

HH-A, a modified honokiol, protects against cerebral ischemia/reperfusion induced brain injury in rodent via Nrf2/HO-1 signaling pathways

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Abstract

Honokiol, a bioactive component of *Magnolia officinalis*, has been shown to protect against ischemic stroke in animal models. However, the poor water solubility of honokiol has hindered its clinical applications. In this study, we introduced a hydrophilic building block on the aromatic ring of honokiol and obtained four new compounds (HH-A, -B, -C and -D) with significantly improved water solubility. We investigated their neuroprotective effects against brain injury in transient middle cerebral artery occlusion/reperfusion (tMCAO/R) mouse and rat models. We found that (S)-6-((3',5-diallyl-2,4'-dihydroxy-[1,1'-biphenyl]-3-yl)amino)-6-oxohexane-1,5-diaminium chloride (i.e., HH-A) significantly reduced the infarct volume and edema of the experimental animal brains showing a distinct efficiency comparing to the other three compounds. HH-A also significantly improved neurological deficits in a dose-dependent manner. In the brain of tMCAO/R rat model, treatment with HH-A resulted in significant upregulations of nuclear factor-erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1). HH-A also significantly reduced the up-regulation of 4-hydroxynonenal (HNE), a major end-product of oxidation. Further, HH-A treatment reduced the increased expression of Bcl-2-associated X protein (Bax) and reverted the reduced expression of Bcl-2, suggesting its anti-apoptotic effect, which is supported by the reduced number of TUNEL positive cells. Collectively, the neuroprotection of HH-A against the brain injury observed in this study may be via targeting the Nrf2/HO-1 signaling pathway.

Introduction

Stroke is the 2nd leading cause of death globally. Ischemic stroke is caused by occlusion of major cerebral artery and its branches leading to a sudden loss of blood supply to specific brain regions where hypoxia injures the brain functions (Lacerte et al., 2023). Intravenous thrombolysis treatment within a few hours after acute ischemic stroke with recombinant tissue plasminogen activator (tPA) to remove clot has shown some beneficial effect including extension of the time interval giving chance for further treatment. The re-introduced blood supply (re-perfusion) contributes re-oxygenation to mitigate the metabolic stress, but re-oxygenation also creates more complex events, mainly through generation of reactive oxidant leading to oxidative stress. It is known that the vast brain injury occurs not during the earlier period of stroke but rather during the period when oxygen was re-introduced through blood re-perfusion (McCord, 1985, Farina et al., 2021). Thus, oxidative stress has become an important pharmacological target in management of ischemic stroke. So far, rPA is the only FDA approved medicine for acute ischemic stroke. Thus, treatment of brain injury occurred during blood re-perfusion period remains as challenge for development of new medicines.

Honokiol is a small-molecule polyphenol isolated from the barks of *Magnolia officinalis* (Sarrica et al., 2018). Honokiol has diverse pharmacological activities e.g., inhibition of oxidative stress (Liu et al., 2018), anti-inflammation (Wang et al., 2018), anti-microbial (Chen et al., 2019) and anti-cancer (Banik et al., 2019), as well as neuroprotective effects in cerebral ischemia/reperfusion injury (Hu et al., 2013). Based on its diverse pharmacological actions, honokiol serves a good leading compound in developing new drugs with better pharmacological properties (Rauf et al., 2021).

Honokiol is poorly dissolved in water restricting its development as a therapeutic drug (Han et al., 2012). To solve this problem, through rational design, we introduced a hydrophilic building block on the aromatic ring of honokiol and obtained several new compounds (HH-A, -B, -C, and -D); all of the new compounds showed greatly improved water solubility. We evaluated the neuroprotective effects of these compounds with transient middle cerebral artery occlusion /reperfusion (tMCAO/R) mouse or rat models. We found that the compound HH-A showed the best neuroprotective effects. The underlying mechanism for the effect of HH-A may be via Nrf2/HO-1 signaling pathway.

Materials and methods

Chemistry

General experimental procedures

All chemical reagents were purchased from commercial suppliers. Thin-layer chromatography (TLC) was performed on silica gel GF-254 plates (Yantai Industrial Research Institute, YanTai, China), and spots were visualized by UV (254 nm). Column chromatography was performed on silica gel (200-300 mesh, YanTai, China).

The target compounds and intermediates were characterized by NMR. ^1H NMR and ^{13}C NMR spectra were obtained using a Bruker 400 or 100 MHz spectrometer in CDCl_3 or $\text{DMSO}-d_6$, with tetramethylsilane (TMS) as an internal standard. Chemical shifts (δ) were reported in ppm: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad singlet and coupling constants (J) in hertz (Hz). Analyses were carried out using a single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) interface. The purity of Compounds A-D was established to be $\geq 95\%$ by HPLC equipped with an Agilent C18 column (4.6 $\times$ 250 mm, 5 μm or 150 $\times$ 4.6 mm, 5 μm) and 220 nm UV detector.

Concrete experimental procedures 3',5-diallyl-3-nitro-[1,1'-biphenyl]-2,4'-diol (compound 1)

To a solution of honokiol (17 g, 63.88 mmol, HWG08031, HWRK CHEM, China) in 26 mL of acetic anhydride with stirring at 0°C was slowly added fuming nitric acid (2.5 mL) and the reaction mixture was stirred for additional 15 min at the same temperature. After that, the reaction solution was poured into ice-water and then extracted with ethyl acetate (200 mL). The organic phase was washed with sat. aq. NaHCO_3 (10 mL) and brine (10 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuum. The residue was purified by column chromatography on silica gel (ethyl acetate/petroleum ether, 1:50) to afford compound 1 (1.0 g, 5%). ^1H -NMR (400 MHz, $\text{MeOD}-d_4$), δ 7.84 (s, 1H), 7.41 (s, 1H), 7.24-7.20 (m, 2H), 6.81 (d, J=8.0 Hz, 1H), 6.04-5.93 (m, 2H), 5.14-4.89 (m, 4H), 3.38-3.31 (m, 4H). ^{13}C -NMR (100 MHz, $\text{MeOD}-d_4$), 154.8, 150.5, 138.1, 136.9, 136.5, 134.0, 133.1, 131.7, 130.8, 128.0, 127.2, 126.1, 122.1, 115.6, 114.2, 114.1, 38.3, 33.8. HRMS (ESI), m/z 310.1095 $[\text{M}-\text{H}]^-$ (Calcd for $\text{C}_{18}\text{H}_{19}\text{NO}_4$, 311.1173).

3', 5-diallyl-3-amino-[1,1'-biphenyl]-2,4'-diol (compound 2)

To a solution of compound 1 (1 g, 3.2 mmol) in ethanol (170 mL) was added $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (6.53 g, 28.9 mmol) and conc. aq. HCl (2 mL) and the reaction was stirred at 80°C for 3 hr. Being cooled to room temperature, it was poured into ice-water, adjusted to pH=7 with sat. aq. NaHCO_3 and extracted with ethyl acetate (200 mL). The resulting mixture was collected and filtered through diatomite to remove insoluble solid. The filtrate was separated into two layers and the organic layer was dried over anhydrous Na_2SO_4 , filtered and concentrated in vacuum. The residue was purified by column chromatography on silica gel (ethyl acetate/petroleum ether, 1:15~1:6) to afford compound 2 (511 mg, 56%). $^1\text{H-NMR}$ (400 MHz, MeOD-d_4), δ 7.16-7.12 (m, 2H), 6.80 (d, $J=8.0$ Hz, 1H), 6.56 (d, $J=2.4$ Hz, 1H), 6.39 (d, $J=2.0$ Hz, 1H), 6.05-5.90 (m, 2H), 5.09-4.89 (m, 4H), 3.38 (d, $J=6.4$ Hz, 2H) 3.22 (d, $J=6.4$ Hz, 2H). $^{13}\text{C-NMR}$ (100 MHz, MeOD-d_4), 154.0, 139.4, 138.2, 137.1, 136.1, 132.1, 130.5, 130.0, 129.8, 127.6, 126.4, 120.4, 115.1, 114.5, 114.1, 113.9, 39.4, 34.0. HRMS (ESI), m/z 282.1503 $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_{18}\text{H}_{19}\text{NO}_2$, 281.1416).

Di-tert-butyl (6-((3',5-diallyl-2,4'-dihydroxy-[1,1'-biphenyl]-3-yl) amino)-6-oxohexane-1,5-diyl) (S)-dicarbamate (compound 3)

To a solution of Boc-L-Lys (Boc)-OH (677 mg, 1.96 mmol) in DMF (20 mL) was added compound 2 (500 mg, 1.75 mmol), HATU (676 mg, 1.78 mmol) and DIEA (700 mg, 5.34 mmol) and the reaction mixture was stirred at room temperature overnight. DMF was evaporated in vacuum. The residue was dissolved in ethyl acetate (50 mL) and washed with 1 N aq. HCl (20 mL) and sat. aq. NaHCO_3 (20 mL). The organic phase was dried over anhydrous Na_2SO_4 , filtered and concentrated in vacuum. The residue was purified by column chromatography (ethyl acetate/petroleum ether, 1:10) to afford compound 3 (273 mg, 25%). $^1\text{H-NMR}$ (400 MHz, MeOD-d_4), δ 7.38 (s, 1H), 7.12-7.16 (m, 2H), 6.84-6.80 (m, 2H), 6.05-5.95 (m, 2H), 5.09-4.98 (m, 4H), 4.16 (s, 1H), 3.38 (d, $J=6.8$ Hz, 2H), 3.31 (d, $J=1.6$ Hz, 2H), 3.05 (t, $J=6.4$ Hz, 2H), 1.88-1.65 (m, 4H), 1.51-1.26 (m, 20H). $^{13}\text{C-NMR}$ (100 MHz, MeOD-d_4), 172.9, 157.2, 156.8, 154.1, 143.3, 137.7, 137.0, 131.7, 130.7, 129.5, 127.8, 127.0, 126.2, 120.8, 114.5, 114.3, 114.1, 79.5, 78.5, 55.4, 39.6, 39.2, 33.9, 31.4, 29.2, 27.4, 27.3, 22.9, 22.3, 13.1. HRMS (ESI), m/z 632.3313 $[\text{M}+\text{Na}^+]^+$ (Calcd for $\text{C}_{34}\text{H}_{47}\text{N}_3\text{O}_7$, 609.3414).

(S)-6-((3',5-diallyl-2,4'-dihydroxy-[1,1'-biphenyl]-3-yl) amino)-6-oxohexane-1,5-diaminium chloride (HH-A)

Compound 3 (273 mg, 0.448 mmol) was added to a 4 M solution of HCl (gas) in ethyl acetate (20 mL, 80 mmol) and the solution was stirred at 0°C for 5 hr. The reaction mixture was concentrated in vacuum and the resulting residual was washed with ether (5 mL) and dried to afford compound A (215 mg, 99.7%). $^1\text{H-NMR}$ (400 MHz, MeOD-d_4), δ 7.43 (s, 1H), 7.19-7.15 (m, 2H), 6.89-6.83 (m, 2H), 6.05-5.92 (m, 2H), 5.09-4.98 (m, 4H), 4.27 (t, $J=6.4$ Hz, 1H), 3.39 (d, $J=6.8$ Hz, 2H), 3.31 (m, 2H), 2.96 (t, $J=7.6$ Hz, 2H), 2.06-2.02 (m, 2H), 1.77-1.75 (m, 2H), 1.62-1.60 (m, 2H). $^{13}\text{C-NMR}$ (100 MHz, MeOD-d_4), 167.8, 154.3, 143.6, 137.6, 137.0, 131.9, 131.2, 130.6, 129.2, 127.8, 127.7, 126.3, 125.7, 121.6, 114.5, 114.4, 114.2, 53.2, 39.1, 38.9, 33.9, 30.9, 26.8, 21.6. HRMS (ESI), m/z 410.2432 $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_{24}\text{H}_{31}\text{N}_3\text{O}_3$, 409.2365).

(S)-6-((2,4'-dihydroxy-3',5-dipropyl-[1,1'-biphenyl]-3-yl)amino)-6-oxohexane-1,5-diaminium chloride (HH-B)

To a solution of compound 4 (300 mg, 0.62 mmol) in methanol (10 mL) was added 5% Pd/C (60 mg) and the mixture was stirred under an atmosphere of H₂ at room temperature for 15 hr. The mixture was filtered, and the filtrate was concentrated to afford compound B (270 mg, 100%). ¹H-NMR (400 MHz, MeOD-d₄), δ 7.44 (d, J=2.4Hz, 1H), 7.19 (d, J=2.0Hz, 1H), 7.13 (dd, J=2.4Hz and 8.0Hz, 1H), 6.88 (d, J=2.0Hz, 1H), 6.81 (d, J=8.0Hz, 1H), 4.25 (t, J=6.4Hz, 1H), 2.97 (t, J=8.0Hz, 2H), 2.60 (t, J=7.6Hz, 2H), 2.54 (t, J=7.6Hz, 2H), 2.05-2.00 (m, 2H), 1.78-1.74 (m, 2H), 1.67-1.58 (m, 6H), 0.99-0.92 (m, 6H). ¹³C-NMR (100 MHz, MeOD-d₄), 167.5, 154.5, 143.1, 134.4, 131.1, 130.8, 129.0, 128.7, 127.5, 127.3, 125.6, 121.2, 114.4, 53.2, 38.9, 37.0, 32.1, 31.0, 26.8, 24.5, 22.8, 21.6, 13.0, 12.7. HRMS (ESI), m/z 414.2738 [M+H]⁺ (Calcd for C₂₄H₃₆N₃O₃, 413.2678).

Di-tert-butyl(6-((3',5-diallyl-4'-hydroxy-2-methoxy-[1,1'-biphenyl]-3-yl) amino)-6-oxohexane-1,5-diyl) (S)-dicarbamate (compound 4) and di-tert-butyl (6-((3',5-diallyl-2,4'-dimethoxy-[1,1'-biphenyl]-3-yl) amino)-6-oxohexane-1,5-diyl) (S)-dicarbamate (compound 5)

To a solution of compound 3 (1.5 g, 2.46 mmol) in acetone (150 mL) was K₂CO₃ (1.69 g, 12.3 mmol) and iodomethane (1.73 g, 12.3 mmol) and the mixture was stirred at room temperature overnight. Solvent was evaporated in vacuum and the residue was partitioned between ethyl acetate (100 mL) and water (100 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered and concentrated in vacuum. The residue was purified by column chromatography on silica gel (ethyl acetate/petroleum ether, 1:4) to afford compound 4 (650 mg, 42%) and compound 5 (900 mg, 57%). Compound 4: MS:624.3 [M+H]⁺. Compound 5: MS: 638.4 [M+H]⁺.

(S)-6-((3',5-diallyl-4'-hydroxy-2-methoxy-[1,1'-biphenyl]-3-yl) amino)-6-oxohexane-1,5-diaminium chloride (HH-C)

Compound 4 (190 mg, 0.30 mmol) was dissolved in a 3.8 M solution of HCl (gas) in ethyl acetate (10 mL, 38 mmol) at 0°C and the mixture was stirred for additional 4 hr at this temperature. Hexane (10 mL) was added and the precipitate was filtered to afford compound C (105 mg, 96%) as a yellow solid. ¹H-NMR (400 MHz, MeOD-d₄), δ 7.84 (s, 1H), 7.36-7.32 (m, 2H), 7.01-6.96 (m, 2H), 6.08-5.90 (m, 2H), 5.08-4.99 (m, 4H), 4.29 (m, 1H), 3.88 (s, 3H), 3.40-3.30 (m, 4H), 2.96 (m, 2H), 2.05-2.01 (m, 2H), 1.77-1.74 (m, 2H), 1.61-1.58 (m, 2H). ¹³C-NMR (100 MHz, MeOD-d₄), 167.6, 157.3, 146.8, 137.3, 136.9, 136.0, 130.0, 129.8, 128.5, 128.0, 127.6, 127.1, 124.8, 121.3, 114.8, 114.2, 110.0, 59.6, 53.2, 39.1, 38.9, 33.8, 31.0, 26.9, 21.6. HRMS (ESI), m/z 424.2643 [M+H]⁺ and 446.2475 [M+Na]⁺ (Calcd for C₂₅H₃₃N₃O₃, 423.2522).

(S)-6-((2,4'-dimethoxy-3',5-dipropyl-[1,1'-biphenyl]-3-yl)amino)-6-oxohexane-1,5-diaminium chloride (HH-D)

Compound 5 (230 mg, 0.91 mmol) was dissolved in a solution of a 3.8 M solution of HCl (gas) in ethyl acetate (10 mL, 38 mmol) at 0°C and the mixture was stirred for additional 4 hr at 0°C. Hexane (10 mL) was added and the precipitate was filtered to afford compound D (170 mg, 95%) as a yellow solid. ¹H-

NMR (400 MHz, MeOD- d_4), δ 7.82 (s, 1H), 7.297.23 (m, 2H), 6.95-6.82 (m, 2H), 6.01-5.96 (m, 2H), 5.11-4.99 (m, 4H), 4.29 (m, 1H), 3.47-3.37 (m, 10H), 2.96 (m, 2H), 2.04-2.01 (m, 2H), 1.76-1.73 (m, 2H), 1.61-1.59 (m, 2H). ^{13}C -NMR (100 MHz, MeOD- d_4), 167.4, 154.6, 147.5, 137.3, 137.0, 135.9, 130.2, 129.9, 128.5, 128.0, 127.3, 127.1, 127.0, 121.3, 114.8, 114.2, 110.0, 59.6, 54.5, 53.2, 39.4, 38.9, 33.8, 31.0, 26.9, 21.6. HRMS (ESI), m/z 438.2760 $[\text{M}+\text{H}]^+$ and 460.2572 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{26}\text{H}_{35}\text{N}_3\text{O}_3$, 437.2678).

Animals

Male C57BL/6 mice (20-23 g BW) and male Sprague Dawley (SD) rats (220-260 g BW) were obtained from the Department of Laboratory Animal Science, Peking University Health Science Center. The animals were given free access to standard diet and tap water. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Peking University Health Science Center following the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals (Permit Number: LA2013-69).

Transient middle cerebral occlusion (tMCAO)/reperfusion model

The tMCAO models in male C57BL/6 mice and Sprague Dawley (SD) rats were established as previously described (Chiang et al., 2011). Briefly, animals were anaesthetized with chloral hydrate (5% for mouse and 10% for rat, 350 mg/kg, *i.p.*) and fixed in the supine position. The body temperature was maintained at 37°C with a warm surgery pad. The right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were surgically exposed, then a siliconized nylon filament with blunted tip was introduced into the ICA through an incision in the external carotid artery and gently advanced 10 ± 1 mm for mice and 20 ± 2 mm for rats until the tip reached the origin of the middle cerebral artery. After the occlusion (1 hr for mice; 1.5 hr for rats), the filament was gently removed to restore blood flow (re-perfusion). Immediately then after, the animals received the treatment by intravenous injection (*i.v.*) with the compounds (HH-A to D) and honokiol. The new compounds were dissolved in saline (1 mg/ml) and honokiol was dissolved in DMSO (10 mg/ml) then adjusted the concentration (1 mg/ml) by saline. The injection volume was 100 μL /25 g BW for mice and 250 μL /250 g body BW for rats. In the sham group, only blood vessels were separated, and no plug was inserted. Twenty-four hours after re-perfusion, the animals were perfused thoroughly with 50 mL cold saline through the left atrium under anesthetized condition. The brains were collected for further histological and biochemical analyses.

Determination of the cerebral infarct volume and water content of the brain

Five coronal brain sections of uniform thickness (2 mm for mouse; 4 mm for rat) were cut with a brain section mold (YUYAN, China). The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Merk) solution at 37°C for 30 min in dark. After staining, the slices were fixed in 4% paraformaldehyde at 4°C overnight. The infarct region was shown by white color and none infarct region by bright red. The stained brain sections were digitized with a digital camera (Powershot G12, Canon), and the images were

analyzed with the Photoshop software. The proportion of the infarct volume were calculated according to the following equation:

$$\text{The proportion of infarct volume (\%)} = \frac{\text{infarct volume} - (\text{whole brain volume} - \text{contralateral hemisphere volume} \times 2)}{\text{contralateral hemisphere volume}} \times 100\%$$

The whole brain was weighed, then dried at 120°C for 8 hr. and weighed again. The brain water content was calculated by the formula as follows:

$$\text{The brain water content (\%)} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\%$$

Neurobehavioral evaluation

Neurologic deficit scores were blindly evaluated according to a seven-point scale evaluation criterion based on a standard scoring system (Zhang et al., 2005): 0, no neurologic deficit; 1, failure to extend left forepaw fully; 2, no turning behavior, fixed tilt to the right when lifting tail in the air; 3, make a big turn to the side of the paralysis; 4, turn to paralytic side, like biting tail shape; 5, cannot walk spontaneously or fall to the paralyzed side; 6, loss of consciousness or no righting reflex; ≥ 2 points without death was a valid model.

Histology staining

Histology staining was performed on 3 mm paraffin sections of the rat brain tissue. TUNEL/DAPI (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling/4',6-diamidino-2-phenylindole) staining was carried out according to the manufacturer's protocols (Wuhan servicebio technology Co., Ltd China). The staining was digitized with a Nikon fluorescence microscope (NIKON ECLIPSE C1) and the images were analyzed with Case Viewer slice scanning software (Wuhan servicebio technology Co., Ltd).

Sample preparations for Western blot analysis

Brain tissues (the infarct regions from rats) were homogenized in ice-cold PBS, and centrifuged at 20,000 x g for 20 min at 4°C. The supernatants were discarded and the pellets were homogenized in RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS. Beyotime, China). The samples were incubated in ice for 30 min and centrifuged at 20,000 x g for 30 min at 4°C. The supernatants were collected for Western blot. Protein contents of the samples were determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, USA).

Western blot analyses

The samples were separated with 10% SDS-PAGE gels and transferred to nitrocellulose membranes in transfer buffer. Membranes were blocked with 5% non-fat milk dissolved in TBST (0.05% Tween-20) and incubated, respectively, with rabbit anti-Keap1 (1:500; CST, USA), rabbit anti-HO-1 (1:500; CST, USA), rabbit

anti-Nrf2 (1:500; Bioss, China), rabbit anti-HNE (1:1000; abcam, NK), mouse anti-Bax (1:1000; Bioss, China), mouse anti-Bcl-2 (1:1000; Bioss, China), mouse anti- β -actin (1:500; Santa Cruz, USA) overnight at 4°C. Membranes were washed 3 times with TBST and then incubated with the HRP-conjugated secondary anti-rabbit IgG or anti-mouse IgG (1:5,000; Zsbio, China) for 1 hr. at room temperature. Membranes were washed 3 times and the images of protein-antibody interaction were captured with a ChemiDoc XRS System (Bio-Rad). The images were analyzed for final determination of protein expression with Image J and normalized to the loading control β -actin.

Statistical analysis

Data were analyzed with GraphPad Prism version 8.0.2 software. All values were expressed as mean $\pm$ SD. Statistical analyses were carried out by Student *t*-test. Multiple group comparisons were performed using two-way ANOVA. Differences were considered significant for $P < 0.05$.

Results

Synthesis of honokiol derivatives HH-A-D

Synthesis of the compounds HH-A-D is described in Scheme 1. To the honokiol, a nitro group was introduced by aromatic nitration with fuming nitric acid, followed by nitro reduction with stannous chloride, amino acylation with Boc-protected L-Lysine and de-protection with hydrogen chloride afforded the hydrochlorides of aromatic amide (compound HH-A). The saturated-chain analogue (compound HH-B) was prepared by palladium-catalyzed hydrogenation of compound A. Methylation of the intermediate compound 3 with methyl iodide after separation generated the mono-methoxy analogue (compound 4) and dimethoxy analogue (compound 5). The target methoxy analogues (HH-C and HH-D) were obtained by Boc-deprotection of their precursors. The newly synthesized compounds HH-A, -B, -C, and -D were easily dissolved in water (>100 mg/mL water).

The protective effect of the new compounds against cerebral injury in tMCAO/R mouse model

The protected effects of the newly synthesized compounds HH-A-D against cerebral injury were investigated with transient middle cerebral artery occlusion/reperfusion (tMCAO/R) mouse model subjected to 1 hr ischemia followed by 24 hr reperfusion. Immediately after reperfusion, the animals received intravenous administration (i.v.) of the new compounds. In this study, we chose the dose of 0.1 mg/kg BW for all the new compounds based on our previous observations that a compound with the same chemical structure as compound HH-A at 0.1 mg/kg BW i.v. showed a significantly better protective effect in tMCAO/R mouse model compared with lower doses such as 10 and 20 μ g/kg BW i.v. (Zhang et al., 2018). For honokiol, a dose of 0.05 mg/kg BW (equal molar amount as compound HH-A) was used. The brains of the experimental animals were harvested 24 hr after the drug treatments. The infarct volume of the brain was measured and presented as % of the whole hemisphere brain volume (Fig. 1a). No cerebral infarction was seen in sham group. The infarct volume was $31.04 \pm 11.16\%$ in vehicle group (saline treatment), which was significantly reduced by treatments with HH-A ($9.17 \pm 1.96\%$, $n = 9$,

P<0.001), HH-B ($12.20 \pm 4.37\%$, n = 8, P<0.001) and honokiol ($16.95 \pm 2.55\%$, n = 6, P<0.05). Interestingly, the infarct volumes observed in the animals received HH-A and HH-B were significantly smaller than those received honokiol (Fig. 1a, P<0.001 and P<0.05, respectively). In addition, HH-A and HH-B also significantly reduced brain water content compared with vehicle group and honokiol group (Fig. 1b). In comparison, HH-D significantly reduced brain edema but showed no effect on infarct volume, while HH-C had no effects on both brain infarction and edema (Fig. 1a). Obviously, acyl amino modification on the parent core structure with L-lysine residue delivered good contribution to the brain protective activities in comparison with honokiol.

The neuroprotective effect of HH-A versus edaravone dexborneol on cerebral injury in tMCAO/R mouse model

Based on the distinct effects of HH-A, the following studies are focused on HH-A. We compared the neuroprotective effects of the HH-A with edaravone dexborneol in the tMCAO/R mouse model. Edaravone dexborneol, comprised of two active ingredients, has been shown as a novel neuroprotective agent with synergistic effects of anti-oxidant and anti-inflammatory in ischemic animal models (Wu et al., 2014). The tMCAO/R mice received treatments with HH-A (0.1 mg/kg BW i.v.) and edaravone dexborneol (3 mg/kg and 0.75 mg/kg BW i.v.) (Wu et al., 2014) immediately after the reperfusion. The neurological deficit scores were used to assess the potential therapeutic effects. Twenty-four hours after the treatments, a significant attenuation of the neurological deficit scores were observed in both groups of the mice received HH-A or edaravone dexborneol compared with the vehicle group (Fig. 2a). The infarct volume was $31.81 \pm 12.2\%$ in vehicle group, $3.73 \pm 1.5\%$ in HH-A treated group. No cerebral infarct was seen in sham group (Fig. 2b, c). These data, together with the data obtained above confirmed the effect of HH-A in reduction of brain damage upon ischemia occlusion (P<0.001 compared with vehicle group for both infarct volume and brain edema). Treatment with HH-A also showed a significantly better effect in reducing infarct volume compared with edaravone dexborneol ($11.00 \pm 3.84\%$, P<0.001) (Fig. 2c).

We also investigated the long-term effects of HH-A treatment on the survival rate and neurological deficit of tMCAO/R mice. Treatment with HH-A at 100 μ g/kg BW i.v. immediately after reperfusion resulted in higher survival rate compared with vehicle group during 14 days post tMCAO/R (Fig. 2d). The neurological behavior of the mice was severely impaired on the 1st day of tMCAO/R and recovered gradually. A significant attenuation of the neurological deficit scores were observed in mice received HH-A in comparison with the vehicle (Fig. 2e).

The neuroprotective effect of HH-A on cerebral injury in tMCAO/R rat model

To further validate the effect of HH-A, we used the tMCAO/R rat model. Immediately after reperfusion, the tMCAO/R rats received treatment with HH-A at 0.05, 0.25, 1 mg/kg BW i.v. once a day for 3 days. The treatments reduced the neurological deficit scores in a dose-dependent manner (Fig. 3a). Next, the neuroprotective effect of HH-A was investigated at the dosage of 0.25 mg/kg BW. The compound was given (i.v.) to the animals immediately after reperfusion and neurological scores were assessed 24 hr after the treatment. A significant attenuation of the neurological deficit scores was observed in the rats

received HH-A compared with the animals received vehicle (Fig. 3b). HH-A treatment also significantly reduced the infarct volume. The infarct volume was $50.33 \pm 12.63\%$ in vehicle group and reduced to 9.00% by HH-A treatment ($P < 0.001$, compared with vehicle group) (Fig. 3d). No cerebral infarct was seen in sham group (Fig. 3c). HH-A treatment also significantly reduced the brain water content compared with vehicle group (Fig. 3e).

The anti-oxidative and anti-apoptotic effects of HH-A through Nrf2/HO-1 pathways in rat

Experimental evidence suggested that the brain injury is mainly occurred during the period when oxygen is re-introduced into the brain tissue, called reperfusion injury. Ischemia-reperfusion leads to increased levels of oxidants that activate nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream protein such as hemo-oxygenase-1 (HO-1) to prevent oxidative stress and cell death. Under homeostatic conditions, Kelch-like ECH-associated protein (Keap1) binds to Nrf2 to maintain Nrf2 in inactive form (McCord, 1985, Farina et al., 2021).

To reveal the molecular mechanisms behind the neuroprotective effects of HH-A on ischemic cerebral injury, we examined Nrf2/HO-1 signaling pathways in the animals after HH-A treatment. Western blot analysis revealed a significantly decreased expression of Keap1 in the brains of tMCAO/R rat model; in contrast, the expression was unchanged in the HH-A treated animals (Fig. 4a). The level of Nrf2 was slightly increased in vehicle group, but was significantly increased in the HH-A treatment group (Fig. 4b). In agreement with the report of literature, expression of HO-1 was increased in vehicle group; while HH-A treatment resulted in a dramatic increase in HO-1 expression (Fig. 4c). HNE (4-hydroxynonenal) is a major end-product of oxidation of polyunsaturated fatty acids, and is an indicator of oxidative damage (Ayala et al., 2014). Western blot analysis showed significantly increased HNE expression in the cerebral ischemia/R injured rat; while the level of HNE was significantly lower in the HH-A treatment group (Fig. 4d).

Based on the fact that oxidative stress and apoptosis are closely linked pathophysiological phenomena (de Vries et al., 2013, Wu et al., 2020), we speculated if HH-A had some anti-apoptosis effect in tMCAO/R animal model. Western blot analysis revealed an increased expression of Bcl-2 (B-cell lymphoma 2) associated X (Bax) and a decreased expression of Bcl-2 in the tMCAO/R rat brain. Interestingly, HH-A treatment reduced the increase of Bax expression and reverted the decrease of Bcl-2 expression (Fig. 5a right panel). In addition, TUNEL staining showed a significantly reduced number of apoptotic cells in the HH-A treatment group compared with vehicle treatment in tMCAO rat brain (Fig. 5b, c).

Discussion

Introduction of amino group into the honokiol parent structure provided a good handle for further modification. Principally, acylation of the hydrophilic amino acid inserted into the honokiol parent structure should increase water solubility of the newly synthesized compounds (Scheme 1). All the 4 compounds (HH-A, -B, -C and -D) after modification became highly water soluble as expected. Particularly

the L-lysine modified analogue of honokiol, HH-A displayed the best neuroprotective effects on the tMCAO/R animal models.

We chose the tMCAO/R animal model is due to that this model has been considered as one of the most closely simulate human ischemic stroke (Fluri et al., 2015). The two major brain pathological changes i.e., infarct volume and edema were used to assess the effect of the compounds that were administrated by i.v. injection immediately after reperfusion. By comparing the parent honokiol structure with the 4 derivatives, we found that HH-A demonstrated higher pharmacological effects against cerebral ischemia/reperfusion injury (Fig 1a, b) and even better than edaravone dexborneol (Fig. 2b, c), though activity remains in HH-B after the double bond reduction on the side chain (Fig. 1a, b). These protected effects are correlated with improvement of brain functions as HH-A significantly reduced the neurological deficit scores, which was also seen with edaravone dexborneol treatment (Fig. 2a). These interesting data prompt us to study the underling mechanisms.

The pathophysiology of cerebral injury induced by ischemia/reperfusion (I/R) is complicated and involves numerous processes (Lin et al., 2016). Among these, oxidative stress and apoptosis are closely linked events (Stocchetti et al., 2015). Increased oxidant due to an imbalance between oxidants and antioxidants leads to the disruption of redox signals and causes molecular damage i.e., oxidative stress (Kalogeris et al., 2016). Oxidative stress is an important step on the path towards neuronal cell apoptosis and death after cerebral I/R injury. Edaravone was approved by FDA to treat amyotrophic lateral sclerosis (ALS) and is used to help people recover from stroke in Japan (Miyaji et al., 2015). The neuroprotective effect of edaravone was proposed via anti-oxidation and anti-inflammation (Edaravone Acute Infarction Study, 2003, Fujiwara et al., 2016, Enomoto et al., 2019, Kawasaki et al., 2020).

Nrf2 is a transcription factor which regulates the expression of a number of anti-oxidant and/or defense proteins (Vomund et al., 2017, Robledinos-Anton et al., 2019) and is a potential pharmacological target in alleviation of oxidative damage of the brain in ischemic stroke (Farina et al., 2021). At homeostatic conditions, Nrf2 is in an inactive form within cells via the negative regulator Keap1 (Yu and Xiao, 2021). Upon oxidative stress, Keap1/Nrf2 interaction is destructed; Nrf2 becomes stable and translocates into nucleus to initiate transcription of antioxidant genes expression including HO-1 (Loboda et al., 2016, Yamamoto et al., 2018). HO-1 is an important factor in intracellular antioxidant stress pathway related to redox balance (Chiang et al., 2021, Yachie, 2021).

We hypothesized that HH-A may exert its neuroprotective effect through anti-oxidation/anti-apoptosis mechanisms. Indeed, Western blot analysis found that HH-A did not change expression of Keap1, but significantly increased Nrf2 and consequently increased expression of HO-1 (Fig. 4a, b, c). These data strongly argue for an activity of HH-A in degradation of Nrf2 (Stewart et al., 2003). The anti-oxidative damage effect of HH-A was also evidenced by significantly decreased HNE level (Fig. 4d). In accordance with the anti-oxidation effect, we found that HH-A treatment significantly reduced the increased expression of Bax and reverted the decreased expression Bcl2 in the brains of the experimental rat brains. Bax is a pro-apoptotic regulator and Bcl-2 is an anti-apoptotic regulator (Pena-Blanco and Garcia-Saez,

2018, Patel et al., 2021). These results strongly suggested the anti-apoptotic effect of HH-A, which was confirmed by the significantly reduced number of apoptotic cells in the brain of tMCAO/R rat model (Fig. 5a, b, c).

In summary, rational designed modifications of the natural honokiol led to the dramatic favorable change in solubility, as water soluble is essential for a therapeutic drug. Most importantly, the new compound HH-A has demonstrated a strong neuroprotective efficacy in cerebral I/R animal models. Our experimental data strongly suggested that the neuroprotective efficacy of HH-A should be attributed to activation of Nrf2/HO-1 pathway. In consideration that pharmacological treatment of brain ischemia is still very limited, our compound has emerged as an attractive new drug candidate. In the clinical management of stroke patients, along with intravenous thrombolysis with r-tPA to remove blood clot and re-establishes blood supply of the brain, HH-A's anti-oxidation and anti-apoptosis effects is expected to prevent the oxidation stress after re-established oxygen supply to the damaged area.

Declarations

Acknowledgements

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Ethical Approval

The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Peking University Health Science Center following the guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals (Permit Number: LA2013-69).

Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

YYZ conceived and designed research. YYZ and PPZ conducted experiments. YYZ and XZ analyzed data. YYZ and YL wrote the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

Not applicable.

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Scheme 1

Scheme 1 is available in the Supplementary Files section.

Figures

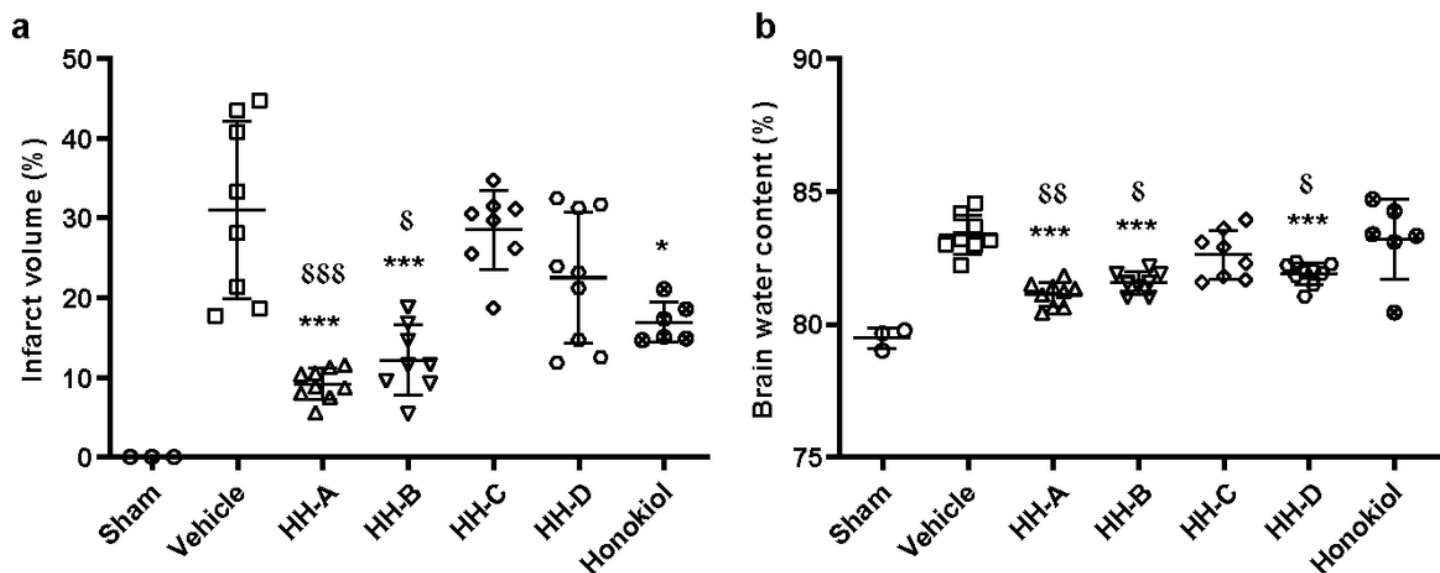


Figure 1

Protective effects of the new compounds against cerebral ischemia/reperfusion injury in tMCAO/R mouse model. (a) Effect of the new compounds on cerebral infarct volume 24 hr after reperfusion. (b) Effect of the new compounds on brain water content 24 hr. after reperfusion. Vehicle group: saline. Animals received each compound with a dose 0.1 mg/kg BW by intravenous injection (*i.v.*). The dose for honokiol was 0.055 mg/kg which is an equal molar amount as compound A. Student *t*-test: * $P < 0.05$, *** $P < 0.001$ versus vehicle group; δ $P < 0.05$, $\delta\delta$ $P < 0.01$, $\delta\delta\delta$ $P < 0.001$ versus honokiol group. Sham group: $n = 3$; Vehicle group $n = 8$; Compound HH-A group: $n = 9$, Compound HH-B group: $n = 8$; Compound HH-C group: $n = 8$, Compound HH-D group: $n = 9$; Honokiol group: $n = 6$.

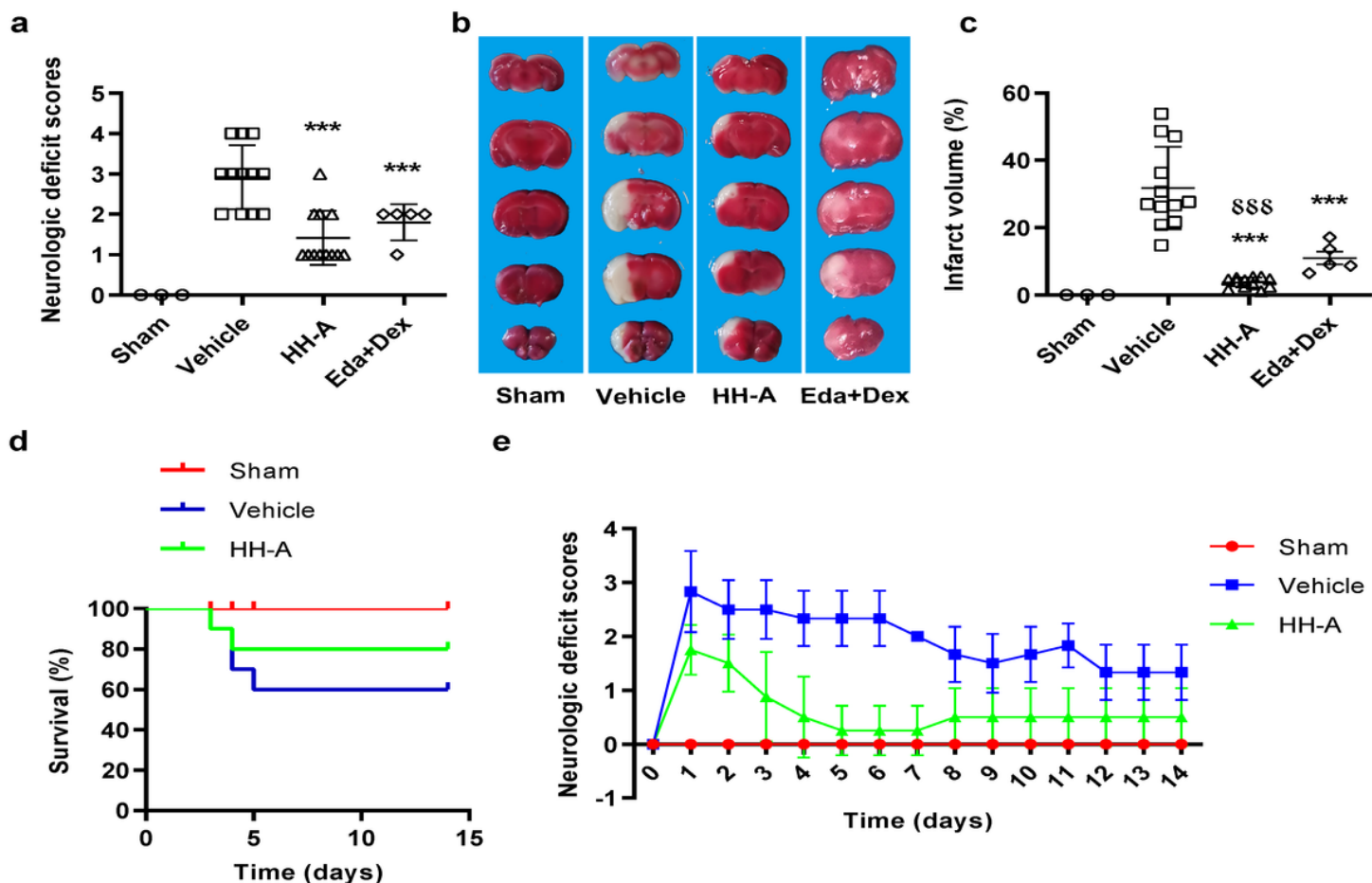


Figure 2

The neuroprotective effect of HH-A against cerebral ischemia/reperfusion injury in tMCAO/R mouse model. (a) Effect of HH-A and edaravone (Eda) dexborneol (Dex) on neurological deficit scores. Student *t*-test: *** $P < 0.001$ versus Vehicle group. Sham group: $n = 3$; Vehicle group $n = 12$; Compound HH-A group: $n = 12$, Eda Dex group: $n = 5$. (b) Representative of TTC-staining of the mice brain sections collected 24 hr. after reperfusion. The white region represents the infarct area and the red region viable tissue. (c) Effect of HH-A and Eda Dex on cerebral infarct volume in mice 24 hr. after reperfusion. Student *t*-test: *** $P < 0.001$ versus Vehicle group; $\delta\delta\delta$ $P < 0.001$ HH-A versus Eda Dex group. Sham group: $n = 3$; Vehicle group $n = 12$; Compound HH-A group: $n = 12$, Eda Dex group: $n = 5$. (d) Survival rate of the mice during 14 day-period of cerebral ischemia/reperfusion injury ($n = 10$). (e) Dynamic changes of neurobehavioral scores in the mice. Two-way ANOVA: $P < 0.01$ HH-A versus Vehicle group. Sham group: $n = 3$; Vehicle group $n = 6$; Compound HH-A group: $n = 8$. Vehicle group: saline. HH-A: 0.1 mg/kg BW.

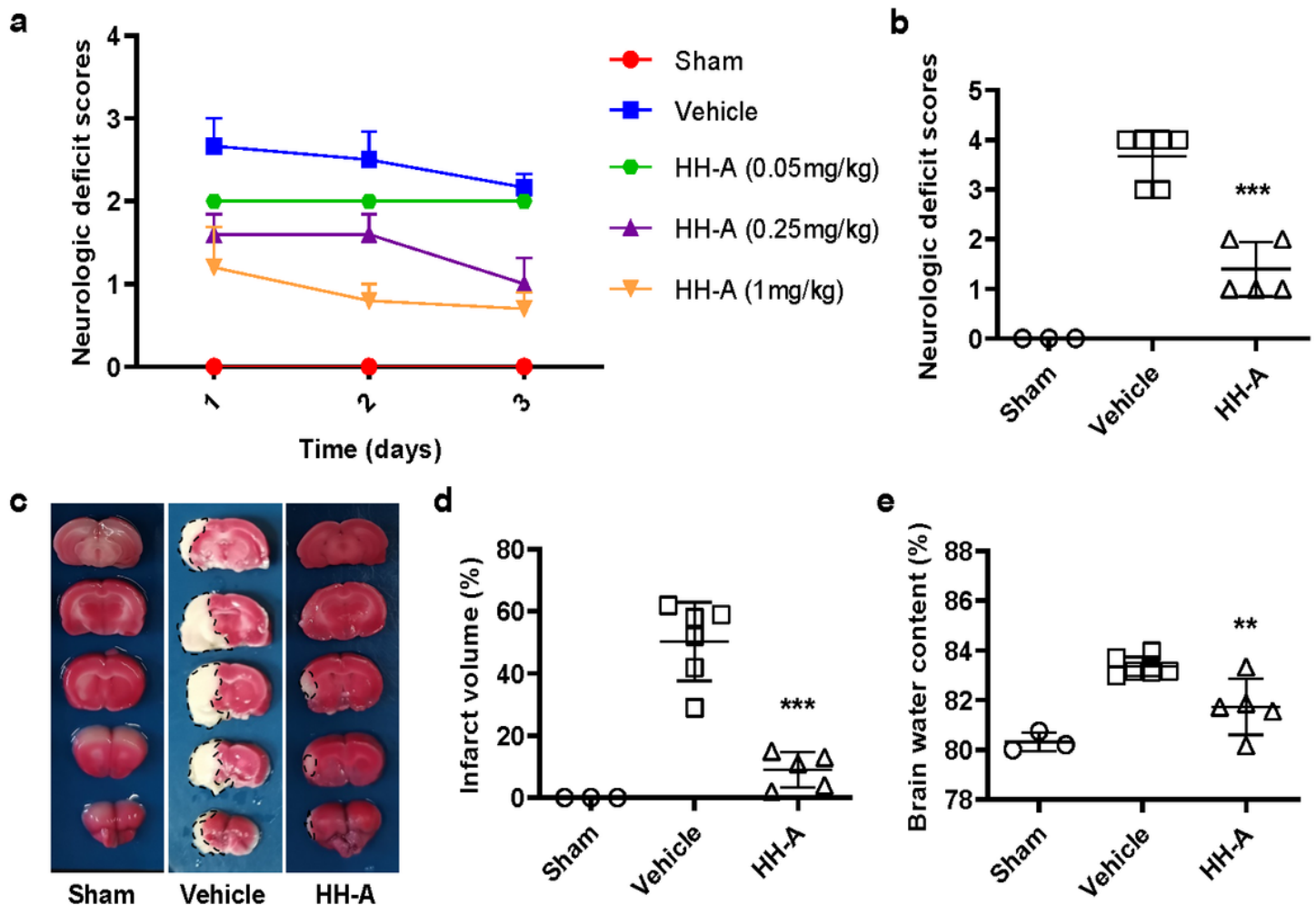


Figure 3

The neuroprotective effect of HH-A against cerebral ischemia/reperfusion injury in tMCAO/R rat model. (a) Dose dependent effect of HH-A (0.05, 0.25 and 1 mg/kg/BW) on neurological deficit scores in 3 day-period of cerebral ischemia/reperfusion. Two-way ANOVA: $P < 0.01$ HH-A (0.25 and 1 mg/kg/BW) versus Vehicle group. Sham group: $n = 3$; Vehicle group $n = 5$; Compound HH-A (0.05 mg/kg/BW) group: $n = 3$; Compound HH-A (0.25 mg/kg/BW) group: $n = 5$; Compound HH-A (1 mg/kg/BW) group: $n = 5$. (b) Effect of HH-A (0.25 mg/kg BW) *i.v.* on neurological deficit scores in rats 24 hr after the treatment. Student *t*-test: *** $P < 0.001$ versus vehicle group. Sham group: $n = 3$; Vehicle group $n = 6$; Compound HH-A group: $n = 5$. (c) Representative of TTC-stained rat brain slices collected 24 hr after HH-A treatment. The white region shows the infarct area and the red region viable tissue. (d) Effect of HH-A on cerebral infarct volume 24 hr after HH-A treatment. Student *t*-test: *** $P < 0.001$ versus vehicle group. Sham group: $n = 3$; Vehicle group $n = 6$; Compound HH-A group: $n = 5$. (e) Effect of HH-A on brain water content 24 hr after HH-A treatment. Student *t*-test: ** $P < 0.01$ versus vehicle group. Sham group: $n = 3$; Vehicle group $n = 6$; Compound HH-A group: $n = 5$. Vehicle group: saline.

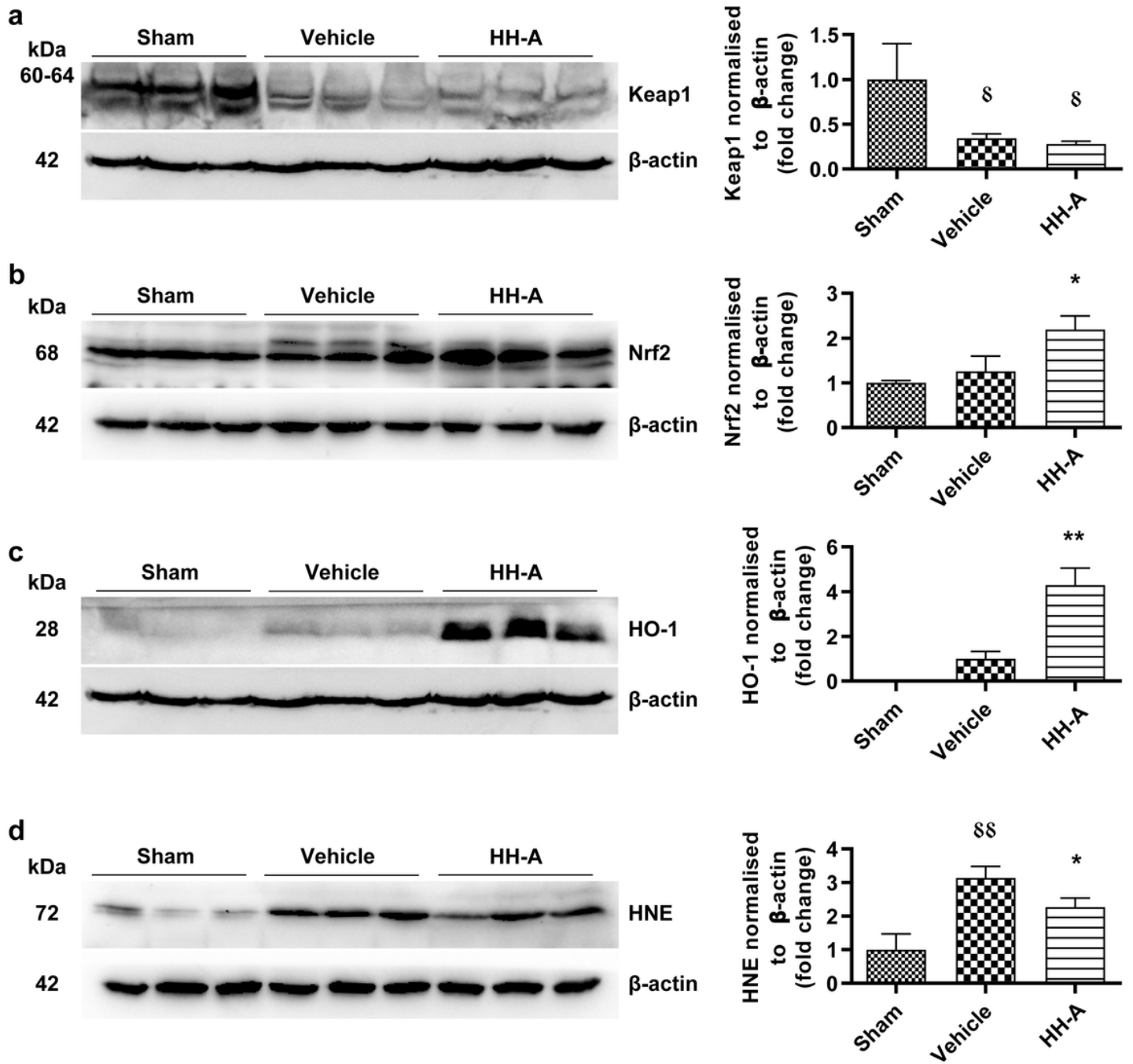


Figure 4

*Effect of HH-A on the expression of Keap1, Nrf2, HO-1 and HNE in the brains of tMCAO/R rat model. (a-d) Western blot analysis (left) and quantitative analysis (right) of Keap1 (a), Nrf2 (b), HO-1 (c) and HNE (d) in the brains 24 hr after of tMCAO/R (n = 3). Student *t*-test: δ $P < 0.05$, $\delta\delta$ $P < 0.01$ compared with Sham group; * $P < 0.05$, ** $P < 0.01$ compared with Vehicle group.*

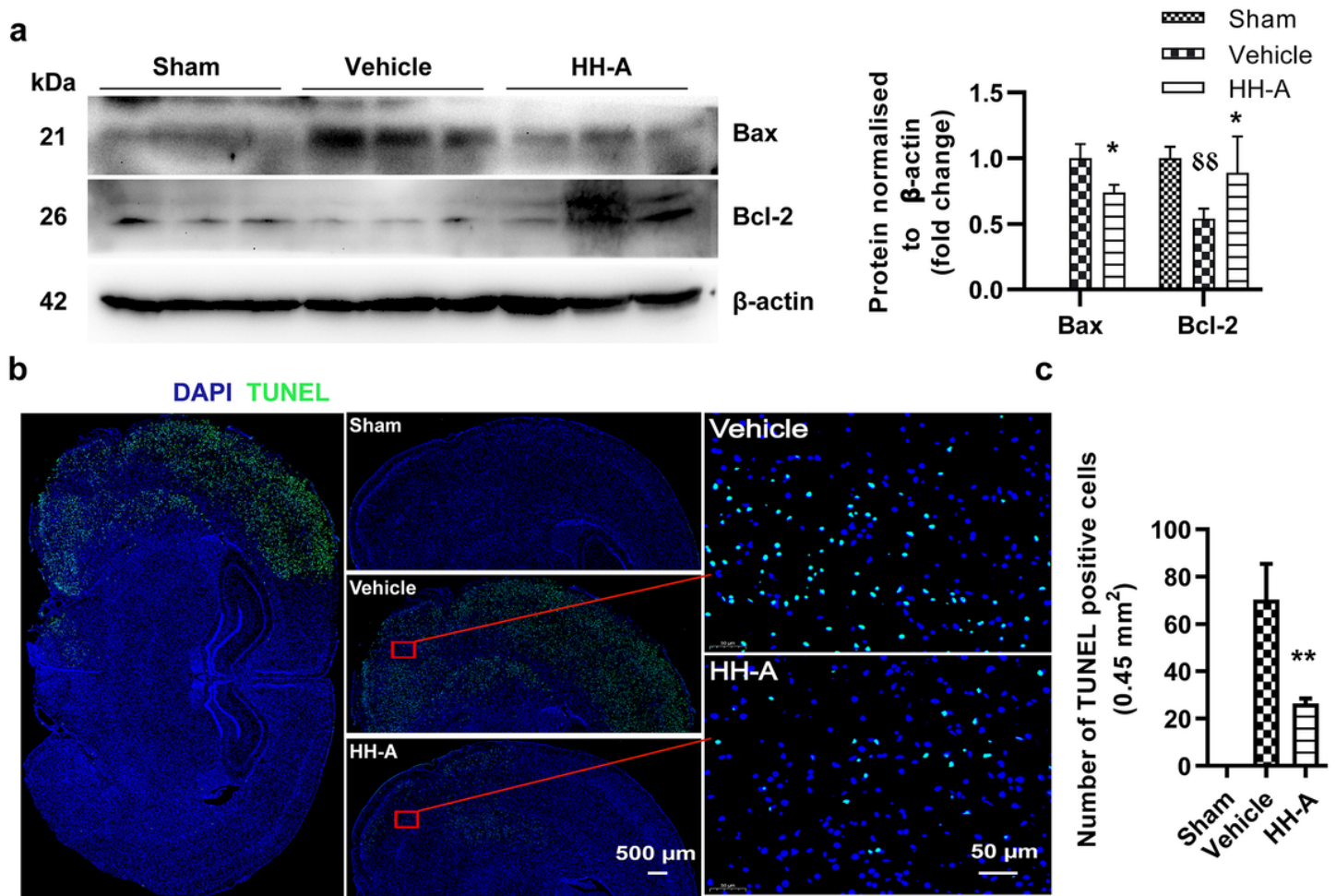


Figure 5

*The anti-apoptosis effect of HH-A against cerebral ischemia/reperfusion injury in tMCAO/R rat model. (a) Right panel: Western blot of Bax and Bcl-2 expression 24 hr after reperfusion. Left panel: quantitative analysis. Student *t*-test: * $P < 0.05$ compared with Vehicle group; $\delta\delta$ $P < 0.01$ compared with Sham group ($n = 3$). (b) TUNEL-stained apoptotic cells (green) in the brain sections collected from sham, vehicle and HH-A group 24 hr after reperfusion. The nuclei were counterstained with DAPI (blue). (c) The number of TUNEL-stained apoptotic cells (green, 0.45 mm²) in the ischemic brain 24 hr after reperfusion. Student *t*-test: ** $P < 0.01$ compared with Vehicle group ($n = 3$).*

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Scheme1.tif](#)
- [Supplementalfile.doc](#)