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RESEARCH ARTICLE

Design, Synthesis and Biological Evaluation of 3-*n*-Butylphthlide-Edaravone Hybrids as Potential Agents for the Treatment of Cerebral Ischemia

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Abstract: A series of 3-*n*-butylphthlide-edaravone hybrids was synthesized and their structures were elucidated on the basis of analytical and spectral (IR, ^{1}H NMR, ^{13}C NMR, MS and elemental analyses) data. These synthesized compounds were evaluated for their *in vitro* antiplatelet by the turbidimetric method and antioxidant activities by the Fenton method. The results indicated that compound **11a** showed similar potency of antiplatelet aggregation as 3-*n*-butylphthlide and stronger ·OH scavenging activity (IC_{50} value of 2.87 mM) than edaravone (IC_{50} value of 3.57 mM).

Keywords: NBP, Edaravone, Antiplatelet, Antioxidant, Anti-cerebral ischemia

Introduction

Stroke due to cerebral ischemia is a serious disease to be a leading cause of long-term disability and mortality worldwide and its impact on human and economic toll is tremendous[1,2]. The pathological process of cerebral ischemia is very complicated. Briefly, the impaired blood flow to brain brings about inadequate influx of oxygen and nutrients and diminishes clearance of metabolic toxins, leading to disastrous metabolic dysfunction causing ultimately neuronal death[3]. Few of drugs are approved for the therapy of cerebral ischemia in clinics to date. The serine protease tissue-type plasminogen activator (t-PA) aimed at restoring cerebral blood flow is the only one approved by FDA for the treatment of

acute cerebral ischemia. Although t-PA is powerful, its utilization is limited by a very narrow therapeutic time window of 4.5 h[4,5]. The neuroprotection therapy seems to be a more practical strategy which uses drugs, such as antiplatelet, antithrombotic or antioxidant agents, to prevent neuronal damage or protect the potentially ischemic tissue during cerebral ischemia[6,7]. However, the efficiencies of these drugs are usually not strong enough, thus it is indispensable for pharmaceutical chemistry researchers to search for more excellent agents to treat cerebral ischemia.

Experimental

Melting points were determined on a WRS-2B digital melting point apparatus and uncorrected. IR spectra were recorded using a Nicolet 380 Fourier-transform infrared (FTIR) spectrophotometer (Thermo, USA) from KBr pellets. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AVANCE III-400 NMR spectrometer (Bruker Biospin Co., Switzerland) with tetramethylsilane (TMS) as the internal standard. MS (ESI) measurement was conducted on an Agilent 1100 LC-MS spectrometer (Agilent, Palo Alto, USA). Elemental analyses were performed on Vario Micro cube (Elementar Co., Germany) instrument. Column chromatography was carried out on silica gel (200-300 mesh) from Qingdao Ocean Chemicals (Qingdao, China). UV/Vis spectra were recorded on a Shimadzu UV-3101PC spectrophotometer at room temperature. ADP (AR grade) was purchased from Sigma-Aldrich and other reagents were obtained commercially from Sinopharm Chemical Reagent Co., Ltd (SCRC) and used without further purification.

3-*n*-Butylphthalide (NBP, **1**), a naphtha component from seeds of *Apium graveolens Linn*, had been approved by the China Food and Drug Administration (CFDA) in 2002 as a new drug for the treatment of cerebral ischemia in clinic. Many basic and clinical studies have shown that NBP possess multiple biological activities such as inhibiting platelet aggregation, reducing thrombus formation, improving mitochondrial function[8-10], *et al*., Edaravone (**2**) is a free radical scavenger which prevents neuronal death and brain edema by scavenging hydroxyl radical and inhibiting lipid peroxidation. It was approved by the Japanese Ministry of Health in 2001 to treat cerebral ischemia[11]. Obviously, NBP and edaravone modulate different targets of cerebral ischemia with distinct mechanisms. Recently, it was reported that edaravone combined with NBP in the treatment of acute cerebral infarction was more effective than each one[12-15], which meant the combination of the two drugs exhibited additive effects. In consideration of the complicated pathological mechanisms of cerebral ischemia, drugs combinations with different mechanisms, rather than a single one, may be rational. Nevertheless, it is troublesome for a patient to take different medicines.

Drug hybridization is an attracting design strategy for drug development and research, which combines the pharmacophores of two or more existing drugs to create a single molecule with multiple pharmacological targets to increase their therapeutic potential. There are numerous examples of hybrid molecules applying this approach[16-18], among which is a well known antihypertensive drug in clinical use, prizidilol (**3**), integrated with hydralazine and a pharmacophoric group responsible for *β*-blocking activity.

The structural basis on which NBP exerts its bioactivities has not yet been elucidated clearly, but some derivatives with substituted groups at position 6 of NBP have been reported to have similar activities as NBP[19,20] (**4**, **5**). In particular, the ring-opening prodrug (**6**) of 6-bromo-3-*n*-butylphthalide (**5**) had been approved by CFDA for clinical trials in China in 2016. As for edaravone, it is well recognized that the pyrazolone sub-structure plays an important role in determining its activities through its two tautomeric forms as

shown in Figure 1[21,22]. In view of the above-mentioned backgrounds, we decided to pack the two pharmacophores of NBP and edaravone into a single chemical entity and test their bioactivities as potential anti-cerebral ischemia agents (Figure 1). Here we describe the synthesis of a series of 3-*n*-butylphthlide-edaravone hybrids and their antiplatelet and antioxidant activities *in vitro* evaluations.

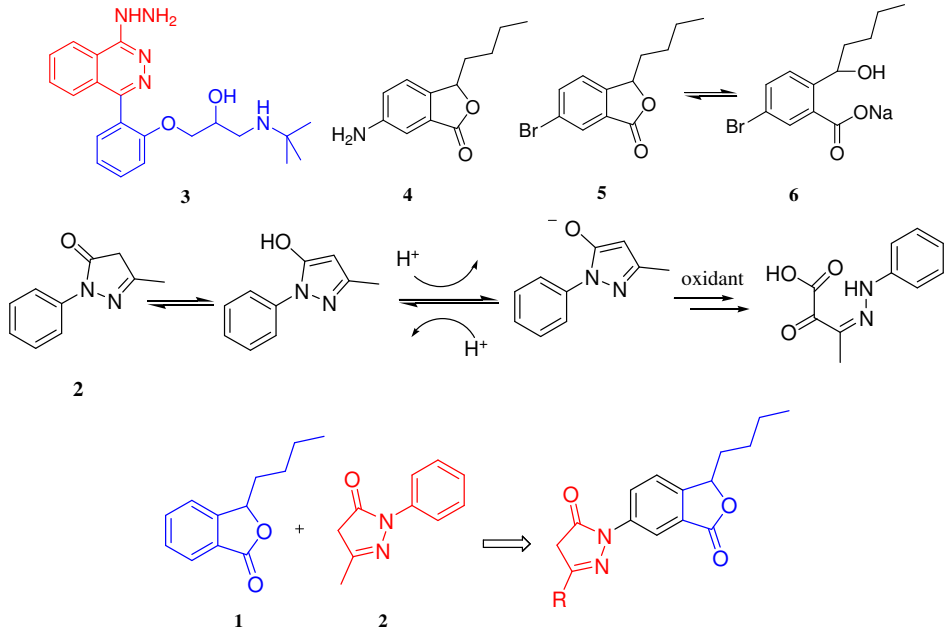


Figure 1. Structrues of compounds related and strategy for the design of 3-*n*-butylphthlide-edaravone hybrids

*Synthesis of intermediate (±)-3-n-butylphthalide (1), (±)-3-n-butyl-6-nitrophtha -lide (8), (±)-3-n-butyl-6-aminophthalide (**4**)*

Compounds **1**, **4**, **8** were synthesized according to previously reported procedure[20,23].

*(±)-3-n-Butyl-6-nitrophthalide (**8**)*

Light yellow solid; yield: 85.92%; m.p. 51.0 °C;-53.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.72(s, 1H, ArH), 8.56(dd, J=8.4Hz, 2.0Hz, 1H, ArH), 7.67(d, J=8.4Hz, 1H, ArH), 5.56(dd, J=8.0，4.0 Hz, 1H, CH), 2.14-2.08(m, 1H, C<u>H</u>H), 1.87-1.79(m, 1H, CH<u>H</u>), 1.54-1.37(m, 4H, CH_2CH_2), 0.92 (t, J=7.1 Hz, 3H, CH_3).

*(±)-3-n-Butyl-6-aminophthalide (**4**)*

Light yellow solid; yield: 88.09%; m.p. 121.9 °C;-124.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.27(s, 1H, ArH), 7.11-7.19(m, 1H, ArH), 6.95-6.98(m, 1H, ArH), 5.37(dd, *J* = 7.2, 4.0 Hz, 1H, CH), 3.95(brs, 2H, NH_2), 1.93-2.00(m, 1H, C<u>H</u>H), 1.66-1.74(m, 1H, CH<u>H</u>), 1.48-1.32(m, 4H, CH_2CH_2), 0.90 (t, J=7.0 Hz, 3H, CH_3).

*Synthesis of (±)-3-n-butyl-6-hydrazinylisobenzofuran-1(3H)-one (**9**)*

To a mixed solution of conc. Hydrochloric acid (8.0 mL) and water (7.0 mL) was added compound **4** (3.08 g, 15 mmol) under stirring and the solution was cooled to 0 °C.

Sodium nitrite (1.09 g, 15.75 mmol) dissolved in water (3.0 mL) was added dropwise to the mixture while the internal temperature was maintained at 0-5 °C. After a check by using starch- iodide paper indicated the presence of excess nitrous acid, the reaction mixture was stirred at 0-5 °C for 0.5 h. A solution of stannous chloride dihydrate (10.14 g, 45 mmol) in 1:1 conc. hydrochloric acid/water (15 mL) was added dropwise to the diazonium salt solution over 0.5 h maintaining an internal reaction temperature at 0-5 °C. The heterogeneous orange mixture was stirred at 0 °C for 1 h before being allowed to warm to ambient temperature and vigorous stirring was maintained for 4 h. The orange precipitate was collected by suction filtration and dissolved in water (60 mL) under stirring at 0 °C and then chloroform (50 mL) was added. The biphasic mixture was basified with 10 M sodium hydroxide to pH 13 before it was transferred to a separatory funnel. The organic layer was separated and dried over magnesium sulfate and filtered. Then it was evaporated on a rotary evaporator to afford the crude product **9** as a light yellow solid in 65.32% yield, mp 86.7-87.9 °C. Compound **9** was used directly in the next step without further purification.

*General procedure for the preparation of compound **10** and **11a-11g***

The corresponding acetylacetone, substituted ethyl acetoacetate or 4-substituted ethyl benzoylacetate derivatives (10 mmol) was added dropwise to a solution of **9** (10 mmol) in acetic acid (30 mL) at room temperature in 0.5 h, then the mixture was heated on an oil bath at 90 °C for 5 h. The excess solvent was evaporated. The residue was dissolved in DCM (30 mL), washed with water (20 mL×3), dried over anhydrous Na_2SO_4 and then evaporated under reduced pressure to give crude product. The pure product was isolated by silica gel column chromatography [Petroleum ether-EtOAc, 6:1 -10:1(v/v)] to give the products **10** and **11a-11g**.

*(±) - 3-n-Butyl-6-(3, 5-dimethyl-1H-pyrazol-1-yl) isobenzofuran-1(3H)-one (**10**)*

Light yellow oil; yield: 76.55%; IR (KBr, cm^{-1}): 2957, 1766, 1560, 1504, 1383, 839, 785; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.88 (s, 1H, ArH), 7.86(dd, J=8.0Hz, 4.0Hz, 1H, ArH), 7.53 (d, *J*=8.0 Hz, 1H, ArH), 6.05 (s, 1H, pyrazole H), 5.54 (dd, *J*=8.0, 4.0 Hz, 1H, CH), 2.37 (s, 3H , pyrazole CH_3), 2.30 (s, 3H, pyrazole CH_3), 2.13-2.03 (m, 1H, C<u>H</u>H), 1.85-1.73 (m, 1H, CH<u>H</u>), 1.52-1.31 (m, 4H, CH_2CH_2), 0.92 (t, *J*=7.6 Hz, 3H, CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 169.72, 149.85, 148.28, 140.96, 139.62, 130.55, 127.09, 122.68, 120.40, 107.95, 81.37, 34.33, 26.75, 22.39, 13.83, 13.45, 12.55; LCMS (ESI): 285.2$[M+1]^+$.

*(±)-1-(1- n-Butyl-1, 3-dihydro-3-oxoisobenzofuran-5-yl)-3-methyl-1H-pyrazol-5(4H)-one **(11a)***

Yellow solid; yield: 58.60%; m.p. 74.6-76.8 °C; IR(KBr,cm^{-1}): 2956, 1762, 1718, 1570, 1496, 777; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.36 (s, 1H, ArH), (dd, J=7.6Hz, 2.0Hz, 1H, ArH), 7.43 (d, *J*=4.0 Hz, 1H, Ar-H), 5.47 (dd, *J*=7.2, 3.6 Hz, 1H, CH), 3.49 (s, 2H, pyrazolone CH_2), 2.23 (s, 3H, pyrazolone CH_3), 2.07-2.01 (m, 1H, C<u>H</u>H), 1.81-1.73 (m, 1H, CH<u>H</u>), 1.52-1.33 (m, 4H, CH_2CH_2), 0.91 (t, *J*=7.0 Hz, 3H,CH_3); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 170.61, 170.24, 157.01, 145.82, 139.06, 126.82, 124.12, 122.10, 115.07, 81.32, 43.09, 34.41, 26.76, 22.39, 17.02, 13.83; LCMS (ESI): 287.1$[M+1]^+$.

*(±)-1-(1- n-Butyl-1, 3-dihydro-3-oxoisobenzofuran-5-yl)-3-propyl-1H-pyrazol-5(4H)-one **(11b)***

Yellow oil; yield: 50.55%; IR (KBr, cm^{-1}): 2959, 1763, 1719, 1569, 1496, 835, 767; ^{1}H NMR (400 MHz,$CDCl_3$) δ: 8.39-8.33 (m, 2H, ArH), 7.39 (d, *J*=8.3 Hz, 1H, ArH), 5.48 (dd, *J*=8.0, 3.6 Hz, 1H, CH), 3.48 (s, 2H, pyrazolone CH_2), 2.47 (t, *J*=7.5 Hz, 2H, CH_2), 2.01 (m,

1H, C<u>H</u>H), 1.80-1.62 (m, 3H, CH_2 and CH<u>H</u>), 1.49-1.27 (m, 4H, CH_2CH_2), 1.01 (t, *J*=7.2 Hz, 3H, CH_3), 0.88 (t, *J*=6.8 Hz, 3H, CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 170.56, 170.24, 160.50, 145.79, 139.18, 126.86, 124.14, 122.06, 115.16, 109.99, 81.28, 41.81, 34.42, 33.09, 26.74, 22.39, 19.78, 13.82, 13.72; LCMS (ESI): 315.2$[M+1]^+$.

*(±)-1-(1- n-Butyl-1, 3-dihydro-3-oxoisobenzofuran-5-yl)-3-isopropyl-1H-pyrazol-5(4H)-one (**11c**)*

Yellow oil; yield: 54.32%. IR(KBr,cm^{-1}): 2961, 1763, 1719, 1496; ^{1}H NMR (400 MHz,$CDCl_3$) δ: 8.38-8.36 (m, 2H,ArH), 7.44 (d, *J*=8.6 Hz, 1H, ArH), 5.48 (dd, *J*=7.6, 4.2 Hz, 1H, CH), 3.48 (s, 2H, pyrazolone CH_2), 2.84-2.77 (m, 1H,*i*-Pr CH), 2.09-2.00 (m, 1H, C<u>H</u>H), 1.82-1.73 (m, 1H, CH<u>H</u>), 1.49-1.33 (m, 4H, CH_2CH_2), 1.28 (s, 3H, *i*-Pr CH_3), 1.25 (s, 3H, *i*-Pr CH_3), 0.91 (t, *J*=7.0 Hz, 3H, *n*-Bu CH_3);^{13}C-NMR (150 MHz,$CDCl_3$) δ 170.63, 170.32, 170.29, 164.97, 145.74, 139.24, 126.79, 124.14, 122.05, 115.08, 81.31, 40.04, 34.39, 30.76, 26.73, 22.38, 19.96, 13.82; LCMS (ESI): 315.2$[M+1]^+$.

*(±)-1-(1-n-Butyl-1, 3-dihydro-3-oxoisobenzofuran-5-yl)-3-phenyl-1H-pyrazol-5(4H)-one (**11d**)*

Yellow solid; yield: 60.26%; m.p. 123.5-125.2 °C. IR(KBr,cm^{-1}): 2956, 1762, 1720, 1492; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.48-8.46 (m, 2H, ArH), 7.82-7.79 (m, 2H, Ar H), 7.50-7.47 (m, 4H, ArH), 5.51 (dd, *J*=7.7, 4.1 Hz, 1H,CH), 3.92 (s, 2H, pyrazolone CH_2), 2.14-2.01 (m, 1H C<u>H</u>H), 1.87-1.74 (m, 1H CH<u>H</u>), 1.56-1.30 (m, 4H,CH_2CH_2), 0.92 (t, *J*=7.0 Hz, 3H ,CH_3);^{13}C-NMR (150 MHz, $CDCl_3$) δ 170.47, 169.48, 164.23, 148.12, 145.65, 139.17, 132.32, 129.43, 128.22, 126.77, 126.32, 126.26, 123.30, 122.36, 116.21, 81.56, 34.40, 26.80, 22.38, 20.98, 13.83; LCMS (ESI): 349.2$[M+1]^+$.

*(±)-1-(1- n-Butyl-1, 3-dihydro-3-oxoisobenzofuran-5-yl)-3-(4-methoxyphenyl)-1H-pyrazol-5(4H)-one (**11e**)*

White solid; yield: 75.40%; m.p. 194.5-195.8°C. IR (KBr, cm^{-1}): 2965, 1766, 1704, 1608, 1496; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.49-8.46 (m, 2H, ArH), 7.75-7.73 (m, 2H, ArH), 7.47 (d, *J*=8.1 Hz, 1H, ArH), 7.01-6.98(m, 2H, ArH), 5.50 (dd, *J*=7.7 Hz, 4.2 Hz, 1H, CH), 3.88 (s, 5H, pyrazolone CH_2 and OCH_3), 2.08-2.00 (m, 1H,C<u>H</u>H), 1.86-1.72 (m, 1H, CH<u>H</u>), 1.52-1.32 (m, 4H, CH_2CH_2), 0.92 (t, *J*=7.1 Hz, 3H,CH_3);^{13}C-NMR (150 MHz, $CDCl_3$) δ 170.30, 170.20, 161.89, 155.02, 145.91, 139.27, 127.73, 126.92, 124.22, 123.17, 122.10, 115.27, 114.40, 81.36, 55.44, 39.76, 34.45, 26.80, 22.41, 13.84; LCMS (ESI): 379.2$[M+1]^+$.

*(±)-1-(1-n-Butyl-1, 3-dihydro-3-oxoisobenzofuran-5-yl)-3-(4-ethoxyphenyl)-1H-pyrazol-5(4H)-one (**11f**)*

Yellow solid; yield: 59.73%. m.p. 176.9-177.4 °C. IR (KBr,cm^{-1}): 2953, 1751, 1702, 1609, 1518; ^{1}H NMR (400 MHz,$CDCl_3$) δ: 8.47 (d, *J*=8.5 Hz, 2H, ArH), 7.73 (d, *J*=8.8 Hz, 2H, ArH), 7.47 (d, *J*=8.2 Hz, 1H, ArH), 6.97 (d, *J*=8.8 Hz, 2H, ArH), 5.50 (dd, *J*=7.6, 4.1 Hz, 1H,CH), 4.11 (q, *J*=8.0 Hz, 2H, ethoxy CH_2), 3.88 (s, 2H, pyrazolone CH_2), 2.10-2.01 (m, 1H, C<u>H</u>H), 1.83-1.75 (m, 1H,CH<u>H</u>), 1.53-1.32 (m, 7H, ethoxy CH_3 and CH_2CH_2), 0.91 (t, *J*=7.0 Hz, 3H ,CH_3).^{13}C-NMR (150 MHz,$CDCl_3$) δ 170.32, 170.26, 161.28, 155.11, 145.88, 139.26, 127.71, 126.86, 124.18, 122.93, 122.11, 115.16, 114.83, 81.38, 63.71, 39.75, 34.43, 26.81, 22.40, 14.67, 13.84; LCMS (ESI): 393.2$[M+1]^+$.

*(±)-1-(1-n-Butyl-1, 3-dihydro-3-oxoisobenzofuran-5-yl)-3-(4-chlorophenyl)-1H-pyrazol-5(4H)-one (**11g**)*

Yellow solid; yield: 43.85%. m.p. 171.3-174.2 °C. IR (KBr,cm^{-1}): 2956, 1762, 1723, 1599; ^{1}H NMR (400 MHz,$CDCl_3$) δ: 8.45 (d, *J*=7.7 Hz, 2H,ArH), 7.74 (d, *J*=8.5 Hz, 2H, ArH), 7.49-7.45 (m, 3H, ArH), 5.50 (dd, *J*=8.0, 4.0 Hz, 1H,CH), 3.89 (s, 2H, pyrazolone CH_2), 2.09-2.01 (m, 1H , C<u>H</u>H), 1.85-1.74 (dd, *J*=11.2, 6.7 Hz, 1H CH<u>H</u>), 1.53-1.33 (m, 4H, CH_2CH_2), 0.92 (t, *J*=7.0 Hz, 3H ,CH_3).^{13}C-NMR (150 MHz, $CDCl_3$) δ 170.21, 169.96, 154.15, 146.23, 139.05, 137.19, 129.33, 128.93, 127.30, 127.02, 124.27, 122.21, 115.38, 81.39, 39.54, 34.44, 26.80, 22.40, 13.83; LCMS (ESI): 383.1$[M+1]^+$.

Biology

Antiplatelet aggregation in vitro

Platelet aggregation was measured by the turbidimetric method in a 96-well microplate[24,25]. Blood was drawn from rabbit carotid artery and mixed with 3.8% sodium citrate (9:1, v/v). Platelet-rich plasma (PRP) was obtained from whole blood centrifugation at 800 rpm for 10 min at room temperature. The remaining blood was further centrifuged at 3000 rpm for another 10 min to prepare platelet-poor plasma (PPP) which was used as a reference solution in aggregation assays. Fresh PRP was added to each well of a flat bottom 96-well microplate and individually incubated at 37 °C for 10 min with DMSO (0.5%) used as negative control, aspirin and NBP as positive controls or the synthesized compounds (all 100 μM final concentration) before the addition of the platelet agonists. The microplate was put on the microplate reader and vibrated for 5 min, then monitored by measuring 655 nm transmission (A_0). The monitoring took place every 30 s. After that ADP (10μM final concentration) was added and 655 nm transmission was monitored every 30 s until it became stable (A). The aggregation rate (AR) = (A_0-A)/ (A_0-Abs PPP). The aggregation inhibition rate (AIR) = [1-(AR sample/ AR negative control)] ×100%.

Hydroxyl radical scavenging effect in vitro

Salicylic acid was used as a probe to detect the hydroxyl radical (·OH) in aqueous media generated by the Fenton reaction[26]. The solution of the tested compounds and salicylic acid was prepared in EtOH while $FeSO_4$ and H_2O_2 were dissolved in distilled water. The four solution were mixed and the total volume of the mixture in each tube was made up to 4 mL by adding the required amount of distilled water. Finally, the 4 mL of samples of selected tested compounds contained following reagents: $FeSO_4$ (2.25 mM), H_2O_2 (2.5 mM), salicylic acid (2.25 mM) and the tested compounds (0.125, 0.25, 0.5, 1, 2 mM). The assay mixtures were incubated at 37 ? for 60 min in a water bath. After which, the absorbance was measured at 510 nm. The suppression ratio for ·OH radical was calculated from the following expression:

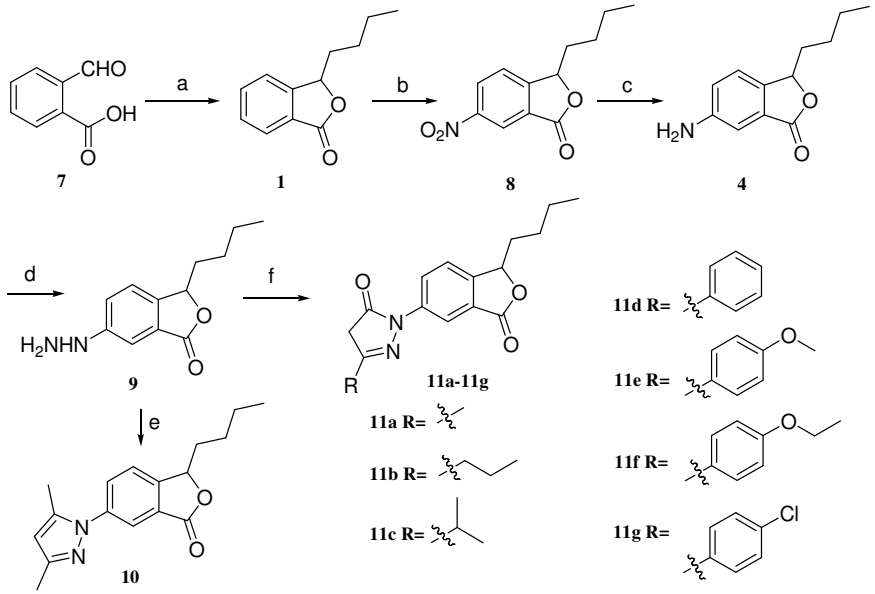
Hydroxyl radical scavenging assay (%) =$[(A_0-A_s)/A_0]\times100$ where A_0 is the absorbance in the absence of the tested compound, A_s is the absorbance in the presence of the tested compound. All the tests were run in triplicate and expressed as the mean. The IC_{50} values of the tested compounds were calculated by SPSS v.16.0.

Results and Discussion

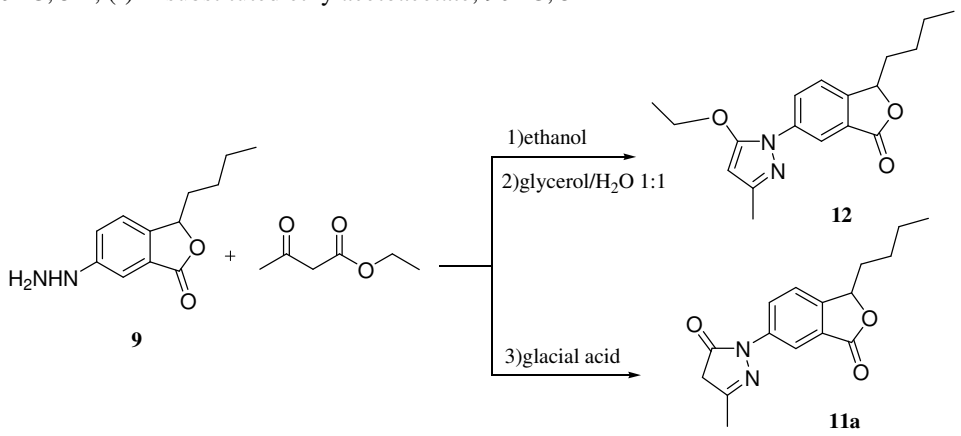
Chemistry

The synthetic routes for the target 3-*n*-butylphthlide-edaravone hybrids **11a**-**11g** and the analogue **10** are outlined in Scheme 1. The 2-carboxybenzaldehyde **7** was reacted with the Grignard reagent *n*-BuMgBr, which was then acidized to provide 3-*n*-butylphthalide **1** (NBP).

NBP was nitrated with concentrated sulfuric acid and fuming nitric acid to afford 3-*n*-butyl-6-nitro-1(3*H*)-isobenzofuranone **8** in good yield. The 3- *n*-butyl-6-amino-1(3*H*)-isobenzofuranone **4** was obtained by reduction of **8** under hydrogen in the presence of Pd/C. Then compound **4** was oxidized under the condition of nitrous acid followed by reduction with stannous chloride ehydrate to generate 3-*n*-butyl-6-hydrazino-1(3*H*)-isobenzofuranone **9**. Finally, the target compound **10** and **11a**-**11g** were synthesized by the condensation reaction between the intermediate **9** and acetylacetone or substituted acetoacetate derivatives respectively. The structures of all the synthesized hybrids **11a**-**11g** and the analogue **10** were confirmed by using spectral techniques such as IR, ^{1}H NMR, ^{13}C NMR and MS.



Scheme 1. Reagents and conditions: (a) (i)*n*-BuMgBr, 0 °C, 4h; (ii)10% HCl, 40 °C, 1 h; (b)98% H_2SO_4, nitrosonitric acid, 2 h; (c) H_2, 10%Pd/C, CH_3OH; (d) (i)$NaNO_2$, 36.5% HCl, 0-5 °C, 0.5 h; (ii)$SnCl_2$, 36.5% HCl, 0-5 °C, 1 h, rt, 3 h; (iii) 10 M NaOH; (e) acetylacetone, 90 °C, 5 h; (f) *R*-substituted ethylacetoacetate, 90 °C, 5h



Scheme 2. Effects of solvents on the main products in the condensation reaction

Interestingly, the different products were produced in various solvents in the process of preparing **11a**-**11g** (Scheme 2). At first, the condensation reaction between **9** and ethylacetoacetate was performed in ethanol or aqueous glycerol solutions, resulting in the formation of the pyrazole derivative **12** as the main product almost without **11a**. However, **11a** was got as a major product when the glacial acid was used as solvent in the reaction. In the ^{1}H NMR spectrum of **12**, the quartet signal at δ 4.19 ppm indicates the presence of two protons of -OCH_2 from -OCH_2CH_3 groups. While the single signal observed at δ 3.49 corresponds to the two protons of CH_2 from the pyrazolone ring in the ^{1}H NMR spectrum of **11a**. Then a simple pyrazole derivative **10** was synthesized to investigate the influence of pyrazole moiety of the hybrid compound on biological activity.

Biology

The inhibitory effects of target compounds **10** and **11a**-**11g** on platelet aggregation induced by adenosine diphosphate (ADP) *in vitro* were determined by the 96-well microplate method. NBP and ASP were used as controls. The assays were repeated six times and data were calculated and expressed as the inhibition rate of platelet aggregation. As shown in Table 1, **11e** was the most active one with 44.12±3.01% inhibition rate which was more potent than NBP (33.82±3.80%) and ASP (39.26±5.04%). Compound **11a** with 31.18±5.56% inhibition rate showed similar potency as NBP. The compounds **10**, **11b** and **11c** showed moderately good activities.

The antioxidant activities of target compounds were determined by scavenging hydroxyl radicals (·OH) according to Fenton method. Edaravone was used as positive control. The results were reported in Table 1, which showed that the ·OH scavenging capacities of **11a** and **11c** (IC_{50} value of 2.87±0.18 mM and 1.71±0.12 mM, respectively) were significantly stronger than that of edaravone (IC_{50} value of 3.57±0.23 mM).

Table 1. Antiplatelet and hydroxyl radicals-scavenging activities of compounds **10**, **11a**-**11g** *in vitro*

Compd.	Inhibition rate[a] (%) of platelet aggregation induced by ADP	IC_{50} [b](mM) ·OH-scavenging ability
10	26.79±2.13*	>100
11a	31.18±5.56*	2.87±0.18&
11b	23.02±4.84	3.77±0.13
11c	22.91±3.92	1.71±0.12&
11d	5.54±2.19	4.53±0.10
11e	44.12±3.01*	>100
11f	6.84±3.67	>100
11g	13.93±4.08	26.95±3.21
NBP	33.82±3.80	-
ASP	39.26±5.04	-
EDA	-	3.57±0.23

*Data were expressed as mean±SD. a. n=6. b. n=3 *p<0.01 versus NBP group, &p<0.01 versus EDA group. ASP = aspirin, EDA= edaravone*

Although it was difficult to explore the detailed structure-activity relationship (SAR) due to the limited number of compounds, we could drive a trend that alkyl groups led to increased activities as compared with substituted phenyl groups on the pyrazolone ring of R. Apart from the inhibitory effect of **11e** on platelet aggregation, the antiplatelet and antioxidant activities of **11a**, **11b** and **11c** were more potent than that of **11d**, **11f** and **11g** respectively. Additionally,

comparison of compound **10** with compound **11a** demonstrated parazole moiety could decrease antioxidant activity as compared with pyrazolone moiety in these hybrids. Among these compounds **11c** and **11e** presented the most potent antioxidant and antiplatelet activities respectively, but they had another poor activity. Taken all together, compound **11a** was a relatively ideal product which exhibited strong both antioxidant and antiplatelet activities. In light of the great significance of antioxidant and antiplatelet drugs to cerebral ischemia, **11a** was chosen for further bioactivity evaluations and we will report later.

Conclusion

A series of 3-*n*-butylphthlide-edaravone hybrids was designed and synthesized and their antiplatelet and hydroxyl radical scavenging activities *in vitro* were screened. The results indicated that **11a** had a similar inhibitory effect on ADP-induced platelet aggregation *in vitro* as NBP but it exhibited more potent activity on scavenging hydroxyl radical than edaravone. Our studies might provide a novel framework for further research for the intervention of cerebral ischemia.

References

1. Broussalis E, Killer M, McCoy M, Harrer A, Trinka E and Kraus J, *Drug Discovery Today*., 2012, **17(7-8)**, 296-309; DOI:10.1016/j.drudis.2011.11.005
2. Pujol Lereis V A, Ameriso S, Povedano G P and Ameriso S F, *J Stroke Cerebrovasc Dis*., 2012, **21(8)**, 868-872; DOI:10.1016/j.jstrokecerebrovasdis.2011.05.009
3. Grupkea S, Halla J, Dobbsb M, Bixb G J and Frasera J F, *Clinical Neurology Neurosurgery*., 2015, 129: 1-9; DOI:10.1016/j.clineuro.2014.11.013
4. Wardlaw J M, Murray V, Berge E, Zoppo G, Sandercock P, Lindley R L and Cohen G, *Lancet.*, 2012, **379(9834)**, 2364-2372; DOI:10.1016/S0140-6736(12)60738-7
5. Fukuta T, Asai T, Yanagida Y, Namba M, Koide H, Shimizu K and Oku N, *FASEB J*., 2017, **31(5),** 1879-1890; DOI:10.1096/fj.201601209R
6. Davis S M and Pennypacker K R, *Neurochem Int*., 2012, **107,** 23-32; DOI:10.1016/j.neuint.2016.12.007
7. Patel R and McMullen P W, *Prog Cardiovasc Dis*., 2017, **59(6),** 542-548; DOI:10.1016/j.pcad.2017.04.005
8. Dong G X and Feng Y P, *Acta Academiae Med Sinicae*., 2002, **24(1)**, 93-97.
9. Zhu X, Li X and Liu J, *Eur J Pharmacol.*, 2004, **500(1-3)**, 221-230; DOI:10.1016/j.ejphar.2004.07.027
10. Peng Y, Zeng X, Feng Y and Wang X, *J Cardiovasc Pharmacol*., 2004, **43(6)**, 876-881; DOI:10.1097/00005344-200406000-00018
11. Toyoda K, Fujii K, Kamouchi M, Nakane H, Arihiro S, Okada Y, Ibayashi S and Iida M, *J Neurol Sci*., 2007, **221**, 11-17; DOI:10.1016/j.jns.2004.03.002
12. Chen Q Y, Zhang Y J and Yu X, *Chin J Clin Pharmacol*., 2016, **32**, 1453-1455.
13. Liang H, Xiao C, Xi Z and Huang P, *China Modern Med*., 2016, **23,**133-135.
14. Li Y, Chen Y, Zhang Q, Li H, Liang B, Li J and Chen X, *Med Pharm J Chin PLA*., 2017, **29**, 86-90.
15. Wang Y, *Chin J Mod Drug Appl*., 2017, **11,** 85-87.
16. Yadav M R, Naik P P, Gandhi H P, Chauhan B S and Giridhar R, *Bioorg Med Chem Lett*., 2013, **23**, 3959-3966; DOI:10.1016/j.bmcl.2013.04.054
17. Jarrahpour A, Shirvani P, Sinou V, Latour C and Brunel J M, *Med Chem Res*., 2016, **25(1)**, 149-162; DOI:10.1007/s00044-015-1474-x

18. Gangarapu K, Thumma G, Manda S, Jallapally A, Jarapula R and Rekulapally S, *Med Chem Res*., 2017, **26(4)**, 819-829; DOI:10.1007/s00044-017-1781-5
19. Wang W, Cha X X, Reiner J, Gao Y, Qiao H L, Shen J X and Chang J B, *Eur J Med Chem*., 2010**, 45**, 1941-1946; DOI:10.1016/j.ejmech.2010.01.036
20. Wang X, Wang L, Huang Z, Sheng X, Li T, Ji H, Xu J and Zhang Y, *Bioorg Med Chem Lett*., 2013, **23**, 1985-1988; DOI:10.1016/j.bmcl.2013.02.035
21. Nakagawa H, Ohyama R, Kimata A, Suzuki T and Miyata N, *Bioorg Med Chem Lett*., 2006, **16(23),** 5939-5942; DOI:10.1016/j.bmcl.2006.09.005
22. Chegaev K, Cena C, Giorgis M, Rolando B, Tosco P, Bertinaria M, Fruttero R, Carrupt P A and Gasco A, *J Med Chem*., 2009, **52(2),** 574-578; DOI:10.1021/jm8007008
23. Min Z, Zhang Y, Zhuang P, Ji H, Lai Y and Peng S, *J China Pharma University*., 2008, **39(5)**, 392-397.
24. Bednar B, Condra C, Gould R J and Connolly T M, *Thromb Res*., 1995, **77(5)**, 453-463; DOI:10.1016/0049-3848(95)93881-Y
25. Chen H F, Li GN, Zhan P and Liu X, *Eur J Med Chem*., 2011, **46(11)**, 5609-5615; DOI:10.1016/j.ejmech.2011.09.030
26. Diez M H, Livertoux A A, Wellman-Rousseau S M and Leroy P, *J Chromatogr B Biomed Sci Appl*., 2001, **763(1-2),** 185-193; DOI:10.1016/S0378-4347(01)00396-6