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Design and Evaluation of Nrf2 Activators with 1,3,4-Oxa/thiadiazole Core as Neuro-protective Agents against Oxidative Stress in PC-12 Cells

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Abstract

Oxidative stress plays vital roles in virous neurodegenerative diseases including Alzheimer's disease. Activation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2), the key regulator of oxidative stress, may provide a new therapeutic strategy for these diseases. Herein we synthesized and evaluated a series of 1,3,4-oxa/thiadiazole core Nrf2 activators as neuroprotective agents. The representative compound **8** exhibited cytoprotective and Nrf2 activation effects in a neuron-like PC-12 cells. Additionally, compound **8** showed good membrane permeability, indicating this compound could penetrate blood-brain barrier (BBB) to reach central nervous system (CNS) as a neuro-protective agent. These results indicated that these Nrf2 activators with 1,3,4-oxa/thiadiazole core could serve as a new chemotype against oxidative stress in neurodegenerative diseases.

Key word: Nrf2 activator; neuro-protective agent; oxidative stress.

Alzheimer's disease (AD) is one of the severest health problems around the world[1]. This neurodegenerative disease is the most common cause of dementia. With the increase of the population of old people, the number of AD patients will escalate rapidly in the coming years.[2, 3]. Only five drugs have been proved by Food and Drug Administration (FDA) for the treatment of AD, including rivastigmine, galantamine, donepezil, memantine, and tacrine (tacrine is now discontinued in the United States). But the effectiveness of these drugs is temporary and varies from person to person. Considering the huge social burden caused by AD, new drugs are urgently needed to treat AD. So far, the etiology of AD has not been well understood. A number of theories of AD pathogenesis are proposed, including cholinergic dysfunction[4], amyloid cascade[5], hyperphosphorylation of tau-protein[6], cell cycle hypothesis[7], brain-derived neurotrophic factor hypothesis[8], and oxidative stress related free radical formation[9], metal dyshomeostasis[10], and mitochondrial dysfunction hypotheses[11]. However, none of the pharmacologic treatments alleviates or prevents the progression of AD.

The relationship between AD and oxidative stress is still obscure. It is difficult to assess that oxidative stress is a cause, a consequence, or just an independent biomarker of AD[12]. Nevertheless, increasing number of studies demonstrate that oxidative stress plays vital roles in the progression of AD. In the brain tissues of AD patients, high level of reactive oxygen species (ROS) directly leads to a range of cellular damages. Nuclear factor erythroid 2-related factor 2 (Nrf2) is the key regulator of cellular antioxidative process. Under normal condition, the newly synthetic Nrf2 proteins are quickly ubiquitylated by Kelch-like ECH-associated protein 1 (Keap1) and then are degenerated through ubiquitin-proteasome pathway. Under stress condition, Nrf2 translocates into nucleus and binds with the antioxidant response element (ARE) to induce the expression of its downstream genes[13], leading to the activation of more than 100 antioxidative proteins, including antioxidant proteins, phase I and II detoxification enzymes, transport proteins, and some transcription factors[14, 15]. Thus, activating Nrf2-ARE pathway could systemically alleviate oxidative stress and Nrf2 is regarded as an attractive therapeutic target for neurodegenerative diseases[16].

Small molecular Nrf2 activators are generally classified into two distinct groups[17-19]: 1, electrophilic activators that can covalently modify Keap1[20-23]; 2, activators interfere Keap1-

Nrf2 protein-protein interaction (PPI)[24, 25]. So far, most of the preclinical and clinical studies of Nrf2 activator are focus on cancer chemoprevention and inflammatory diseases in lung or kidney. However, the use of Nrf2 activators in central nervous system is limited by the potential long-term toxicity or poor pharmacokinetics properties[18, 26].

Previously, we identified a series of compounds with 1,2,4-oxadiazole core as Nrf2 activators[27, 28]. The most potent activator, compound **1** (figure 1), upregulated gene and protein level of Nrf2 as well as its downstream genes, including NQO1, GCLM, and HO-1, *in vivo*. Moreover, it remarkably suppressed inflammatory symptom in the LPS-challenged mouse model and the DSS-induced murine colitis[27, 29]. However, the low aqueous solubility of compound **1** limits its further development. In this study, on the basis of compound **1**, we synthesized and evaluated a series of compounds with 1,3,4-oxadiazole or 1,3,4-thiadiazole core. These compounds displayed similar Nrf2 inductive activities compared with the activators with 1,2,4-oxadiazole core. More importantly, replacing 1,2,4-oxadiazole moiety by 1,3,4-oxadiazole led to increased aqueous solubility and lower lipophilicity. Moreover, we explored the potential neuro-protective effects of these 1,3,4 oxa/thiadiazole core Nrf2 activators in PC-12 cells. The results showed that the most potent compound **8** can upregulate the mRNA and protein levels of Nrf2 and its downstream genes and protect PC-12 cells against the H_2O_2-induced damage.

The previous SAR studies about the ring A and C can be briefly descripted as: 1, on ring A, electron-withdrawing groups can improve the ARE-induced activity; 2, *para*-substitution on ring A is more preferable than *meta*- and *ortho*-substitutions[27]; 3, for the ring C, introduction of electron-donating groups enhances the ARE-induced activity[30]. However, the significance of the B ring, not only for the Nrf2 activation, but also for its effects on the physicochemical properties, has not been fully discussed before. Comparing with 1,2,4-oxadiazole, 1,3,4-oxadiazole isomer shows an order of magnitude lower lipophilicity (log D) and higher aqueous solubility[31]. To further improve the solubility of the 1,2,4-oxadiazole core Nrf2 activators, especially with the aim to optimize their BBB permeability, in the present study, we synthesized and evaluated a series of compounds bearing 1,3,4-oxa/thiadiazole core.

Derivatives were synthesized through two steps. Firstly, ethyl benzimidazole-5-carboxylate was treated with hydrazine hydrate and refluxed in ethanol for 48 hours to give

benzimidazole-5-carbohydrazide (**2**). Then, the target compounds **3**-**12** were cyclized from **2** with substituted benzoic acid. The ARE inductivities of compound **3**-**12** were evaluated by luciferase reporter assay in HepG2-ARE-C8 cells[27]. As shown in table 1, most of the compounds with 1,3,4-oxadiazole core exhibited ARE inductivity at all tested concentrations. Compared to compound **3**, the nitro and *n*-butyl substituted compounds (**9** and **8**) displayed stronger Nrf2 induced activities. Compounds with halogen substituents in ring A (**4**, **5**, and **6**) exhibited similar activities with compound **3**. However, under the high concentrations (40 μM and 80 μM), the ARE inductivity of the bromo substituted compound **4** went through a downward turn. It is noteworthy that similar activity-declining results have been found during the SAR study of chlorine and bromine substituted Nrf2 activators with 1,2,4-oxadiazole core [27, 30] (figure. S1). Compounds with electron-donating group (**7**, **11**, and **12**) showed reduced ARE inductivities. The results indicated that the electron-withdrawing group was more preferable for this series of Nrf2 activators. However, compound **10** with electron-withdrawing cyano group and compound **8** with electron-donating *n*-butyl group were two exceptions. Compound **10** just maintained 2~3-fold ARE inductivities under all test concentration. Similarly, the cyano-substituent 1,2,4-oxadiazole core Nrf2 activators did not exhibit good ARE inductivities, too[27]. Compound **8** exhibited a strong inductivity, with 20.05 ± 2.40-fold upregulation of the ARE level at 40 μM. Overall, the 1,3,4-oxadiazole core Nrf2 activators displayed slightly less ARE inductivities than the corresponding 1,2,4-oxadiazole core activators. However, compound **8** displayed closed ARE inductivity with compound **1** in a preliminary screening (Figure S2). Meanwhile, compounds with 1,3,4-oxadiazole core shared similar SAR features with 1,2,4-oxadiazole core Nrf2 activators. Thus, we suggested that the replacement of five-member ring B was tolerable in structural optimization of physicochemical properties of the Nrf2 activators.

In order to verify this result, we further synthesized compounds with 1,3,4-thiadiazole core (**13 - 16**). Their Nrf2 inductivities were also evaluated by luciferase reporter assay. As shown in table 1, Compound **13** (unsubstituted), **14** (p-fluorine substituent), and **16** (p-nitrogen substituent) shown slightly reduced Nrf2 inductivities comparing with 1,2,4- and 1,3,4-oxadiazole core compounds. But the *n*-butyl substituted compound **15** just exhibited very limited activity. As a cell-based assay, the luciferase report gene assay is always affected by

the status of the tested cells, especially under high induced-folds. However, in this work, we believed the large errors may be caused by two additional reasons: 1, cytotoxicity under high concentration of the compounds may affect the cell condition. The potential toxicity of nitro-substituted compounds was found in this work and previous study. The large errors of compound **9** and **16** may be caused by their potential cytotoxicity. 2, the low solubility of several compounds under high concentration may be responsible for the errors, too.

In the previous studies, the Nrf2 activators with 1,2,4-oxadiazole core ameliorated the oxidative stress both *in vitro* and *in vivo* [27-30]. However, their biological effects on neurocytes have not been elucidated. In order to explore the potentially protective effect of these compounds on central nervous system (CNS), several representative compounds were evaluated in a neuron-like cell line PC-12. Firstly, the cytotoxicity of representative compounds **8**, **9**, **14**, and **16** toward PC-12 cells were determined by MTT assay. As shown in figure 2, all the four compounds did not display significant cytotoxicity to the cells. The IC_{50} values of compounds **8** and **9** (1,3,4-oxadiazole core) were greater than 100 μM, while the IC_{50} values of compounds **14** and **16** (1,3,4-thiadiazole core) were above 500 μM. Additionally, all the four compounds did not show remarkable cytotoxicity against other neuron-like SH-SY5Y cells at 100 μM (figure S3). Next, we determined the protective effects of the four compounds against the H_2O_2-induced damage in PC-12 cells, a well-established cellular model of neurodegenerative disorders[20, 32]. PC-12 cells were pretreated with compounds for 6 hours and subsequently were treated with medium that contained 1000 μM H_2O_2 for additional 12h. As shown in figure 3, under the low concentration (5 μM), the two nitro-contain compounds, **9** and **16**, displayed good protective effects against H_2O_2-induced damage. However, under the higher concentrations (10 and 20 μM), the protective effects of the two compounds vanished. This result indicated the nitro group may lead to potential cytotoxicity under oxidative stress condition. Two other tested compounds **8** and **14**, by contract, did not display obvious cytoprotective activity in 5 μM. Nevertheless, the two compounds gave excellent performance under the high concentrations (10 and 20 μM). Therefore, compound **8** was selected for further mechanism investigation.

With the aim of confirming the Nrf2 inductivity of **8** in PC-12 cells, we further examined the concentration-dependent effect of **8** on the mRNA level of *Nrf2* and its downstream genes,

NQO1 and *HO-1*, by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. As shown in figure 4, compound **8** can dose-dependent increase the expressions of the three genes. Then we evaluated the protein levels of Nrf2, NQO1 and HO-1 in PC-12 cell after the treatment of compound **8**. As shown in figure 5, compound **8** led to moderate up-regulation of these proteins in a dose-dependent manner. Taken together, these results exhibited that compound **8** can activate the Nrf2-ARE pathway in both mRNA level and protein level in PC-12 cells. And these results were consistent with previously published results using compound **1** in HCT116 cells[27].

Membrane permeability is a significant property for CNS drugs[33, 34]. In an effort to identify the membrane permeability of the representative compound **8**, we determined its permeability (*Pe* value) via parallel artificial membrane permeability assay (PAMPA)[35]. Five known compounds were also involved as internal standards (table S1). Generally, a compound with a *Pe* value greater than 4×10^{-6} cm/s can be regarded to be able to penetrate blood-brain barrier (BBB)[35]. In the PAMPA experiment, **8** displayed good membrane permeability, with the *Pe* value of 12.35×10^{-6} cm/s, indicating this compound could reach CNS. With the aim of evaluating the effect of rearranging 1,2,4-oxadiazole to 1,3,4-oxadiazole, we determined some physicochemical properties of compound **5** which had same structural moieties of ring A and C with compound **1**. The $LogD_{7.4}$ value and intrinsic aqueous solubility of compound **5** were determined on a Gemini profiler instrument (pION) by the "goldstandard" Avdeef-Bucher potentiometric titration method[27, 30]. In comparison with **1**[27], compound **5** exhibited a lower $LogD_{7.4}$ value (2.89 vs 3.37) and an improved intrinsic solubility (102.50 μM vs 66.90 μM). We inferred that the greater dipole moment and polarity of 1,3,4-oxadiazole toward 1,2,4-oxadiazole was the reason for the lower lipophilicity and increased solubility[31].

In summary, a series of Nrf2 activators with 1,3,4-oxa/thiadiazole core were designed and synthesized, and the representative compound **8** displayed cytoprotective and Nrf2 activation effects in a neuron-like PC-12 cells. Additionally, compound **8** showed good membrane permeability, indicating this compound could reach CNS as a neuro-protective agent. Moreover, these results deepen our understanding of the SAR study on this series of Nrf2 activators. While the modification of the five-member heterocycle (ring B) of this series of compounds was not previously discussed[27, 30], this study reveals that replacing ring B by other five-member ring is

tolerable in maintaining Nrf2 inductive activity. More importantly, the 1,3,4-oxadiazole core resulted in improved solubility and lower lipophilicity. Altogether, these results provide a new chemical tool to evaluate the therapeutic effect of Nrf2 activation in neurodegenerative diseases.

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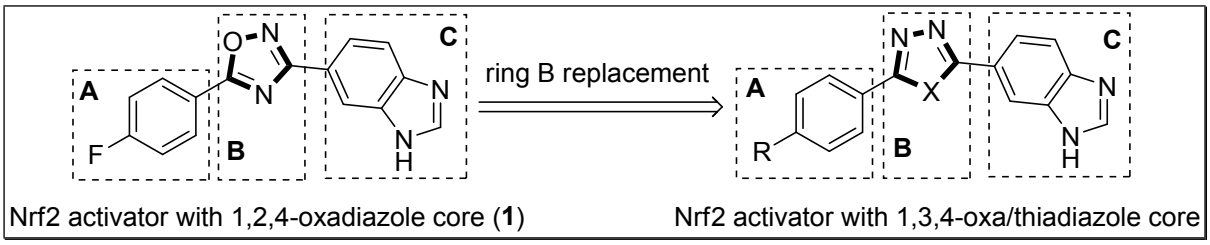


Figure 1. The molecular design strategy of the target compounds.

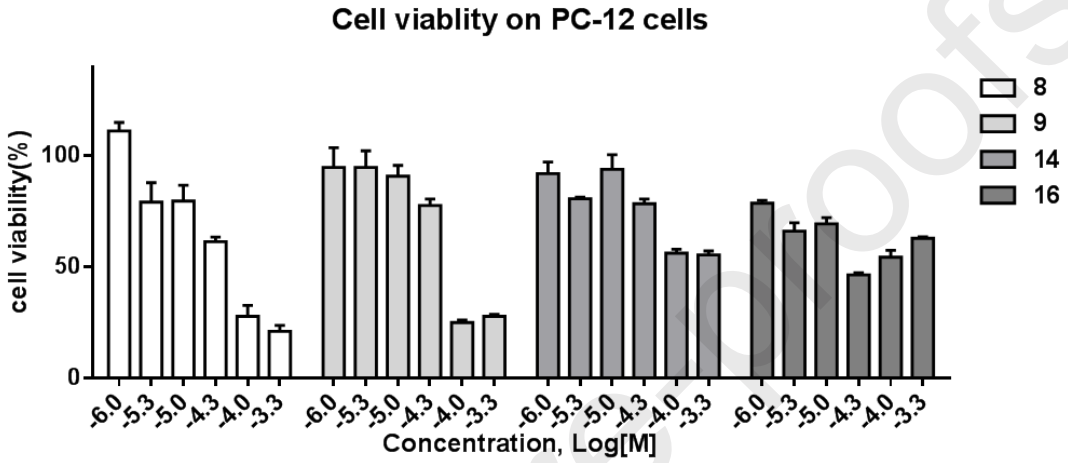


Figure 2. Cytotoxicity evaluation of representative compounds (**8**, **9**, **14**, and **16**) in PC-12 cells. The cells were plated in a 96-well plate for 12 h and subsequently treated with different concentrations (1, 5, 10, 50, 100, and 500 μM) of the selected derivatives for another 24 h. The cell viability was measured by the MTT assay. All data represent the mean ± SEM of three independent experiments.

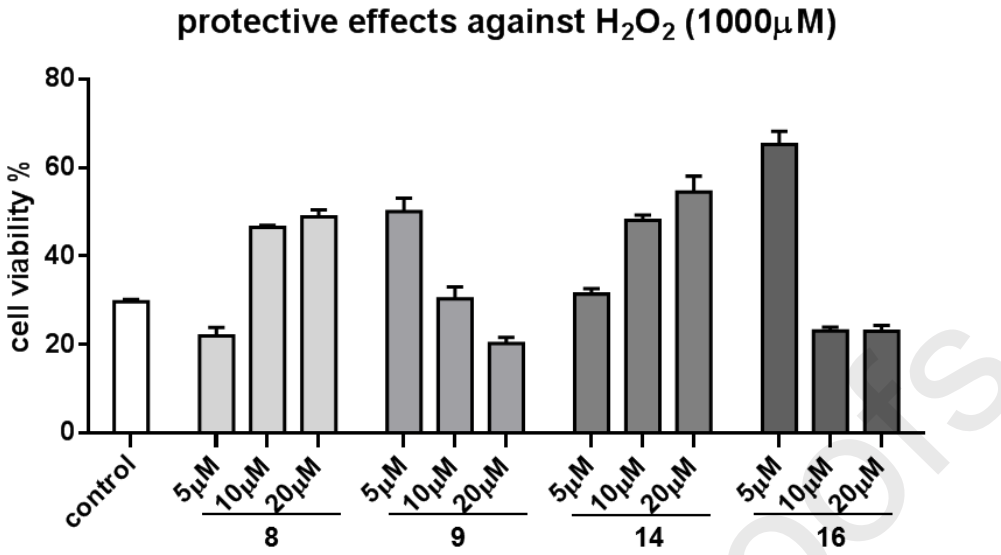
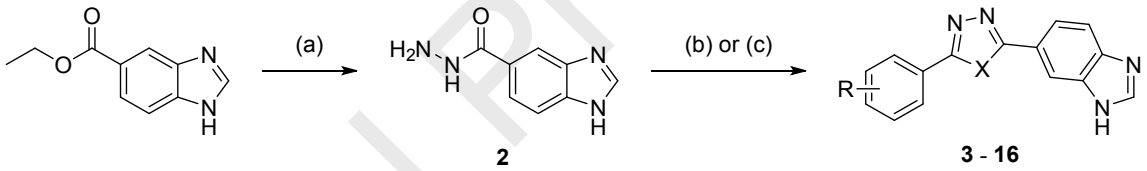


Figure 3. Protective effects of representative compounds against H_2O_2-induced damage in PC-12 cells. Cell viability was determined using the MTT assay. The viability of untreated cells is defined as 100%. All data represent the mean ± SD of three independent experiments.



Scheme 1. Synthetic Route of the 1,3,4-oxadiazole and 1,3,4-thiodiazole core derivatives. Reagents and conditions: (a) hydrazine hydrate, EtOH, reflux, 48h; (b) substituted benzoic acid, CDI, THF, $POCl_3$, reflux, 5h; (c) substituted benzoic acid, CDI, P_2S_5, DMF. 6h.

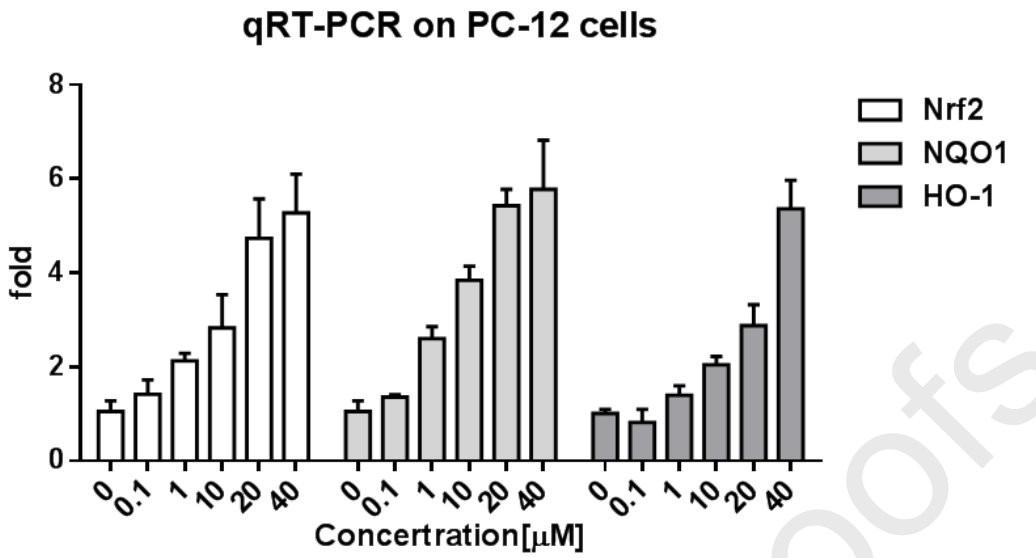


Figure 4. Quantitative real-time PCR analysis of Nrf2, NQO1, and HO-1 in PC-12 cells. All genes transcription levels were determined after treatment with different concentrations of **8** for 12 h.

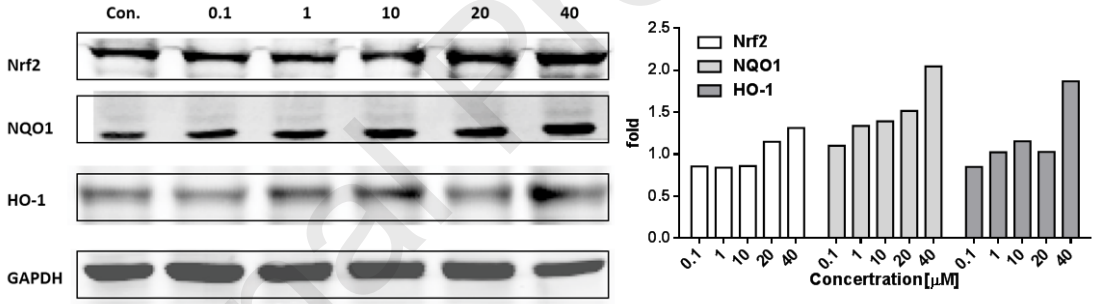


Figure 5. Dose-dependent increase of Nrf2 and its downstream protein NQO1 and HO-1 after treatment with **8.** PC-12 cells were treated with **8** at different concentrations (0.1 μM, 1 μM, 10 μM, 20 μM, 40 μM) for 12 h. GAPDH was determined as the loading control.

Table 1, Nrf2 inductivity of compound **3** to **16** in Luciferase reporter assay.

cpd.	X	R	ARE-inducing activity in luciferase reporter assay[a]					
			0.1 μM	1 μM	10 μM	20 μM	40 μM	80 μM
1	-	-	2.49 ± 0.40	10.23 ± 0.66	14.21± 0.66	27.28 ± 1.26	40.81 ± 0.22	44.40 ± 0.89
3	O	H	1.70 ± 0.40	2.35 ± 0.95	6.70 ± 0.95	9.15 ± 2.25	13.48 ± 0.67	19.10 ± 1.65
4	O	3-F	1.45 ± 0.10	2.38 ± 0.33	1.35 ± 0.15	1.65 ± 0.70	1.75 ± 0.10	1.63 ± 0.02
5	O	4-F	1.58 ± 0.03	1.28 ± 0.08	2.60 ± 0.25	6.83 ± 0.68	8.73 ± 1.53	24.40 ± 1.10
6	O	4-Br	2.05 ± 0.55	4.85 ± 0.60	7.98 ± 1.53	8.08 ± 0.03	6.23 ± 0.52	7.98 ± 0.28
7	O	4-Ph	0.90 ± 0.05	1.60 ± 0.30	1.58 ± 0.13	3.15 ± 0.30	5.18 ± 0.63	9.10 ± 1.60
8	O	4-*n*-Bu	1.80 ± 0.50	6.73 ± 0.88	14.75 ± 0.80	15.88 ± 0.07	24.58 ± 1.18	19.55 ± 0.45
9	O	4-NO_2	1.50 ± 0.45	1.90 ± 0.35	7.68 ± 0.37	12.23 ± 3.48	20.05 ± 2.40	33.83 ± 0.93
10	O	4-CN	1.40 ± 0.00	1.65 ± 0.80	1.33 ± 0.73	2.35 ± 0.55	1.78 ± 0.18	2.83 ± 1.18
11	O	4-Me	1.40 ± 0.35	1.45 ± 0.15	3.25 ± 0.80	3.35 ± 1.25	4.95 ± 1.40	6.60 ± 1.80
12	O	3-OMe	1.64 ± 0.55	1.72 ± 0.08	1.72 ± 0.53	1.83 ± 0.50	2.41 ± 1.02	7.50 ± 2.35
13	S	H	1.70 ± 0.05	2.03 ± 0.23	8.95 ± 3.15	8.18 ± 0.18	13.83 ± 4.88	15.70 ± 2.45
14	S	4-F	1.60 ± 0.40	3.25 ± 0.95	13.10 ± 2.15	14.90 ± 2.15	22.30 ± 0.95	9.90 ± 0.80
15	S	4-*n*-Bu	1.08 ± 0.18	2.33 ± 0.23	2.30 ± 0.45	1.68 ± 0.03	1.33 ± 0.03	1.43 ± 0.23

16	S	4-NO_2	2.20 ± 0.20	5.75 ± 0.40	19.45 ± 5.45	17.43 ± 3.48	22.18 ± 5.48	24.63 ± 3.78
t-BHQ	-	-	1.68 ± 0.38	1.70 ± 0.40	ND	ND	5.10 ± 1.05	4.73 ± 0.58

[a]The inductivity of the compound is calculated compared to the blank control, and data are presented as mean ± SD of two separate experiments.

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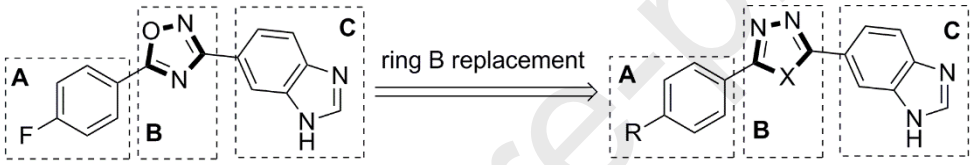
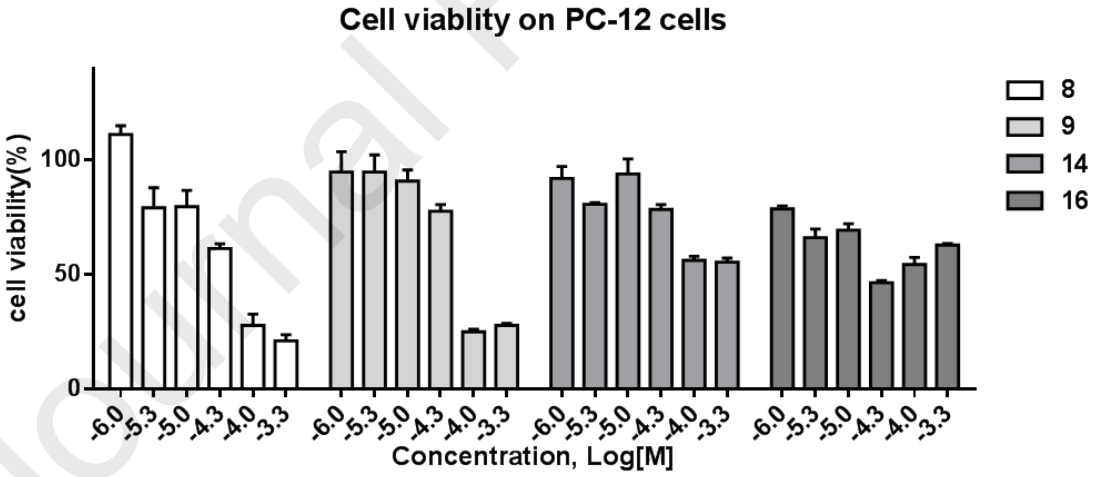
Declaration of interests

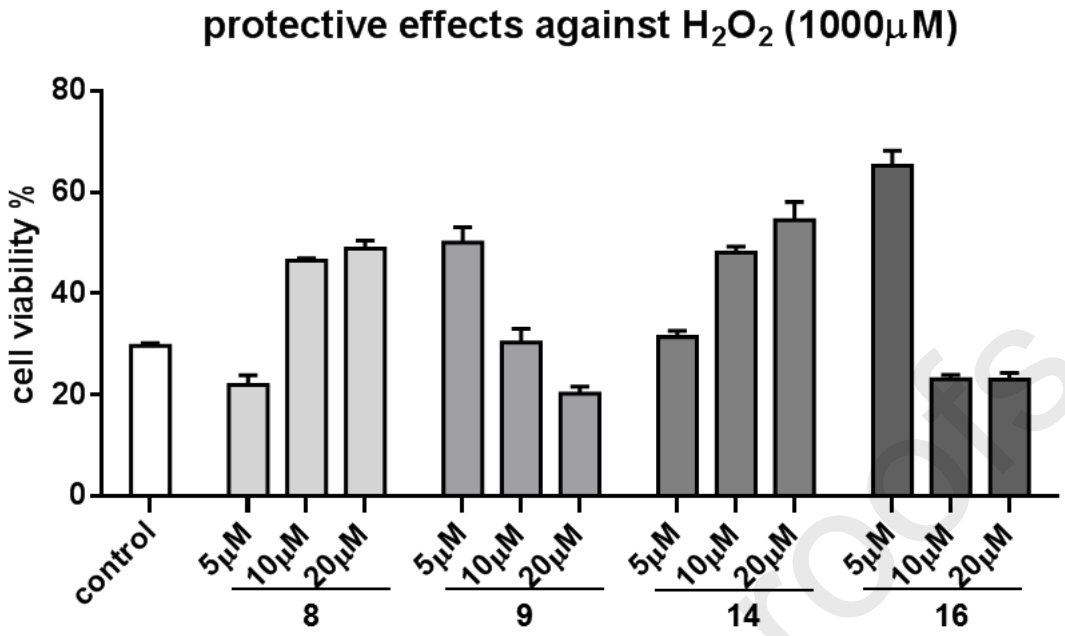
☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

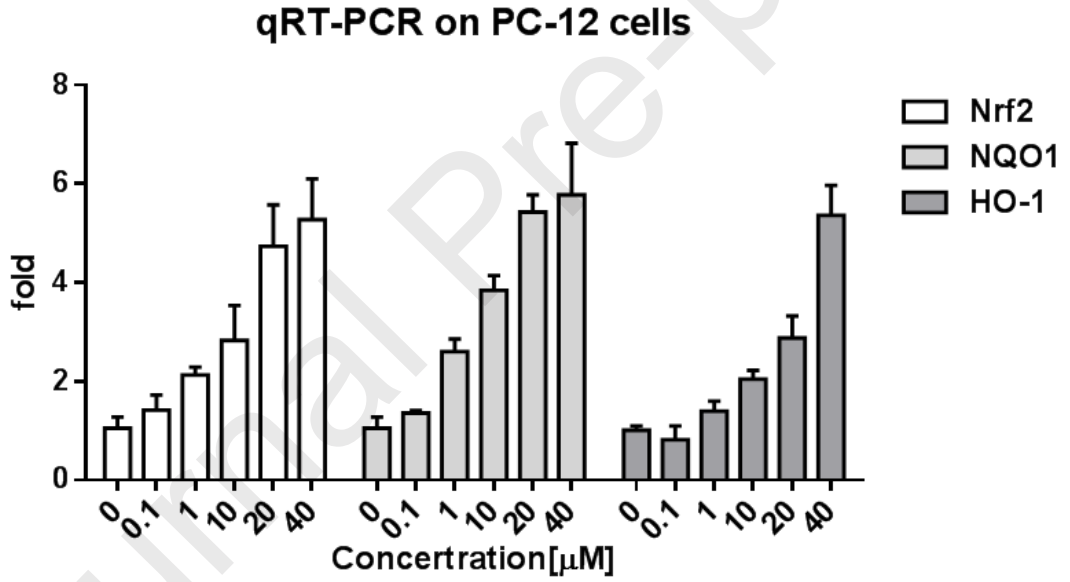
☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

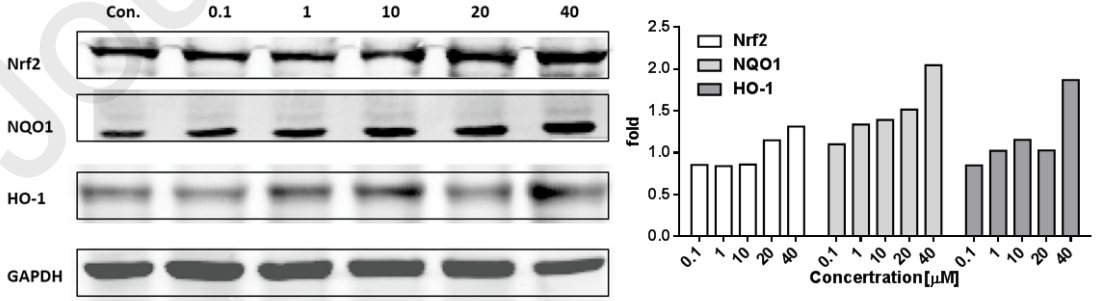


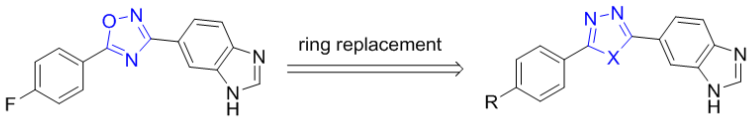
Nrf2 activator with 1,2,4-oxadiazole core (**1**) Nrf2 activators with 1,3,4-oxa/thiadiazole core











Nrf2 activator with 1,2,4-oxadiazole core

Nrf2 activator with 1,3,4-oxa/thiadiazole core

Nrf2 activation
PC-12 cells protection
increased solubility
BBB permeability
low lipophilicity