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Novel antioxidants' synthesis and their anti-oxidative activity through activating Nrf2 signaling pathway

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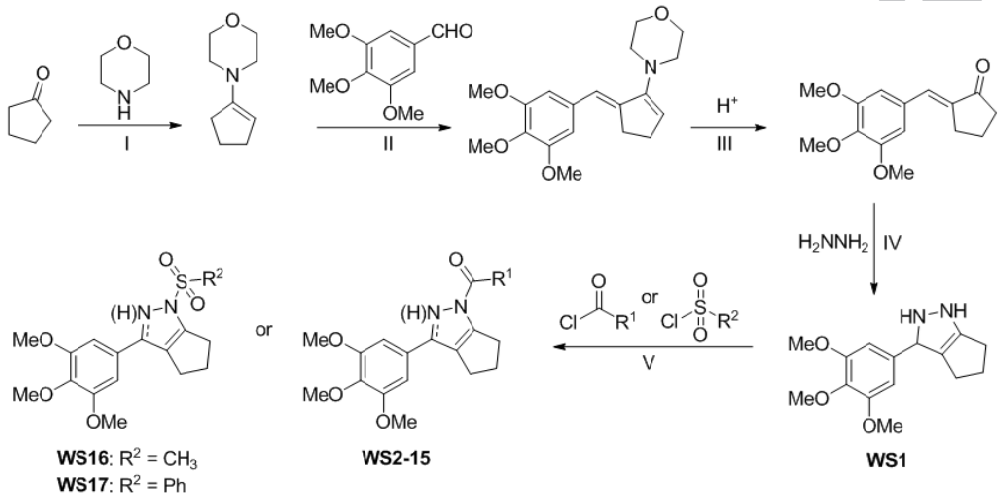
Abstract:

Novel structure compounds (**WS**) containing 3,4,5-trimethoxyphenyl and acyl pyrazole were designed and synthesized based combination principles. Among them, **WS13** was screened out to possess desirable anti-oxidative activity in vitro. Cell survival assay and apoptosis experiment in H_2O_2 induced PC12 cells injury model all showed that its cytoprotection exhibited a concentration-effect manner. **WS13** at 10 μM could remove ROS with equal effiency to edaravone. Further, it clearly activated Nrf2 nuclear translocation and upregulated GCLC mRNA transcription and protein expression in dose-dependent manner, and its cytoprotection was reversed by GCLC protein inhibitor. In total, **WS13** with further promotion can serve as Nrf2-GCLC activator in anti-oxidative therapy.

Key words: Antioxidant; Nrf2; GCLC; PC12

Acute or chronic accumulation of oxidant in cells from external and internal is extremely harmful to human health.[1] Through damaging cell lipids, protein and nucleic acids, oxidative stress is closely associated with numerous disease such like cancer, Alzheimer and chronic heart failure.[2-4] Human bodies are equipped with multi layers of anti-oxidative mechanisms, which can clean up internal oxidants in physiological condition. Once internal oxidants were over-loaded or attacked by external stimulation, cells resulted in oxidative stresses or inflammatory reactions.[5-8] At molecule level, the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway has drawn much attentions in recent years because it plays important roles in the oxidative stress related physiological processes.[9-12] And

the pathway is generally considered to be composed of Keap1, cis-antioxidant response element, and downstream phase II detoxifying enzymes and anti-oxidant enzymes coding genes. Glutamate-cysteine ligase catalytic subunit (GCLC), part of glutamate cysteine ligase (GCL) which limiting the rate of synthesizing glutathione (GSH) and regulated by Nrf2/ARE signaling pathway, is one of the most general indices used to indicate the activation of Nrf2-ARE pathway. The pathway activation usually provides cells with enhanced capacity in eliminating reactive oxygen species (ROS) and in coping with oxidative stress. But up to now, few anti-oxidative targeted agents have been approved for clinic therapy. So many researchers focus on developing novel Nrf2-ARE signaling modulators.



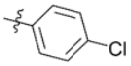
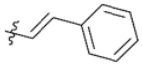
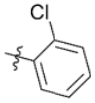
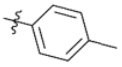
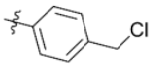
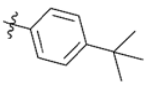
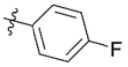
Compound	R1	Compound	R1
WS2		WS9	
WS3		WS10	
WS4		WS11	
WS5		WS12	
WS6		WS13	
WS7		WS14	
WS8		WS15	

Figure 1. Synthetic routes and compounds' structures. Synthetic conditions: (I) sol. cyclohexane; cat. TsOH; temp. 90 ^{o}C; reflux 5-6 h. (II) sol. alcohol; temp. 78 ^{o}C; reflux 0.5-1.5 h. (III) reagent HCl (aq); temp. room temperature; stir to pH ~2. (IV) sol. alcohol; temp. 78 ^{o}C; reflux 2 h. (V) sol. THF; reagent Et_3N; temp. 0 ^{o}C; stir 2 h.

Evidences show that 3,4,5-trimethoxy benzyl may be a novel Nrf2-ARE activator.[13-17] For instance, Jianguo Fang reported that piperlongumine analogues which containing 3,4,5-trimethoxy benzyl can serve as Nrf2 activator.[13] Further, Roberto Motterlini found that there were structure-activity relationships between methoxyl chalcones and its abilities inducing heme oxygenase-1 (HO-1) protein.[14] When its aromatic rings' hydrogens were replaced by 3,4,5-trimethoxy, the inducing activities peaked. Azhar Ariffin also analyzed the correlation of new hydrazone compounds bearing a 3,4,5-trimethoxybenzyl moiety and their antioxidant activities.[15] And there are other literatures reported some novel natural products containing 3,4,5-trimethoxybenzyl having anti-oxidant property.[16,17] So it's believed that 3,4,5-trimethoxybenzyl is beneficial for activating Nrf2-ARE signaling pathway. Acyl pyrazoles were rarely investigated as synthetic modules, whereas some compounds showed anticancer, antioxidant or anti-inflammatory activities.[18-21] Further, acyl pyrazoles were kind of similar to edaravone which can eliminate free radicals and intracellular ROS.[22] Therefore, based on combination principles, a series of novel compounds containing 3,4,5-trimethoxyphenyl and acyl pyrazole moieties were designed and synthesized. Then their cytoprotection in H_2O_2 induced PC12 cells injury model was tested, as well their abilities to clear ROS and activate the Nrf2-ARE signaling pathway. The synthetic routes and all compounds' structures were displayed in **Figure 1**.

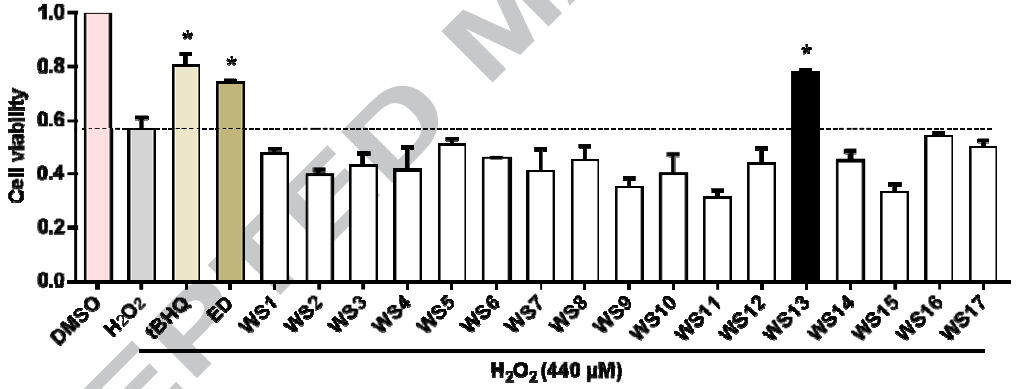


Figure 2. Compounds' cytoprotection on PC12 cells in H_2O_2 damage model. PC12 cells were pretreated for 24 h with **WS** compounds (10 μM), tBHQ (10 μM), or ED (Edaravone) (10 μM), then another 24h exposure in H_2O_2 (440 μM), finally added MTT and measured the OD value in 490nm. Values are means ± SEM (n = 3 or 4). *$p < 0.01$ vs H_2O_2, one way ANOVA, followed by Tukey's multiple comparison test.

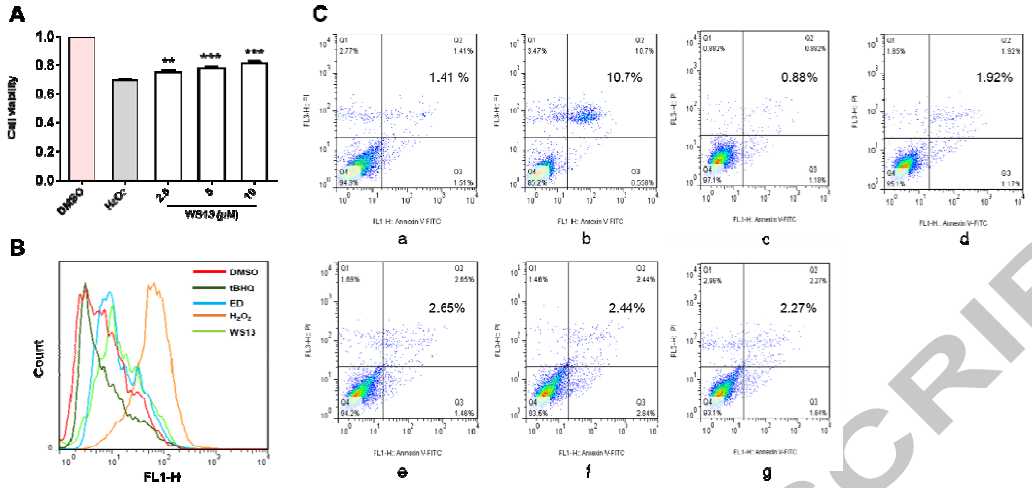


Figure 3. **WS13** resisted the H_2O_2 induced damage in PC12 cells. (A): **WS13** raised PC12 cell viability dose-dependently in H_2O_2 induced cell injury model. PC12 cells were pre-treated with **WS13** in different doses (2.5, 5, 10 μM) for 24 h and then treated with H_2O_2 for 24 h. The cell viability was measured by MTT assay. Values are means ± SEM (n = 3 or 4). **P < 0.01, ***P < 0.001 vs H_2O_2, one way ANOVA, followed by Tukey's multiple comparison test. (B): **WS13** pre-incubation decreased ROS level in H_2O_2 treated PC12 cells. PC12 cells were pre-treated with 10 μM of **WS13**, ED (Edaravone), or tBHQ; then treated with H_2O_2 for 5 h, and the ROS level was measured by flow cytometry. (C): **WS13** protected PC12 cells from apoptosis in H_2O_2 induced cell damage model. PC12 cells were pre-treated with tBHQ (10 μM), Edaravone (10 μM) or **WS13** (2.5, 5, 10 μM) for 24 h, and then followed by another 24 h exposed in H_2O_2 (440 μM). The apoptosis was detected with flow cytometry. (C-a) DMSO; (C-b) H_2O_2; (C-c) tBHQ; (C-d) Edaravone; (C-e-g) **WS13** at 2.5 μM, 5 μM and 10 μM.

Pre-incubation with these compounds for 24 hours, only **WS13** presented a valuable protection against oxidative damage in PC12 cells induced by H_2O_2 (**Figure 2**). Cell viability in **WS13** treatment group was significantly higher than that in model group. Then, the concentration-effect relationship of **WS13** was also investigated as showed in **Figure 3A**. When **WS13** was applied at 2.5, 5 and 10 μM level, a gradually improved protection against oxidative damage in PC12 cells induced by H_2O_2 was observed. Annexin V-FITC/PI double staining test with flow cytometry (**Figure 3C**) also showed that **WS13** performed protective effect at a dose of 2.5, 5 and 10 μM in dose-dependent manner. Further, **WS13** at 10 μM significantly eliminated endocellular ROS level with the same effiency of Edaravone (**Figure 3B**).

Hypothetically, compounds **WS13** may execute its cytoprotection through scavenging free radicals directly and activating the anti-oxidative signaling pathway at the same time. But DPPH free radical scavenging experiment indicated **WS13** showed about half fold lower effect than edaravone or tBHQ (**Figure S1**), which mean that **WS13** may execute the anti-oxidant activities partly through clearing free radical directly but mainly through activating anti-oxidative signaling pathway.

Since **WS13** didn't mainly work directly on free radical, its ability to activate the anti-oxidative signaling pathway was investigated. As showed in **Figure 4**, immunofluorescence experiment revealed that Nrf2 nuclear translocation was activated by **WS13** at a dose of 10 μM.

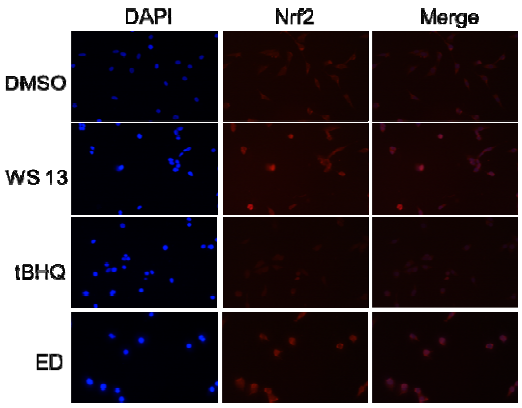


Figure 4. WS13 activated Nrf2 nuclear-translocation. PC12 cells were incubated with tBHQ, ED or **WS13** at dose of 10 μM for 6 h, and then stained with Nrf2 antibody and DAPI.

RT-PCR and western-blotting experiments all indicated that GCLC protein coding genes in the Nrf2-ARE signaling pathway was activated. As exhibited in **Figure 5A**, GCLC mRNA level was up-regulated by **WS13** at 2.5, 5 and 10 μM level. Further, western-blotting experiments also revealed that **WS13** up-regulated GCLC protein expression in PC12 cells and followed a concentration-dependent manner (**Figure 5B**).

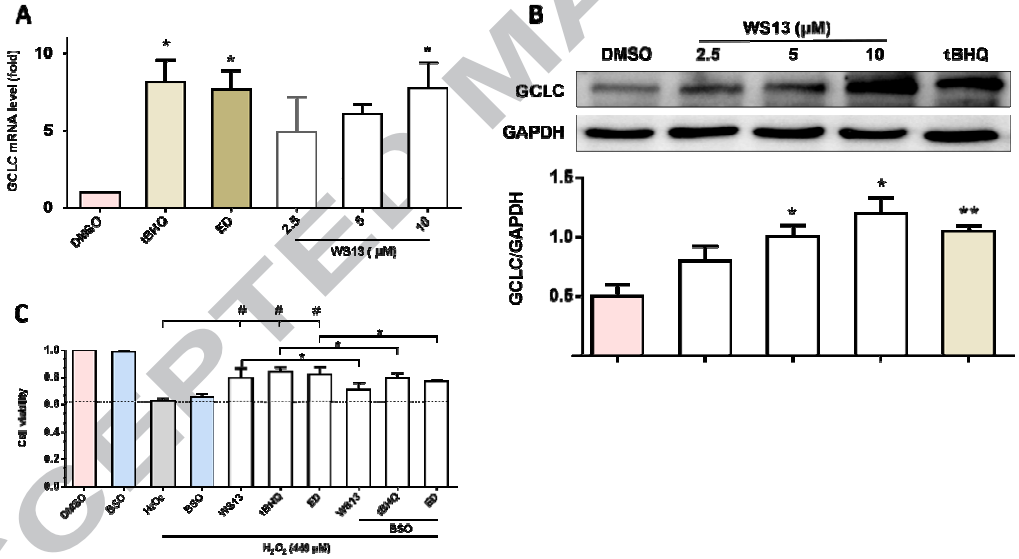


Figure 5. **WS13** increased the expression of GCLC. (A): **WS13** induced the mRNA expression of GCLC. PC12 cells were incubated with **WS13** in different doses for 24 h and then evaluated the mRNA level of GCLC by RT-PCR experiment. Values are means ± SEM (n = 3 or 4). *$p < 0.05$ vs control, one way ANOVA, followed by Tukey's multiple comparison test. (B): **WS13** induced the expression of GCLC. PC12 cells were incubated with **WS13** in 2.5, 5, 10 μM and tBHQ in 10 μM for 24 h, and the GCLC was determined by Western-blotting experiment and representative result was present. Values are means ± SEM (n = 3 or 4). *$p < 0.1$, **$p < 0.01$ vs DMSO, one way ANOVA, followed by Tukey's multiple comparison test. (C): BSO diminished the protected effect of **WS13** on H_2O_2 induced cell damage. PC12 cells were incubated with GCLC inhibitor BSO for 2 h, then treated with **WS13**, tBHQ or ED in 10 μM for 24 h. Finally MTT assay measured the OD values in 490 nm. Values are

means ± SEM (n = 3 or 4). *p < 0.1; #p < 0.1 vs H_2O_2, one way ANOVA, followed by Tukey's multiple comparison test.

To make sure if **WS13** executes anti-oxidative protection through activating GCLC protein expression, its specific enzyme inhibitor namely BSO is applied to this study. As showed in **Figure 5C**, it's obvious that BSO had no influence on cell viability in H_2O_2 induced model group, while **WS13** applied alone raised the cell viability. But, when BSO and **WS13** were applied together, the cytoprotection was constrained. This result showed that **WS13** executed its anti-oxidative activities through, at least partly, activating GCLC protein expression.

In summary, seventeen novel compounds containing 3,4,5-trimethoxyphenyl and acyl pyrazole moieties were designed and synthesized, and **WS13** was screened out to exhibit an inspirable protection against H_2O_2 induced PC12 cell injury in concentration-dependent manner. Mechanism study showed that through activating Nrf2 pathway, upregulating GCLC mRNA transcription and protein expression, **WS13** eliminate ROS with equal effiency to edaravone and executed its cytoprotection. However, including **WS13**, the activity of these compounds still need to be promoted and further research of activity in vivo is required.

Acknowledgment

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Supplementary data

Supplementary data (complete structures and characterization information of all the compounds) associated with this article can be found in the online version.

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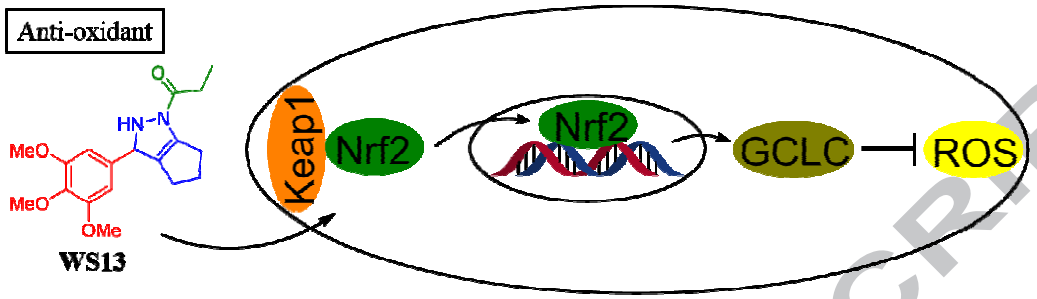
Graphical abstract



Abstract: Novel structure compounds (**WS**) containing 3,4,5-trimethoxyphenyl and acyl pyrazole were designed and synthesized based combination principles. Among them, **WS13** was screened out to possess desirable anti-oxidative activity in vitro. Cell survival assay and apoptosis experiment in H_2O_2 induced PC12 cells injury model all showed that its cytoprotection exhibited a concentration-effect manner. **WS13** at 10 μM could remove ROS with equal effiency to edaravone. Further, it clearly activated Nrf2 nuclear translocation and upregulated GCLC mRNA transcription and protein expression in dose-dependent manner, and its cytoprotection was reversed by GCLC protein inhibitor. In total, **WS13** with further promotion can serve as Nrf2-GCLC activator in anti-oxidative therapy.