

### Supporting Information

Simply combining Fasudil and Lipoic acid in a novel multitargeted chemical entity  
potentially useful in central nervous system disorders

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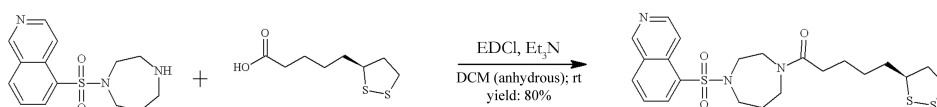
## 1. General

All reagents were commercially available and were used without a further purification unless stated. Column chromatography was generally performed on silica gel (200-300 mesh) and reactions were monitored by thin layer chromatography (TLC) using UV light and phosphomolybdic acid to monitor the course of the reactions. The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR data were recorded on Bruker 400 M spectrometer, given chemical shift as ppm referenced to  $\text{CDCl}_3$  with 7.26 for  $^1\text{H}$  and 77.10 for  $^{13}\text{C}$ , and to  $\text{DMSO}-d_6$  with 2.50 for  $^1\text{H}$  and 49.0 for  $^{13}\text{C}$ . In the case of multiplet, the signals were reported as intervals. Signals were abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants were expressed in Hz. In the HPLC, MeOH and  $\text{H}_2\text{O}$  were employed as two flow phases while flow rate was set at 1 mL/min. HPLC was run with 90% MeOH for 10 mins at 254 nm. Tetrahydropalmatine (No.110726-200610, purity 98.0%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade ammonium acetate was purchased from Aladdin (Shanghai, China). HPLC-grade acetonitrile and methanol purchased from Merck (Darmstadt, Germany) were used for HPLC analysis and plasma sample preparation. All other reagents were of analytical grade. Dullbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibico-BRL (Grand Island, NY, USA). 3-(3, 4- dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated.

## 2. Synthesis

Synthesis of (R)-5-(1, 2-dithiolan-3-yl)-1-(4-(isoquinolin-5-ylsulfonyl) -1,4-diazepan-1-yl) pentan-1-one (L-F 001). To a mixture of LA (100 mg) and Fasudil·HCl (158 mg, 1.0 equiv) in dry dichloromethane (5 mL) was injected triethylamine (0.18 mL, 2.5 equiv), and then added EDCI (140 mg, 1.5 equiv) in one portion. The reaction solution was stirred at room temperature under Ar atmosphere overnight before it was diluted with EtOAc (30 mL), and quenched with sat. aq.  $\text{NH}_4\text{Cl}$  (3 mL).

The phases were separated, and the aqueous phase was extracted with EtOAc (5 mL×3). The combined phases were washed with brine (3 mL×1), dried with Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The residue was purified by silica column chromatography (methanol/dichloromethane 1/30-1/10) to give a yellow sticky oil (186 mg, 80%). HPLC RT=2.00 mins; purity: 98%. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.36 (s, 1H), 8.69-8.67 (m, 1H), 8.40 (d, J = 6.1 Hz, 1H), 8.33 (dd, J = 7.3, 4.3 Hz, 1H), 8.22 (dd, J = 8.2, 3.3 Hz, 1H), 7.73-7.68 (m, 1H), 3.75-3.69 (m, 1H), 3.65-3.56 (m, 4H), 3.50-3.48 (m, 1H), 3.43 (dd, J = 11.0, 5.1 Hz, 3H), 3.36 (t, J = 6 Hz 1H), 3.21-3.05 (m, 3H), 2.48-2.41 (m, 2H), 2.31-2.19 (m, 2H), 2.00-1.96 (m, 5.5 Hz, 2H), 1.92-1.87 (m, 1H), 1.70-1.62 (m, 5H), 1.49-1.38 (m, 2H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 172.1, 171.9, 153.2, 145.0, 144.9, 134.0, 133.9, 133.5, 133.0, 132.8, 131.3, 129.0, 125.8, 117.2, 117.1, 56.3, 56.3, 49.8, 49.5, 48.7, 48.1, 47.6, 46.7, 46.6, 44.4, 40.1, 38.4, 34.6, 32.7, 32.3, 29.1, 28.8, 27.7, 24.6, 24.5; IR (KBr, cm<sup>-1</sup>) : ν 2930, 1640, 1428, 1326, 1212, 1146; LRMS([M+H]<sup>+</sup>): 480.2.



Scheme 1. Synthesis of L-F 001.

### 3. Evaluation of the ROCK inhibitory activities

The IC<sub>50</sub> value of L-F 001 for inhibition of ROCK 1/2 kinase was evaluated by a fluorescence resonance energy transfer based Z'-LYTE kinase assay kit as previously reported<sup>1</sup> (Invitrogen, Carlsbad, CA). The reaction was performed on a 384-well plate with a 10 μL reaction volume per well containing 2 μM peptide substrate in 50 mM HEPES, pH 7.5, 0.01% Brij-35, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, and an appropriate amount of ROCK 1/2 kinase with a serial 3-fold dilution of L-F 001. The final reaction concentration of ATP was 75 μM. After 1 h incubation, a reaction was developed and terminated, and the fluorescence ratio was calculated according to the manufacturer's protocol. A dose-response curve was fitted using Prism 5.0 (GraphPad

Software Inc., San Diego, CA).

#### 4. Binding conformation of L-F 001 and ROCK 1

The X-ray crystallographic structure of ROCK 1 (PDB ID: 2ESM) was retrieved from RCSB Protein Data Bank (PDB). The binding pocket was predicted by MOE (Molecular Operating Environment, Canada) and MOE's default docking protocol was employed to dock L-F 001 and Fasudil to binding pocket.

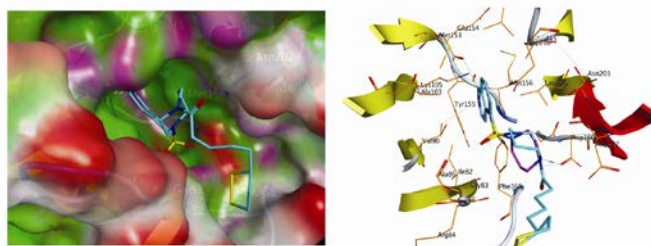


Fig. S1. Binding conformation of L-F 001 in the ATP pocket of ROCK 1 (PDB ID: 2ESM). L-F 001 is displayed in blue and Fasudil is labeled in red, while the protein key residues are reported in orange.

#### 5. Cell culture and drug treatment

HT 22 murine hippocampal neuronal cells were maintained in DMEM supplemented with 10% (v/v) FBS and incubated at 37 °C under 5% CO<sub>2</sub>. To study the protective effect of LA, Fasudil, L-F 001 on Glu-induced neuronal death, cells were seeded in 96-well plates (10,000 cells/well), and 3 wells were used for each treatment group. The fresh solution of LA, Fasudil, L-F 001 (100 mM in DMSO) was diluted in DMEM supplemented with 10% (v/v) FBS immediately before adding to each well at the desired final concentrations (3, 10, 30 μM). For the experiments, cells were preincubated with LA, Fasudil, L-F 001 for 30 min, and followed by treatment with glutamate (Glu) for 24 h. Control group was treated with 0.1% (v/v) DMSO as vehicle control.

#### 6. Reduction of the actin stress formation

HT 22 cells were washed with phosphate-buffered saline (PBS), fixed with 4%

paraformaldehyde for 15 min, and permeabilized with 0.1% TritonX-100 for 5 min at room temperature. Next, the cells were incubated with rhodamine-phalloidin (Cytoskeleton, Denver, CO) for 30 min. After washed for 3 times, a Hoechst 33258 solution (Sigma–Aldrich, St. Louis, MO) was added to counterstain the nucleus for 15 min in the dark. Finally, cells were placed under a laser scanning confocal microscope (LSM710, Carl Zeiss, Germany) for image acquisition.

## **7. Measurement of vasorelaxant effects**

Male Sprague-Dawley (SD) rats ( $200 \pm 20$  g) were supplied by the Center of Experimental Animals, Sun Yat-sen University (Guangzhou, China, Certificate No. SCXK 20110029). The rats were housed in standard cages under controlled temperature conditions of  $21\text{ }^{\circ}\text{C}$  with free access to food and water until 12 h prior to experiments. The pharmacokinetic study was approved by Sun Yat-sen University of Animal Experimentation Ethics Committee. The vasorelaxant effects were examined by a rat thoracic aorta assay. Briefly, animals were anesthetized with 0.1% sodium pentobarbital (50 mg/kg). The aorta was quickly isolated and cleaned of fat and adherent connective tissue. Aortic rings 2–3 mm in length were prepared and mounted horizontally in 5 mL organ bath containing the Krebs–Henseleit (K-H) solution at  $37^{\circ}\text{C}$ , bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . The ring was allowed to equilibrate for 30 min under 2 g resting tension. Then, KCl (80 mM) was used to induce a similar sustained contraction of thoracic aorta rings with a peak tension in each group. Following, LA, Fasudil, L-F 001 were cumulatively added into the organ bath in 10 min intervals respectively. DMSO was also added into the bath as a control. The contractile responses were expressed as a percentage of the responses following and before the application of the testing compounds. Results are expressed as mean  $\pm$  S.E.M. Statistical analysis was performed with repeated measures of the ANOVA. Differences were accepted as statistically significant at P values  $< 0.05$ .

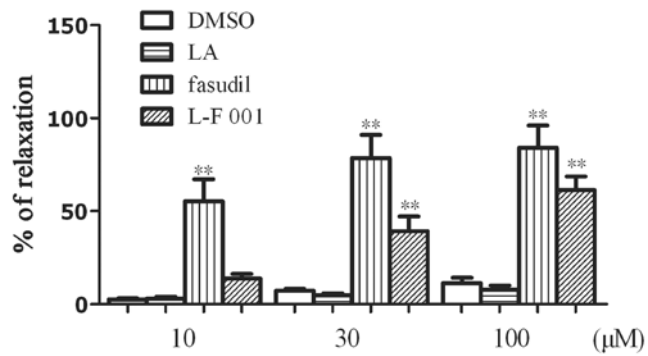


Fig. S2. L-F 001 dilated aortic rings pre-contracted by KCl. L-F 001, Fasudil, LA (10, 30, 100 μM) were administered cumulatively to the aorta precontracted by KCl (80 mM). All values represent mean  $\pm$  S.E.M. \*\*  $p < 0.01$  vs. Control group (n = 3).

## 8. Measurement of ROS

Intracellular ROS formation was measured by fluorescence using H<sub>2</sub>DCF-DA. This non-fluorescent dye freely permeates into cells, where it de-esterifies to form the ionized free acid (dichlorofluorescein), which reacts with ROS to form the fluorescent 2',7'-dichlorofluorescein (DCF)<sup>2</sup>. Briefly, after treatment, cells were washed and then stained with H<sub>2</sub>DCF-DA in serum-free medium for 30 min at 37 °C in the dark. DCF fluorescence was analyzed by flow cytometry. Quantitative analysis was presented as the percentage of DCF-positive cells in each group.

## 9. Estimation of intracellular GSH

Intracellular GSH concentration was tested by a GSH assay kit (48 T, Nanjing Jiancheng, China). By reacting with dithiobisnitrobenzoic acid, reduced glutathione (GSH) could form a yellow compound, which is quantifiable at 405 nm and reflect the content of the reduced GSH indirectly. In brief, after treatment, whole-cell lysate was prepared and tested according to manufacturer's instructions. All GSH values were normalized to per μg protein of each sample.

## 10. Protection against Glu-induced toxicity

Cell viability was measured by MTT assay. Cells were seeded on 96-well plates at a density of  $1 \times 10^4$  for 24 h. After treated with LA, Fasudil, L-F 001 (3-30  $\mu\text{M}$ ) and Glu as mentioned, 10  $\mu\text{L}$  of MTT (5 mg/mL) was then added to each well and the mixture was incubated for 2 h at 37  $^\circ\text{C}$ . MTT reagent was then replaced with DMSO (100  $\mu\text{L}$  per well) to dissolve the formazan crystals. The mixture was shaken at 37  $^\circ\text{C}$  for 15 min, and then the absorbance was determined at 570 nm using a microplate reader. Results were expressed as the percentage of MTT reduction and the absorbance of control cells was set as 100%.

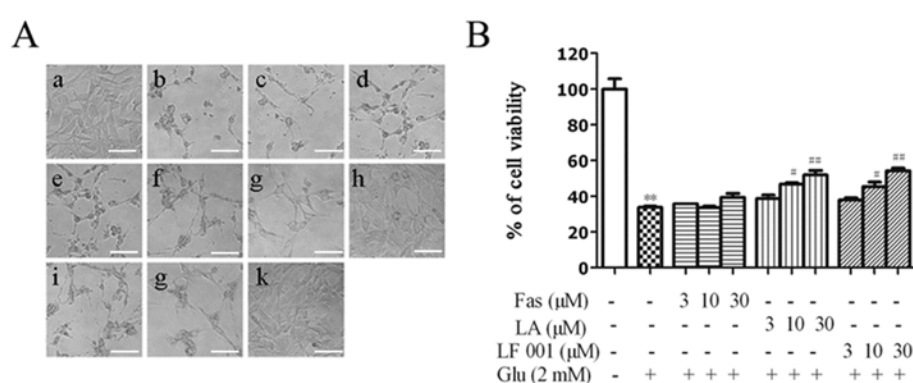


Fig. S3. LF-001 dose-dependently prevents HT 22 cell death caused by Glu. HT 22 cells were pretreated with LA, Fasudil or L-F 001 for 0.5 h followed by exposed to Glu for 24 h. A) HT 22 cells were photographed under phase-contrast optics; B) Cell viability was tested by MTT assay, a) CT, b) Glu, c-e) Fasudil (3, 10, 30  $\mu\text{M}$ ), f-h) LA (3, 10, 30  $\mu\text{M}$ ), i-k) L-F 001(3, 10, 30  $\mu\text{M}$ ). \*\* $P < 0.01$  compared with the control (n = 6); # $P < 0.05$  compared with the Glu group (n = 6); ## $P < 0.01$  compared with the control (n = 6).

### 11. Evaluation of the BBB permeability of L-F 001

BBB penetration of compounds was evaluated using a parallel artificial membrane permeation assay (PAMPA). The porcine brain lipid extract (PBL) was obtained from Avanti Polar Lipids. The donor microplate and the acceptor microplate were both obtained from Millipore. The acceptor 96-well microplate (Corning Incorporated) was



filled with 300  $\mu$ L of PBS/EtOH (7:3), and the filter membrane was impregnated with 4  $\mu$ L of PBL in dodecane (20 mg/mL). Compounds were dissolved in DMSO at 5 mg/mL and diluted 50-fold in PBS/EtOH (7:3) to achieve a concentration of 100 mg/mL, 200  $\mu$ L of which was added to the donor wells. The acceptor and donor plate were assembled to form a “sandwich”, and incubated for 10 h at room temperature. After incubation, the donor plate was carefully removed and the concentration of compound in the acceptor wells was determined using a UV plate reader. Assay validation was made by comparing experimental permeabilities of 13 commercial drugs with reported values. A plot of experimental data versus bibliographic values gave a good linear correlation,  $P_e$  (exp.) = 1.4574  $P_e$  (bibl.) -1.0773 ( $R^2 = 0.9427$ ). From this equation and considering the limit reported by Yang Sun *et al*<sup>4</sup>. The results were obtained by at least three independent runs, and the results are given as the mean  $\pm$  standard deviation.  $P_e$ , the effective permeability coefficient( $\text{cm}\times\text{s}^{-1}$ ), can be calculated as previously<sup>3</sup>.

## 12. Pharmacokinetics in rats

L-F 001 and Fasudil were administered to groups of three male rats by oral gavage (30 mg/kg). L-F 001 stock solution was prepared in Cremophor EL/Ethanol (10/90, v/v) at 3 mg/mL. The animals were killed by decapitation at  $T_{\text{max}}$  of each drug. Plasma and brain homogenate were collected at intervals up to 24 h and were stored until they were analyzed by LC/MS-MS.

Table S1 Pharmacokinetic parameters of L-F 001 after a single oral administration of 30 mg/kg, each value represents the mean  $\pm$  S.D. (n=6).

| Compound | $C_{\text{max}}$  | $T_{1/2}$ (h) | $T_{\text{max}}$ (h) | $AUC_{0\rightarrow t}$ (ng•h/mL) | $AUC_{0\rightarrow\infty}$ (ng•h/mL) |
|----------|-------------------|---------------|----------------------|----------------------------------|--------------------------------------|
| L-F 001  | 651.1 $\pm$ 380.4 | 1.3 $\pm$ 0.8 | 0.7 $\pm$ 0.4        | 793.6 $\pm$ 287.1                | 805.5 $\pm$ 286.1                    |

### 13. Preparation of plasma and brain homogenate samples

To a 30  $\mu\text{L}$  of the plasma sample, a 60  $\mu\text{L}$  of IS solution was added. Then the mixture was vortexed for 2 min and centrifugation at 13,000 rpm for 10 min at 4  $^{\circ}\text{C}$ . Subsequently, the supernatant liquor was centrifuged at 13,000 rpm for 5 min at 4  $^{\circ}\text{C}$ . Finally, same volume water was added to the supernatant liquor before analysis.

Brain homogenate was homogenized with 5 volumes of saline by a tissue homogenizer. A volume of 0.1 mL of tissue homogenizer was transferred to microcentrifuge tubes and isolated by centrifugation at 8000 rpm for 10 min. Next 30  $\mu\text{L}$  supernatant liquor was transferred and 60  $\mu\text{L}$  IS solution was added. The following procedure was same as the preparation of plasma.

### 14. Statistical analysis

All quantitative data and experiments described in this study were repeated at least three times. Data were presented as mean  $\pm$  S.D. of multiple independent experiments. Statistics were analyzed with one-way ANOVA followed by a least significant difference test (SPSS 17.0 software). Statistical difference was considered at  $P < 0.05$ .

### 15. References:

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- S2. Y. Huang, M. Jin, R. Pi, J. Zhang, M. Chen, Y. Ouyang, A. Liu, X. Chao, P. Liu, J. Liu, C. Ramassamy and J. Qin, *Neuroscience letters*, 2013, **535**, 146-151.
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# 16. <sup>1</sup>HNMR <sup>13</sup>CNMR spectra

