Supporting Information

Polar Recognition Group Study of Keap1-Nrf2 Protein-Protein Interaction Inhibitors

Meng-Chen Lu,[a] Shi-Jie Tan,[a] Jian-Ai Ji,[a] Zhi-Yun Chen,[a] Zhen-Wei Yuan,[a] Qi-Dong You[a,b *] and Zheng-Yu Jiang[a,b *]

[a]State Key Laboratory of Natural Medicines and Jiang Su Key Laboratory of Drug Design and Optimization, China Pharmaceutical University, Nanjing 210009, China; [b]Department of Medicinal Chemistry, School of Pharmacy, China Pharmaceutical University, Nanjing 210009, China

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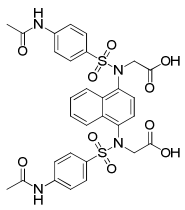
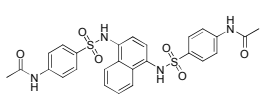
S1. Materials and methods

1. Chemistry

Reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm silica gel plates with fluorescent indicator (GF_{254}) and visualized under UV light. Melting points were determined with a Melt-Temp II apparatus. The ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AV-300 instrument using deuterated solvents with tetramethylsilane (TMS) as an internal standard. ESI-mass and high resolution mass spectra (HRMS) were recorded on a Water Q-Tof micro mass spectrometer. Purity (≥ 95%) of target compounds was determined by the HPLC study performed on Agilent C18 (4.6 mm × 150 mm, 3.5 μm) column using a mixture of solvent methanol/water 70:30 methanol:water with 1‰ TFA) at the flow rate of 0.5 mL/min and peak detection at 254 nm under UV. The ^{1}H NMR spectrums of all the titled compounds exhibited a doubling of signals, which was also reported previously by Jain et al.'s[1] and our group[2]. Considering the elements of symmetry suggested by the planar structure, this phenomenon was quite surprising and interesting. Jain et al. has specifically demonstrated this phenomenon and gave suficient explanation on it.[1]

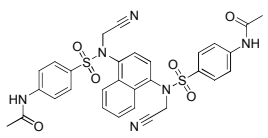


2,2'-(naphthalene-1,4-diylbis(((4-acetamidophenyl)sulfonyl)azanediyl))diacetic acid (2): To a solution of compound 6 (696 mg, 1 mmol) in MeOH/H_2O (8 mL/4 mL) was added LiOH (1.5 g). After 6 h stirring at room temperature, the reaction mixture was then diluted in 30 mL water and quenched with 2 M hydrochloric acid to pH 5. The crude product was obtained through filtration. Recrystallization from ethyl acetate/n-hexane give the compound 11 as a light pink soild, yield 68%; m.p.: 288-290°C; ^{1}H-NMR (300 MHz, DMSO, δ) 10.74 (s, 1H), 10.47 (s, 1H), 8.35 (dd, 1H, J = 6.23, 3.08 Hz), 8.18 (dd, 1H, J = 6.36, 3.06 Hz), 7.90 (d, 2H, J = 8.61 Hz), 7.74 (d, 2H, J = 8.73 Hz), 7.58-7.55 (m, 6H), 7.11 (s, 1H), 6.82 (s, 1H), 4.48-4.28 (m, 4H), 2.17(s, 3H), 2.09 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.37, 169.40, 169.17, 143.52, 143.35, 137.02, 133.20, 132.85, 131.96, 130.86, 128.95, 128.71, 128.58, 126.74, 126.26, 125.99, 125.61, 124.99, 124.64, 118.94, 118.30, 118.18, 54.34, 54.07, 43.81, 24.09; HRMS (ESI): found 686.1614 ($C_{30}H_{32}N_5O_{10}S_2$, $[M+NH_4]^+$, requires 686.1585); HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 3.3 min, 98.5%.

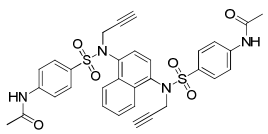


N,N'-((((4-(hydrosulfonylamino)naphthalen-1-yl)amino)sulfonyl)bis(4,1-phenylene))diacetamide (3): To a solution of 4-Nitronaphthalen-1-amine (1.81 g, 10 mmol) in THF was added Pd/C. The reaction mixture was stirred under hydrogen for 4h.

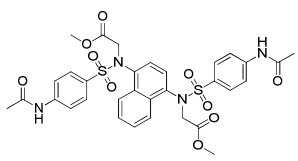
The solution was filtered to remove the catalyst. The filtrate was concentrated under reduced pressure to give the light yellow oil. The crude product, naphthalene-1,4-diamine, was used without further purification. 4-acetamidobenzenesulfonyl chloride (5.14 g, 22 mmol) and pyridine (2.37 g, 30 mmol) was added to the solution of toluene (20 mL) and naphthalene-1,4-diamine. The reaction mixture was stirred at 100 °C for 2 h under nitrogen. After cooling to room temperature, reaction mixture was then diluted in 20 mL petroleum ether. After filtration, the solid was collected and washed with 1 M hydrochloric acid. Recrystallization from acetonitrile gave the pink solid, yield 76%; m.p.: 200-201°C; ^{1}H-NMR (300 MHz, DMSO, δ) 10.34 (s, 2H), 10.05 (s, 2H), 7.99 – 7.97 (m, 2H), 7.69 – 7.66 (m, 4H), 7.56 – 7.53 (m, 4H), 7.41 – 7.38 (m, 2H), 7.00 (s, 2H), 2.07 (s, 6H); HRMS (ESI): found 570.1491 ($C_{26}H_{28}N_5O_6S_2$, $[M+NH_4]^+$, requires 570.1476).



N,N'-((((cyanomethyl)(4-((cyanomethyl)(hydrosulfonyl)amino) naphthalen-1-yl)amino)sulfonyl)bis(4,1-phenylene))diacetamide (4): To a solution of compound 3 (1.104 g, 2 mmol) in DMF (5 ml) was added K_2CO_3 (830 mg, 6 mmol) followed by bromoacetonitrile (600 mg, 5 mmol). After 3h stirring at room temperature, the reaction mixture was then diluted in 30 mL water and quenched with 2 M hydrochloric acid to pH 5. The crude product was obtained through filtration. Recrystallization from ethyl acetate/n-hexane give compound 4 as a light gray soild, yield 53%; m. p. :204-205 °C; 1H-NMR (300 MHz, DMSO, δ) 10.45 (s, 2H), 8.08 (m, 2H), 7.81 (m, 10H), 7.14 (s, 1H), 7.05 (s, 1H), 4.96 (s, 2H), 5.00 (s, 2H), 2.12 (s, 6H); HRMS (ESI): found 631.1434 (C30H27N6O6S2, [M+H]+, requires 631.1428). HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 8.9 min, 98.0%.

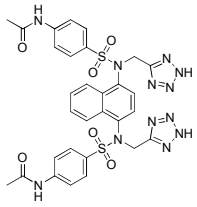


N,N'-((((4-(hydrosulfonyl(prop-2-yn-1-yl)amino)naphthalen-1-yl)(prop-2 -yn-1-yl)amino)sulfonyl)bis(4,1-phenylene))diacetamide (5): Compound 5 was synthesized according to the procedure of compound 4, light gray solid, yield 72%; m.p.: 126-127°C; 1H-NMR (300 MHz, DMSO, δ) 10.40 (s, 2H), 8.23 (m, 2H), 7.66 (m, 12H), 7.02 (s, 1H), 6.90 (s,1H), 4.54 (s,4H), 2.16 (s, 3H), 2.10 (s, 3H); HRMS (ESI): found 629.1526 (C32H29N4O6S2, [M+H]+, requires 629.1523). HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 7.1 min, 96.7%.

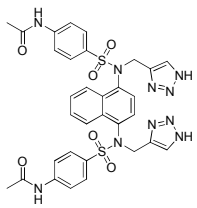


Dimethyl 2,2'-(naphthalene-1,4-diylbis(((4-acetamidophenyl) sulfonyl)azanediyl))diacetate (6): To a solution of compound 3 (1.104 g, 2 mmol) in DMF (5 ml) was added K_2CO_3 (830 mg, 6 mmol) followed by

methyl bromoacetate (765 mg, 5 mmol). After 3 h stirring at room temperature, the reaction mixture was then diluted in 30 mL water and quenched with 2 M hydrochloric acid to pH 5. The crude product was obtained through filtration. Recrystallization from ethyl acetate/n-hexane give compound 6 as a light brown soild, yield 64%; m. p. :131-132 °C; 1H-NMR (300 MHz, DMSO, δ) 10.48 (s, 2H), 8.30 (dd, 1H, *J* = 6.3, 3.1 Hz), 8.17 (dd, 1H, *J* = 6.6, 3.2 Hz), 7.85 (d, 2H, *J* = 8.6 Hz), 7.73 (d, 2H, *J* = 8.7 Hz), 7.58 (m, 6H), 7.09 (s, 1H), 6.88 (s, 1H), 4.52 (m, 4H), 3.64 (s, 6H), 2.05 (s, 6H); HRMS (ESI): found 697.1644 (C32H33N4O10S2, [M+H]+, requires 697.1633). HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 5.8 min, 98.3%.

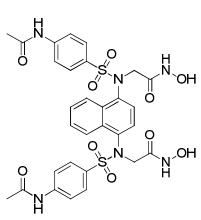


N,N'-(((((2H-tetrazol-5-yl)methyl)(4-(((2H-tetrazol-5-yl)methyl)(hydrosulfonyl)amino)naphthalen-1-yl)amino)sulfonyl)bis(4,1-phenylene))diacetamide (7): To a solution of compound 4 (630 mg, 1 mmol) in DMF (5 ml) was added NH_4Cl (161 mg, 3 mmol) followed by NaN_3 (163 mg, 2.5 mmol). The reaction mixture was stirred at 100 °C for 6 h under nitrogen. After cooling to room temperature, the reaction mixture was then diluted in 30 mL water and quenched with 2 M hydrochloric acid to pH 5. The crude product was obtained through filtration. Recrystallization from ethyl acetate/n-hexane give compound 7 as a light yellow soild, yield 67%; m. p. :227-228 °C; 1H-NMR (300 MHz, DMSO, δ) 10.46 (s, 1H), 10.43 (s, 1H), 8.12 (m, 1H), 8.06 (m, 1H), 7.84 (m, 2H), 7.76 (m, 2H), 7.56 (m, 6H), 6.79 (s, 1H), 6.75 (s, 1H), 5.29-4.97 (m, 4H), 2.16 (s, 3H), 2.03 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.42, 169.21, 153.65, 153.35, 143.84, 143.80, 136.44, 136.00, 133.02, 132.93, 129.94, 129.18, 129.06, 126.92, 126.14, 123.96, 118.57, 118.49, 45.36, 44.92, 24.22, 24.14; HRMS (ESI): found 717.1771 (C30H29N12O6S2, [M+H]+, requires 717.1769). HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 2.6 min, 98.0%.

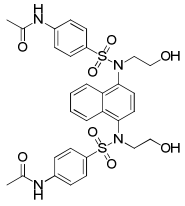


N,N'-(((((1H-1,2,3-triazol-4-yl)methyl)(4-(((1H-1,2,3-triazol-4-yl)methyl)(hydrosulfonyl)amino)naphthalen-1-yl)amino)sulfonyl)bis(4,1-phenylene))diacetamide (8): To a solution of compound 5 (628 mg, 1 mmol) in DMF (5 ml) was added NH_4Cl (161 mg, 3 mmol) followed by NaN_3 (163 mg, 2.5 mmol). The reaction mixture was stirred at 100 °C for 6 h under nitrogen. After cooling to room temperature, the reaction mixture was then diluted in 30 mL water and quenched with 2 M hydrochloric acid to pH 5. The crude product was obtained through filtration. Recrystallization from ethyl acetate/n-hexane give cmpound 8 as a light yellow soild, yield 63%; m. p. :166-168 °C; 1H-NMR (300 MHz, DMSO, δ) 10.43 (m, 2H), 8.01 (m, 2H), 7.86 (m, 4H), 7.73 (m, 4H), 7.63 (m, 2H), 7.49 (m, 2H), 6.79 (m, 2H), 5.13 (dd, 2H, *J* = 14.2, 2.9 Hz), 4.77 (m, 2H), 2.15(s, 6H); HRMS

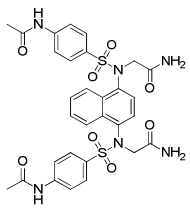
(ESI): found 715.1866 (C32H31N10O6S2, [M+H]+, requires 715.1864). HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 3.9 min, 98.1%.



2,2'-(naphthalene-1,4-diylbis(((4-acetamidophenyl)sulfonyl)azanediyl))bis(N-hydroxyacetamide) (9): To a solution of compound 6 (696 mg, 1 mmol) in MeOH (10 mL) was added $NH_2OH{\cdot}HCl$ (4.17 g) followed by NaOH (1.2 g). After 6 h stirring at room temperature, the reaction mixture was then diluted in 30 mL water and quenched with 2 M hydrochloric acid to pH 5. The crude product was obtained through filtration. Recrystallization from ethyl acetate/n-hexane give compound 9 as a light yellow soild, yield 64%; m. p. :203-204 °C; 1H-NMR (300 MHz, DMSO, δ) 10.49 (s, 2H), 10.38 (s, 2H), 8.92 (m, 2H), 8.25 (s, 2H), 7.81 (d, 2H, *J* = 8.1 Hz), 7.72 (d, 2H, *J* = 8.25 Hz), 7.57 (s, 6H), 6.92 (m, 2H), 4.25 (m, 4H), 2.11(s, 6H); HRMS (ESI): found 699.1558 (C30H31N6O10S2, [M+H]+, requires 699.1538). HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 4.1 min, 97.2%.



N,N'-((((4-(hydrosulfonyl(2-hydroxyethyl)amino)naphthalen-1-yl)(2-hydroxyethyl)amino)sulfonyl)bis(4,1-phenylene))diacetamide (10): To a solution of $LiAlH_4$ (75.9 mg, 2 mmol) in THF (5 mL) was added a solution of compound 6 (696 mg, 1 mmol) in THF (5 mL) dropwisely. After 1 h stirring in an icewater bath, 4 mL water was added to the reaction mixture. After stirring 20 min at room temperature, 4 mL of 15% NaOH solution was added to the reaction mixture dropwisely, followed by another 20 min stirring. Then the reaction mixture was quenched with 12 mL water. The crude product was obtained through extraction. Recrystallization from ethyl acetate/n-hexane give compound 10 as a light gray soild, yield 61%; m. p. :157-159 °C; 1H-NMR (300 MHz, DMSO, δ) 10.44 (s, 1H), 10.40 (s, 1H), 8.26 (m, 2H), 7.86 (m, 4H), 7.68 (d, 6H, *J* = 4.86 Hz), 6.99 (s, 1H), 6.79 (s, 1H), 4.77 (m, 4H), 3.92 (m, 4H), 2.18(s, 6H); HRMS (ESI): found 641.1730 (C30H33N4O8S2, [M+H]+, requires 641.1734). HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 3.9 min, 96.0%.



N,N'-((((2-amino-2-oxoethyl)(4-((2-amino-2-oxoethyl)(hydrosulfonyl)amino)naphthalen-1-yl)amino)sulfonyl)bis(4,1-phenylene))diacetamide (11): To a solution of compound 3 (1.104 g, 2 mmol) in DMF (5ml) was added K_2CO_3 (830 mg, 6 mmol) followed by bromoacetamide (690 mg, 5 mmol). After 3 h stirring at room temperature, the reaction mixture was then diluted in 30 mL water and quenched with 2 M hydrochloric acid to pH 5. The crude product was obtained through filtration. Recrystallization from ethyl acetate/n-hexane give compound 11 a light pink soild, yield 58%; m.

p. :186-188 °C; 1H-NMR (300 MHz, DMSO, δ) 11.03 (s, 1H), 10.62 (s, 1H), 8.41 (s, 1H), 8.20 (s, 1H), 8.0 (m, 1H), 7.76 (m, 1H), 7.53 (m, 4H), 7.35 (s, 4H), 7.18 (s, 4H), 7.0 (m, 1H), 6.65 (s, 1H), 4.25 (m, 4H), 2.15 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.45, 169.19, 168.57, 143.89, 143.60, 136.93, 133.26, 132.86, 131.13, 129.95, 129.14, 128.83, 126.34, 125.21, 124.91, 124.58, 118.29, 118.04, 54.15, 53.88, 24.13; HRMS (ESI): found 667.1644 ($C_{30}H_{31}N_6O_8S_2$, $[M+H]^+$, requires 667.1639). HPLC (70 : 30 methanol : water with 1‰ TFA): t_R = 3.6 min, 96.6%.

2. Molecular Docking

The Ligandfit docking tool in Discovery Studio, which has been validated for this target previously[2-3], was used to predict the binding mode of the designed small molecular inhibitor. The docking site was derived from the position of the small molecular ligand co-crystallized in the binding site of Keap1 (PDB code: 4IQK).

3. Physicochemical properties Studies

The pKa and partition coefficient (log D, pH 7.4) were determined according to the methods of Avdeef and Tsinman[4] on a Gemini Profiler instrument (pION) by the "goldstandard" Avdeef-Bucher potentiometric titration method[5]. The experiment was carried out as previously reported.[6] The intrinsic solubility was determined by using the pH-metric method, which is a new potentiometric acid-base titration method. The potentiometric solubility data were obtained with the pSOL Model 3 instrument (pION INC., Cambridge, MA, USA) and subsequently processed with the accompanying computer program, pS. Detailed procedures can be found in the references[7].

4. Biology

4.1 FP Competition Assay

The FP assay was carried out as previously reported.[2] The experiments were performed in the black nonbinding surface Corning 3676 384-well plates. The values were measured using a SpectraMax Multi-Mode Microplate Reader (Molecular Devices) with excitation and emission wavelengths of 485 and 525 nm, respectively. All aqueous solutions were prepared using deionized water collected from a Millipore water purification system. The plates were loaded with 40μL of assay solution per well, consisted of 10 μL of 4 nM FITC-9mer Nrf2 peptide amide and 10 μL of 12 nM Keap1 Kelch domain protein, 10 μL of HEPES buffer, and 10 μL of an inhibitor sample of varying concentrations. In this FP assay, the concentration of DMSO is less than 1%. The plate was covered and rocked for 30 min at room temperature prior to FP measurements. FP was determined by measuring the parallel and perpendicular fluorescence intensity ($F_{\parallel}$ and $F_{\perp}$) with respect to the

linearly polarized excitation light. We elected to use polarization in our quantitative analysis. The percentage inhibition of the competitor at each concentration point was determined by using equation %inhibition = 1- (Pobs-Pmin) / (Pmax-Pmin). The values of Pmax, Pmin, and Pobs in the equations refer to the polarization of the wells containing Keap1 and the probe, the polarization of the free probe, and the observed polarization for the wells containing the inhibitors at a range of concentrations under the assay conditions. The IC_{50} of an inhibitor was determined from the plot of %inhibition against inhibitor concentration analyzed by GraphPad Prism 6.0 software.

4.2 Cell Culture Conditions and ARE-Luciferase Activity Assay.

HepG2 cells stably transfected with a luciferase reporter (HepG2-ARE-C8) were kindly provided by Professor Dr. A. N. Tony Kong (Rutgers University, Piscataway, NJ) and Prof. Rong Hu (China Pharmaceutical University, Nanjing). Cells were maintained in modified RPMI-1640 medium (GiBco, Invitrogen Corp., USA) with 10% fetal bovine serum (FBS) (GiBco, Invitrogen Corp., USA) and penicillin/streptomycin in a 37°C incubator with 5% CO_2. HCT116 cells (Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were cultured in McCoy's 5A (Sigma-Aldrich, #M4892, USA) supplemented with 10% (v/v) FBS and penicillin/streptomycin.

The experimental procedures were carried out as reported previously.[2] Generally, HepG2-ARE-C8 cells were plated in 96-well plates at a density of 4 × 10^4 cells/well and incubated overnight. The cells were exposed with different concentrations of test compounds, with tBHQ serving as a positive control, DMSO as a negative control, and the luciferase cell culture lysis reagent as a blank. After 12 h of treatment, the medium was removed and 100μL of cold PBS was added into each well. Then the cells were harvested in the luciferase cell culture lysis reagent. After centrifugation, 20 μL of the supernatant was used for determining the luciferase activity according to the protocol provided by the manufacturer (Promega, Madison, WI). The luciferase activity was measured by a Luminoskan Ascent (Thermo Scientific, USA). The data were obtained in triplicates and expressed as fold induction over control.

4.3 RNA Extraction and qRT-PCR Analysis.

We extracted total RNA of HCT116 cells using TRIzol reagent (Invitrogen). The experimental procedure of quantitative real-time RT-PCR was previously reported[2]. The RNA was reversely transcripted by using PrimeScript RT reagent kit following the manufacturer's instructions. The primers used for PCR can be found in supporting information. Quantitative real-time RT-PCR analysis of Nrf2 targeted genes NQO1, HO-1, and GCLM were performed by using the StepOne

System Fast Real Time PCR system (Applied Biosystems). The values are expressed as the fold of the control. β-Actin was used for normalization.

Primers used for qRT-PCR are shown as follows:

HO-1

Sense primer: ATGGCCTCCCTGTACCACATC

Antisense primer: TGTTGCGCTCAATCTCCTCCT

NQO-1

Sense primer: CGCAGACCTTGTGATATTCCAG

Antisense primer: CGTTTCTTCCATCCTTCCAGG

GCLM-1

Sense primer: TTGGAGTTGCACAGCTGGATTC

Antisense primer: TGGTTTTACCTGTGCCCACTG

4.4 Western blot analysis.

Anti-NQO1 (sc-271116) antibodies and γ-GCS (sc-22755) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-action (AP0060) and anti-Nrf2 (BS1258) were purchased from Bioworlde (Bioworlde, USA). Anti-HO-1 (#5853S) were bought from Cell Signaling Technology (USA). Isolation of cell fractions and Western blotting were performed as previously reported.[2] Briefly, the extracts were separated by SDS-PAGE and then electrotransferred to PVDF membranes (Perkin Elmer, Northwalk, CT, USA). Membranes were blocked with 1% BSA for 1 h followed by incubation with a primary antibody at 4 ℃ overnight. Then they were washed and treated with a DyLight 800 labeled secondary antibody at 37 ℃ for 2 h. The membranes were screened through the odyssey infrared imaging System (LI-COR, Lincoln, Nebraska, USA).

S2. Densitometric analysis of the relative ratios of each proteins in western blot assay.

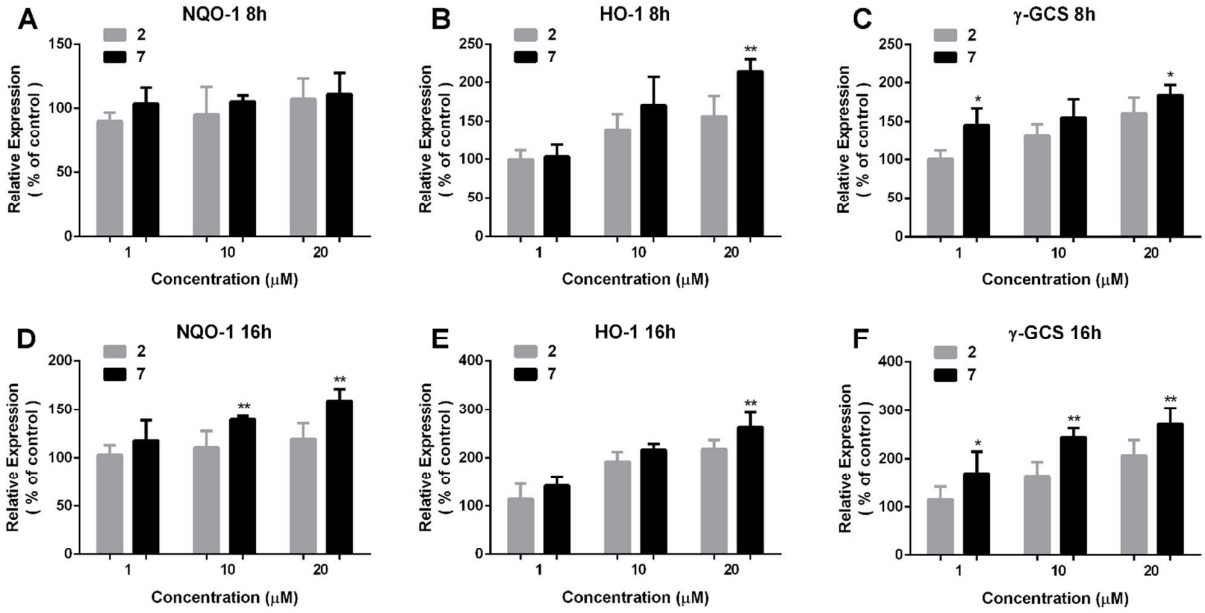


Figure S1. Densitometric analysis was performed to determine the relative ratios of each proteins. The data were normalized with β-actin expression and are expressed as the mean ± SEM of three individual experiments, ***P<0.001, **P<0.01, and*P<0.05. Data were analyzed by Image J 1.44p. Expression of **(A)** NQO1, **(B)** HO-1 and **(C)** γ-GCS after 8 h treating with **2** and **7**, respectively. Expression of **(D)** NQO1, **(E)** HO-1 and **(F)** γ-GCS after 16 h treating with **2** and **7**, respectively.

References

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