116.2, 102.4, 99.5, 94.3, 31.5, 30.1, 19.1.

Intermediate **10** (1 mmol) was dissolved in 2-butanone (15 mL). K_2CO_3 (5 mmol), KI (1 mmol) and 1-methylpiperazine (5 mmol) were added to the above solution and the reaction mixture was refluxed with stirring for 1.5 h. After the reaction was completed, the solvent was evaporated in vacuo. The final product **11** was obtained as yellow powder after purification by silica gel column chromatography eluting with DCM–CH_3OH (10:1, v/v). Yield 75%; mp > 225 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 10.18 (s, 1H), 6.27 (d, J = 1.8 Hz, 1H), 6.14 (d, J = 1.7 Hz, 1H), 3.2–2.2 (m, 8H), 2.67 (t, J = 6.9 Hz, 2H), 2.54 (s, 3H), 2.44 (s, 3H), 2.44(t, J = 6.9 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.9, 160.1, 157.5, 154.8, 149.8, 117.0, 102.5, 99.4, 94.2, 55.9, 53.8, 51.4, 44.5, 23.8, 18.9. HRMS calcd for $C_{17}H_{23}N_2O_4$ (M + H^+) 319.1658; found. 319.1646.

4.1.5. Synthesis of 5,7-dihydroxy-4-methyl-3-(2-(4-methylpiperazin-1-yl)-2-oxoethyl)-2H-chromen-2-one (**17a**)

To a solution of **12** (3 mmol) in absolute ethanol (5 mL) was added ethylate sodium (2 mmol) in absolute ethanol (2 mL). The mixture was heated to 60 °C slowly followed by the addition of **13a** (2 mmol) in ethanol (2 mL). The reaction was stirred at room temperature for 2 d and then was refluxed for 2 h. Diethyl ether (Et_2O) was added to the mixture when cooled to room temperature. After filtration and concentration, brown oil **14a** was obtained without purification. 10 mL of methanol saturated with dry HCl gas was added to the mixture of pholoroglucinol (2 mmol) and **14a**. The mixture was stirred at room temperature until pholoroglucinol was completely disappeared monitored by LC-MS. The mixture was concentrated in vacuo. Intermediate **15a** was obtained as brown powder after recrystallization in ethanol. Yield 37% for two steps; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.55 (s, 1H), 10.27 (s, 1H), 6.29 (d, J = 2.3, 1H), 6.17 (d, J = 2.3, 1H), 3.61 (s, 3H), 3.59 (s, 2H), 2.49 (s, 3H).

Intermediate **15a** (0.5 mmol) and K_2CO_3 (1 mmol) were dissolved in 10 mL of ethanol/H_2O (1:1, v/v). The mixture was refluxed for 3 h, diluted by H_2O (5 mL) followed by neutralization with 1 N HCl until pH 3–4, and then left overnight. Intermediate **16a** was obtained as pale-yellow needle crystal after filtration. Yield 68%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.34 (s, 1H), 10.54 (s, 1H), 10.26 (s, 1H), 6.29 (d, J = 1.8 Hz, 1H), 6.16 (d, J = 1.8 Hz, 1H), 3.49 (s, 3H), 2.47 (s, 2H).

To a solution of **16a** (0.2 mmol), BOP (0.24 mmol) and TEA (0.3 mmol) in THF (5 mL) was added 1-methylpiperazine (0.4 mmol). The mixture was stirred at room temperature for 1.5 h and some precipitate was formed. After filtration compound **17a** was obtained as white powder. Yield 78%; mp > 300 °C decomposed. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.48 (s, 1H), 10.23 (s, 1H), 6.29 (d, J = 1.8 Hz, 1H), 6.15 (d, J = 2.1 Hz, 1H), 3.57 (s, 2H), 3.57 (brs, 2H), 3.42 (brs, 2H), 2.39 (s, 3H), 2.34 (brs, 2H), 2.24 (brs, 2H), 2.19 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.62, 161.01, 160.19, 157.60, 154.89, 150.89, 114.75, 102.54, 99.45, 94.21, 54.81, 54.36, 45.63, 44.79, 41.30, 30.91, 19.28. HRMS calcd for $C_{17}H_{21}N_2O_5$ (M + H^+) 333.1450; found. 333.1439.

4.1.6. Synthesis of 5,7-dihydroxy-4-methyl-3-(3-(4-methylpiperazin-1-yl)-3-oxopropyl)-2H-chromen-2-one (**17b**)

Compound **17b** was prepared in a similar manner to the synthesis of compound **17a**, substituting **13a** with **13b**. The intermediate **15b** was obtained as pale-yellow in 33% yield; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.45 (s, 1H), 10.18 (s, 1H), 6.25 (d, J = 2.1 Hz, 1H), 6.13 (d, J = 2.4 Hz, 1H), 3.58 (s, 3H), 2.74 (t, J = 8.4 Hz, 2H), 2.52 (s, 3H), 2.41 (t, J = 8.4 Hz, 2H).

The intermediate **16b** was obtained as white powder in 57% yield; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.21 (d, J = 1.8 Hz, 1H), 5.98 (d, J = 2.1 Hz, 1H), 2.63 (t, J = 8.1 Hz, 2H), 2.52 (s, 3H), 2.06 (t, J = 8.1 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.7, 161.3, 161.2, 154.9, 149.9, 142.5, 117.7, 102.5, 100.1, 92.8, 36.1, 23.7, 18.5.

The final product **17b** was obtained as off-white powder. Yield 79%; mp > 250 °C decomposed. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.29 (brs, 2H), 6.23 (d, J = 2.4 Hz, 1H), 6.11 (d, J = 2.1 Hz, 1H), 3.39–3.38 (m, 4H), 2.66 (t, J = 7.2 Hz, 2H), 2.49 (s, 3H), 2.37 (t, J = 7.2 Hz, 2H), 2.20–2.17 (m, 4H), 2.11 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.9, 157.5, 157.1, 154.8, 154.1, 149.6, 118.2, 102.2, 99.4, 94.2, 54.5, 54.1, 45.3, 44.6, 40.7, 31.1, 22.7, 18.7. HRMS calcd for $C_{18}H_{23}N_2O_5$ (M + H^+) 347.1067; found. 347.1596.

4.1.7. Synthesis of 7-hydroxy-5-methoxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (**20**)

The intermediate compound **1** was prepared in a similar manner to the synthesis of compound **5**, substituting piperazine with 1-methylpiperazine. To a solution of **1** (20 mmol) in pyridine (20 mL) at 0 °C was added TsCl (50 mmol) slowly. The mixture was stirred at room temperature for 2 h. When the reaction was completed as monitored by LC-MS, the pyridine was evaporated in vacuo and water (50 mL) was added to the residue followed by extraction with ethyl acetate (3 × 30 mL). The organic layers were combined, dried over anhydrous $MgSO_4$, and evaporated in vacuo. Intermediate **23** was obtained as yellow powder after chromatography by silica gel eluting with DCM–CH_3OH (20:1, v/v). Yield 89%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.75 (d, J = 8.3 Hz, 2H), 7.62 (d, J = 8.3 Hz, 2H), 7.48 (d, J = 8.2 Hz, 2H), 7.44 (d, J = 8.2 Hz, 2H), 7.08 (d, J = 2.4 Hz, 1H), 6.69 (d, J = 2.4 Hz, 1H), 2.88 (s, 4H), 2.40 (s, 6H), 2.34 (s, 4H), 2.32 (s, 3H), 2.17 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.2, 151.6, 147.9, 146.7, 146.4, 145.6, 139.1, 135.6, 130.6, 130.6, 130.4, 128.3, 128.2, 114.9, 112.6, 109.2, 55.1, 49.1, 46.0, 21.2, 21.1, 17.2.

To a solution of compound **23** (15 mmol) in acetone (100 mL) was added K_2CO_3 (22.5 mmol) and BnBr (15 mmol) with stirring at room temperature. When the reaction was completed as monitored by LC-MS, the insoluble substrate was filtered off and the filtrate was evaporated in vacuo. The intermediate **24** was obtained as light-yellow powder after chromatography by silica gel eluting with DCM–CH_3OH (20:1, v/v). Yield 87%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.78–7.71 (m, 4H), 7.65–7.58 (m, 2H), 7.58–7.47 (m, 7H), 7.20 (d, J = 2.4, 1H), 6.69 (d, J = 2.4, 1H), 4.74 (s, 2H), 3.67 (t, J = 10.8, 2H), 3.52 (t, J = 12.5, 2H), 3.41–3.37 (m, 2H), 3.05 (s, 3H), 3.02 (brs, 2H), 2.48 (s, 3H), 2.45 (s, 3H), 2.44 (s, 3H).

Intermediate **24** (12 mmol) was dissolved THF (100 mL). The mixture was added with TBAF (12 mmol) in ice bath and kept at 0 °C for 4 h with stirring. Then the solvent was evaporated in vacuo and the intermediate **25** was obtained as yellow powder after chromatography by silica gel eluting with DCM–CH_3OH (15:1, v/v). Yield 87%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.74 (d, J = 8.3, 2H), 7.62–7.53 (m, 5H), 7.42 (d, J = 8.1, 2H), 6.17 (d, J = 1.5, 1H), 6.01 (d, J = 1.9, 1H), 4.71 (s, 2H), 3.65 (brs, 4H), 3.39 (brs, 4H), 2.99 (s, 3H), 2.72 (s, 3H), 2.37 (s, 3H).

To a solution of **25** (9 mmol) in acetone (70 mL) was added K_2CO_3 (15 mmol) and Me_2SO_4 (15 mmol) successively with stirring at room temperature. 5 min later, the mixture was refluxed for 2 h. When the reaction was completed as monitored by LC-MS, the insoluble substrate was filtered off and the filtrate was evaporated in vacuo. The intermediate **26** was obtained as yellow powder after chromatography by silica gel eluting with DCM–CH_3OH (15:1, v/v). Yield 82%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.82 (d, J = 8.3, 2H), 7.65–7.46 (m, 7H), 6.68 (d, J = 2.3, 1H), 6.62 (d, J = 2.3, 1H), 4.74 (s, 2H), 3.76 (s, 3H), 3.67–3.37 (m, 8H), 3.00 (s, 3H), 2.60 (s, 3H), 2.42 (s, 3H).

TBAF (12 mmol) was added to the solution of **26** (6 mmol) in THF (30 mL) and the mixture was stirred at room temperature. When the reaction was completed as monitored by LC-MS, the solvent was evaporated in vacuo. The intermediate **27** was obtained as yellow powder after chromatography by silica gel eluting with DCM–CH_3OH (15:1, v/v). Yield 85%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.59–7.52 (m, 5H), 6.02 (s, 1H), 5.85 (s, 1H), 4.67 (s, 2H), 3.71 (s, 3H), 3.68 (brs, 4H), 3.32–3.30 (m, 2H), 2.97 (s, 3H), 2.74–2.72 (m, 2H), 2.56 (s, 3H).

To the solution of **27** (4 mmol) in CH_3OH (20 mL) was added Pd/C (8 mmol) and $HCOONH_4$ (20 mmol). The reaction mixture was refluxed for 40 min. After filtration, the filtrate was concentrated in vacuo and the final product **20** was obtained as pale-yellow powder after chromatography by silica gel eluting with DCM–CH_3OH (20:1, v/v). Yield 91%; mp 199–201 °C. ^{1}H NMR (300 Hz, DMSO-d_6) δ 10.428 (s, 1H), 6.347 (d, J = 2.1 Hz, 1H), 6.266 (d, J = 1.8 Hz, 1H), 3.812 (s, 3H), 3.60–2.20 (m, 8H), 2.586 (s, 3H), 2.204 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.5, 159.2, 157.6, 154.2, 148.6, 129.2, 103.1, 96.2, 95.0, 55.9, 55.3, 48.9, 46.1, 17.0. HRMS calcd for $C_{16}H_{21}N_2O_4$ ($M + H^+$) 305.1501; found. 305.1503.

4.1.8. Synthesis of 5-hydroxy-7-methoxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (*21*)

The intermediate **28** was prepared from **23** in a similar manner to the synthesis of **25** from **24** as pale-yellow powder after chromatography through silica gel eluting with DCM–CH_3OH (20:1, v/v). Yield 84%; ^{1}H NMR (400 Hz, DMSO-d_6) δ 7.76 (d, J = 4.0 Hz, 2H), 7.45 (d, J = 4.0 Hz, 2H), 6.45 (s, 1H), 6.33 (s, 1H), 2.93 (brs, 4H), 2.60 (s, 3H), 2.44 (brs, 4H), 2.23 (s, 3H).

The intermediate **29** was prepared as yellow solid in a similar manner to the synthesis of **24** from **23**. Yield 89%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.79 (d, J = 7.6, 2H), 7.65–7.32 (m, 12H), 6.81 (s, 1H), 6.69 (s, 1H), 5.09 (s, 2H), 4.67 (s, 2H), 3.72–3.34 (m, 8H), 2.97 (s, 3H), 2.56 (s, 3H), 2.42 (s, 3H).

The intermediate **30** was prepared as light yellow solid in a similar manner to the synthesis of **27** from **26**. Yield 92%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.58 (s, 1H), 7.68–7.31 (m, 10H), 6.51 (d, J = 1.9, 1H), 6.34 (d, J = 1.9, 1H), 5.17 (s, 2H), 4.69 (s, 2H), 3.64 (brs, 4H), 3.33 (brs, 4H), 2.98 (s, 3H), 2.59 (s, 3H).

The intermediate **31** was prepared as light yellow solid in a similar manner to the synthesis of **26** from **25**. Yield 84%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.71–7.30 (m, 10H), 6.65 (d, J = 2.1, 1H), 6.58 (d, J = 2.0, 1H), 5.21 (s, 2H), 4.80 (s, 2H), 3.83 (s, 3H), 3.71 (brs, 4H), 3.39 (brs, 4H), 3.03 (s, 3H), 2.60 (s, 3H).

The final compound **21** was obtained through the similar operation as **20**. Yield 95%; mp 184–186 °C. ^{1}H NMR (300 Hz, DMSO-d_6) δ 10.67 (s, 1H), 6.36 (d, J = 2.4 Hz, 1H), 6.32 (d, J = 2.4 Hz, 1H), 3.76 (s, 3H), 2.86 (brs, 4H), 2.64 (s, 3H), 2.41 (brs, 4H), 2.21 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.3, 158.1, 157.7, 154.4, 149.1, 129.2, 103.6, 98.2, 92.0, 55.4, 48.9, 46.1, 16.9. HRMS calcd for $C_{16}H_{21}N_2O_4$ ($M + H^+$) 305.1501; found. 305.1498.

4.1.9. General procedure for the synthesis of compounds *33a–v*

To a solution of **28** (1 mmol), PPh_3 (2.5 mmol) and various commercially available alcohol (2.5 mmol) in dry THF (1 mL) was added DEAD (2.5 mmol) dropwise with intensive stirring at room temperature. 20 min later, the reaction mixture was concentrated in vacuo and the corresponding intermediates **32a–v** were obtained after chromatography by silica gel eluting with DCM–CH_3OH. The final products **33a–v** were prepared in a similar manner to the synthesis of compound **27** from **26** and characterized after purification by silica gel chromatography eluting with DCM–CH_3OH.

4.1.9.1. 5-Ethoxy-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (*33a*).

33a was gray powder. Yield 78.6%; mp > 218 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 6.37 (d, J = 1.6 Hz, 1H), 6.31 (d, J = 1.6 Hz, 1H), 4.06 (q, J = 6.8 Hz, 2H), 2.76 (s, 3H), 2.64 (s, 3H), 1.40 (t, J = 6.8 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.4, 158.4, 157.6, 154.2, 148.7, 129.2, 103.1, 96.6, 94.9, 64.4, 55.3, 48.8, 46.0, 17.2, 14.3. HRMS calcd for $C_{17}H_{23}N_2O_4$ ($M + H^+$) 319.1658; found 319.1639.

4.1.9.2. 5-Butoxy-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (*33b*).

33b was white powder. Yield 85%; mp > 220 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (s, 1H), 6.33 (s, 1H), 6.26 (s, 1H), 3.98 (s, 2H), 2.62 (s, 3H), 2.37 (s, 3H), 1.75 (s, 2H), 1.46 (dd, J = 14.1, 7.2 Hz, 2H), 0.94 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 162.52, 160.68, 160.57, 156.13, 153.16, 128.98, 104.70, 97.56, 96.18, 70.04, 55.70, 48.49, 44.29, 32.28, 20.61, 18.26, 14.19. HRMS calcd for $C_{19}H_{27}N_2O_4$ ($M + H^+$) 347.1971; found 347.1965.

4.1.9.3. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-(octyloxy)-2H-chromen-2-one (*33c*).

33c was pale-yellow powder. Yield 67%; mp 85–87 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.49 (s, 1H), 6.35 (d, J = 2.0 Hz, 1H), 6.28 (d, J = 2.0 Hz, 1H), 3.99 (t, J = 6.0 Hz, 2H), 2.90 (brs, 8H), 2.63 (s, 3H), 2.56 (s, 3H), 1.80–1.74 (m, 2H), 1.49–1.41 (m, 2H), 1.29–1.23 (m, 8H), 0.88–0.85 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.68, 158.67, 157.75, 154.34, 130.72, 128.39, 102.91, 96.84, 95.08, 68.66, 53.86, 47.52, 43.82, 31.19, 28.65, 28.58, 28.48, 25.77, 22.05, 17.34, 13.94. HRMS calcd for $C_{23}H_{35}N_2O_4$ ($M + H^+$) 403.2597; found 403.2597.

4.1.9.4. 7-Hydroxy-5-isopropoxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (*33d*).

33d was yellow powder. Yield 85%; mp 73–75 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.37 (s, 1H), 6.35 (d, J = 2.0 Hz, 1H), 6.24 (d, J = 2.1 Hz, 1H), 4.70–4.61 (m, 1H), 2.62 (s, 3H), 2.33 (s, 3H), 1.34 (s, 2H), 1.32 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.4, 157.6, 157.2, 154.4, 148.9, 129.1, 103.6, 97.4, 94.8, 70.7, 55.1, 48.6, 45.7, 21.6, 17.5. HRMS calcd for $C_{18}H_{25}N_2O_4$ ($M + H^+$) 333.1814; found 333.1816.

4.1.9.5. 5-(3-Chloropropoxy)-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (*33e*).

33e was pale-yellow powder. Yield 89%; mp > 270 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.51 (s, 1H), 6.36 (d, J = 1.6 Hz, 1H), 6.28 (d, J = 1.5 Hz, 1H), 4.11 (t, J = 5.8 Hz, 2H), 3.82 (t, J = 6.5 Hz, 2H), 3.24–2.66 (m, 8H), 2.61 (s, 2H), 2.30 (s, 3H), 2.27–2.21 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.47, 158.17, 157.61, 154.24, 148.65, 129.04, 103.06, 96.64, 95.20, 65.64, 54.86, 48.34, 45.26, 42.15, 31.47, 17.33.HRMS calcd for $C_{18}H_{24}ClN_2O_4$ ($M + H^+$) 367.1425; found 367.1423.

4.1.9.6. 5-(2-(Dimethylamino)ethoxy)-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (*33f*).

33f was yellow powder. Yield 89%; mp > 225 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1H), 6.45 (d, J = 1.9 Hz, 1H), 6.40 (d, J = 2.0 Hz, 1H), 4.37 (brs, 2H), 3.8–2.8 (m, 8H), 3.43 (brs, 2H), 2.73 (s, 6H), 2.72 (s, 3H), 2.66 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 162.75, 160.86, 160.10, 156.33, 152.74, 129.98, 105.20, 98.32, 96.91, 67.74, 58.95, 56.33, 45.91, 45.42, 18.38. HRMS calcd for $C_{19}H_{28}N_3O_4$ ($M + H^+$) 362.2080; found 362.2060.

4.1.9.7. 5-(Cyclopentyloxy)-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (*33g*).

33g was yellow powder. Yield 70%; mp > 240 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.52 (s, 1H), 6.37 (s, 1H), 6.28 (s, 1H), 4.87 (brs, 1H), 3.15–2.67 (brs, 8H), 2.61 (s, 3H), 2.57 (s, 3H), 1.92–1.91 (m, 2H), 1.83–1.79 (m, 2H), 1.76–1.59 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.5, 157.7, 157.3, 154.4, 149.3, 128.3, 103.2, 97.4, 94.7, 80.0, 54.0,

47.5, 43.8, 32.1, 23.6, 17.4. HRMS calcd for $C_{20}H_{27}N_2O_4$ (M + H^+) 359.1971; found 359.1969.

*4.1.9.8. 5-(Cyclohexyloxy)-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (**33h**).* **33h** was yellow powder. Yield 73%; mp 80–82 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.47 (s, 1H), 6.41 (d, J = 1.9 Hz, 1H), 6.26 (d, J = 2.0 Hz, 1H), 4.46–4.42 (m, 1H), 3.66–2.74 (m, 8H), 2.65 (s, 6H), 1.95–1.94 (m, 2H), 1.71–1.69 (m, 2H), 1.58–1.28 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.71, 157.80, 157.21, 154.55, 129.08, 128.16, 103.34, 97.28, 94.83, 75.44, 53.87, 47.10, 43.83, 30.78, 25.06, 22.68, 17.88. HRMS calcd for $C_{21}H_{29}N_2O_4$ (M + H^+) 373.2127; found 373.2109.

*4.1.9.9. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-((tetrahydrofuran-3-yl)oxy)-2H-chromen-2-one (**33i**).* **33i** was pale-yellow powder. Yield 77%; mp 163–165 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.55 (s, 1H), 6.34 (d, J = 2.0 Hz, 1H), 6.30 (d, J = 2.0 Hz, 1H), 5.09 (s, 1H), 3.92–3.78 (m, 4H), 2.83 (brs, 8H), 2.60 (s, 3H), 2.51 (s, 3H), 2.29–2.20 (m, 1H), 2.09–2.01 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.4, 157.6, 156.8, 154.4, 149.0, 128.7, 103.4, 97.6, 95.2, 78.3, 92.0, 66.4, 54.3, 47.7, 44.3, 32.2, 17.3. HRMS calcd for $C_{19}H_{25}N_2O_5$ (M + H^+) 361.1763; found 361.1750.

*4.1.9.10. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-((1-methylpiperidin-4-yl)oxy)-2H-chromen-2-one (**33j**).* **33j** was pale-yellow powder. Yield 45%; mp 127–129 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.31 (s, 1H), 6.35 (d, J = 2.1 Hz, 1H), 6.22 (d, J = 2.2 Hz, 1H), 4.47–4.40 (m, 1H), 2.63 (s, 3H), 2.58–2.51 (m, 2H), 2.28–2.20 (m, 2H), 2.19 (s, 3H), 2.17 (s, 3H), 1.99–1.92 (m, 2H), 1.77–1.66 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.3, 157.5, 156.9, 154.4, 148.6, 129.3, 103.5, 97.2, 94.9, 55.4, 52.1, 48.9, 46.1, 45.8, 30.1, 17.8. HRMS calcd for $C_{21}H_{30}N_3O_4$ (M + H^+) 388.2236; found 388.2226.

*4.1.9.11. Tert-butyl 4-((7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2-oxo-2H-chromen-5-yl)oxy)piperidine-1-carboxylate (**33k**).* **33k** was pale-yellow powder. Yield 77%; mp 107–109 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.47 (d, J = 1.8 Hz, 1H), 6.31 (d, J = 2.0 Hz, 1H), 4.64–2.61 (m, 1H), 3.65–3.54 (m, 2H), 3.26 (m, 2H), 3.18–2.65 (m, 8H), 2.62 (s, 3H), 2.59 (s, 3H), 1.99–1.90 (m, 2H), 1.61–1.58 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.76, 157.71, 156.73, 154.42, 153.86, 149.17, 128.38, 103.35, 97.47, 95.12, 78.78, 73.03, 53.82, 47.27, 43.58, 29.97, 28.04, 17.83. HRMS calcd for $C_{25}H_{36}N_3O_6$ (M + H^+) 474.2604; found 474.2597.

*4.1.9.12. 5-(Cyclopropylmethoxy)-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (**33l**).* **33l** was pale-yellow powder. Yield 89%; mp > 238 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.48 (s, 1H), 6.31 (d, J = 2.1 Hz, 1H), 6.28 (d, J = 2.1 Hz, 1H), 3.86 (d, J = 7.5 Hz, 2H), 3.5–2.5 (m, 8H), 2.69 (s, 3H), 2.63 (s, 3H), 1.33–1.23 (m, 1H), 0.62–0.57 (m, 2H), 0.39–0.35 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 162.63, 160.92, 160.64, 156.26, 153.36, 129.42, 105.02, 97.88, 96.34, 75.02, 56.09, 48.94, 44.88, 18.33, 11.11, 3.92. HRMS calcd for $C_{19}H_{25}N_2O_4$ (M + H^+) 345.1814; found 345.1797.

*4.1.9.13. 5-((2,4-Dimethylbenzyl)oxy)-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (**33m**).* **33m** was pale-yellow powder. Yield 81%; mp 155–157 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.53 (s, 1H), 7.09 (s, 2H), 6.99 (s, 1H), 6.46 (d, J = 2.1 Hz, 1H), 6.31 (d, J = 2.1 Hz, 1H), 5.06 (s, 2H), 2.89 (brs, 8H), 2.58 (s, 3H), 2.53 (s, 3H), 2.29 (s, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.5, 158.2, 157.7, 154.3, 137.5, 136.0, 129.4, 128.6, 125.6, 103.1, 97.3, 95.2, 70.7, 54.1, 47.5, 44.2, 20.9, 17.5. HRMS calcd for $C_{24}H_{29}N_2O_4$ (M + H^+) 409.2127; found 409.2122.

*4.1.9.14. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-(pyridin-2-ylmethoxy)-2H-chromen-2-one (**33n**).* **33n** was white powder. Yield 81%; mp 166–168 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.522 (s, 1H), 8.59 (d, J = 4.8 Hz, 1H), 7.85 (td, J = 7.8, 1.8 Hz, 1H), 7.56 (d, J = 8.4 Hz, 1H), 7.37 (dd, J = 6.6, 4.8 Hz, 1H), 6.44 (d, J = 2.4 Hz, 1H), 6.31 (d, J = 2.4 Hz, 1H), 5.22 (s, 2H), 3.18–2.65 (m, 8H), 2.62 (s, 3H), 2.49 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.5, 157.9, 157.7, 155.7, 154.3, 149.2, 137.0, 123.2, 122.2, 103.3, 97.5, 95.5, 71.6, 54.39, 47.7, 45.1, 17.5. HRMS calcd for $C_{21}H_{24}N_3O_4$ (M + H^+) 382.1767; found 382.1756.

*4.1.9.15. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-(thiazol-2-ylmethoxy)-2H-chromen-2-one (**33o**).* **33o** was yellow powder. Yield 88%; mp > 230 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.71 (s, 1H), 9.22 (s, 1H), 8.11 (s, 1H), 6.61 (d, J = 1.7 Hz, 1H), 6.41 (d, J = 1.8 Hz, 1H), 5.50 (s, 2H), 3.38 (s, 2H), 2.89 (s, 8H), 2.59 (s, 3H), 2.56 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.56, 157.63, 157.35, 155.71, 154.20, 143.27, 133.09, 128.77, 103.18, 97.59, 95.66, 62.78, 54.10, 47.55, 43.78, 17.55. HRMS calcd for $C_{19}H_{22}N_3O_4S$ (M + H^+) 388.1331; found 388.1318.

*4.1.9.16. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-(2-(pyrrolidin-1-yl)ethoxy)-2H-chromen-2-one (**33p**).* **33p** was pale-yellow powder. Yield 91%; mp 207–209 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.63 (s, 1H), 6.42 (d, J = 1.6 Hz, 1H), 6.34 (d, J = 1.6 Hz, 1H), 4.30 (brs, 2H), 3.02 (brs, 12H), 2.66 (s, 3H), 2.60 (s, 3H), 1.86 (brs, 4H). ^{13}C NMR (100 MHz, CD_3OD) δ 161.34, 159.24, 158.32, 154.75, 150.88, 128.59, 103.64, 97.04, 95.61, 66.56, 56.65, 54.94, 54.36, 54.06, 53.87, 48.10, 44.22, 22.84, 22.53, 16.90. HRMS calcd for $C_{21}H_{30}N_3O_4$ (M + H^+) 388.2236; found 388.2218.

*4.1.9.17. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-(2-(piperidin-1-yl)ethoxy)-2H-chromen-2-one (**33q**).* **33q** was yellow powder. Yield 86%; mp 110–112 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.49 (s, 1H), 6.35 (d, J = 1.1 Hz, 1H), 6.27 (d, J = 1.1 Hz, 1H), 4.08 (t, J = 4.6 Hz, 2H), 2.76 (t, J = 4.6 Hz, 2H), 2.62 (s, 3H), 2.33 (s, 3H), 1.50–1.49 (m, 4H), 1.44–1.27 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.5, 158.3, 157.6, 154.2, 149.0, 128.9, 103.1, 96.9, 95.1, 66.3, 56.8, 54.8, 54.1, 48.3, 45.2, 25.3, 23.6, 17.2. HRMS calcd for $C_{22}H_{32}N_3O_4$ (M + H^+) 402.2393; found 402.2380.

*4.1.9.18. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-(2-morpholinoethoxy)-2H-chromen-2-one (**33r**).* **33r** was yellow powder. Yield 87%; mp > 215 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 6.40 (d, J = 1.7 Hz, 1H), 6.32 (d, J = 1.8 Hz, 1H), 4.11 (t, J = 4.8 Hz, 2H), 3.59 (t, J = 4.0 Hz, 4H), 3.3–2.8 (m, 8H), 2.77 (t, J = 4.0 Hz, 2H), 2.70 (s, 3H), 2.67 (s, 3H), 2.48 (brs, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.78, 158.46, 157.78, 154.25, 149.67, 128.17, 103.00, 96.96, 95.11, 66.18, 66.06, 56.70, 53.57, 53.38, 46.96, 43.11, 17.36. HRMS calcd for $C_{21}H_{30}N_3O_5$ (M + H^+) 404.2185; found 404.2164.

*4.1.9.19. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-(2-(thiophen-2-yl)ethoxy)-2H-chromen-2-one (**33s**).* **33s** was pale-yellow powder. Yield 68%; mp 103–105 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 7.37 (dd, J = 4.3, 1.8 Hz, 1H), 6.99 (d, J = 4.2 Hz, 2H), 6.38 (d, J = 1.8 Hz, 1H), 6.28 (d, J = 1.9 Hz, 1H), 4.28 (t, J = 5.9 Hz, 2H), 3.34 (t, J = 5.9 Hz, 2H), 2.51 (s, 6H). ^{13}C NMR (100 MHz, CD_3OD) δ 161.17, 159.25, 158.76, 154.80, 151.85, 140.42, 127.67, 126.53, 125.33, 123.50, 103.45, 96.46, 95.19, 69.53, 54.29, 47.07, 42.85, 29.20, 16.92. HRMS calcd for $C_{21}H_{25}N_2O_4S$ (M + H^+) 401.1535; found 401.1537.

*4.1.9.20. 7-Hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-5-(2-(pyridin-2-yl)ethoxy)-2H-chromen-2-one (**33t**).* **33t** was white powder.

Yield 86%; mp 90–92 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.39 (s, 1H), 8.51 (d, J = 4.2 Hz, 1H), 7.74 (t, J = 7.4 Hz, 1H), 7.37 (d, J = 7.7 Hz, 1H), 7.26–7.23 (m, 1H), 6.39 (d, J = 1.5 Hz, 1H), 6.25 (d, J = 1.6 Hz, 1H), 4.43 (t, J = 6.0 Hz, 2H), 3.25 (t, J = 5.9 Hz, 2H), 2.31 (s, 3H), 2.19 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.3, 158.3, 158.2, 157.5, 154.2, 149.1, 148.6, 136.5, 129.2, 123.5, 121.7, 103.0, 96.6, 95.0, 67.8, 55.3, 48.8, 46.1, 36.7, 16.9. HRMS calcd for $C_{22}H_{26}N_3O_4$ (M + H^+) 396.1923; found 396.1915.

*4.1.9.21. Tert-butyl (2-((7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2-oxo-2H-chromen-5-yl)oxy)ethyl)carbamate (**33u**).* **33u** was pale-yellow powder. Yield 63%; mp 114–116 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 7.02 (s, 1H), 6.31 (s, 1H), 6.27 (s, 1H), 3.99 (s, 2H), 3.39 (s, 2H), 2.60 (s, 3H), 2.20 (s, 3H), 1.39 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD) δ 162.4, 160.7, 160.1, 158.4, 156.1, 152.7, 129.8, 105.1, 97.8, 96.6, 80.3, 68.7, 56.3, 49.1, 45.4, 40.8, 28.8, 18.3. HRMS calcd for $C_{22}H_{32}N_3O_6$ (M + H^+) 434.2291; found 434.2286.

*4.1.9.22. Tert-butyl (3-((7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2-oxo-2H-chromen-5-yl)oxy)propyl)carbamate (**33v**).* **33v** was pale-yellow powder. Yield 59%; mp 164–166 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 6.36 (s, 1H), 6.30 (s, 1H), 4.00 (s, 2H), 3.14 (s, 2H), 2.68 (s, 3H), 2.64 (s, 3H), 1.90 (s, 2H), 1.37 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD) δ 152.92, 151.20, 150.86, 149.01, 146.59, 143.34, 117.44, 95.37, 88.14, 86.81, 70.47, 58.28, 46.59, 39.57, 35.57, 29.06, 21.15, 19.28, 8.85. HRMS calcd for $C_{23}H_{34}N_3O_6$ (M + H^+) 448.2448; found 448.2445.

*4.1.10. Procedure for the synthesis of compounds **35a** and **35b***

To a solution of intermediate **32u** or **32v** (0.3 mmol) in 10 mL DCM was added CH_3COOH (2 mL). The mixture was stirred at 40 °C for 1 h. The corresponding intermediate **34a** or **34b** was obtained after purification by silica gel chromatography eluting with DCM–CH_3OH. Then to the solution of intermediate **34a** or **34b** in THF (5 mL) was added TBAF (0.6 mmol) under stirring at 50 °C. The corresponding product **35a** or **35b** was obtained after purification by C18 chromatography eluting with CH_3CN–H_2O.

*4.1.10.1. 5-(2-Aminoethoxy)-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-on (**35a**).* The final product **35a** was pale-yellow powder. Yield 53%; mp 148–150 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.79 (s, 1H), 8.20 (s, 2H), 6.43 (d, J = 2.0 Hz, 1H), 6.35 (d, J = 2.1 Hz, 1H), 4.24 (t, J = 4.8 Hz, 2H), 3.43 (s, 6H), 3.32 (t, J = 4.8 Hz, 3H), 3.17 (s, 2H), 2.85 (s, 3H), 2.66 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.9, 158.6, 158.3, 157.8, 154.3, 128.0, 103.2, 97.2, 95.7, 65.7, 53.4, 46.7, 42.6, 38.1, 17.4. HRMS calcd for $C_{17}H_{24}N_3O_4$ (M + H^+) 334.1767; found 334.1785.

*4.1.10.2. 5-(3-Aminopropoxy)-7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2H-chromen-2-one (**35b**).* The final product **35b** was yellow powder. Yield 45%; mp > 245 °C decomposed. ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.34 (d, J = 2.2 Hz, 1H), 6.2 (d, J = 1.7 Hz, 1H), 4.07 (t, J = 5.6 Hz, 2H), 2.91 (t, J = 7.0 Hz, 2H), 2.62 (s, 3H), 2.20 (s, 3H), 2.05–1.99 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 162.78, 160.14, 156.30, 153.28, 129.87, 126.91, 104.81, 98.10, 96.73, 67.09, 55.55, 48.36, 43.92, 38.25, 28.32, 18.32. HRMS calcd for $C_{18}H_{26}N_3O_4$ (M + H^+) 348.1923; found 348.1911.

*4.1.11. Procedure for the synthesis of compounds **37a** and **37b***

To a solution of **34a** (0.2 mmol) in 10 mL DCM was added TEA (0.4 mmol) and various acyl chlorides (0.3 mmol) with stirring at room temperature. The intermediate **36a** or **36b** was obtained after purification by silica gel chromatography eluting with DCM–CH_3OH. The final product **37a** or **37b** was synthesized in a similar manner to the synthesis of **35a** from **34a**.

*4.1.11.1. N-(2-((7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2-oxo-2H-chromen-5-yl)oxy)ethyl)methanesulfonamide (**37a**).* The final product **37a** was pale-yellow powder. Yield 62%; mp 189–191 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.28 (t, J = 5.6 Hz, 1H), 6.36 (d, J = 2.2 Hz, 1H), 6.31 (d, J = 2.1 Hz, 1H), 4.08 (t, J = 5.2 Hz, 2H), 3.42–3.38 (m, 2H), 2.95 (s, 3H), 2.67 (s, 3H), 2.61 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 162.47, 160.74, 159.91, 156.10, 152.81, 129.76, 105.05, 97.97, 96.70, 69.31, 56.19, 49.43, 45.30, 43.27, 39.92, 18.40. HRMS calcd for $C_{18}H_{26}N_3O_6S$ (M + H^+) 412.1542; found 412.1533.

*4.1.11.2. N-(2-((7-hydroxy-4-methyl-3-(4-methylpiperazin-1-yl)-2-oxo-2H-chromen-5-yl)oxy)ethyl)-4-methylbenzenesulfonamide (**37b**).* The final product **37b** was white solid. Yield 57%; mp > 250 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 7.85 (t, J = 5.7 Hz, 1H), 7.69 (d, J = 8.1 Hz, 2H), 7.37 (d, J = 8.0 Hz, 2H), 6.30 (d, J = 2.1 Hz, 1H), 6.29 (d, J = 2.1 Hz, 1H), 4.01 (t, J = 4.9 Hz, 2H), 3.8–2.8 (m, 8H), 3.19–3.15 (m, 2H), 2.79 (s, 3H), 2.62 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.7, 158.1, 157.8, 154.2, 142.6, 137.4, 129.6, 128.0, 126.4, 103.0, 96.7, 95.2, 67.3, 53.4, 46.7, 42.7, 41.9, 20.9, 17.5. HRMS calcd for $C_{24}H_{30}N_3O_6S$ (M + H^+) 488.1855; found 488.1845.

4.2. Biology

Cell culture and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) assay. PC12 cells were purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 5% fatal bovine serum (FBS) and 5% equine serum, and L-glutamine (2 mM). Cultures were maintained at 37 °C in 5% CO_2 in a humidified incubator. For cell viability assay, PC12 cells were incubated in 96-well plates at a density of 5×10^3 cells per well for 24 h. Then compounds at dose of 10 μM and damage factor were added in the cells. OGD assay: the cells were treated by low glucose medium and 5 mM sodium dithionite for 24 h. Then 5 mg/mL MTT was added and maintained for 4 h. Absorbance was measured using an Ultramark microplate reader at a wavelength of 570 nm. The cell viability was expressed as a percentage of the absorbance density value of the control cultures.

4.2.1. Animals

Male Sprague–Dawley rats (age, 7 weeks; weighting, 260–280 g) were purchased from Experimental Animal Center of Chinese Academy of Medical Sciences (Beijing China). All animals were handled in accordance with the standards established in the *Guide for the Care and Use of Laboratory Animals* published by the Institute of Laboratory Animal Resources of the National Research Council (United States) and approved by the Animal Care and Use Committee of the Peking Union Medical College and the Chinese Academy of Medical Sciences.

4.2.2. Focal brain ischemia

Transient middle cerebral artery occlusion (TMCAO) was performed as previously described with some modifications. Briefly, under 10% chloral hydrate (4 mL/kg, intraperitoneal injection), a 4–0 nylon thread, the tip of which was burned (diameter 0.36 mm), was inserted into the right common carotid artery and advanced until the origin of the right MCA was occluded. After 60 min of the occlusion, the thread was removed to allow reperfusion, and then the rats (n = 12) were returned to the chamber.

To investigate the effect of **20** on MCAO, saline or **20** at doses of 1.5, 3.0, 6.0 mg/kg or edaravone at dose of 6.0 mg/kg was intravenous injection at the same time of reperfusion. The rats were assessed for neurologic deficits at 24 h after reperfusion according to longa's 5-point scale. A score of 0: no neurological deficit; a score

of 1: failure to extend left forepaw fully; a score of 2: circling to the left; a score of 3: falling to the left; and a score of 4: did not walk spontaneously and had a depressed level of consciousness. The animals without symptoms of neurological impairment or dying after the surgery were rejected and other rats were recruited.

The animals were killed at 24 h after reperfusion. Brains of the animals were removed and cut into 2 mm-thick slices, for a total of six slices per animal. The slices were immersed in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffered saline (PBS) at 37 °C for 30 min and fixed in 4% phosphate buffered formalin. Images of the slices were obtained with a scanner and a computer. The infarct area and the total area were calculated by tracing the areas on the computer screen with Image J. The percentage of the infarct area was expressed as the infarct area to total area.

To assess the brain water content, brain samples were dried in an oven at 110 °C for 24 h, and water content of these samples was then measured by the (weight-dry)/weight.

For histological analysis, the rats were anesthetized with 10% chloral hydrate and then perfused with PBS followed by 4% buffered paraformaldehyde 24 h. The brain of each rat was then removed, postfixed, and embedded in paraffin. Coronal sections of 8 μM were stained with 0.5% cresyl fast violet.

4.2.3. TUNNEL staining

Detection of cell death *in situ* DNA fragmentation is one of the wide accepted markers by which apoptotic cells are recognized. To detect DNA fragmentation of cell nuclei, TUNEL reaction was applied to the paraffin sections using the In Situ Cell Death Detection Kit, peroxidase (POD) (Roche Applied Science, Germany). The brain sections were incubated with TUNEL reaction according to the manufacturer's protocol. After dewaxing and rehydration, brain slices were treated with protease K (20 μg/mL) for 15 min at room temperature. Then slices were incubated with reaction buffer containing TdT enzyme (37 °C, 1 h). After washing with stop/wash buffer, slices were treated with anti-digoxigenin conjugate for 30 min at room temperature, and subsequently developed color in alkaline phosphatase substrate.

4.2.4. Electron microscopy

Processing and electron microscope (EM) were conducted as previously described (Jasinska et al., 2006). Briefly, anesthetized animals were perfused with 0.1 M PBS, followed by 3.75% acrolein in buffered 2% paraformaldehyde (PF). Then the brains were removed, postfixed with 2.5% glutaraldehyde for 2 h, washed with 0.1 M PBS, and then exposed to 1% osmium tetraoxide for 2 h. After several subsequent washes with water, the tissues were dehydrated with gradient alcohol and embedded in EPON resin. Randomly selected ultrathin sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (H-7650, HITACHI, Tokyo, Japan).

4.2.5. Western blot analysis

The animals were anesthetized with 10% chloral hydrate and then perfused with saline at 24 h after reperfusion, and the cortex and hippocampus samples from the rats were homogenized thoroughly and then lysed in a RIPA lysis buffer (50 mmol·L^{-1} Tris (pH 7.4), 150 mmol L^{-1} NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfonate (SDS)). Protein concentrations were measured with a bicinchoninic acid (BCA) kit. The lysates were solubilized in SDS sample and separated by SDS-PAGE, then transferred to polyvinyl difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked by 3% bull serum albumin (BSA) and incubated with anti-Bcl-2, anti-Bax, anti-β-actin (Santa Cruz, CA, California, USA), anti-Caspase-3 (Cell Signaling, Beverly, MA, USA), followed by horseradish peroxides (HRP)-conjugated secondary antibody (Santa Cruz, CA, California, USA), and detected with the Enhanced Chemiluminescence (ECL) plus detection system (Molecular Device, Lmax). The density of each band was quantified using image analysis software (Science Lab 2005 Image Guage; Fuji Film Co. Ltd, Tokyo, Japan).

4.2.6. Statistic analysis

Results were expressed as mean ± SD. Statistical evaluation was performed using one-way analysis of variance. Significant difference was further performed in conjunction with the Student–Newman–Keuls method. Statistical significance was accepted at $P < 0.05$ ($n = 12$).

4.3. General procedure for pharmacokinetic investigation

Male Sprague–Dawley rats (260–280 g) were intravenously injected with compound **20** at dose of 6 mg/kg. Then the rats were anesthetize by ether and blood samples (approximately 1.5 mL) were collected from the jugular vein into the tubes containing sodium heparin at 3.5, 7, 15, 30, 60 and 120 min. Plasma was prepared by centrifugation of blood at 4000 rpm for 5 min, and then stored at −70 °C until analysis. The rats were perfused with saline after the blood samples were collected, and the cortex and hippocampus were separated and homogenated with 2 volumes (v/w) of Milli-Q water. The samples were centrifuged at 13,000 rpm for 10 min, and the supernatant was collected and stored at −70 °C until analysis. Test tubes containing rat plasma or cortex (200 μL) and the internal standard (100 μL of 1 μg/mL) were subjected to protein precipitation with acetonitrile (200 μL) using a vortex mixer for 1 min and centrifuged at 10,000 rpm for 10 min. Supernatant was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in an aliquot of the mobile phase (200 μL) and filtered using 0.45 μM Teflon filters before LC-MS analysis. An Alltima C18 column (150 mm × 4.6 mm, 5 μm) with a C18 guard column were applied, using a mixture of acetonitrile and 0.02 M ammonium formate with a gradient elution (0–15 min, acetonitrile of 10%–80%). The flow rate was set at 0.8 mL·min^{-1} and the detective wavelength fixed at 332 nm. An Agilent 1200 HPLC system coupled to Agilent 6130 mass spectrometer (Aglient, USA) equipped with ESI were used for the study. A positive mode was applied with selected ion monitoring mode (SIM); the selected ions for compound **20** and the internal standard (IS), were 304.9 ([M + H]) and 240.8 [M + H] respectively. Quantitation was achieved by constructing a calibration curve by weighted linear regression of the ratio of the analyte peak area to that of the added internal standard. The standard calibration curve for compound **20** was linearly proportional to the concentration from 0.1 μg/mL to 30 μg/mL in plasma and from 1 ng/mL to 2000 ng/mL in cortex, respectively. All the detection limits of compound **20** were 0.05 ng.

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Appendix A. Supplementary data

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