A novel NOX2 inhibitor attenuates human neutrophil oxidative stress and ameliorates inflammatory arthritis in mice

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Supplementary Materials and Methods

1.1. Synthesis of CYR5099

A mixture of 1-methyl-1*H*-benzo[*f*]indole-4,9-dione (**A**, Mp: 188-190 ^{o}C. (lit. 191-192 ^{o}C), 0.42 g, 2.0 mmol) [1] and hydroxylamine hydrochloride (0.70 g, 10.0 mmol) in EtOH (10 mL) was refluxed for 36 h. After cooling, the reaction mixture was poured into ice water. The crude product was filtered, washed with ice water and crystallized from EtOH to give 4-(Hydroxyimino)-1-methyl-1*H*-benzo[*f*]indol-9(4*H*)-one **(B)** (0.35 g, 78% yield). Mp: 217-219 ^{o}C. IR (KBr): 3308, 1629, 1604, 1583, 1413, 1263, 1197, 695 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.08 (s, 3H, NMe), 7.17 (d, 1H, *J* = 2.4 Hz, ArH), 7.39 (m, 1H, ArH), 7.57-7.61 (m, 1H, ArH), 7.63-7.67 (m, 1H, ArH), 8.13-8.15 (m, 1H, ArH), 8.26-8.29 (m, 1H, ArH), 12.66 (s, 1H, NOH). ^{13}C NMR (400 MHz, DMSO-d_6): δ 36.48, 110.87, 121.23, 123.23, 125.68, 126.23, 128.86, 131.37, 131.87, 132.59, 134.05, 141.52, 174.30. Anal. Calcd for $C_{13}H_{10}N_2O_2$: C, 69.01; H, 4.46; N, 12.38. Found: C, 69.05; H, 4.55; N, 12.30.

A mixture of **B** (0.23 g, 1.0 mmol) and sodium hydride (60% in oil, 0.12 g, 3.0 mmol) in dry DMF (8.0 mL) was stirred at room temperature for 15 min, then 1,4-dibromobutane (0.86 g, 4.0 mmol) was added. After 15 min, the dimethylamine (40% in water, 3 mL) was added, and the reaction mixture was stirred for 15 min. The solvent was removed in vecuo and the residue was extracted with water and CH_2Cl_2 three times. The organic layer evaporated in vecuo and purified by column chromatography, using a gradient eluant (MeOH/CH_2Cl_2, 1/10 to 1/5) in 77% yield as a white solid **(CYR5099)**. Mp: 173-175 ^{o}C. IR (KBr): 1637, 1601, 1411, 1271, 980, 781, 689 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.94-2.00 (m, 2H, $OCH_2CH_2\underline{CH_2}CH_2N$), 2.06-2.14 (m, 2H, $OCH_2\underline{CH_2}CH_2CH_2N$), 2.77 (s, 6H, $N(\underline{CH_3})_2$), 3.08-3.12 (m, 2H, $OCH_2CH_2CH_2\underline{CH_2}N$), 4.12 (s, 3H, $N\underline{CH_3}$), 4.47 (t, 2H, *J* = 5.6 Hz,

O$\underline{CH_2}$$CH_2CH_2CH_2N$), 6.99 (d, 1H, *J* = 2.4 Hz, ArH), 7.07 (d, 1H, *J* = 2.8 Hz, ArH), 7.50-7.58 (m, 2H, ArH), 8.22-8.25 (m, 1H, ArH), 8.28-8.31 (m, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.29, 26.46, 36.98, 42.81 (2C), 57.74, 74.41, 111.38, 121.34, 123.73, 126.17, 127.10, 129.14, 131.44, 131.74, 131.90, 133.70, 142.54, 175.58. Anal. Calcd for $C_{19}H_{23}N_3O_2 \cdot 0.9$ HBr · 0.1 H_2O: C, 55.89; H, 5.96; N, 10.30. Found: C, 55.69; H, 5.96; N, 10.22.

1.2. Preparation of cultured cells

The human promyelocytic leukemic HL-60 cells were purchased from ATCC. The colorectal adenocarcinoma HT-29 cell was a kind gift from Dr. Tzu-Hao Wang (Chang Gung Memorial Hospital). HL-60 and HT-29 cells were respectively cultured in RPMI 1640 medium supplemented with 20% and 10% fetal bovine serum (FBS) and 1× Antibiotic-Antimycotic. Both cells were grown in a humidified atmosphere at 37°C with 5% CO_2. HL-60 cells were differentiated as neutrophil-like cells by a 5-day treatment with 1.3% DMSO in the growth medium (dHL-60 cells) [2].

1.3. Lentivirus infection

Viral supernatants of shRNA against $p47^{phox}$ or luciferase were obtained from RNAi core (Academia Sinica, Taiwan). After plating HL-60 cells (1 x 10^6) in 1 ml in a 6-well plate, viral supernatants were added to each well, followed by centrifugation at 1,000g for 1 h. Transfected cells were cultured at 37°C for 12 h and then incubated in growth medium containing puromycin (2 μg/ml) for a further 7 days.

1.4. Measurement of intracellular $O_2^{\bullet-}$ generation

The measurement of intracellular $O_2^{\bullet-}$ generation was previously described [3].

The levels of 2-hydroxyethidium (2-OH-E^+) were determined by high-performance liquid chromatography–mass spectrometry (HPLC–MS).

1.5. Assays of NADPH oxidase 1 (NOX1)-dependent ROS generation

The NOX1-dependent ROS generation was measured using luminol in HT-29 cells, which highly expressed NOX1 but not other NOX types [4, 5]. HT-29 cells (7×10^5 cells/ml) were pretreated with DMSO, CYR5099, DPI, or apocynin for 5 min and then incubated with 37.5 μM luminol in the presence of 6 U/ml horseradish peroxidase at 37°C.

1.6. Fractionation of plasma membrane proteins

Cells (1×10^8) were homogenized in 2 ml of the Homogenization Buffer Mix on ice for 30-50 times. The isolation of plasma membrane proteins was performed using the Plasma Membrane Protein Extraction Kit (BioVision, CA, USA) according to the manufacturer's protocol.

1.7. Homology remodeling of human gp91^{phox}

The human gp91^{phox} protein sequence was from Uniprot protein database (Uniprot_P04839). The crystal structures of C-terminal human gp91^{phox} and FAD-binding region of *C. stagnale* gp91^{phox} were obtained from Protein Data Bank (PDB 3A1F and 5O0X, respectively). Partial human gp91^{phox} structure from S291 to F570 was modelled using MODELLER (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2018, San Diego: Dassault Systèmes, 2016). The best model was chosen according to the discrete optimised protein energy score that was verified by measuring the compatibility score of each residue in the given 3D

environment and then checked using Ramachandron plot and LOOPER. The final model was used in molecular docking studies.

References

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Supplementary Figure Legends

Supplementary Fig. 1. The synthetic process of CYR5099.

Supplementary Fig. 2. Remodeling of human gp91^{phox} (A) Superimpose of gp91^{phox} templates (green: 3A1F, yellow: 5O0X). The RMSD value is 1.1704 Å. (B) The remodeling structure of human gp91^{phox} with FAD-binding region.

Supplementary Fig. 3. CYR5099 inhibits superoxide anion generation in activated human neutrophils. (A) 2-Hydroethidine-labeled human neutrophils were incubated with DMSO or CYR5099 (1-10 μM) for 5 min and then activated with fMLF (0.1 μM)/CB (1 μg/ml) for another 5 min. 2-OH-E^{+} production was determined by HPLC–MS. All data are shown as the mean ± S.E.M. (n = 3). $^{**}p < 0.05$; $^{***}p < 0.001$. (B) The representative chromatograms.

Supplementary Fig. 4. CYR5099 inhibits the zymosan A-induced ROS generation in human neutrophils. Human neutrophils were incubated with DMSO or CYR5099 (5 or 10 μM) for 5 min before stimulation with zymosan A (250 μg/ml). Luminol was used to detect total ROS. Chemiluminescence levels were calculated and data are shown as the mean ± S.E.M. (n = 3). $^{***}p < 0.001$.

Supplementary Fig. 5. CYR5099 does not show cytotoxicity. Human neutrophils were incubated with DMSO or CYR5099 (5-15 μM) for 5 min and then activated with (A) fMLF (0.1 μM)/CB (1 μg/ml) or (B) PMA (10 nM) for another 10 min. (C) Human neutrophils were pretreated with PMA (10 nM) for 5 min and then incubated with DMSO or CYR5099 (10 or 15 μM) for another 10 min. LDH release was expressed as percentage of enzyme liberated by incubation with 0.1% Triton X-100. All data are expressed as the mean ± S.E.M. (n = 3).

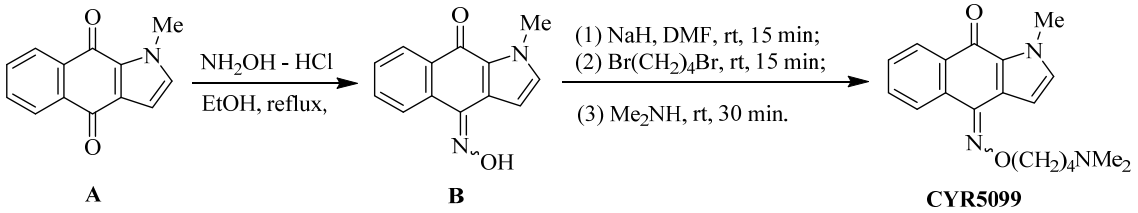
Supplementary Fig. 6. CYR5099 does not inhibit $p47^{phox}$ translocation in human neutrophils. Human neutrophils were incubated with DMSO or CYR5099 (5 or 10 μM) for 5 min, and then activated PMA (10 nM). (A) Total cell lysates and plasma membrane lysates were assayed by Western blot using antibodies against total or phosphorylated $p47^{phox}$. (B) The membrane-bound $p47^{phox}$ or phosphorylation of $p47^{phox}$ were quantified expressed as a relative ratio (fold of PMA alone).

Supplementary Fig. 7. CYR5099 does not inhibit NOX1-dependent ROS generation in HT-29 cells. HT-29 cells were incubated with DMSO, (A) CYR5099 (5 or 10 μM), (B) DPI (0.3 or 3 μM), or (C) apocynin (10 or 30 μM) for 5 min. Luminol

was used to detect total ROS. Chemiluminescence levels were calculated and data are shown as the mean ± S.E.M. (n = 3). ***$p < 0.001$.

Supplementary Fig. 8. CYR5099 does not show further inhibitory effect on PMA-activated superoxide anion generation in p47^{phox}-knockdown dHL-60 cells. (A) The levels of p47^{phox} and GAPDH in sh-control and sh-p47^{phox} dHL-60 cells were assayed by Western blot. (B) Sh-control or sh-p47^{phox} dHL-60 cells were incubated with DMSO or CYR5099 (5 or10 μM) for 5 min and then activated with PMA (10 nM). Ferricytochrome c reduction was monitored by a spectrophotometer at 550 nm. All data are expressed as mean ± S.E.M. (n = 3). **$p < 0.01$; ***$p < 0.001$; n.s. (no significance).

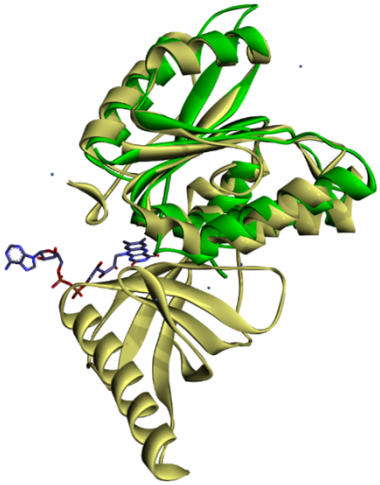
Supplementary Fig. 1



A **B** **CYR5099**

Supplementary Fig. 2

(A)



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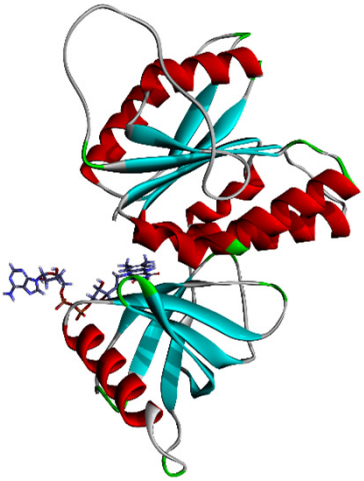
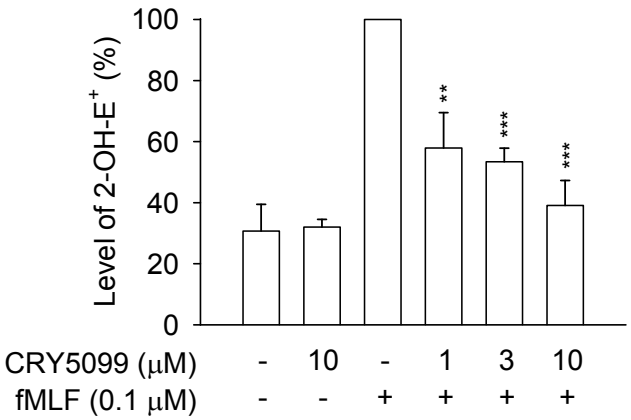


Supplementary Fig. 3

(A)



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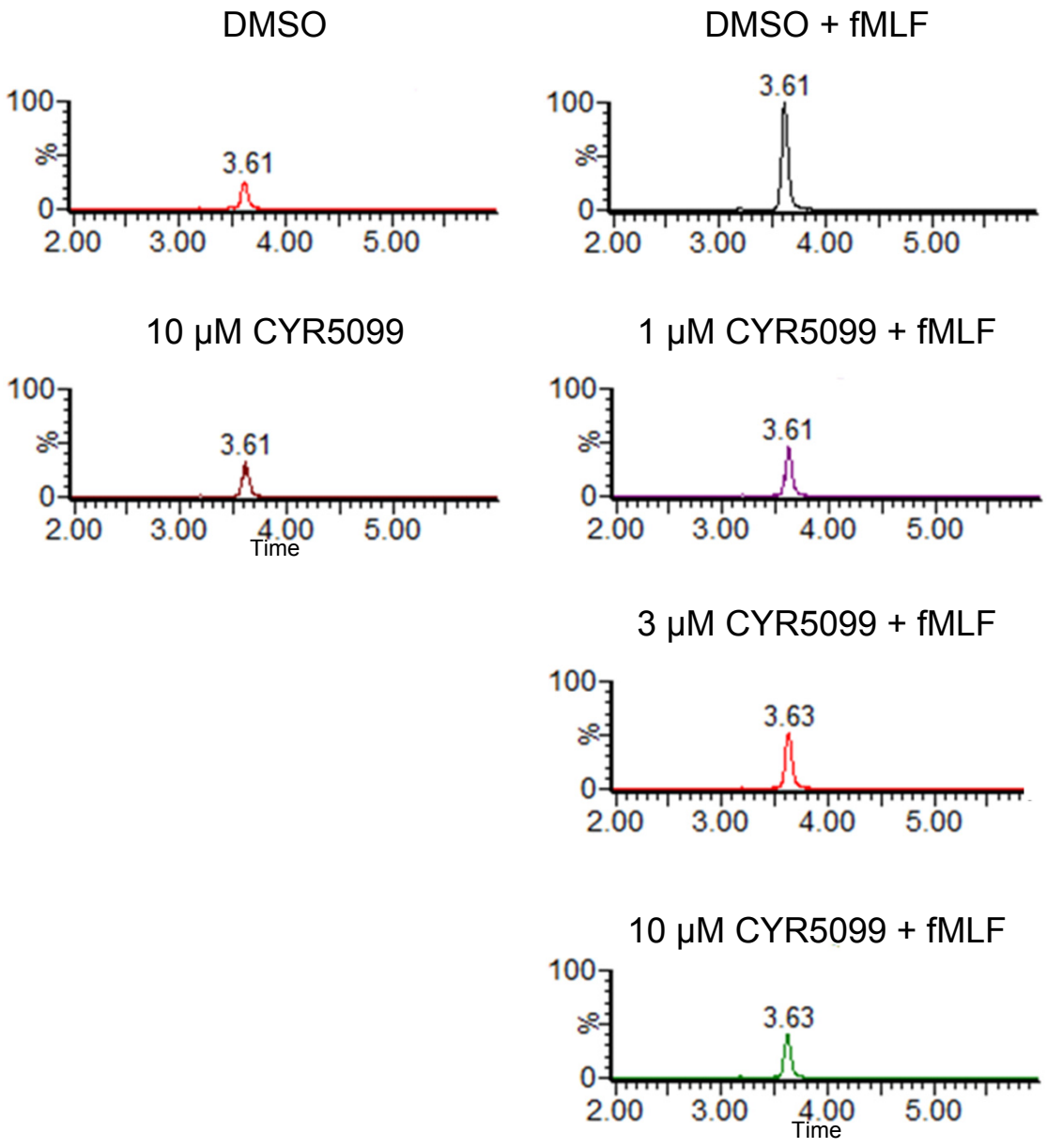

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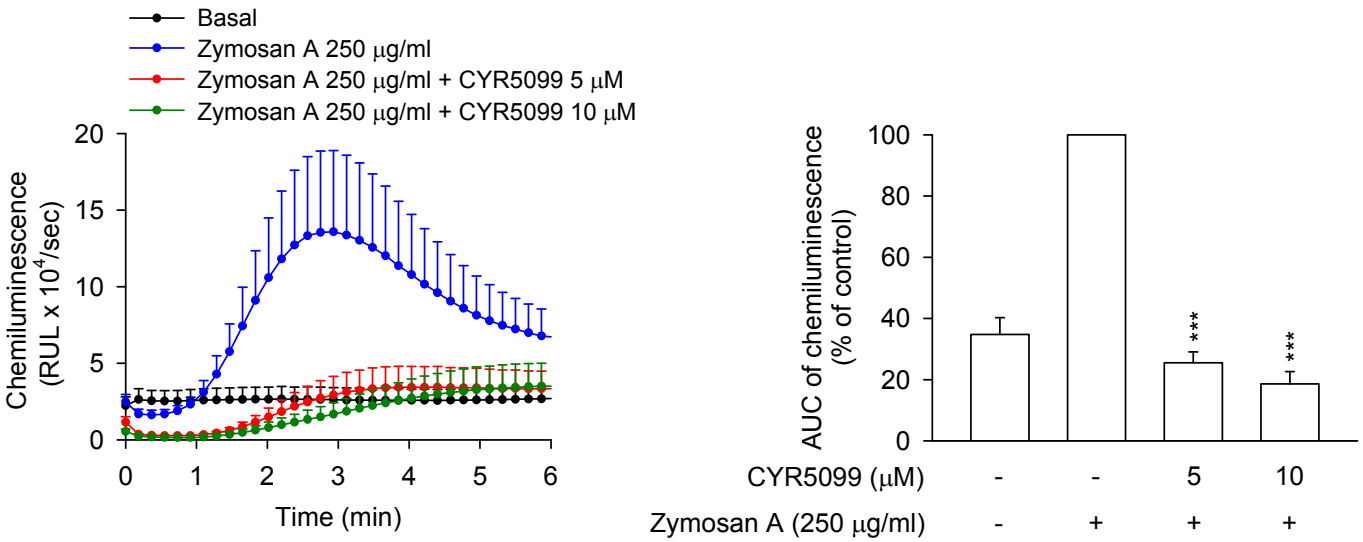
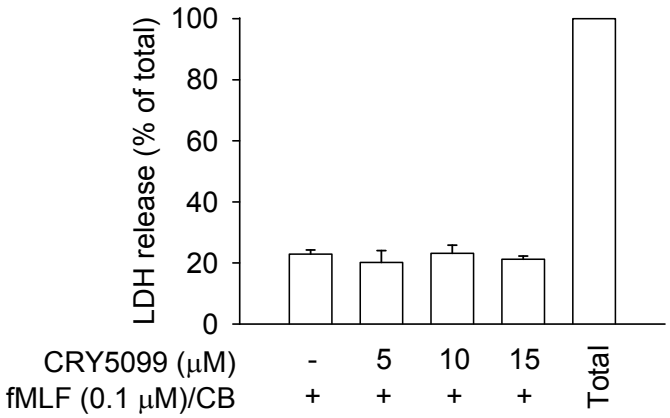


Supplementary Fig. 5

(A)



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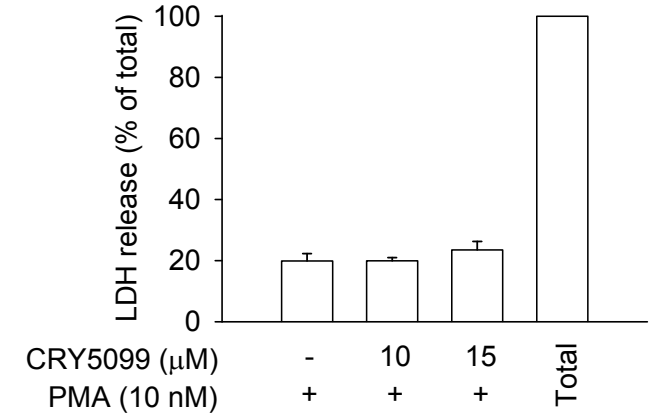

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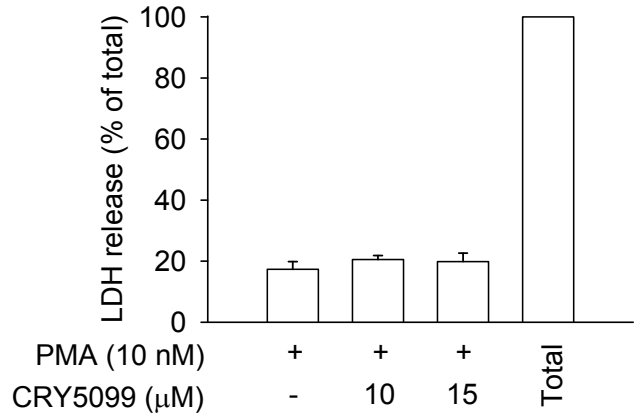
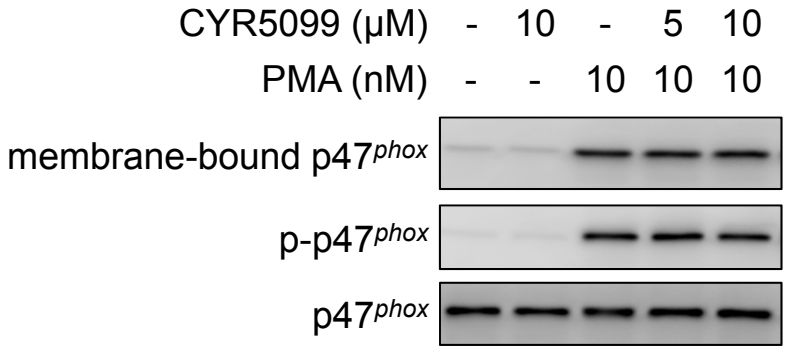


Supplementary Fig. 6

(A)



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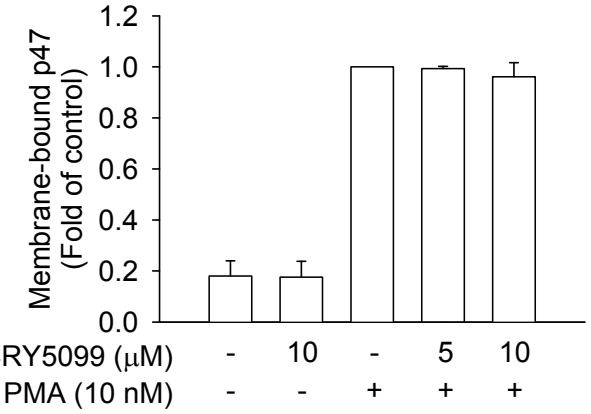
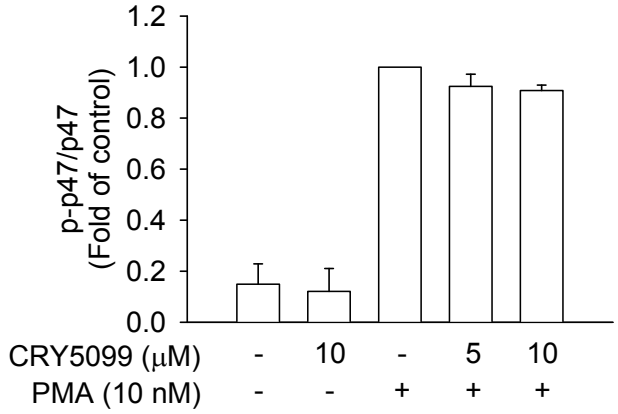
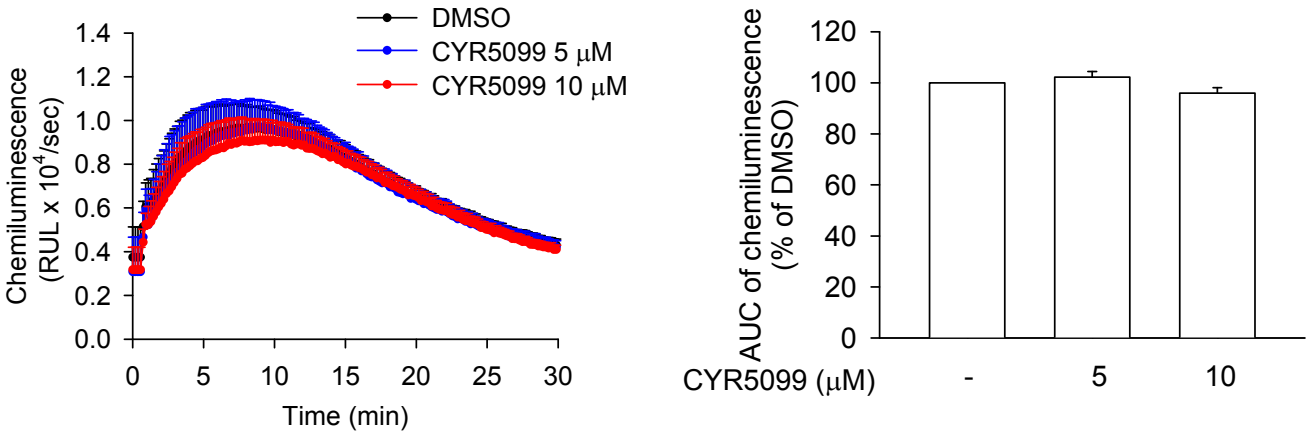




Supplementary Fig. 7

(A)



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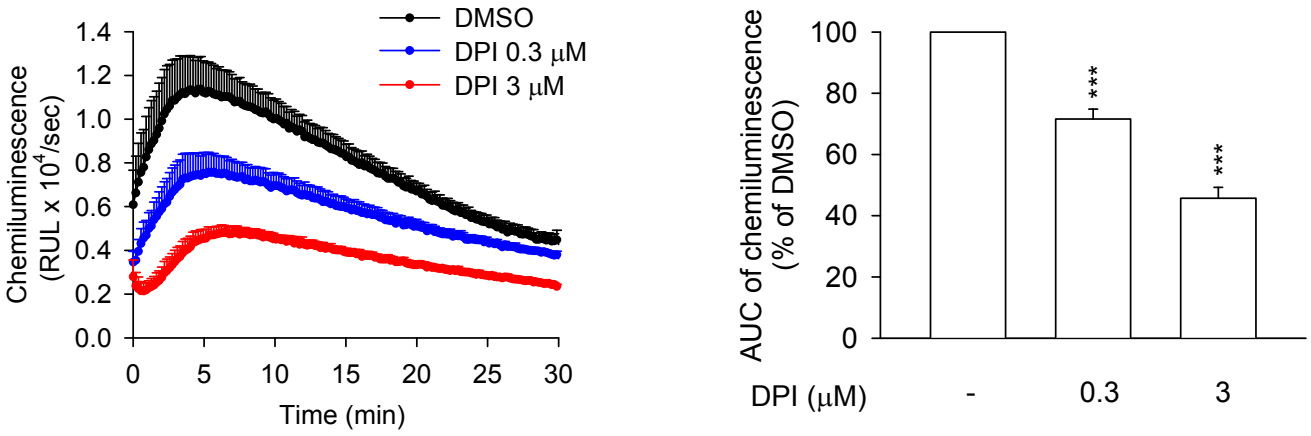

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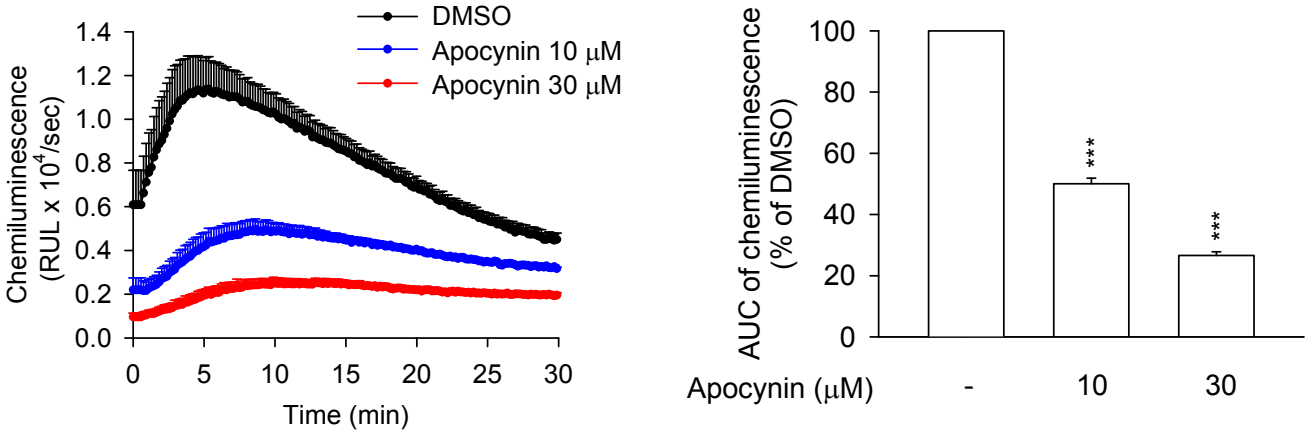
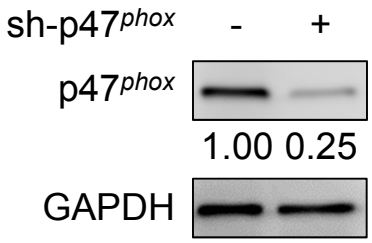


Supplementary Fig. 8

(A)



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