

## Electronic Supporting Information

### Structure-based Discovery of an Immunomodulatory Inhibitor of TLR1-TLR2 Heterodimerization from a Natural Product-like Database†

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**1. Materials and reagents.** All chemicals were purchased from Sigma-Aldrich and were used as acquired. Pam<sub>3</sub>CSK<sub>4</sub> (N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteiny]-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-l ysine.3HCl) and rhodamine-labeled Pam<sub>3</sub>CSK<sub>4</sub> were purchased from InvivoGen (San Diego, CA, USA). Lipofectamine<sup>®</sup> 2000 was purchased from Invitrogen (Carlsbad, CA, USA). pNF-κB-luc reporter gene was purchased from Beyotime (Beyotime, Shanghai, China). pRL-TK vector and dual-luciferase reporter assay system were obtained from Promega Corporation (Madison, WI, USA). pZERO-TLR1 was purchased from InvivoGen (San Diego, CA) and pCMV-Flag-TLR2 was purchased from Addgene. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All compounds with the purity of 92-98% were purchased from InterBioScreen (Moscow, Russia) and dissolved in dimethyl sulfoxide (DMSO).

## **2. Molecular docking and virtual screening.**

**Model construction.** The initial model of TLR1/2 was derived from the X-ray crystal structure of the TLR1/2 heterodimer co-crystallized with the a tri-acylated lipopeptide (PDB: 2Z7X),<sup>1</sup> using the molecular conversion procedure implemented in the ICM-pro 3.6-1d program (Molsoft).<sup>2</sup> The molecular conversion procedure implemented in ICM-pro 3.6-1d program can read, build, convert, refine, analyze and superimpose molecules, plus provide target evaluation to generate three dimensional models. Hydrogen and missing heavy atoms were added to the receptor structure, also atom types and partial charges were assigned. The model was then subjected to local energy minimization to identify the optimal position by using the ICM biased probability Monte Carlo algorithm<sup>3</sup> and analytical derivatives in the internal coordinates. The optimization gradient was 45 kcal/mol/Å<sup>3</sup>.

**High throughput molecular docking.** A chemical library containing over 90,000 natural product or natural product-like compounds (ZINC natural product database) was docked to the molecular model of TLR1/2 *in silico*. Molecular docking was performed using the virtual library screening (VLS) module in the ICM-Pro 3.6-1d program (Molsoft). In the ICM fast docking and VLS procedure, the receptor all-atom model was converted into energy potential maps calculated on a fine 3D grid (0.5 Å cell). The grid potential maps account for van der Waals, hydrogen-bonding, hydrophobic, and electrostatic interactions between ligand and receptor. The search area for molecular docking was restricted to the interaction domain of TLR1/2. Each compound in the library was assigned the MMFF<sup>4</sup> force field atom types and charges then subjected to Cartesian minimization. During the docking analysis, the ligand was represented by an all-atom model and considered fully flexible in the potential field of the receptor, the binding pose and internal torsions were sampled by the BPMC minimization procedure, which involved local energy minimization after each random move. Each compound was docked to the protein complex binding pocket and a score from the docking was assigned to each compound according to the weighed component of the ICM scoring function (see below). Each compound was docked three times to ensure the convergence of the Monte Carlo optimization, and the

minimum score of each ligand from the three independent docking experiments was retained and used for ranking. The docking procedure takes about 30 s of time per compound on an Intel Xeon 2.8 GHz CPU using a 100 processor Linux cluster. A permissive cut-off score of  $-30.0$  was chosen in order to weed out low-affinity ligands and to reduce the number of compounds tested *in vitro*. 17 compounds were purchased for *in vitro* biological testing.

ICM full-atom ligand-receptor complex refinement and scoring. Once the ligand-receptor complexes are generated by molecular docking, they have to be subjected to complex refinement and scoring. According to the ICM method,<sup>5</sup> the molecular system was described using internal coordinates as variables. Energy calculations were based on the ECEPP/3 force field with a distance-dependent dielectric constant. The biased probability Monte Carlo (BPMC) minimization procedure was used for global energy optimization. This procedure consisted of four iterative steps. The BPMC global-energy-optimization method consists of 1) a random conformation change of the free variables according to a predefined continuous probability distribution; 2) local-energy minimization of analytical differentiable terms; 3) calculation of the complete energy including non-differentiable terms such as entropy and solvation energy; 4) acceptance or rejection of the total energy based on the Metropolis criterion and return to step (1). The binding between the small molecules and TLR1/2 were evaluated with a full-atom ICM ligand binding score<sup>6</sup> from a multi-receptor screening benchmark as a compromise between approximated Gibbs free energy of binding and numerical errors. The scoring function should give a good approximation of the binding free energy between a ligand and a receptor, as well as a function of different energy terms based on a force-field. The ICM scoring function is weighed according to the following parameters (i) internal force-field energy of the ligand, (ii) entropy loss of the ligand between bound and unbound states, (iii) ligand-receptor hydrogen bond interactions, (iv) polar and non-polar solvation energy differences between bound and unbound states, (v) electrostatic energy, (vi) hydrophobic energy and (vii) hydrogen bond donor or acceptor desolvation. The lower the ICM score, the higher the chance the ligand is a binder. The score was calculated by:

$$S_{\text{bind}} = E_{\text{int}} + T\Delta S_{\text{Tor}} + E_{\text{vw}} + \alpha_1 E_{\text{el}} + \alpha_2 E_{\text{hb}} + \alpha_3 E_{\text{hp}} + \alpha_4 E_{\text{sf}}$$

whereas  $E_{\text{vw}}$ ,  $E_{\text{el}}$ ,  $E_{\text{hb}}$ ,  $E_{\text{hp}}$ , and  $E_{\text{sf}}$  are van der Waals, electrostatic, hydrogen bonding, and nonpolar and polar atom solvation energy differences between bound and unbound states, respectively.  $E_{\text{int}}$  is the ligand internal strain,  $\Delta S_{\text{Tor}}$  is its conformational entropy loss upon binding, and  $T = 300$  K, and  $\alpha_i$  are ligand- and receptor independent constants.<sup>7</sup>

**3. Cell culture.** RAW 264.7 cells (mouse leukaemic monocyte macrophage cell line) and HEK293 cells (human embryonic kidney 293 cell line) were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 mg/mL), incubated at 37 °C in a 5% CO<sub>2</sub> humidified incubator, and passaged three times a week.

**4. TNF- $\alpha$  and IL-6 Quantikine<sup>®</sup> ELISA Assay.** RAW 264.7 cells were placed in 24-well plates at a density of 20,000 cells per well and grown overnight. After 24 h, cells were treated with vehicle, CU-CPT22 and compounds for 4 h, and were subsequently co-treated with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for an additional 12 h. The conditioned medium was collected and the levels of secreted TNF- $\alpha$  and IL-6 were determined by using immunoassay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, 50  $\mu$ L of Assay Diluent was added to each well, followed by adding 50  $\mu$ L of the conditioned medium in to the wells. The plate was incubated at 25 °C for 2 h to allow the secreted TNF- $\alpha$  or IL-6 to bind to the immobilized antibodies specific for TNF- $\alpha$  or IL-6. The wells were then washed with wash buffer 5 times and incubated with 100  $\mu$ L of TNF- $\alpha$  or IL-6 Conjugate at 25 °C for further 2 h. The plate was washed 5 times before the addition of 100  $\mu$ L of Substrate Solution to each well. After 30 min incubation at 25 °C in the dark, 100  $\mu$ L of Stop Solution was added to each well. The color intensity was measured by using SpectraMax M5 microplate reader (Molecular Devices, California, USA) with excitation at 450 nm and emission at 570 nm.

**5 Dual-luciferase reporter assay.** RAW 264.7 cells and HEK293T cells were seeded at the density of  $2 \times 10^5$  cells per well in a 24-well plate overnight. RAW 264.7 cells were co-transfected with 0.8  $\mu$ g pNF- $\kappa$ B-luc and 0.8  $\mu$ g pRL-TK as a transfection efficiency control. HEK293T cell were co-transfected with pZERO-TLR1, pCMV-Flag-TLR2, pNF- $\kappa$ B-luc and pRL-TK. The plasmids and Lipofectamine<sup>®</sup> agent were diluted in Opti-MEM reduced serum medium according to Lipofectamine<sup>®</sup> DNA transfection reagent protocol, respectively. The diluted DNA was mix together with diluted Lipofectamine<sup>®</sup> agent at the ratio of 1:1 and incubated at 25 °C for 20 min. 100  $\mu$ L of DNA-lipofectamine complexes was transferred to each well. After 4 h incubation, the medium was changed and the cells were cultivated in the completed medium for further 48 h. The cells were pre-treated with vehicle, CU-CPT22 or compound **1** in low FBS medium for 4 h before stimulation with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for 12 h. Cell lysates were collected by using passive lysis buffer according to the dual luciferase assay protocol. Sample light output was collected by using SpectraMax M5 microplate reader. Resulting data were aligned relative to pRL-TK values before normalization to control.

**6. Western blotting assay.** RAW 264.7 cells were harvested after pre-incubation with vehicle, CU-CPT22 or compound **1** for 4 h and stimulation with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for 12 h. Proteins samples were extracted with radio-immunoprecipitation assay buffer (RIPA) lysis buffer. 30  $\mu$ g of total proteins were separated by 10% SDS-PAGE followed by transferring onto a PVDF membrane. After blocking with 5 % non-fat milk, the membranes were probed with specific primary antibodies at 4 °C overnight and then corresponding secondary antibody at 25 °C for 1 h. The specific proteins bands were visualized by using an ECL advanced western blotting detection kit (Amersham Life Sciences, UK). The primary antibodies used were: anti-phospho-I $\kappa$ B $\alpha$ , anti-I $\kappa$ B $\alpha$ , anti-phospho-IKK $\alpha/\beta$ , anti-IKK $\alpha$ , anti-IKK $\beta$  and anti-GAPDH.

**7. Fluorescence polarization assay.** The binding experiment was performed in a 384-well black plate as described previously.<sup>8</sup> Briefly, 0.2  $\mu\text{M}$  TLR1 protein and 0.2  $\mu\text{M}$  TLR2 protein in 20  $\mu\text{L}$  Tris buffer (pH 7.2) was added to each well, followed by the addition of 0.4  $\mu\text{L}$  (1  $\mu\text{g}/\text{mL}$ ) rhodamine-labeled Pam<sub>3</sub>CSK<sub>4</sub> and incubation for 30 min at 25 °C. 2  $\mu\text{L}$  of compound **1** or CU-CPT22 at different concentrations were added to the wells, and the plate was incubated for further 30 min. Fluorescence polarization was measured by using FP Dual module in SpectraMax M5 microplate reader with excitation of 488 nm and emission of 566 nm.

**8. Immunoprecipitation assay.** RAW 264.7 cells were pre-treated with vehicle, CU-CPT22 or compound **1** for 4 h and then treated with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) in a 6-well plate for 12 h. The immunoprecipitation assay was performed according to the instructions from the manufacturer. 200  $\mu\text{g}$  of the cell lysates were mixed with 1  $\mu\text{g}$  of anti-TLR1 antibody and incubated overnight at 4 °C under constant rotation. To recover immunoprecipitated complexes, 50  $\mu\text{L}$  of protein A agarose beads were added to the lysates and incubated for additional 4 h of incubation at 4 °C under constant rotation. The beads were harvested by centrifugation, and the supernatant was discarded. The beads were washed with RIPA buffer for three times to remove non-specific binding protein. The beads with bound proteins were eluted by boiling in 2X SDS loading buffer at 95 °C for 5 min. The eluted proteins were subjected to electrophoresis SDS-PAGE. The signals of bands were detected by probing with anti-TLR1 and anti-TLR2 antibodies.

**9. Pull down assay.** HEK293T cells were seeded at the density of  $5 \times 10^5$  cells per well in a 6-well plate overnight. HEK293T cells were co-transfected with 1  $\mu\text{g}$  of pZERO-TLR1 and pCMV-Flag-TLR2 for 6 h by using Lipofectamine® agent. After 6 h transfection, the cells were grown in the DMEM containing 10% FBS for 12 h. Cells were pre-treated with vehicle, CU-CPT22 or compound **1** for 4 h and then treated with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) in a 12-well plate for 12 h. Cell lysates were harvested and the protein concentration in the supernatant was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). 30  $\mu\text{g}$  of each protein sample were immunoprecipitated with anti-Flag magnetic beads according to the manufacturer's protocol. The beads were equilibrated and resuspended with 5 packed gel volumes of TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4) twice, after removal of the TBS buffer in the appropriate magnetic separator, 30  $\mu\text{g}$  of each protein sample were added to anti-Flag magnetic beads and incubated for 1 h at room temperature with gentle mixing to capture the FLAG fusion proteins. After 1 h, the beads were collected in the appropriate magnetic separator and the supernatant was removed. The protein-binding beads were washed with 20 packed gel volumes of TBS buffer twice to remove non-specifically bound proteins, and the FLAG protein was eluted from the magnetic beads with 0.1 M glycine HCl, pH 3.0. Protein samples were subjected to the SDS-PAGE and analysed by Western blotting with anti-TLR1 (1:1,000) or anti-TLR2 antibodies (1:1,000).

**10. Phagocytosis assay.**  $3 \times 10^6$  RAW 264.7 cells were seeded in a 6-well plate for 24 h. Cells were pre-treated with vehicle or compound **1** (4  $\mu\text{M}$ ) at 37 °C for 4 h. 20  $\mu\text{L}$  of suspended Rainbow calibration (BD

Biosciences, San Jose, CA) particles diluted in 200 mL PBS was added to the wells (per mL of culture medium-to-beads ratio 1:20) with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) to stimulate heterodimerization of TLR1 and TLR2. After 12 h treatment to allow the uptake of fluorescence-labeled particles, phagocytosis was terminated by placing the plate on ice. Cells were collected, washed with cold PBS and analyzed in the FL1 channel on a Flow Cytometry (BD FACS Canto™, BD Biosciences, San Jose, USA). Percentage of macrophage that had taken up one or more microspheres was identified.

**11. Cell viability assay.** RAW 264.7 cells were placed in 96-well plates at a density of 4,000 cells per well and grown for 24 h. Cells were incubated with vehicle as background control, Triton X-100 (0.1 %) as maximum LDH control, Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL), CU-CPT22 or compound **1** for 4 h. The cells pre-treated with CU-CPT22 or compound **1** were then co-treated with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for a further 12 h. After treatment, the activities of LDH released from the cells were determined by using a cytotoxicity detection kit (Roche Molecular Biochemicals) following a modification of the manufacturer's protocol. In brief, 70 µL of supernatant was dispensed into a new 96-well plate and centrifuged at 350g for 5 min. 50 µL volume of supernatant was taken from each well and added to LDH substrate for 30 min reaction at 25 °C in the dark. The absorbance was monitored in SpectraMax M5 microplate reader at 490 nm and 600 nm.

**12. Statistical analysis.** For statistical analysis, all data were analyzed with one-way analysis of variance (ANOVA) followed by the Dunnett's method for multiple comparisons by using GraphPad Prism 6.0. A significant difference was defined as  $P < 0.05$ .

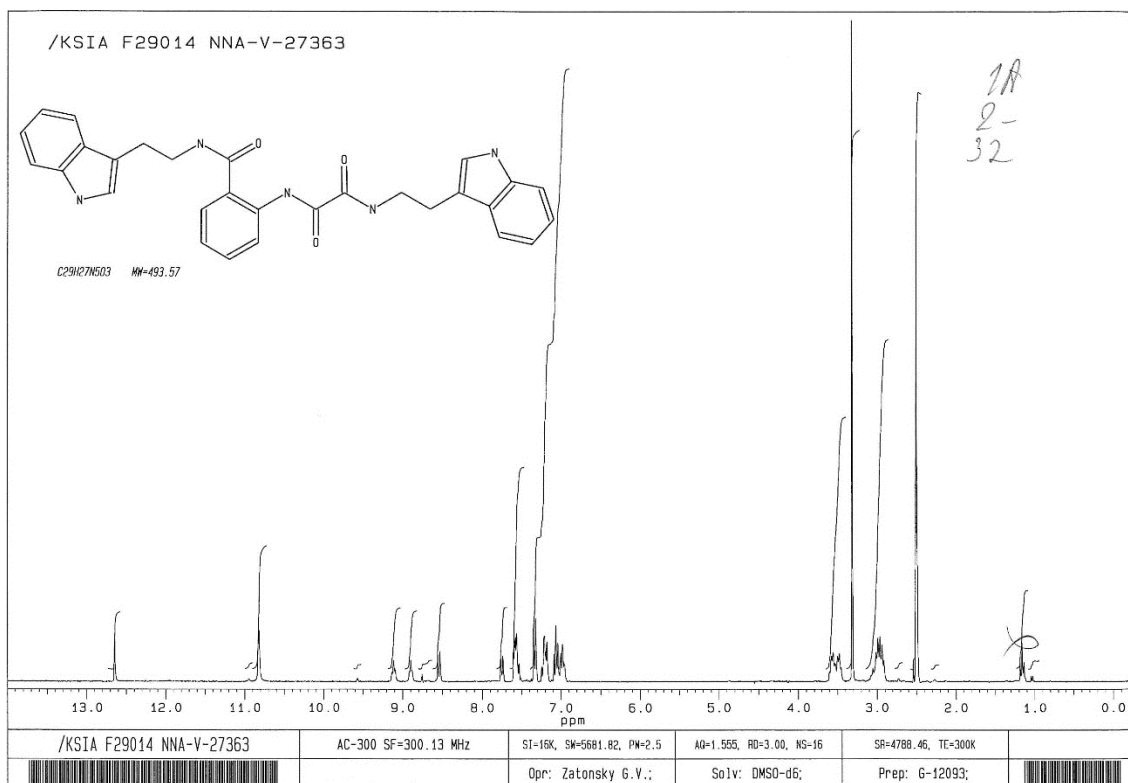
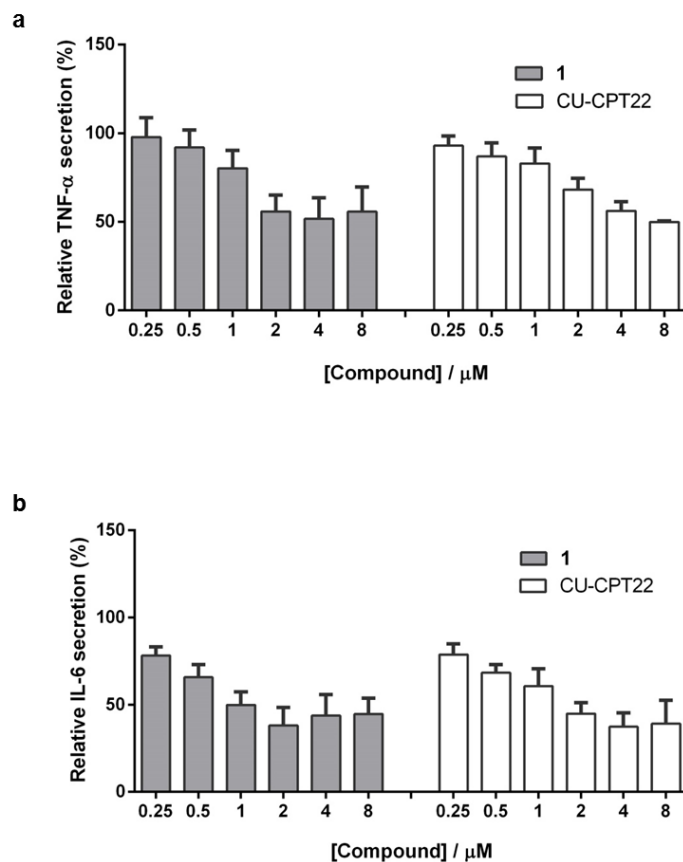


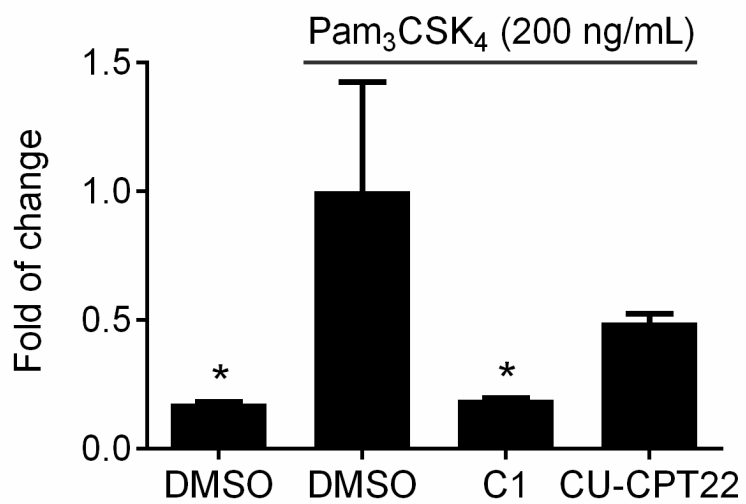
Fig. S1 <sup>1</sup>H-NMR spectrum of compound 1.



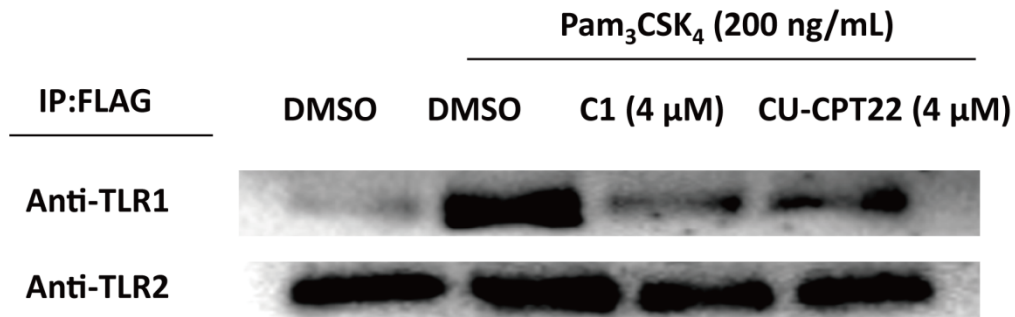
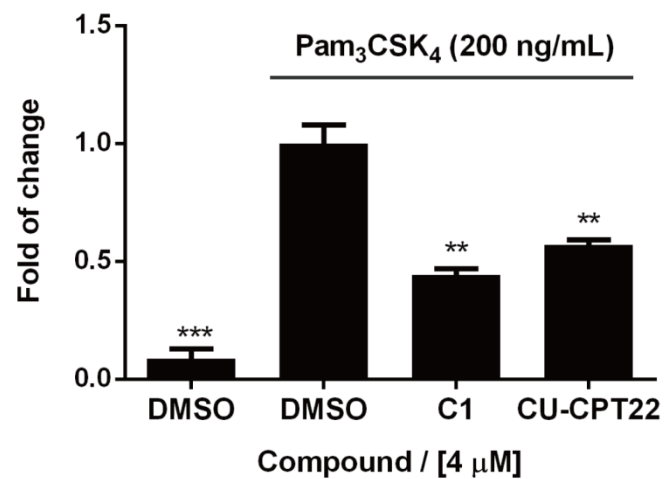
**Fig. S2** Dose response effect of compound **1** and CU-CPT22 on Pam<sub>3</sub>CSK<sub>4</sub>-induced pro-inflammatory cytokine secretion in RAW 264.7 cells. RAW 264.7 cells were pre-treated with the indicated concentrations of compound **1** or CU-CPT22 for 4 h, followed by stimulation of Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for a further 12 h. (a) Compound **1** and CU-CPT22 inhibits TNF- $\alpha$  secretion in Pam<sub>3</sub>CSK<sub>4</sub>-stimulated RAW 264.7 cells. IC<sub>50</sub> values of compound **1** and CU-CPT22 are *ca.* 6.1  $\mu\text{M}$  and *ca.* 6.5  $\mu\text{M}$ . (b) Compound **1** and CU-CPT22 inhibits the IL-6 secretion in Pam<sub>3</sub>CSK<sub>4</sub>-stimulated RAW 264.7 cells. IC<sub>50</sub> values of compound **1** and CU-CPT22 are *ca.* 1.9  $\mu\text{M}$  and *ca.* 2.0  $\mu\text{M}$ . Error bars represent the standard deviations of the results from three independent experiments.



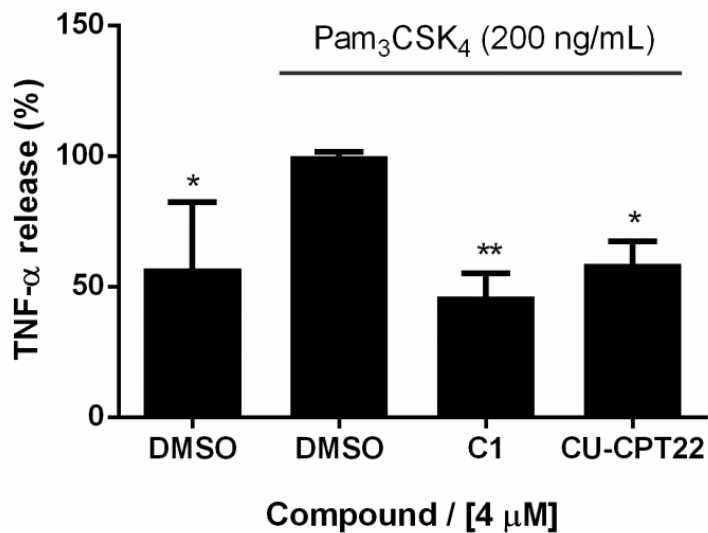




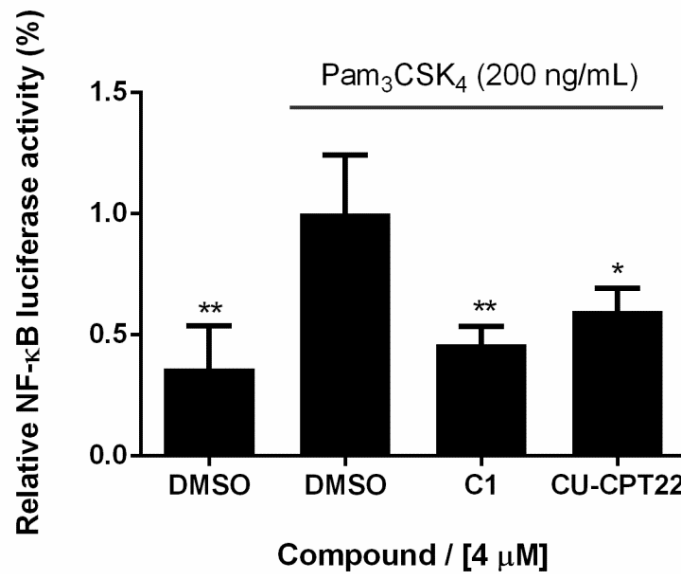
**Fig. S3** Densitometry analysis of compound **1** on inhibition of TLR1-TLR2 heterodimerization. The results were analysed using One-way ANOVA. Significantly different at  $*P < 0.05$ . Error bars represent the standard deviations of the results from three independent experiments.

**A****B**

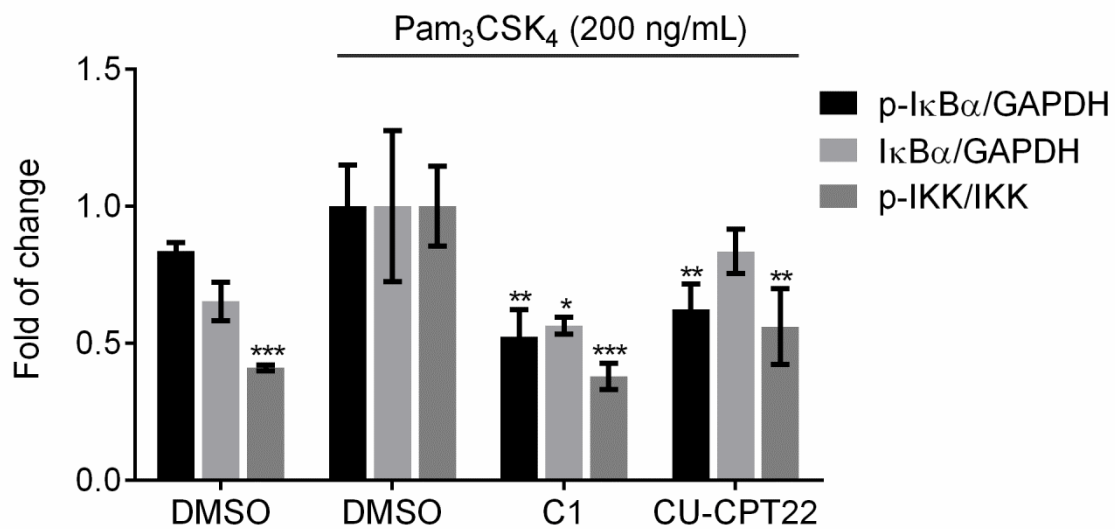
**Fig. S4** Effect of compound **1** on the inhibition of Pam<sub>3</sub>CSK<sub>4</sub>-induced TLR1-TLR2 heterodimerization in HEK293T cells. HEK293T cells transfected with pZERO-TLR1 and pCMV-Flag-TLR2 were pre-treated with compounds (4 μM) for 4 h, followed by stimulation with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for a further 12 h. Pam<sub>3</sub>CSK<sub>4</sub>-induced TLR1-TLR2 dimerization was pulled down by using anti-Flag magnetic beads and evaluated by Western blotting. The results were analysed using One-way ANOVA. Significantly different at \**P* < 0.05. Error bars represent the standard deviations of the results from two independent experiments.



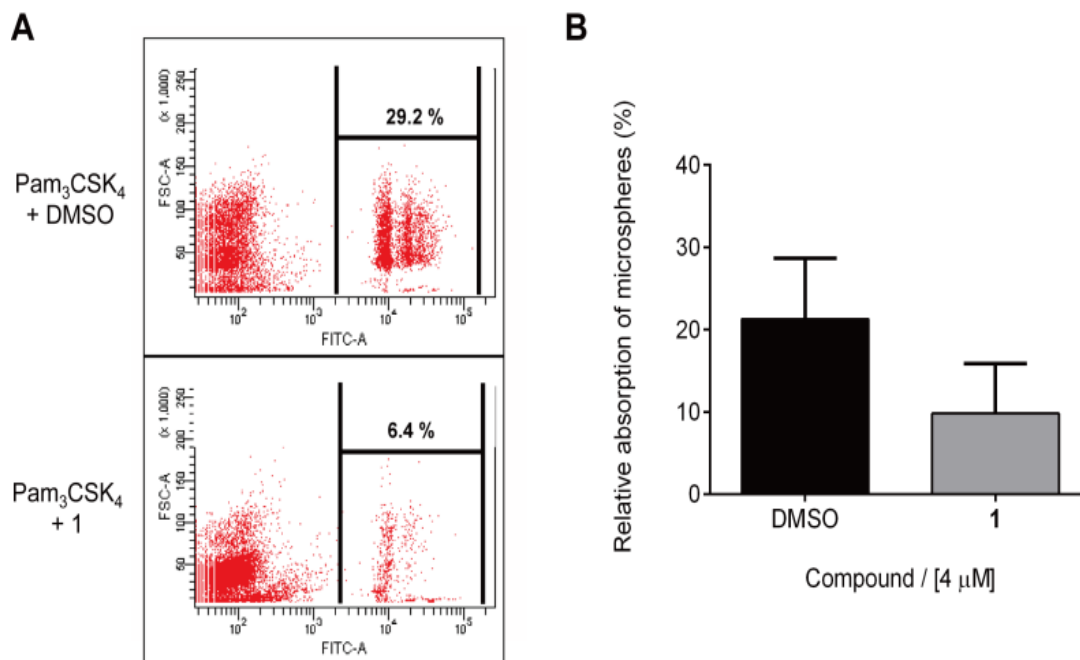
**Fig. S5** Effect of compound **1** on the inhibition of Pam<sub>3</sub>CSK<sub>4</sub>-induced TNF-α release in HEK293T cells. HEK293T cells transfected with pZERO-TLR1 and pCMV-Flag-TLR2 were pre-treated with compounds (4 μM) for 4 h, followed by stimulation with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for a further 12 h. Pam<sub>3</sub>CSK<sub>4</sub>-induced TNF-α release was measured by TNF-α Quantikine® ELISA Assay. The results were analysed using One-way ANOVA. Significantly different at \**P* < 0.05. Error bars represent the standard deviations of the results from three independent experiments.



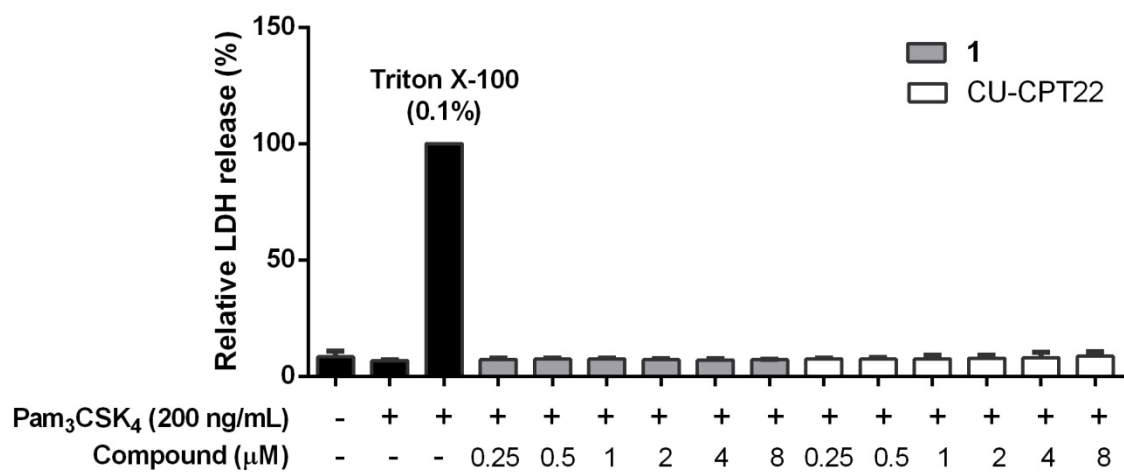
**Fig. S6** Effect of compound 1 on the inhibition of Pam<sub>3</sub>CSK<sub>4</sub>-induced induced NF-κB transcriptional activity in HEK293T cells. HEK293T cells co-transfected with pZERO-TLR1, pCMV-Flag-TLR2 and p NF-κB-TA-Luc were pre-treated with compounds (4 μM) for 4 h, followed by stimulation with Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for a further 12 h. Pam<sub>3</sub>CSK<sub>4</sub>-induced NF-κB-driven transcription activity was measured by using the dual-luciferase reporter system. The results were analysed using One-way ANOVA. Significantly different at \**P* < 0.05. Error bars represent the standard deviations of the results from three independent experiments.



**Fig. S7** Densitometry analysis of compound **1** on inhibition of Pam<sub>3</sub>CSK<sub>4</sub>-induced NF-κB signaling pathway. The results were analysed using One-way ANOVA. Significantly different at \* $P < 0.05$ . Error bars represent the standard deviations of the results from three independent experiments.



**Fig. S8** Effect of compound **1** on the phagocytic ability in RAW 264.7 cells. (A) Drug-treated RAW 264.7 cells were incubated with compound **1** and fluorescently-labeled particles for 24 h. Phagocytosis of RAW 264.7 cells was assessed by flow cytometry. (B) Statistical results of relative absorption of microspheres. Error bars represent the standard deviations of the results from three independent experiments.



**Fig. S9** Dose response analysis of LDH release of compound **1** and CU-CPT22 against RAW 264.7 cells. RAW 264.7 cells were pre-treated with compound **1** or CU-CPT22 for 4 h, followed by the stimulation of Pam<sub>3</sub>CSK<sub>4</sub> (200 ng/mL) for further 4 h. Cell viability was measured by the release of the LDH from the cells. Error bars represent the standard deviations of the results from three independent experiments.



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