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Novel hybrids of 3-*n*-butylphthalide and edaravone: Design, synthesis and evaluations as potential anti-ischemic stroke agents

Xiao Sheng [a,b,c,e,†], Kai Hua [a,d,†], Chunyu Yang [a,b,c], Xiaoli Wang [a,b,c], Hui Ji [a,d,*], Jinyi Xu [a,b,c,*], Zhangjian Huang [a,b,c,*], Yihua Zhang [a,b,c]

[a] State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, PR China
[b] Jiangsu Key Laboratory of Drug Discovery for Metabolic Diseases, China Pharmaceutical University, Nanjing 210009, PR China
[c] Center of Drug Discovery, China Pharmaceutical University, Nanjing 210009, PR China
[d] Department of Pharmacology, China Pharmaceutical University, Nanjing 210009, PR China
[e] Department of Medicinal Chemistry, School of Pharmacy, Fudan University, Shanghai 201203, PR China

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ABSTRACT

Fourteen hybrids (**10a–g**, **11a–g**) of 3-*n*-butylphthalide (NBP) and edaravone (Eda) analogues have been designed and synthesized as potential anti-ischemic stroke agents. In vitro biological studies showed that compounds **10d** and **10g** exhibited more potent anti-platelet aggregation than ticlopidine (Ticlid), aspirin (ASP) and NBP. Compound **10g** more significantly prevented H_2O_2-mediated neuronal cell (PC12) death than NBP, Eda or NBP together with Eda. Meanwhile, **10g** also possessed potent radical scavenging effects on hydroxyl radical (·OH) and superoxide anion radical ($\cdot O_2^-$). Our findings may provide new insights into the development of these hybrids, like **10g**, for the intervention of ischemic stroke.

Stroke, is one of the common cardiovascular and cerebrovascular diseases, and has become a chief cause of adult disability and the second cause of death worldwide.[1] Among strokes, ischemic - stroke occupies approximately 80% of all, seriously harming to human health.[2] It is reported that platelet-mediated thrombosis can cause blood vessel blockage,[3] leading to interrupted blood supply to the brain, thereby causing the brain tissue energy depletion, metabolic dysfunction, and ischemic brain damage.[4]

Currently, therapeutic strategies for ischemic stroke include two aspects: (1) preventing and/or correcting the inducing factors of cerebral ischemia; (2) recovering brain perfusion after ischemia to improve cerebral blood and to block ischemic injury cascade, thus, preventing neuronal damage. In view of the complicated pathological mechanisms of ischemic stroke, the drugs with different mechanisms, rather than a single antiplatelet activity, are needed. In this regard, a hybrid multitargeting prodrug that will release each component in vivo, to furnish the desired pharmacological effects at the same prodrug dose, warrants further exploration, especially in the development of anti-ischemic stroke drugs.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) (Fig. 1) is a novel free radical scavenger, and was approved by the Japanese Ministry of Health in 2001, mainly for the treatment of ischemic stroke. Edaravone exerts neuroprotective effects through scavenging free radicals, inhibiting lipid peroxidation and oxidative damage to brain cells, endothelial cells and nerve cells, reducing cerebral ischemia and edema, and thus decreasing tissue damage. Furthermore, edaravone can also effectively improve neurological deficits caused by the acute cerebral infarction.[5]

It is demonstrated that edaravone has three tautomeric forms: the amine (Eda), keto (**1**), and enol (**2**) forms. Edaravone exerts free radical scavenging effects through a one-electron transfer mechanism. The pyrazolone ring enol (**2**) loses a proton to form pyrazolone ring conjugated anionic form (**3**). Subsequently, the anion **3** transfers an electron to free radicals, such as peroxyl (LOO·) and hydroxyl radicals (·OH), to furnish a radical intermediate (**3A**), which finally forms a stable oxidation product (**OPB**: 2-oxo-3-(phenylhydrazono)-butanoic acid), generating free radical scavenging effects (Fig. 1).[6]

The racemic 3-*n*-butylphthalide (NBP) (Fig. 2), as a natural drug extracted from celery seed, was approved for the treatment of

* Corresponding authors. Tel.: +86 25 86021369; fax: +86 25 86635503 (H.J.); tel.: +86 25 83271445; fax: +86 25 83302827 (J.X.); tel.: +86 25 83271072; fax: +86 25 83271015 (Z.H.).
E-mail addresses: huijicpu@163.com (H. Ji), jinyixu@china.com (J. Xu), cpudahuang@163.com (Z. Huang).
† These authors contributed equally to this work.

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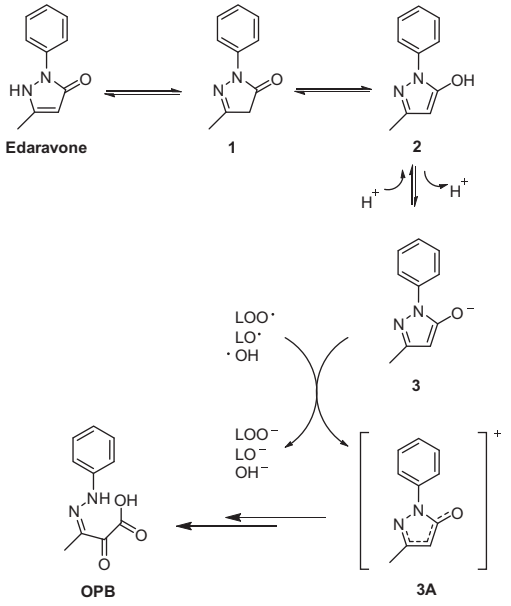


Figure 1. Edaravone and its two tautomeric forms, as well as a one-electron transfer mechanism underlying its free radical scavenging activity.

ischemic stroke by State Food and Drug Administration (SFDA) of China in 2002. It has been reported that NBP can have effects on multiple points of pathological processes in ischemic stroke, possessing a variety of biological activities such as anti-platelet aggregation, anti-thrombosis, improving cerebral microcirculation and reducing infarct volume.[7] **HPBA** (Fig. 2) is the ring-opening derivative of NBP, could cyclize to NBP in vivo.[8] Recently, our research group has designed and synthesized several groups of hybrids of **HPBA** with other small active molecules, and some hybrids such as (**R**/**S**)-**ZJM-289** and compound **4** (Fig. 2) possess more potent therapeutic efficacy for intervention of ischemic stroke than NBP.[9–11]

Given that edaravone and NBP have been clinically used for the treatment of ischemic stroke via different mechanisms, it is therefore of interest to determine whether coupling edaravone and NBP would provide a hitherto unknown class of hybrids that possess potent and synergetic anti-ischemic stroke activity. As part of this

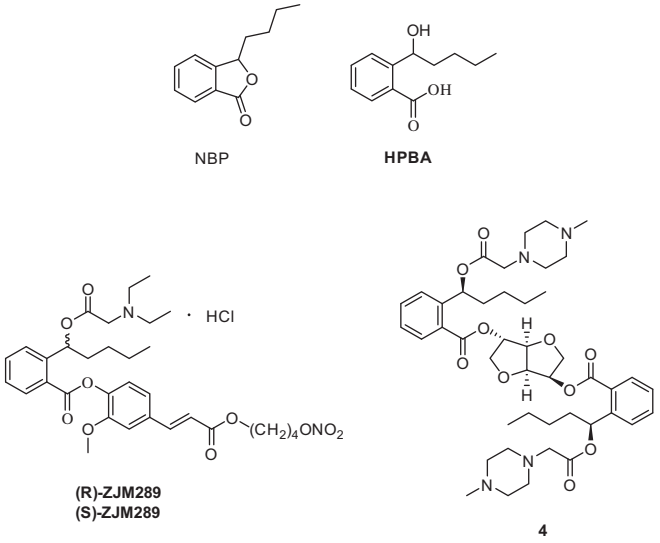


Figure 2. Structrues of NBP, **HPBA**, (**R**/**S**)-**ZJM289** and **4**.

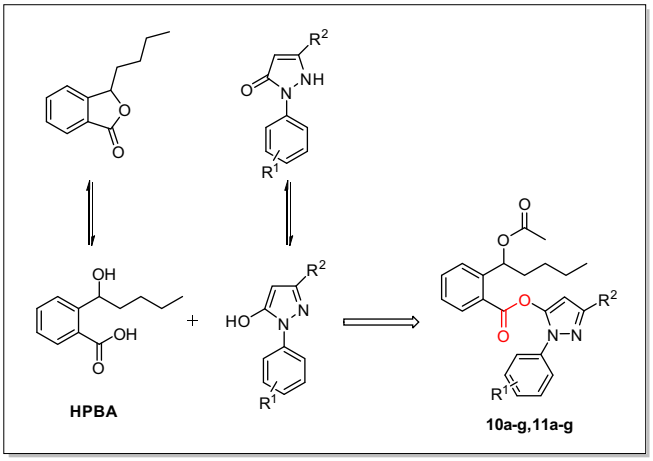


Figure 3. Rationale for the design of hybrids **10a**–**g** and **11a**–**g**.

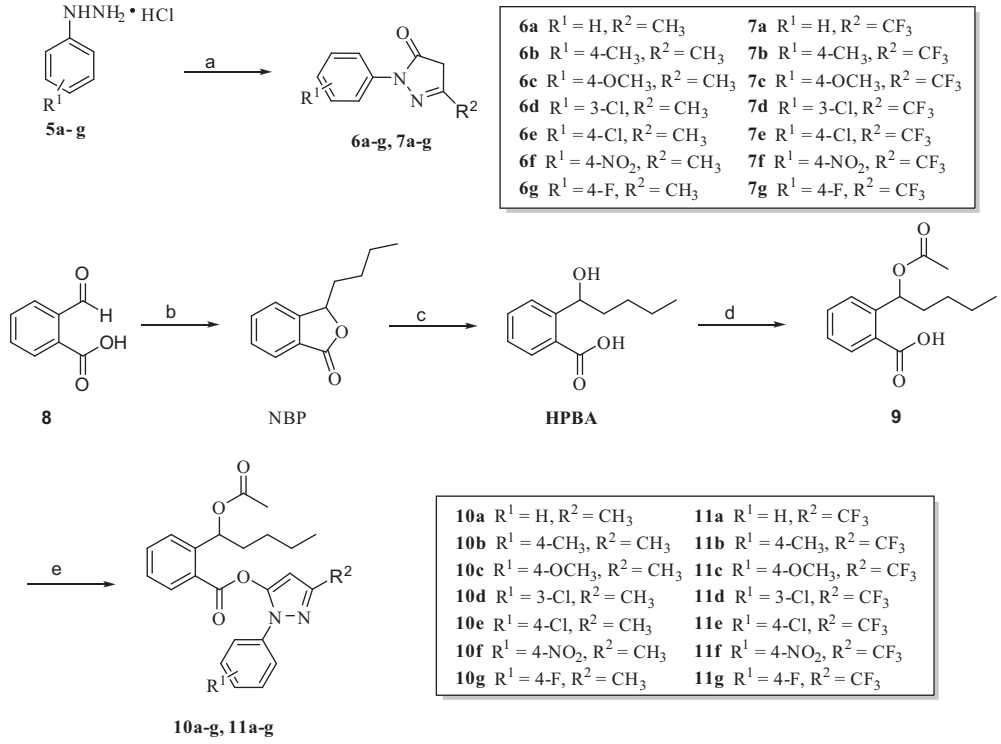
ongoing program, the hydroxyl group of enol tautomer of edaravone or its trifluoromethyl analogue was coupled with the carboxylic acid group of **HPBA** to form a group of hybrids (**10a**–**g** and **11a**–**g**, Fig. 3). We hypothesized that these hybrids, under esterase-induced hydrolysis in vivo, could spontaneously generate edaravone or edaravone analogues and **HPBA**, which forms NBP after a cyclization reaction, generating complementary anti-ischemic stroke effects. Herein, we described the synthesis and biological evaluations for these hybrids.

The synthesis of the target compounds **10a**–**g** and **11a**–**g** was depicted in Scheme 1. Edaravone derivatives **6a**–**g** and **7a**–**g** were obtained in 50–70% yields from the reactions of substituted phenyl hydrazine hydrochloride with acetylacetic ether or trifluoroacetyl ethyl acetate in the presence of sodium acetate under reflux.

Starting from the reaction of 2-formylbenzoic acid **8** with Grignard reagent *n*-BuMgBr, the consequent acidification gave NBP in 85% yield. Subsequent ring-opening of NBP in the presence of sodium hydroxide produced **HPBA** in 92% yield. Without further purification, the hydroxyl at the side chain of **HPBA** was reacted with acetyl chloride, providing intermediated **9** in the yield of 77%. Compound **9** was reacted with oxalyl chloride in anhydrous dichloromethane to give the intermediate acyl chloride, followed by the esterification with edaravone derivatives in the presence of triethylamine to offer target compounds **10a**–**g** and **11a**–**g** in 43–60% yields. All target compounds were purified by column chromatography and their structures were characterized by IR, MS, ^{1}H NMR, ^{13}C NMR and HR-MS.[12,13]

The inhibitory effects of target compounds on adenosine diphosphate (ADP)- or arachidonic acid (AA)-induced platelet aggregation in vitro were evaluated using Born's turbidimetric method, and ticlopidine hydrochloride (Ticlid) and aspirin (ASP) were used as positive controls (Table 1).[14,15] As comparison, the inhibitory effects of NBP, Eda or combination administration of NBP together with Eda groups were also investigated. The assay was repeated six times, and data were calculated and expressed as the inhibition rate of platelet aggregation.[11,16]

As shown in Table 1, for inhibiting the ADP-induced platelet aggregation, **10c** (30.17%), **10d** (35.20%), **10e** (29.33%), **10f** (33.87%), **10g** (31.80%), **11a** (26.93%), **11b** (25.48%) and **11d** (31.24%) showed more potent inhibitory effect than Ticlid (11.53%), NBP (22.95%), Eda (24.77%) and NBP together with Eda (28.06%). And **10d**, **10g** inhibited the AA-induced platelet aggregation with inhibition rates of 46.11% and 59.87%, respectively, significantly superior to all reference compounds ASP (16.16%), NBP (14.10%), Eda (25.47%) and NBP together with Eda (42.45%) in vitro.



Scheme 1. Synthetic routes of the target compounds **10a**–**g** and **11a**–**g**. Reagents and conditions: (a) AcONa, AcOH, reflux, N_2, 5–10 h; (b) (i) *n*-BuMgBr, Et_2O, −5 to 0 °C, 5 h; (ii) 1 M HCl, rt, 0.5 h; (c) (i)NaOH, CH_3OH–H_2O, 50 °C, 0.5 h; (ii) 1 M HCl, −10 to 0 °C; (d) CH_3COCl, Et_3N, DMAP, dry CH_2Cl_2, −10 °C, 7 h; (e) (i) $(COCl)_2$, dry CH_2Cl_2, rt, 8 h; (ii) **6a**–**g** or **7a**–**g**, Et_3N, dry CH_2Cl_2, 0 °C–rt, 4–6 h.

Table 1
Inhibitory effects of **10a**–**g** and **11a**–**g** (0.1 mM) on ADP- or AA-induced platelet aggregation in vitro ($n = 6$, $\bar{x} \pm s$)

Compound	ADP (%)	AA (%)
Ticlopidine	11.53 ± 3.20	
Aspirin		16.16 ± 3.16
NBP	22.95 ± 2.45	14.10 ± 3.17
Eda	24.77 ± 3.60	25.47 ± 4.05
NBP + Eda	28.06 ± 1.82	42.45 ± 4.87
10a	23.01 ± 2.89	15.39 ± 5.50
10b	21.32 ± 2.81	20.49 ± 5.12
10c	30.17 ± 3.79	29.36 ± 4.90
10d	35.20 ± 4.10***	46.11 ± 4.26
10e	29.33 ± 1.79	40.32 ± 3.47
10f	33.87 ± 4.11***	39.61 ± 4.86
10g	31.80 ± 4.59***	59.87 ± 6.03***
11a	26.93 ± 3.85	29.29 ± 3.81
11b	25.48 ± 3.56	40.67 ± 4.54
11c	19.88 ± 2.22	37.66 ± 5.86
11d	31.24 ± 3.10	21.21 ± 3.88
11e	23.22 ± 3.22	26.58 ± 2.91
11f	15.43 ± 1.66	21.30 ± 1.31
11g	13.60 ± 2.55	27.52 ± 4.23

PRP were preincubated with tested compounds (0.1 mM) at 37 °C for 5 min followed by the addition of ADP (10 μM) or AA (1 mM). Data are expressed as mean ± SD of each group ($n = 6$) and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey test.
*** $P < 0.05$ versus NBP + Eda.

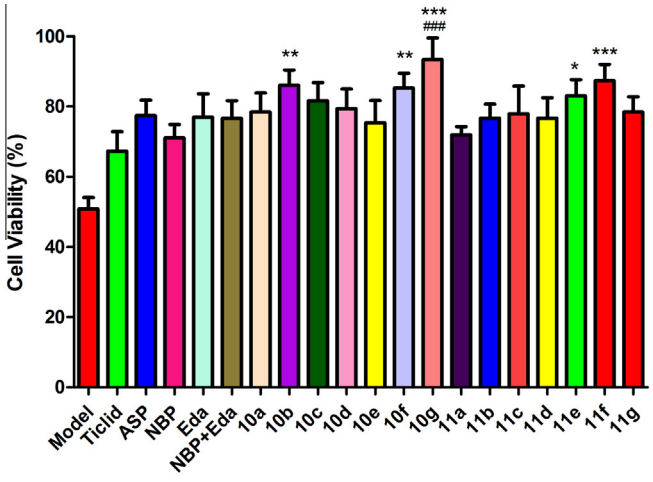


Figure 4. Protective effects of the target compounds on nerve cells damage in vitro. PC12 cells were pre-treated with vehicle, all positive controls (10 μM) or target compounds (10 μM) for 1 h, and then co-cultured with H_2O_2 (800 μM) for 3 h. The cell viability was determined by MTT assay. Results were obtained from independent experiments and were expressed as mean ± SD ($n = 6$). *$P < 0.05$ versus NBP group; **$P < 0.01$ versus NBP group; ***$P < 0.001$ versus NBP group; ###$P < 0.001$ versus NBP + Eda group.

Since these hybrids are the precursors of edaravone or its trifluoromethyl analogue, which act as radical scavengers generating protection activity,[17] we established the hydrogen peroxide induced PC12 cells injury model in vitro, and preliminarily investigated the protective effects of all target compounds on nerve cells injury caused by the treatment of H_2O_2, using Ticlid, ASP, NBP, Eda and NBP together with Eda as positive controls.[18,19]

As shown in Figure 4, the protective effects of **10b** (86.02%), **10f** (85.22%), **10g** (93.33%), **11e** (82.95%) and **11f** (87.31%) were superior to those of all positive controls, Ticlid (67.22%), ASP (77.37%), NBP (71.01%), Eda (76.89%) and NBP together with Eda (76.60%) at the same concentration, and **10g** exhibited the greatest protective effects in all test compounds. All these results supported us in selecting **10g** as the candidate compound for further investigation.

Given that **10g** is composed of two moieties, NBP's precursor **9** and edaravone's precursor **6g**, we investigated their individual contribution to the overall inhibitory activity against ADP-induced or AA-induced platelet aggregation and protective effects on nerve cells damage caused by the treatment of H_2O_2 in vitro.

It is shown that compounds **6g**, **9** or **6g** together with **9** all displayed moderate anti-platelet aggregation activity (inhibition rates are 13.46% and 24.34%, 17.73% and 27.73%, 16.21% and 25.03%, in two respective models), but less than **10g** (31.80% and 59.87% Fig. 5A and B). Similarly, **10g** exhibited better nerve cell protective activity (cell viability percentages are 93.33% and 88.06% at the dose of 10 μM and 1 μM, respectively, Fig. 5C) than its moiety compounds **6g**, **9** or **6g** together with **9**. These data suggested that the improved inhibitory activity against ADP-induced or AA-induced platelet aggregation and protective effect against H_2O_2-induced cell damage in vitro may derive from the synergistic effects of its two moieties.

Furthermore, we evaluated the hydroxyl radical (·OH) and superoxide anion radical ($\cdot O_2^-$) scavenging activity of **10g** in vitro using Griess assay and NBT assay, respectively.[20,21] It was found that the ·OH scavenging activity of **10g** (65.5100 U/mL) was significantly superior to that of Ticlid (15.0616 U/mL), ASP (30.6956 U/mL), NBP (16.2026 U/mL), Eda (25.2753 U/mL) and NBP together with Eda (36.8347 U/mL) at the same concentration of 10 μM. (Fig. 5D) The $\cdot O_2^-$ scavenging activity of **10g** (141.4900 U/L) had an advantage over that of Ticlid (26.9592 U/L), ASP (79.3793 U/L), NBP (36.7429 U/L) and Eda (97.6942 U/L), and was comparable to that of NBP together with Eda (140.7870 U/L) at the same concentration of 10 μM. (Fig. 5E). Notably, the ·OH or $\cdot O_2^-$ scavenging effect of **6g** (31.7447 U/mL or 117.5790 U/L), **9** (17.6015 U/mL or 56.1954 U/L), and **6g** together with **9** (44.0229 U/mL or 133.1410 U/L) were all less than **10g** at the same dosage. These may also suggest that the two moieties have synergistic effects on scavenging the ·OH or $\cdot O_2^-$ free radical in vitro.

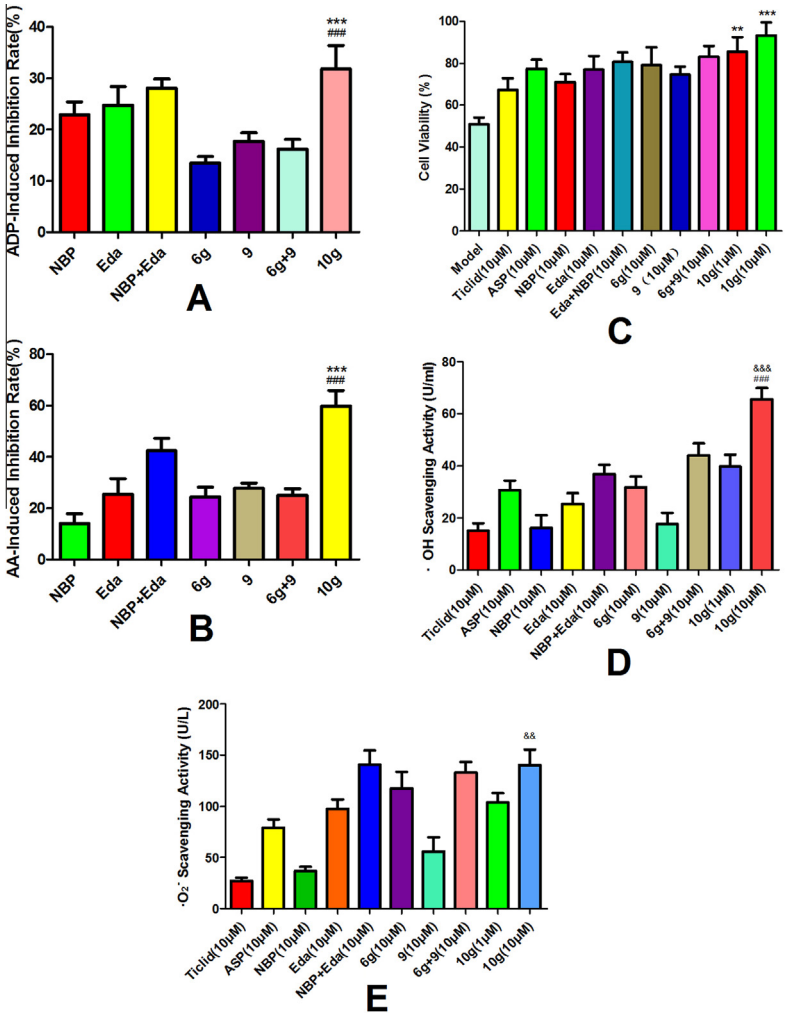


Figure 5. (A) Inhibition of ADP-induced rabbit platelet aggregation by selected compounds in vitro. (B) Inhibition of AA-induced rabbit platelet aggregation by selected compounds in vitro. Rabbit platelet suspensions were pre-incubated with tested compounds (0.1 mM) at 37 °C for 5 min followed by addition of ADP (10 μM) or AA (1 mM) was added and incubated with the drug-platelet suspension in the indicated group. (C) Protective effects of selected compounds on nerve cells damage in vitro. PC12 cells were pre-treated with model, all positive controls(10 μM), selected compounds (10 μM) and compound **10g** (1–10 μM) for 1 h, and then co-cultured with H_2O_2 (800 μM) for 3 h. The cell viability was determined by MTT assay. (D) ·OH scavenging effects of selected compounds in PC12 cells. PC12 cells were pre-treated with all positive controls (10 μM), selected compounds (10 μM) and compound **10g** (1–10 μM) for 1 h, then co-cultured with 1 mM H_2O_2/20 μM Fe^{2+} (pH = 7.4). The reduced activity of ·OH was detected by Griess assay. (E)·O_2^- scavenging effects of selected compounds in PC12 cells. PC12 cells were pre-treated with all positive controls (10 μM), selected compounds (10 μM) and compound **10g** (1–10 μM) for 1 h, then co-cultured with DMSO, 468 μM NADH and 300 μM NBT at the same concentration. The reduced activity of $\cdot O_2^-$ was detected by NBT assay. Data are expressed as mean ± SD of each group ($n = 6$) for six separate experiments. Data was analysed by one-way analysis of variance (ANOVA) followed by post hoc Turkey test. $^{\&\&}P$ <0.01 versus NBP + Eda group, $^{\&\&\&}P$ <0.001 versus NBP + Eda group, $^{**}P$ <0.01 versus compound **9**, $^{***}P$ <0.001 versus compound **9**, $^{\#\#\#}P$ <0.001 versus **6g** + **9** group.

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Among target compounds **10a**–**g**, the substituent on the phenyl ring in edaravone moiety affected the inhibitory activity of platelet aggregation, and electron withdrawing substituted compounds were more active than electron donating groups. As to **11a**–**g**, the relationship between electronic effect of substitution and inhibitory activity of platelet aggregation is contrary to **10a**–**g**. Interestingly, when the phenyl ring of edaravone derivatives substituted with electron donating groups, the inhibitory activity of platelet aggregation of the target compounds **11a**–**c** with 3-trifluoromethyl pyrazolone moiety was also higher than **10a**–**c** with 3-methyl pyrazolone. Meanwhile, when the phenyl ring of edaravone derivatives substituted with electron withdrawing groups, such as chlorine atom, fluorine atom, and nitro group, the inhibitory activity of platelet aggregation of the target compounds **10d**–**g** with 3-methyl pyrazolone moiety is much higher than that of **11d**–**g**. However, the antioxidant activity did not show the same rules, the target compounds showed varying degrees of antioxidant activity, and the anti-platelet aggregation and antioxidant activity of **10g** is optimal in all. From these results we can speculate that those various substituents may have different abilities to modulate the structure, stability, metabolism and penetrability of the hybrids, affecting the releasing of NBP and edaravone analogues, and leading to varied bioactivities of the hybrids.

Altogether, a series of novel hybrids of edaravone analogues and NBP ring-opening compound were designed and synthesized in the present study. Among them, **10d**, **10g** have stronger inhibitory effects on ADP- induced and AA-induced platelet aggregation than Ticlid, ASP, NBP, Eda and NBP together with Eda in vitro. Furthermore, we also found that **10g** exhibited a potent inhibitory activity against the H_2O_2-mediated cytotoxicity in PC12 cells and could scavenge free radical (·OH) and ($\cdot O_2^-$), a major contributor to secondary brain injury induced by reperfusion after occlusion of brain vessels, which is more potent than those of NBP, Eda, **6g**, **9**, even than the co-administration of two active fragment **6g** and **9** in further study. Notably, **10g** has an ester bond between its two fragments, which may make it has a better liposolubility to enter into cells easily or the breaking of the ester bond may take a long time so as to prolong the duration of action. All these suggest that **10g** may undergo rapid hydrolysis by esterases to yield edaravone analogue **6g** and NBP ring-opening derivative **9**, which can undergo a conversion to produce NBP. So it would exhibit edaravone-dependent and NBP-dependent activities to exert synergistic effect and to enhance its therapeutic potency. In summary, **10g** may be a potential agent for the intervention of ischemic stroke and such hybrids based on edaravone and NBP ring-opening derivatives may represent a novel class of candidates for the treatment of ischemic stroke.

Acknowledgements

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12. General procedure for the synthesis of the target compounds **10a**–**g** and **11a**–**g**: acetylacetic ether and trifluoroacetyl ethyl acetate (25 mmol) were added to a mixture solution of **5a**–**g** (25 mmol) and sodium acetate (26 mmol) in acetic acid (20 mL). The reaction mixture was stirred at reflux temperature for 5–10 h. After cooling, the mixture was added to a saturated solution of $NaHCO_3$ until its pH value is adjusted to 7 and extracted with ethyl acetate for three times. The combined organic layer was then dried, filtered, concentrated and the residue was purified by column chromatography (PE/EtOAc = 6:1, v/v) to give light yellow solid **6a**–**g** and **7a**–**g** in 50–70% yield. To a solution of **9** (4.7 mmol) in anhydrous dichloromethane (20 mL), oxalyl chloride (6.24 mmol) was added and stirred at room temperature for 8 h, then solvent of the mixture was evaporated to obtain the corresponding acyl chloride. A solution of **6a**–**g** and **7a**–**g** (7.2 mmol), triethylamine (9.6 mmol) in anhydrous dichloromethane (20 mL) was stirred for 10 min under 0 °C. Then the acyl chloride of **9** obtained above in anhydrous dichloromethane (20 mL) was added dropwise to the solution, the reaction mixture was stirred at room temperature for 4–6 h and then poured into water, extracted with ethyl acetate for three times, the combined organic phase was then dried, filtered, and evaporated to dryness. The residue was purified by column chromatography (PE/EtOAc = 10:1, v/v) to give pure **10a**–**g** and **11a**–**g** in 43–60% yield.
13. Analytical data for **10g**: mp 67–69 °C. ESI-MS *m*/*z*: 425 $[M+H]^+$; 447 $[M+Na]^+$. IR (cm^{-1}, KBr): 3076, 2957, 2923, 2856, 1752, 1731, 1601, 1546, 1417, 1390, 1146, 1096, 939 783, 750, 661. ^{1}HNMR ($CDCl_3$, 300 MHz, δ): 0.87 (t, 3H, CH_3, J = 7.2 Hz), 1.26–1.41 (m, 4H, 2 × CH_2), 1.69–1.85 (m, 2H, CH_2), 2.07 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 6.25 (s, 1H, C=CH), 6.44–6.49 (m, 1H, CH), 7.07–7.14 (m, 2H, ArH), 7.31–7.36 (m, 1H, ArH), 7.52–7.62 (m, 4H, ArH), 7.91 (d, 1H, ArH, J = 7.8 Hz). ^{13}C NMR ($CDCl_3$,75 MHz, δ):170.2, 163.2, 161.7, 159.9, 149.1, 145.6, 144.4, 133.7, 130.5, 127.4, 126.5, 125.5, 125.3, 125.2, 116.1, 115.8, 95.9, 72.6, 36.3, 27.9, 22.3, 21.1, 14.4, 13.9. HR-MS (ESI) for $C_{24}H_{25}FN_2O_4$ ($[M+Na]^+$) calcd 447.1696, found: 447.1709.
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16. Blood samples were withdrawn from rabbit carotid artery and mixed with 3.8% trisodium citrate (9:1 v/v), followed by centrifuging at 500 rpm for 10 min. The supernatants were collected and used as platelet rich plasma (PRP). Additional samples were centrifuged at 3000 rpm for 10 min and the supernatants were collected as platelet poor plasma (PPP). The effect of individual compounds on the ADP-induced and AA-induced platelet aggregation was measured by Born's turbidimetric method using a Platelet-Aggregometer (LG-PABER-I Platelet-Aggregometer, Beijing). Briefly, PRP (240 μL) was pre-treated in duplicate with vehicle, different concentrations of all positive drugs and all individual compounds for 5 min and exposed to 10 μM of ADP or 1.0 mM of AA incubated at 37 °C for 5 min, respectively. The formation of platelet aggregation was monitored longitudinally by optical density. Compounds under study or vehicle alone were added to the PRP samples 5 min before addition of the aggregating agent. The antiplatelet aggregatory activity of all positive control and all individual compounds was evaluated as percent inhibition of platelet aggregation compared to positive controls that had been pre-treated with vehicle alone and exposed to the inducer samples.
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19. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated horse serum (Hyclone), 5% fetal bovine serum (GIBCO), 1.0 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere (Thermo Scientific, 3110, OH, U.S.). During the exponential phase of growth, PC12 cells (20,000 cells/well) were cultured in 96-well plates that had been coated with

poly-L-lysine for 24 h. When the concentration of H_2O_2 is 800 mM in 3 h, the cell survival rate was 50.85%, so we choose this concentration as the best concentration of damage. The cells were treated in triplicate with different concentrations (1–10 μM) of the tested compounds for 1 h and exposed to 800 μM H_2O_2 for 3 h. After replacement with fresh medium, the cells were incubated for 4 h and the cell viability was determined by MTT.

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21. PC12 cells were grown as the same as before, and the cells were treated in triplicate with all referential controls and different concentrations (1–10 μM) of **10g** for 1 h. Then we exposed them to 1 mM H_2O_2/20 μM Fe^{2+} (pH = 7.4). After replacement with fresh medium, the cells of every couple was collected in 0.5 mL of phosphate buffer (pH = 7.4), we detected the reduced activity of ·OH by Griess assay. On the other hand, 0.5 mL every test tube was added 2.0 mL of DMSO, 2.0 mL of 468 μm NADH, 1.0 mL of 300 μm NBT at the same concentration. Then we detected the reduced activity of $\cdot O_2^-$ by DMSO, which was used as solvent for completely solubilizing the formazan compounds formed in the original NBT assay. The inhibition effect of $\cdot O_2^-$ or ·OH scavenging (U/L or U/mL) was calculated. Results were expressed as the mean ± standard deviation (SD) of triplicate determinations.