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PII: S0223-5234(17)30742-0

DOI: 10.1016/j.ejmech.2017.09.028

Reference: EJMECH 9743

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 9 August 2017

Revised Date: 5 September 2017

Accepted Date: 15 September 2017

Please cite this article as: L. Huang, J. Wang, L. Chen, M. Zhu, S. Wu, S. Chu, Y. Zheng, Z. Fan, J. Zhang, W. Li, D. Chen, X. Yang, S. Wang, P. Qiu, J. Wu, Design, synthesis, and evaluation of NDGA analogues as potential anti-ischemic stroke agents, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.09.028.

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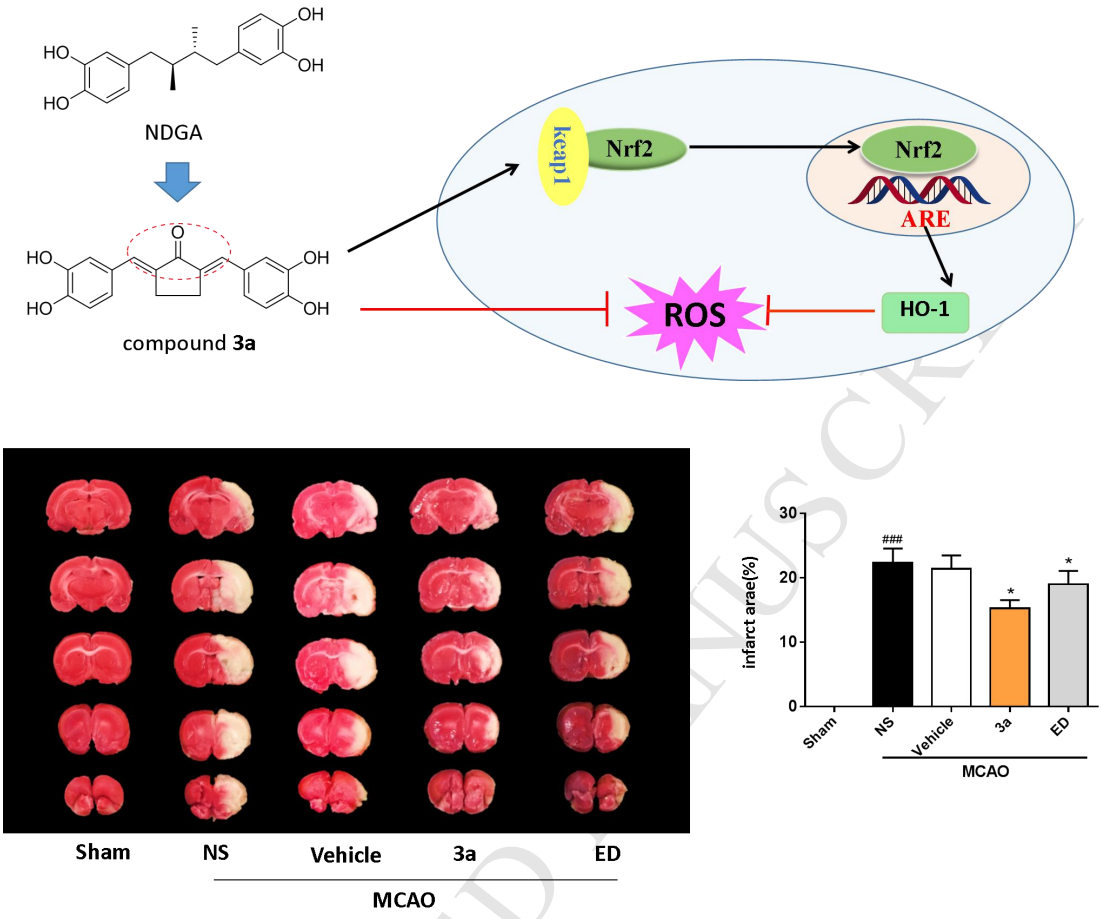

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Design, Synthesis, and Evaluation of NDGA Analogues as Potential Anti-Ischemic Stroke Agents

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Abstract

Exogenous supplementation of antioxidants with ROS scavenging activity would be a potential therapy to cerebral ischemia-reperfusion injury in stroke. In the present study, a series of NDGA analogues with attenuation oxidative stress by directly scavenging ROS and indirectly through keap1/Nrf2/ARE pathway activation were designed and synthesized. All analogues were found to effectively remove ROS directly by DPPH radical scavenging assay, and compound **3a** conferred potent protection from the oxidative injury in PC12 cells via promoting Nrf2 to translocate into nucleus and increasing expression of heme oxygenase-1(HO-1), where strongly reduced intracellular ROS level indirectly. More importantly, **3a** significantly reduced brain infarction after cerebral ischemia-reperfusion injury in rats subjected to transient middle cerebral artery occlusion

(MCAO). Overall, our findings shown compound **3a** could serve as a promising compound for the treatment of stroke.

Key words: Synthesis; ROS; Keap1/Nrf2/ARE pathway; Anti-ischemic stroke agent

1. Introduction

Stroke, a common cause of morbidity and mortality across the world, is the fourth-leading cause of death in the United States[1,2]. According to a recent study, ischemic stroke accounts for 87% of all strokes[3]. Currently, the treatment of ischemic stroke is used for thrombolytic therapy in generally, which brings the second brain injury, namely, cerebral ischemia-reperfusion injury[4-6]. Oxidative stress has been considered as the most important reason among a series of mechanisms related to the pathogenesis of cerebral ischemia-reperfusion injury. Because oxidative stress can accelerate reactive oxygen species (ROS) generation, which exacerbate ischemic brain damage extremely[7]. On the other hand, overproduction of ROS can not be neutralized in ischemic condition because the antioxidant defense system in ischemic tissues is interrupted[8]. Hence, exogenous supplementation of antioxidants with ROS scavenging activity would be a potential therapy to cerebral ischemia-reperfusion injury. At present, there are two broad categories of antioxidants: (1)The antioxidants with scavenging reactive oxygen species directly, like edaravone, resveratrol, carnosic acid, rosmarinic acid and so on[9-10]. (2)The antioxidants with scavenging reactive oxygen species indirectly, namely activating cellular endogenous antioxidant signaling pathways and promoting the transcription of a broad range of cytoprotective genes to remove ROS[11], where Keap1/Nrf2/ARE is one of the important antioxidant signaling pathways, such as TBHQ, xanthohumol, quercetin, etc[12-13]. Despite these two types of antioxidants have been reported in many studies, in addition to edaravone and other very few antioxidants, many antioxidants failed to put into clinical to treatment of cerebral ischemia-reperfusion injury. Hence in this sense, the development of novel antioxidant agents with ROS scavenging activity will have the big rise space and be of importance in treating cerebral ischemia-reperfusion injury.

Natural products and their synthetic analogues have been shown to be invaluable resources in drug discovery due to various great bioactivities and less side effects[14,15]. For example, Nordihydroguaiaretic Acid (NDGA) or 4,4'-(2,3-Dimethylbutane-1,4-diyl)dibenzene-1,2-diol is naturally occurring antioxidant lignan isolated from creosote bush, *Larrea tridentate,* which has shown to have promising applications in the treatment of multiple diseases[16-21], like cardiovascular, immune and neurological systems, cancer, etc. Besides, many studies have indicated that NDGA has important antioxidant properties,

such as becoming a ROS scavenger and an activators of cellular intrinsic keap1/Nrf2/ARE antioxidant pathway[22,23], which showed it could be a promising antioxidants for drug discovery. Curcumin, is polyphenol yellow pigment in dietary spice turmeric from *Curcuma longa*, contains α,β-unsaturated ketone moiety(Michael acceptors) that is able to modify cysteine thiols present in Keap1, which could active Nrf2 signaling pathway, and further sensitized the transcription of phase II genes and these genes encode enzymes (HO-1, SODs, GCLC, etc.) to remove ROS indirectly[24,25]. Accordingly, we hypothesize that hybrids of NDGA and α,β-unsaturated ketone scaffold may have potent antioxidant activity against cerebral ischemia-reperfusion injury. Hence, in this study, a series of hybrids of NDGA and α,β-unsaturated ketone scaffold were designed and synthesized, and investigated the underlying antioxidative mechanism in a neuronlike rat pheochromocytoma cell line, PC12. In addition, **3a** was found to attenuate brain injury after transient middle cerebral artery occlusion (MCAO) in rats.

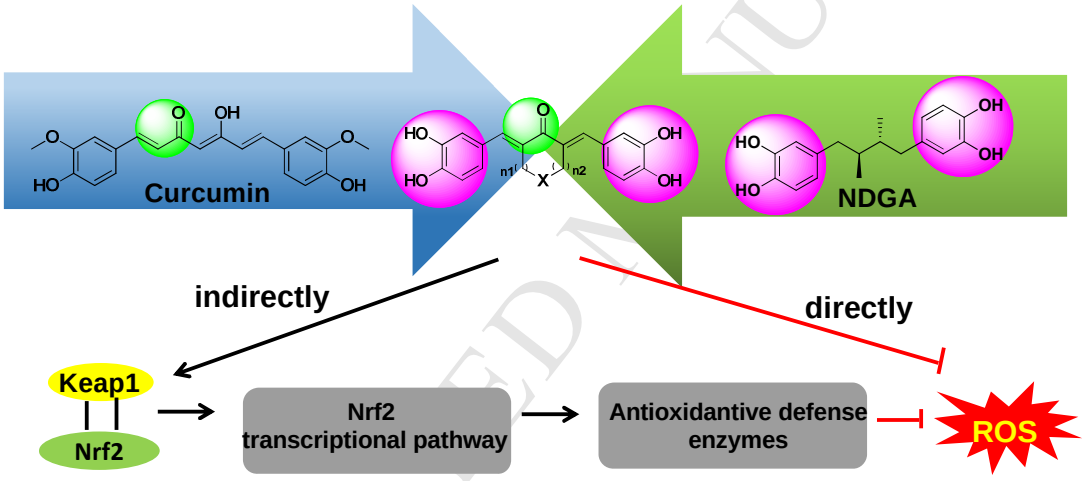


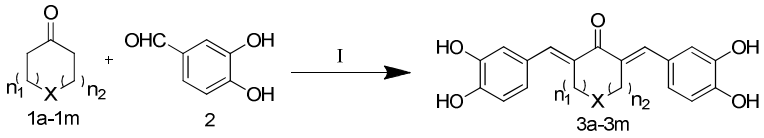
Figure 1. Design of NDGA derivatives with attenuation oxidative stress by directly scavenging ROS and indirectly through keap1/Nrf2/ARE pathway activation.

2. Results and discussion

2.1. Chemistry

The synthetic pathway adopted to synthesize the NDGA derivatives were as described as in Figure 2. All compounds(**3a-3m**) were obtained in reaction of appropriate ketone(**1a-1m**) with 3,4-dihydroxybenzaldehyde(**2**) at room temperature under HCl gas. All compounds were purified by column chromatography and were obtained in medium yields (30%-70%) after purification. The products were characterized by analysis and comparison of their spectral data including high performance liquid chromatography-mass spectrometry (HPLC-MS) and ^{1}H-NMR. These characteristic data including color, yield,

melting points, HPLC-MS and ^{1}H-NMR spectrum of compounds were presented in chemistry synthetic section.



Comp.	X	n_1	n_2	Comp.	X	n_1	n_2
3a	CH_2	1	0	**3h**	S	1	1
3b	CH_2	1	1	**3i**	NCH_2CH_2Ph	1	1
3c	/ [a]	0	0	**3j**	$NCOOCH_2Ph$	1	1
3d	$NCH_2CH_2CH_3$	1	1	**3k**	$NCOOCH_3$	1	1
3e	NCH_3	1	1	**3l**	NCH_2Ph	1	1
3f	NH	1	1	**3m**	$NCHCH_2CH_2$	1	1
3g	O	1	1				

Figure 2. Synthesis and structures of NDGA derivatives. Synthetic conditions: (I) HCl gas, EtOH, room temperature. [a] "/" represents none.

2.1 Scavenging free radicals *in vitro*

Due to NDGA and its derivatives belong to phenolic compounds, and thus we first investigated their antioxidant activity *in vitro*[26]. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay is considered as a valid and easy assay to evaluate the radical-scavenging activity of antioxidants, because DPPH radicals are more stable and easier to handle than oxygen free radicals[27,28]. In this experiment, the DPPH radical scavenging activities of NDGA derivatives **3a-3m** were determined. As shown in Figure 3, all analogues were found to effectively scavenge DPPH radical via chemical ways, and showed the radical scavenging activity similar to NDGA. Meanwhile, we found that **A1,** the methylated derivative of **3a,** can not scavenge free radicals directly (Figure S1 in the supporting information). In all, all NDGA derivatives can act as direct radical scavengers.

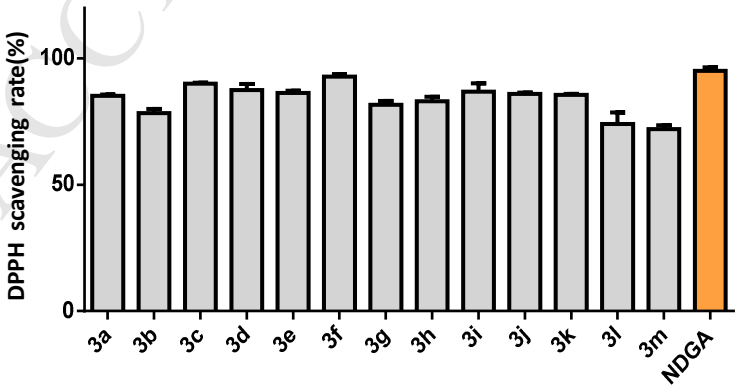


Figure 3. DPPH radical scavenging rate of NDGA derivatives (20 mg/L). Data are expressed as the mean ± SD, n = 3.

2.2 Protection of NDGA analogues from H_2O_2-induced PC12 cells damage

Hydrogen peroxide(H_2O_2), an endogenous cellular signaling molecule, has been widely used as an inducer of oxidative stress in many cellular models[29]. And treatment with compounds for 24 h followed by H_2O_2 insult generally as an experimental method for studying antioxidant activity was used in this test[30-33]. Additionally, we chose H_2O_2-induced PC12 cell damage model to evaluate the cytoprotection of NDGA analogues. As shown in Figure 4A, pre-incubation with these compounds for 24 h, only **3a**, **3f** and **3g** presented good protection of PC12 cells. Notably, compounds pre-incubation for 1 h, all analogues and NDGA showed valuable protection against oxidative damage in PC12 cells induced by H_2O_2 insult (Figure 4B), while **A1** exhibited no protection (Figure S2 in the supporting information). The reason why these compounds pre-incubation for 1 h has potent protection may be that they bear four phenolic hydroxyl groups, which make them remove reactive oxygen species directly. In addition, only a few analogues pre-incubation for 24 h showed protection against H_2O_2 insult, it may be due to their effect of activating antioxidant signal pathway. Moreover, **3a**, **3f** and **3g** were found to have better advantageous cytoprotection at both time points. Furthermore, the cytotoxicity of the three compounds toward the PC12 cells at 5 μM after incubation time were determined by the MTT assay. As shown in figure 4C, when the PC12 cells were treated with drugs for 24 h, there is not apparent toxicity of the tested compounds; while treatment with for 72 h, three compounds and NDGA exhibited a degree of toxicity (Figure 4D). Based on the above results, **3a** exhibited lower cytotoxicity and better protection among all compounds.

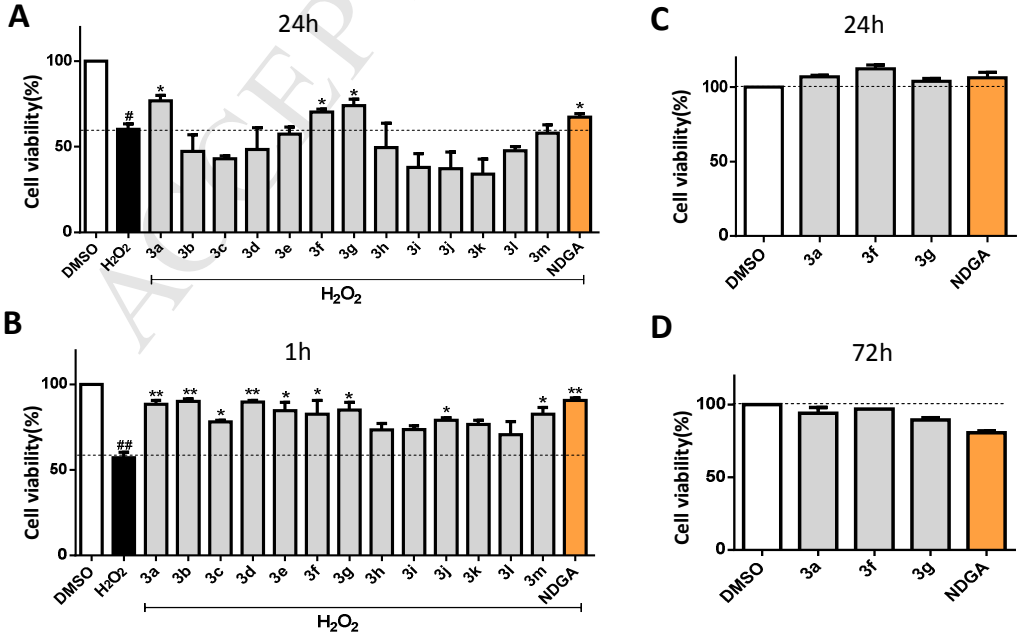


Figure 4. Compounds'cytoprotection on PC12 cells in H_2O_2 damage model(A,B) and cytotoxicity screening of three active compounds in PC12 cells (C,D). PC12 cells were pretreated for 24 h (A) or 1h (B) with NDGA and its derivatives **3a-3m** (5 μM), then another 24 h exposure in H_2O_2 (400 μM), finally determined by the MTT assay. The viability of untreated cells is defined as 100%. The cells were treated with the 5 μM of NDGA analogues (**3a, 3f, 3g**) and NDGA for 24 h (C) or 72 h (D), and the cytotoxicity of compounds was determined by the MTT assay. Data are expressed as the mean ± SD, n = 3. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs DMSO, $^{**}P < 0.01$, $^{*}P < 0.05$ vs H_2O_2. one way ANOVA, followed by Tukey's multiple comparison test.

2.4 Protection of PC12 cells from H_2O_2-induced damage by **3a**.

As compound **3a** exhibited the better protection**,** this compound was selected for the further study. As shown in Figure 5A, B, PC12 cells treated with H_2O_2 only showed about 40% cell death compared with the control group. However, when the cells were treated with **3a** for 1 h or 24 h followed by H_2O_2 insult, the population of viable cells increased in a dose-dependent manner. Malondialdehyde(MDA), a byproduct of polyunsaturated fatty acid peroxidation caused by ROS, is regarded as a significant biomarker of oxidative stress[34,35]. As shown in Figure 5C and D, pretreatment of PC12 cells with **3a** for 1 h or 24 h significantly reduced the MDA in a dose-dependent manner. Stimulation of the cells with H_2O_2 could produced a burst of ROS in the cells, so we then examined whether 3a could prevent ROS accumulation in PC12 cells. As illustrated in Figure 5E, F, it is found that pretreatment of the cells with **3a** for 1 h or 24 h remarkably reduced the ROS accumulation. Moreover, prevention of ROS accumulation in PC12 cells by compound **3a** might account for their cytoprotection against oxidative injury. Taken together, compound **3a** can significantly protect PC12 cells from H_2O_2-induced cell injury.

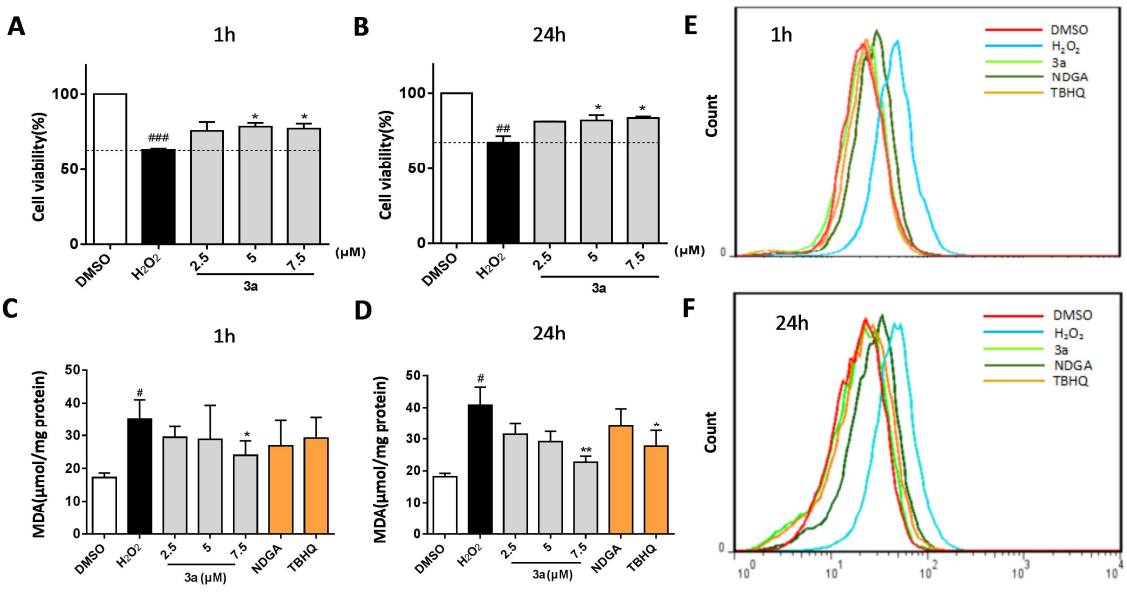


Figure 5. Protection of compound **3a** by scavenging ROS and reducing MDA against H_2O_2-induced PC12 cell damage. (A,B) **3a** protected PC12 cells from H_2O_2-induced cell injury. PC12 cells were pretreated with **3a** in different doses (2.5, 5, 7.5 μM) for 1 h or 24 h, then treated with 400 μM H_2O_2 for 24 h, and determined by the MTT assay. (C,D) **3a** reduced the MDA induced by H_2O_2 in PC12 cells. PC12 cells were pre-incubated with **3a** at 2.5, 5, 7.5 μM, NDGA (5 μM) or TBHQ (5 μM) for 1 h or 24 h, then treated with 700 μM H_2O_2 for 16 h, finally determined by the manufacturer's instructions. (E,F) **3a** decreased ROS level in H_2O_2 treated PC12 cells. PC12 cells were pre-treated with 5 μM of **3a**, NDGA or TBHQ, then treated with H_2O_2 for 3 h, and the ROS level was measured by flow cytometry. Data are expressed as the mean ± SD, n = 3. $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.005$ vs DMSO, $^{**}P < 0.01$, $^{*}P < 0.05$ vs H_2O_2, one way ANOVA, followed by Tukey's multiple comparison test.

2.5 **3a** activated the Nrf2 signaling pathway

Promoting Nrf2 to translocate into nucleus by antioxidants has been considered as the mechanism of preventing PC12 cells from H_2O_2-induced damage[36,37]. Therefore immunofluorescence assay is used to examined whether **3a** could promote Nrf2 to accumulate in nuclei. As shown in Figure 6A, the blue staining and the red staining represent nuclei and Nrf2, respectively. Compared with the blank control group, **3a**-treated group showed strong red fluorescent light in the nucleus, and it revealed that Nrf2 entered and concentrated in the nucleus after treatment by **3a**. The similar phenomenon occurred in positive group both NDGA and TBHQ. The data supported that **3a** can induce Nrf2 translocation into the nucleus.

Next, we examined the effect of **3a** on the expression of HO-1 in PC12 cell. A dose-dependent manner could be observed for **3a** to enhance the expression of HO-1 (Figure 6B), indicating the potent Nrf2-ARE inductivity of **3a** at a cell-based level. Moreover, **3a** at 10 μM exhibited even more stronger promoting effect than that of NDGA and TBHQ. To investigate whether the expression of HO-1 caused by the **3a** is responsible for the cytoprotective effects against H_2O_2-derived oxidative cell death, its specific inhibitor, ZnPP was utilized in this study[38]. PC12 cells were treated by ZnPP (15 μM) in for 1 h before the addition of **3a**. As shown in Figure 6C, it was obvious that ZnPP had no significant adverse effect on cell viability in H_2O_2-induced model group, while **3a** increased the cell viability. However, when ZnPP and **3a** were applied together, the cytoprotection of 3a was suppressed. This result showed that **3a** executed its anti-oxidative activities through, at least partly, activating HO-1 protein expression. In all, these results showed that **3a** induced the Nrf2 translocation into the nucleus and further activated HO-1 expression to exhibit an inspirable protection against H_2O_2 induced PC12 cell injury.

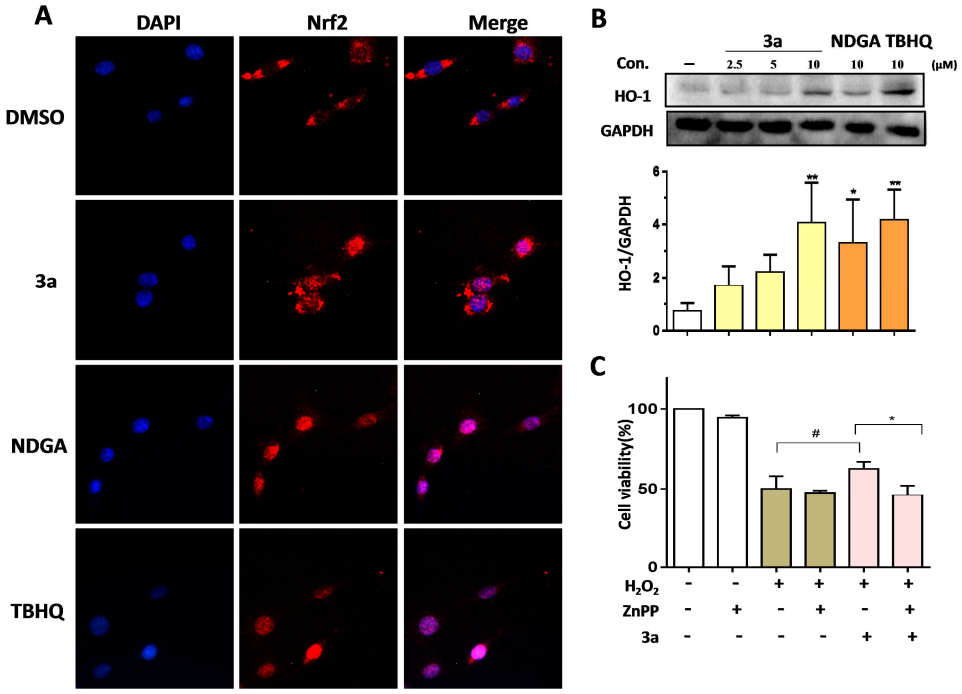


Figure 6. Activating the Nrf2 signaling pathway by **3a**. (A) The nucleus translocation of Nrf2 caused by **3a.** PC12 cells were incubated with **3a**, NDGA and TBHQ at 5 μM for 6 h, and then stained with Nrf2 antibody and DAPI. (B) Elevating the expression level of protein HO-1 by **3a**. PC12 cells were incubated with **3a** at 2.5, 5, 10 μM , NDGA or TBHQ at 10 μM for 24 h, and the HO-1 was determined by western blotting experiment. Data are expressed as the mean ± SD, n = 3. **P < 0.01, *P < 0.05 vs DMSO, one way ANOVA, followed by Tukey's multiple comparison test. (C) ZnPP diminished the protected effect of **3a** on H_2O_2 induced cell damage. PC12 cells were incubated with HO-1 inhibitor ZnPP for 1 h, then treated with **3a** at 5 μM for 24 h. Finally MTT assay measured the OD values in 490 nm. Data are expressed as the mean ± SD, n = 3. $^{\#}$P < 0.1, *P < 0.05, one way ANOVA, followed by Tukey's multiple comparison test.

2.6 **3a** attenuated brain injury after cerebral ischemia-reperfusion injury in rats subjected to transient middle cerebral artery occlusion (MCAO)

We further evaluated for the neuroprotective activity of **3a** *in vivo* in a rat model of transient focal cerebral ischemia by intraluminal occlusion of the middle cerebral artery (MCAO), which has considered to the most common reason for inducing I/R-related brain injury in the clinic[39]. The efficacy of **3a** was evaluated by preinjection into lateral ventricles. The infarct size of individual rats were evaluated by the 2,3,5-triphenyltetrazolium chloride (TTC) staining. As shown in Figure 7A and B, there were no obvious infarction damage in the sham group, while the infarct area in the model group and vehicle group of rats significantly increased, as shown in the white region of rat brain sections. However, intracerebroventricular administration of **3a** significantly reduced the infarct sizes of I/R rats. The treatment with edaravone also showed a protective effect on

infarction damage. Furthermore, the effect of **3a** was more pronounced than that of edaravone. Additionally, treatment with **3a** and edaravone markedly reduced the neuroloigic scoring in the brains from ischemic rats (Figure 7C). These results suggested the potential of **3a** for the treatment of ischemic brain damage.

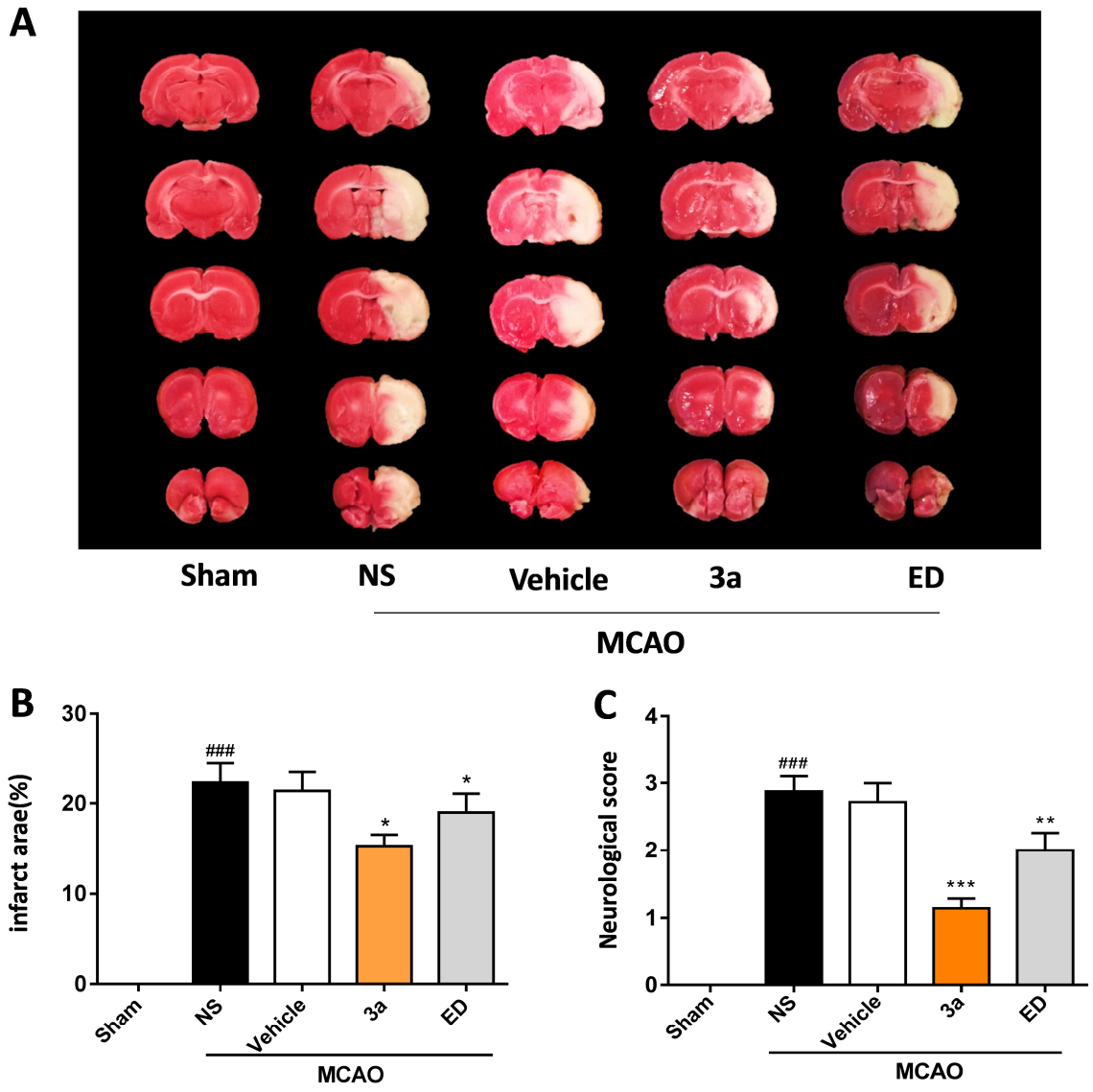


Figure 7. Effect of **3a** on middle cerebral artery occlusion(MCAO)-induced focal cerebral ischemia in rats. (A) Representative images of TTC-stained brain slices. The corresponding (B) infarct areas and (C) neuroloigical score levels in brain tissues. Cerebral infarction in sham-operated (sham) or MACO reperfusion rats from a representative animal that received normal saline (NS), vehicle, 0.2 mg/kg **3a** or ED(edaravone) by intraventricular injection. Data are expressed as the mean ± SD, $n \geq 6$. $^{###}P < 0.001$ vs sham-operated group, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs vehicle-treated group. one way ANOVA, followed by Tukey's multiple comparison test.

3. Conclusion

It is well known that generation of excessive ROS during reperfusion contribute to brain injury associated with stroke. Administration of anti-ischemic stroke agent with ROS scavenging activity at the time of reperfusion could potentially decrease the risk, which may a valid neuroprotective strategy. The case of edaravone, an effective free radical scavenger approved for the treatment of patients with acute stroke, proves the viability of this approach. Also, researches showed that many small molecules could activate Nrf2 pathway to confer protection against ROS and prevent the ischemia-reperfusion injury[40-42]. Hence, exogenous supplementation of agents with attenuation oxidative stress by directly scavenging ROS and indirectly through keap1/Nrf2/ARE pathway activation would be a potential therapy to cerebral ischemia-reperfusion injury.

In this study, a series of hybrids of NDGA and α,β-unsaturated ketone scaffold were designed and synthesized. All analogues can effectively scavenge DPPH radicals. Then their cytoprotection in H_2O_2 induced PC12 cells injury model was tested. Notably, after pre-incubation with compounds for 1 h, all analogues showed valuable cytoprotection against oxidative damage in PC12 cells; and after pre-incubation for 24 h, compounds **3a**, **3f** and **3g** also displayed excellent cytoprotection. Among them, compound **3a** was screened out to exhibit low cytotoxicity and an inspirable protection against H_2O_2 induced PC12 cell injury in a concentration-dependent manner. **3a** can remarkably remove the ROS accumulation, and reduce the MDA in a dose-dependent manner. Furthermore, **3a** can induce Nrf2 nuclear translocation and hence upregulate HO-1 protein expression, conferring protection of PC12 cells against H_2O_2 insult. Meanwhile, using a rat model of ischemic stroke, also showed that preinjection of **3a** can significantly decrease the infarct area and neuroloigical scoring in the ischemic brain.

In summary, our work presented a series of novel NDGA analogus with antioxidant activity. **3a** may confer protection for PC12 cells against oxidative insults through double mechanisms, including scavenging free radicals directly and activating the Nrf2 anti-oxidative signaling pathway at the same time, and serve as a promising compound for the treatment of ischemia-reperfusion injury in the stroke.

4. Experimental

4.1. Chemistry

All chemical reagents were obtained commercially from Sigma-Aldrich(St Louis, Missouri, USA), Aladdin (Shanghai, China) and used without further purification unless otherwise noted. Reactions were monitored by TLC using silica gel GF254, the

chromatograms were conducted on silica gel (200-300 mesh) and observed under UV light at 254 and 365 nm. Melting points (mp) are uncorrected and were measured in open capillary tubes on a Fisher-Johns melting apparatus. Mass spectra (MS) were recorded on an Agilent 1100 LC-MS (Agilent, Palo Alto, CA, USA). ^{1}H NMR spectra were obtained from 600 MHz spectrometer (Bruker Corporation, Switzerland) with TMS as an internal standard. The chemical data of compounds are presented as follows:

4.1.1

(2Z,5E)-2,5-bis(3,4-dihydroxybenzylidene)cyclopentanone (3a): Green powder, 53.6% yield, mp>300°C. (Lit.[43] mp>300°C). ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.563 (s, 2H, 3-OH×2), 9.211 (s, 2H, 4-OH×2), 7.238 (s, 2H, Ar-CH=C×2), 7.113 (d, *J*=1.8Hz, 2H, Ar-H^{2}×2), 7.006 (dd, *J*=1.8Hz, *J*=8.4Hz, 2H, Ar-H^{6}×2), 6.834 (d, *J*=8.4Hz, 2H, Ar-H^{5}×2), 3.001 (s, 4H, CH_2-O-CH_2). LC-MS m/z: 325.10$(M+H)^+$, calcd for $C_{19}H_{16}O_5$: 324.10.

4.1.2

(2Z,6E)-2,6-bis(3,4-dihydroxybenzylidene)cyclohexanone (3b): Orange powder, 45.7% yield, mp 242.7-243.8°C. (Lit.[43] 244-246°C). ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.438 (s, 2H, 3-OH×2), 9.131 (s, 2H, 4-OH×2), 7.446 (s, 2H, Ar-CH=C×2), 6.980 (d, *J*=1.8Hz, 2H, Ar-H^{2}×2), 6.873 (dd, *J*=1.8Hz, *J*=8.4Hz, 2H, Ar-H^{6}×2), 6.799 (d, *J*=8.4Hz, 2H, Ar-H^{5}×2), 2.845 (t, *J*=4.8Hz, 4H, CH_2-C-CH_2), 1.725 (t, *J*=4.8Hz, 2H, C-CH_2-C). LC-MS m/z: 339.18$(M+H)^+$, calcd for $C_{20}H_{18}O_5$: 338.12.

4.1.3

(1E,4E)-1,5-bis(3,4-dihydroxyphenyl)penta-1,4-dien-3-one (3c): Green powder, 58.9% yield, mp242.7-245.4°C(Lit.[43] 221-223°C). ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.646 (s, 2H, 3-OH×2), 9.171 (s, 2H, 4-OH×2), 7.568(d, *J*=16.2Hz, 2H, Ar-CH=C×2), 7.155 (d, *J*=1.8Hz, 2H, Ar-H^{2}×2), 7.083(dd, *J*=1.8Hz, *J*=1.8Hz, 2H, CO-CH=C×2), 7.002(d, *J*=15.6Hz, 2H, Ar-H^{5}×2), 6.800(d, *J*=8.4Hz, 2H, Ar-H^{6}×2). LC-MS m/z: 299.11$(M+H)^+$, calcd for $C_{17}H_{14}O_5$: 298.08.

4.1.4

(3E,5E)-3,5-bis(3,4-dihydroxybenzylidene)-1-propylpiperidin-4-one (3d): Yellow powder, 58.9% yield, mp 232.2-233.8°C. ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.877 (s, 2H, 3-OH×2), 9.439 (s, 2H, 4-OH×2), 7.719 (s, 2H, Ar-CH=C×2), 6.942 (s, 2H, Ar-H^{2}×2), 6.902 (d, *J*=18.6Hz, 4H, Ar-H^{5}×2, Ar-H^{6}×2), 4.576 (d, *J*=37.2Hz, 4H, CH_2-N-CH_2), 3.513-3.479 (m, 2H, N-CH_2), 1.698 (d, *J*=7.2Hz, 2H, N-C-CH_2), 0.895 (t, *J*=14.4Hz, 3H, CH_3). LC-MS m/z: 382.17$(M+H)^+$, calcd for $C_{22}H_{23}NO_5$: 381.16.

4.1.5

(3E,5E)-3,5-bis(3,4-dihydroxybenzylidene)-1-methylpiperidin-4-one (3e): Drown powder, 51.3% yield, mp>300°C. (Lit.[44] mp>300°C). ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.886 (s, 2H, 3-OH×2), 9.403 (s, 2H, 4-OH×2), 7.708 (s, 2H, Ar-CH=C×2), 6.965 (d, *J*=1.2Hz, 2H, Ar-H^{2}×2), 6.900-6.879(m, 4H, Ar-H^{5}×2, Ar-H^{6}×2), 4.609(d, *J*=44.4Hz, 4H,

CH_2-N-CH_2), 3.009 (s, 3H, CH_3). LC-MS m/z: 354.17$(M+H)^+$, calcd for $C_{20}H_{19}NO_5$: 353.13.

4.1.6

(3E,5E)-3,5-bis(3,4-dihydroxybenzylidene)piperidin-4-one (3f): Yellow-green powder, 49.7% yield, mp>300°C. (Lit.[43] mp>300°C). ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.832 (s, 2H, 3-OH×2), 9.687 (d, *J*=19.8Hz, 1H, NH), 9.425 (s, 2H, 4-OH×2), 7.690 (s, 2H, Ar-CH=C×2), 6.937 (s, 2H, Ar-H^2×2), 6.901 (d, *J*=8.4Hz, 2H, Ar-H^6×2), 6.872 (d, *J*=8.4Hz, 2H, Ar-H^5×2), 4.428 (s, 4H, CH_2×2). LC-MS m/z: 340.04$(M+H)^+$, calcd for $C_{19}H_{17}NO_5$: 339.11.

4.1.7

(3E,5E)-3,5-bis(3,4-dihydroxybenzylidene)dihydro-2H-pyran-4(3H)-one (3g): Green powder, 50.8% yield, mp 262.4-264.7°C. (Lit.[43] mp>300°C). ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.563 (s, 2H, 3-OH×2), 9.241 (s, 2H, 4-OH×2), 7.461 (s, 2H, Ar-CH=C×2), 6.807 (d, *J*=7.8Hz, 2H, Ar-H^6×2), 6.805 (s, 2H, Ar-H^2×2), 6.756 (d, *J*=8.4Hz, 2H, Ar-H^5×2), 4.836 (s, 4H, CH_2-O-CH_2). LC-MS m/z: 341.10$(M+H)^+$, calcd for $C_{19}H_{16}O_6$: 340.19.

4.1.8

(3Z,5Z)-3,5-bis(3,4-dihydroxybenzylidene)dihydro-2H-thiopyran-4(3H)-one (3h): Drown powder, 43.9% yield, mp 223.3-224.6°C. (Lit.[43] 223-225°C). ^{1}H-NMR (600MHz, *d*-DMSO), δ: 7.436 (s, 2H, Ar-CH=C×2), 6.941 (d, *J*=1.8Hz, 2H, Ar-H^2×2), 6.857 (dd, *J*=8.4Hz, *J*=1.8Hz, 2H, Ar-H^6×2), 6.808 (d, *J*=7.8Hz, 2H, Ar-H^5×2), 3.946 (s, 4H, CH_2-S-CH_2). LC-MS m/z: 356.98$(M+H)^+$, calcd for $C_{19}H_{16}O_5S$: 356.07.

4.1.9

(3E,5E)-3,5-bis(3,4-dihydroxybenzylidene)-1-phenethylpiperidin-4-one (3i): Green powder, 58.7% yield, mp150.8-152.8°C. ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.886 (s, 2H, 3-OH×2), 9.462 (s, 2H, 4-OH×2), 7.719 (s, 2H, Ar-CH=C×2), 7.327 (t, *J*=4.8Hz, 4H, Ar-$H^{6'}$, Ar-$H^{2'}$, Ar-$H^{3'}$, Ar-$H^{5'}$), 7.262-7.239 (m, 1H, Ar-$H^{4'}$), 7.021 (s, 2H, Ar-H^2×2), 6.904-6.871 (m, 4H, Ar-H^5×2, Ar-H^6×2), 4.657 (d, *J*=4.8Hz, 4H, CH_2-N-CH_2), 3.587 (s, 2H, CH_2×2), 3.099 (t, *J*=16.2Hz, 2H, CH_2). LC-MS m/z: 444.17$(M+H)^+$, calcd for $C_{27}H_{25}NO_5$: 443.17.

4.1.10

(3E,5E)-benzyl 3,5-bis(3,4-dihydroxybenzylidene)-4-oxopiperidine-1-carboxylate (3j): Green powder, 50.8% yield, mp250.4-251.5°C. ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.646 (s, 4H, 3-OH×2), 9.285 (s, 2H, 4-OH×2), 7.541 (s, 2H, Ar-CH=C×2), 7.242 (d, *J*=1.8Hz, 2H, Ar-$H^{2'}$, Ar-$H^{6'}$), 7.231 (t, *J*=5.4Hz, 1H, Ar-$H^{4'}$), 7.079 (t, *J*=7.2Hz, 2H, Ar-$H^{3'}$, Ar-$H^{5'}$), 6.974 (s, 2H, Ar-H^2×2), 6.857 (d, *J*=7.8Hz, 4H, Ar-H^5×2, Ar-H^6×2), 5.017 (s, 2H, CH_2), 4.795(d, *J*=30.6Hz, 4H, CH_2-N-CH_2). LC-MS m/z: 474.25$(M+H)^+$, calcd for $C_{27}H_{23}NO_7$: 473.15.

4.1.11

(3E,5E)-ethyl3,5-bis(3,4-dihydroxybenzylidene)-4-oxopiperidine-1-carboxylate(3k): Orange powder, 43.9% yield, mp280.5-283.6°C. ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.640 (s, 2H, 3-OH×2), 9.276 (s, 2H, 4-OH×2), 7.510 (s, 2H, Ar-CH=C×2), 6.939 (s, 2H, Ar-H^{2}×2), 6.881-6.843 (m, 4H, Ar-H^{5}×2, Ar-H^{6}×2), 4.734 (s, 4H, CH_2-N-CH_2), 4.002-3.966 (m, 2H, CH_2), 1.050 (t, *J*=13.8Hz, 3H, CH_3). LC-MS m/z: 412.06$(M+H)^+$, calcd for $C_{22}H_{21}NO_7$: 411.13.

4.1.12

(3E,5E)-1-benzyl-3,5-bis(3,4-dihydroxybenzylidene)piperidin-4-one (3l): Yellow-green powder, 50.8% yield, mp158.4-160.2°C. ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.868 (s, 2H, 3-OH×2), 9.408 (s, 2H, 4-OH×2), 7.737 (s, 2H, Ar-CH=C×2), 7.554 (d, *J*=6.6Hz, 2H, Ar-H$^{2'}$, Ar-H$^{6'}$), 7.386-7.344 (m, 3H, Ar-H$^{3'}$, Ar-H$^{4'}$, Ar-H$^{5'}$), 6.9005 (d, *J*=2.4Hz, 2H, Ar-H^{2}×2), 6.868 (d, *J*=8.4Hz, 2H, Ar-H^{6}×2), 6.816 (dd, *J*=1.8Hz, *J*=1.8Hz, 2H, Ar-H^{5}×2), 4.512 (s, 6H, CH_2×3). LC-MS m/z: 430.17$(M+H)^+$, calcd for $C_{26}H_{23}NO_5$: 429.16.

4.1.13

(3E,5E)-1-cyclopropyl-3,5-bis(3,4-dihydroxybenzylidene)piperidin-4-one (3m): Orange powder, 50.8% yield, mp 247.0-249.3°C. ^{1}H-NMR (600MHz, *d*-DMSO), δ: 9.884 (s, 2H, 3-OH×2), 9.456 (s, 2H, 4-OH×2), 7.716 (s, 2H, Ar-CH=C×2), 6.967 (s, 2H, Ar-H$^{2'}$×2), 6.921-6.891 (m, 4H, Ar-H$^{5'}$×2, Ar-H$^{6'}$×2), 4.605 (s, 4H, CH_2-N-CH_2), 1.190 (t, *J*=15.6Hz, 1H, CH), 0.483 (d, *J*=3.0Hz, 4H, CH_2×2). LC-MS m/z: 380.16$(M+H)^+$, calcd for $C_{22}H_{21}NO_5$: 379.14.

4.2. General procedure for synthesis of NDGA analogues **3a-3m**

A mixture of 3,4-dihydroxybenzaldehyde (**2**, 10 mmol) and appropriate ketone (5 mmol **1a**: cyclopentanone, **1b**: cyclohexanone, **1c**: acetone, **1d**: 1-propylpiperidin-4-one, **1e**: 1-methylpiperidin-4-one, **1g**: dihydro-2H-pyran-4(3H)-one, **1h**: dihydro -2H-thiopyran-4(3H)-one, **1i**: 1-phenethylpiperidin-4-one, **1j**: benzyl 4-oxopiperidine -1-carboxylate, **1k**: ethyl 4-oxopiperidine-1-carboxylate, **1l**: 1-benzylpiperidin-4-one, **1m**: 1-cyclopropylpiperidin-4-one)was stirred at room temperature. HCl (gas) was bubbled into the solution to catalyze the reaction. Completion of the reaction was monitored by thin layer chromatography. 4-Piperidone hydrochloride hydrate (**1f**) and 3,4-dihydroxybenzaldehyde were dissolved in the mixture solvent of ethanol and water (10:1) different with others. The crude mixture was cooled and poured into cold water (20 mL) to precipitate the product. The filter residue was purified by silica gel chromatography using hexane and ethyl acetate gradient to obtain desired products **3a-3m**.

4.3 Biological evaluation

4.3.1. DPPH radical scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of NDGA analogues was measured as described by Ikram Dib et al[45] with slight modifications. The diluted NDGA analogues solution (20 mg/mL) were prepared in ethanol. 0.15 mM DPPH• solution was prepared in ethanol. A volume of 120 μL of DPPH• solution (0.15 mM) was added to 80 μL NDGA analogues solution(Ai). The control was 80 μL NDGA analogues solutions diluted in 120 μL ethanol solution(Aj). A blank preparation was obtained by adding 120 μL of DPPH• solution to 80 μL of ethanol solution(Ac). These solution mixtures were incubated for 30 min at 25 °C, then after, absorbance was measured at 517 nm. Measurements were carried out in triplicate for each experiment. Antioxidant activity was calculated using the equation: % scavenging=[1-(Ai -Aj)/Ac] ×100.

4.3.2. Cell Culture

PC12 cells, a rat pheochromocytoma cell line, were provided by the Cell Storage Center of Wuhan University (Wuhan, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal bovine serum(FBS), (containing 100 U/mL penicillin and 100 U/ mL streptomycin), and incubated at 37 °C with 5 % CO_2.

4.3.3. MTT assay

MTT assay was utilized to measure PC12 cells cytotoxicity and viability. PC12 cells (5×10^3 cells/well) were plated in 96-well plates and allowed to attach overnight. After appropriate treatment, The cells were treated with MTT solution (5 mg/mL) for 4 h at 37 °C. The formazan crystals were dissolved in 120 μL DMSO and the optical density (OD) was measured using a Microplate Reader at 490 nm. The cell viability was given in a percentage of the OD value of the control cultures. *In vitro* cell cytotoxicity assay, the cell viability was determined after treated with NDGA analogues and NDGA for 24 h or 72 h. *In vitro* antioxidant activities, the cells were pre-treated with NDGA analouges and NDGA for 1 h or 24 h, H_2O_2 was added for an additional 24 h.

4.3.4. Intracellular ROS analysis

PC12 cells were seeded in 6-well plates at a density of 3×10^5/mL and allowed to attach overnight. After 1 h or 24 h of pre-incubation with compounds, H_2O_2 was added for 3 h. Then, cells were incubated with DCFH-DA (10 mM) at 37 °C for 30 min. Then cells were digested with enzyme and rinsed with PBS, and re-suspended in 500 mL PBS. After filtration, the suspension was analyzed by Flow Cytometry .

4.3.5. Measurement of MDA

PC12 cells were incubated with 3a and NDGA for 1 h or 24 h. Then another 16 h exposure in H_2O_2. The supernatants were collected through centrifuge at 1600×g for 10 min at 4 °C. The supernatants were removed to measure the levels of MDA according to the manufacturer's instructions.

4.3.6. Immunostaining of Nrf2

Cells were cultured in a 6-well plates with clean-glass cover slips. After treatment, the cells were fixed by 4% paraformaldehyde for 20 min at 4 °C. The cells were then washed three times with PBS and incubated in 1% Triton X-100 for 15 min and 1% BSA for 1 h at room temperature. Cells were then incubated overnight at 4 °C on addition of anti-Nrf2 primary antibody solution (1:300 in 3% BSA). After rewashed in PBS, the cells were allowed to react with PE-labeled secondary antibody (1:300 in 3% BSA) for 1h in a dark room and counterstained 4,6-diamidino-2-phenylindole dihydrochloride(DAPI) for 5 min. Images were captured under the fluorescence microscope.

4.3.7. Western Blot Analysis

PC12 cells treated with 3a, NDGA and TBHQ for 24 h and lysed with ice-cold RIPA lysis buffer. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamidegel (SDS-PAGE) and then transferred onto a Polyvinylidene Fluoride (PVDF) membrane blocking with 5% skim milk. The blots were incubated with antibodies against HO-1 (1:300), GADPH (1:1000) overnight at 4 °C and were washed three times with 1×TBST. Then, the blots were incubated for 1 h at room temperature with a 1:1000 dilution of horseradish peroxidase-labeled anti-rabbit IgG and washed three times with 1×TBST. Target proteins were detected by Image J software.

4.3.8. Focal Cerebral Ischemia

Animals:Male Sprague-Dawley rats (250-280g) were obtained from the Shanghai Slaccas Lab Animal Co., Ltd. The 35 adult rats were randomly divided into five groups (n = 7): (A) sham control; (B) normal saline (NS) group; (C) vehicle (polymer:DMSO:distilled water at 50mg:1mL:3mL)+MCAO; (D) 0.2 mg/kg 3a+MCAO; (E) 0.2 mg/kg edaravone +MCAO. Rats were administered by intracerebroventricular injection with the isovolumetric normal saline, vehicle, 3a nanoparticles and edaravone nanoparticles 2 h before MCAO. All animal experiments and care were performed according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996).

Preparation of 3a/edaravone-nanoparticles: the 3a/edaravone-loaded nanoparticles were prepared as described previously[46]. The concentration of the 3a(3a nanoparticles) and edaravone (edaravone nanoparticles) were 0.2 mg/kg.

MCAO:Male Sprague-Dawley rats (250-280g) were used male as the object of middlecere bral artery occlusion (MCAO)method. Firstly, animals were anesthetized with 10% chloral hydrate (0.35mL/100kg; intraperitoneally). After disinfection with 75% alcohol, making a appropriate incision on the neck. Then, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed and separated. ICA and CCA were clamped with artery clips temporarily. Cut an incision on ECA, insert thread, until we feel resistance to stop (about 1.8 cm). The thread fixed and the incision stitched. After 2 h of MCAO, the rats achieved reperfusion for 72 h.

4.3.9. Neurological deficit score and TTC staining.

Neurological score was determined 72 h after MCAO using Longa's method[47]. Briefly, the test as follows: 0: no deficit; 1: forelimb weakness and torso turning to the ipsilateral side when held by the tail; 2: circling to the affected side when held by the tail on the bench; 3: unable to bear weight on the affected side or spontaneous circling to the affected side; 4: no spontaneous locomotor activity or barrel rolling. For TTC staining, the brains were quickly removed from rats and was cut into slices. Five the slices were bathed in the TTC solution at 37 °C for 30 minutes. The TTC-stained brain slices were photographed with a digital camera. The infarct area was calculated by Image-Pro plus.

4.3.10. Statistical analysis

Data were expressed as mean ± standard deviation of three independent experiments. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences were established at $P < 0.05$.

Acknowledgement

Thank you for the nano-dosage form of the compound produced by Professor Yingzheng Zhao of wenzhou medical university. This work was supported by the National Natural Science Foundation of China (Grant Nos. 81402839 and 81272462), the ZheJiang Province Natural Science Fund of China (Grant Nos. LY17H160059). The Opening Project of Zhejiang Provincial Top Key Discipline of Pharmaceutical Sciences.

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Highlights:

1. A series of NDGA derivatives containing α,β-unsaturated ketone scaffold were designed and synthesized base combination principles.
2. Compound **3a** could confer protection of PC12 cells against H_2O_2 insult by directly scavenging ROS and indirectly through keap1/Nrf2/ARE pathway activation.
3. The effect of **3a** was more pronounced than that of edaravone after MCAO in rats.

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