Supporting information

Chemoproteomics reveals Sofalcone inhibits the inflammatory response of Caco-2 cells by covalently targeting HMGB1

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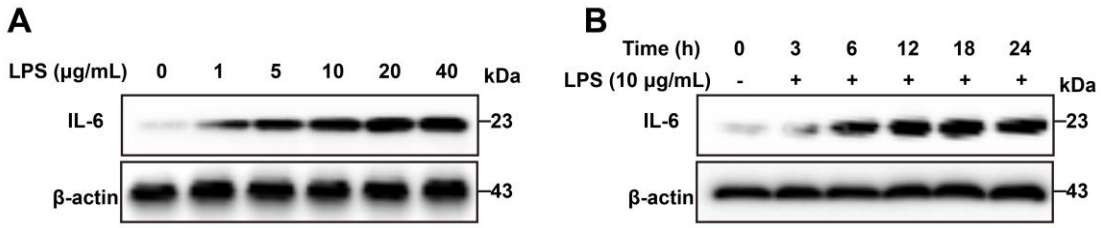

Fig. S1. LPS-induced inflammation in Caco-2 cells. (A) IL-6 expression in Caco-2 cells induced by different concentrations of LPS for 24 h. (B) IL-6 expression in Caco-2 cells induced by 10 μg/ml LPS at different times.

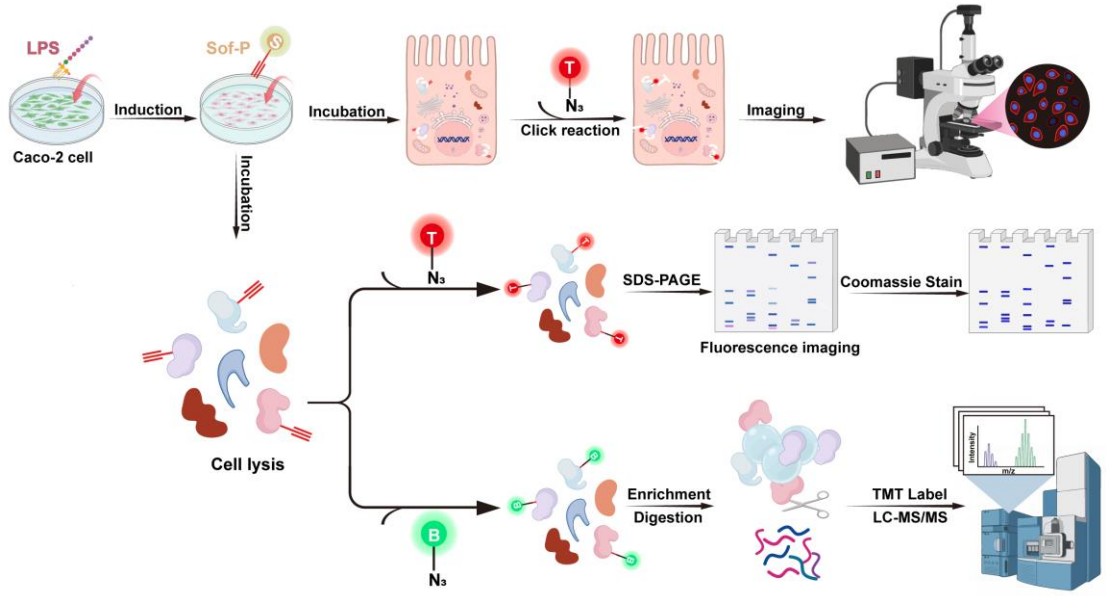

Fig. S2. Overall workflow for identifying protein targets of Sof based on ABPP strategy.

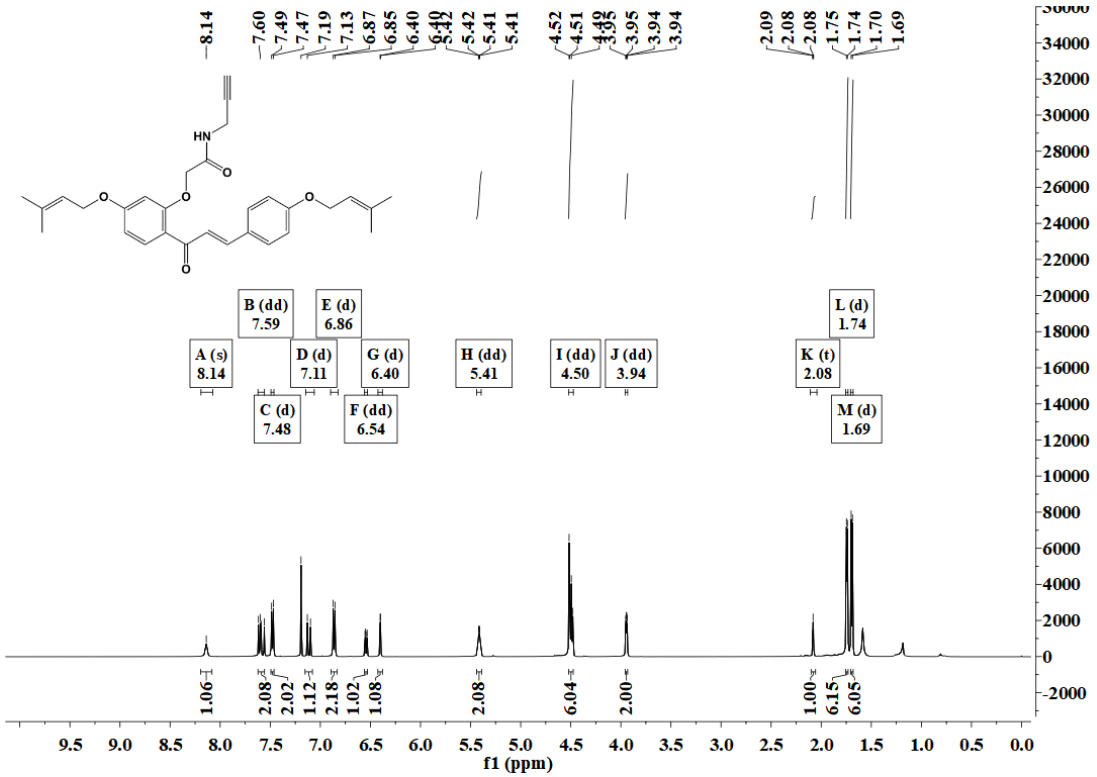

Fig. S3. ^{1}H-NMR of Sof-P.

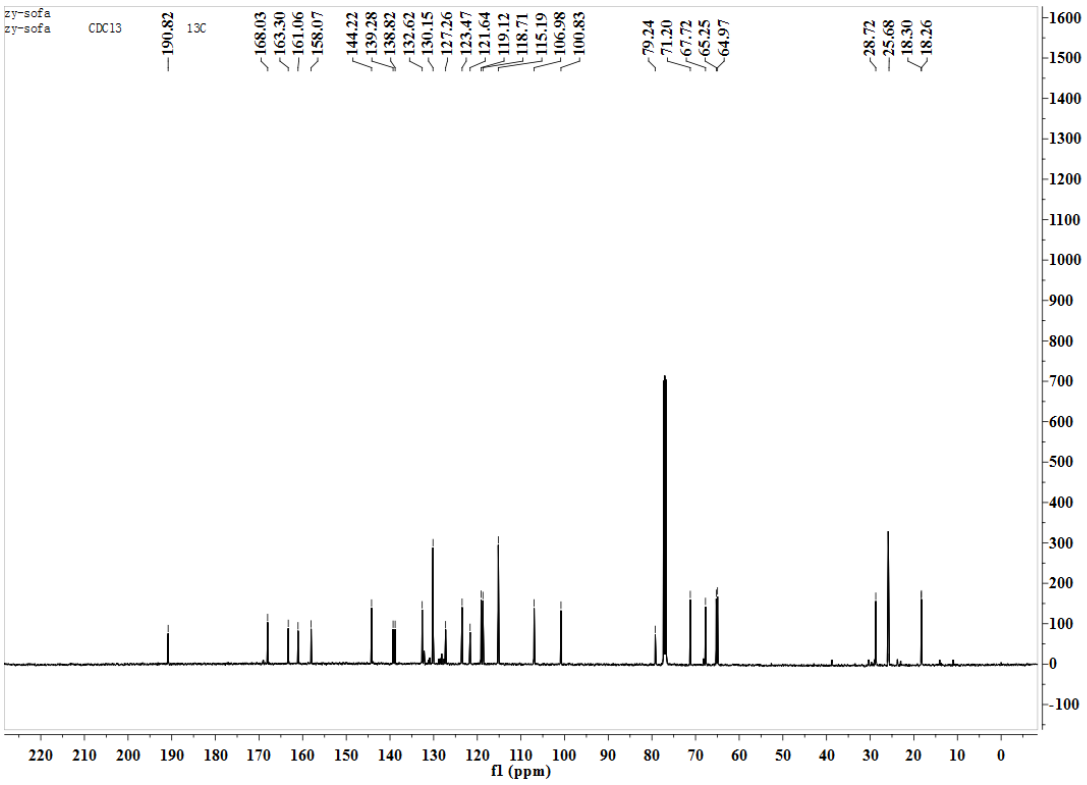

Fig. S4. ^{13}C-NMR of Sof-P.

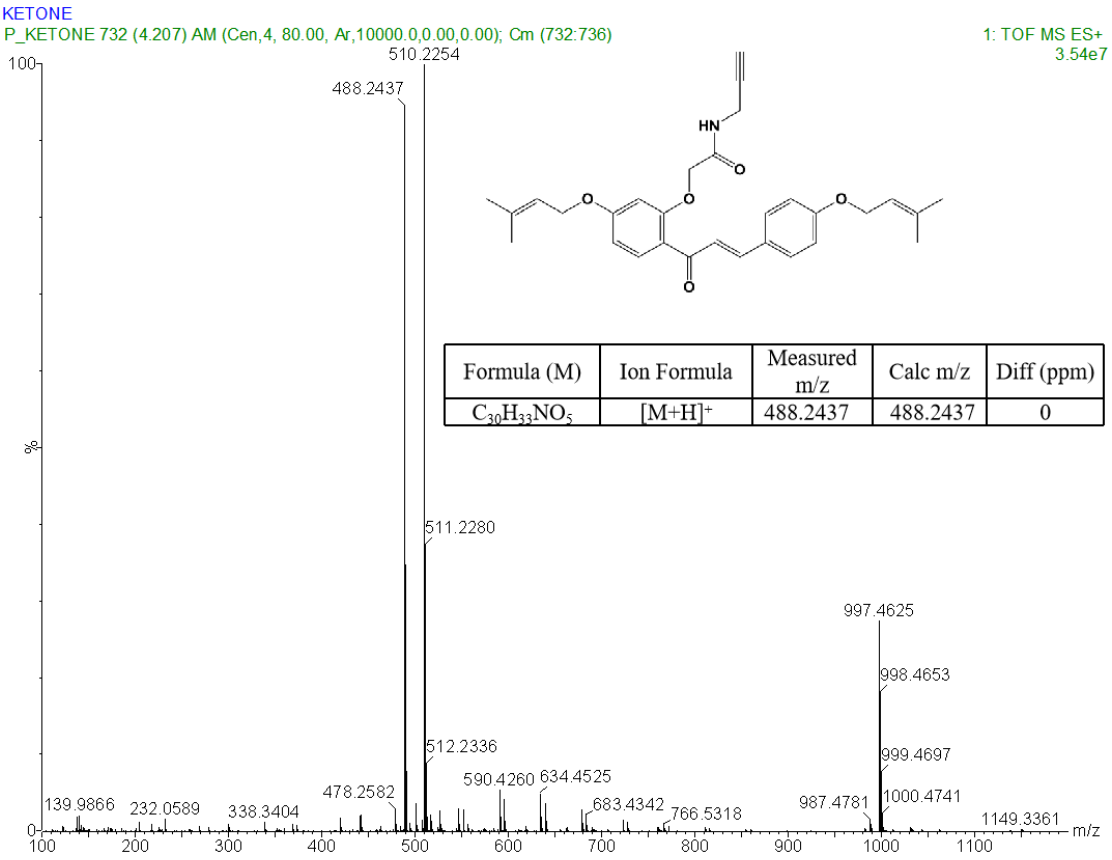

Fig. S5. High-resolution mass spectrum of Sof-P.

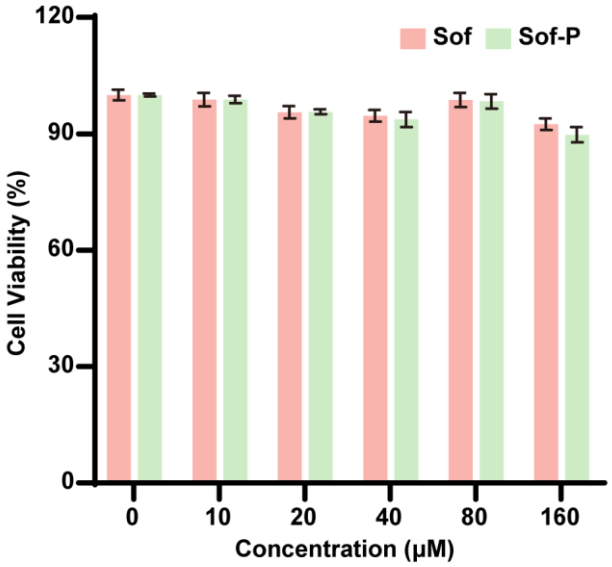

Fig. S6. Cytotoxicity assessment of Sof and Sof-P in Caco-2 cells after incubation for 24 h.

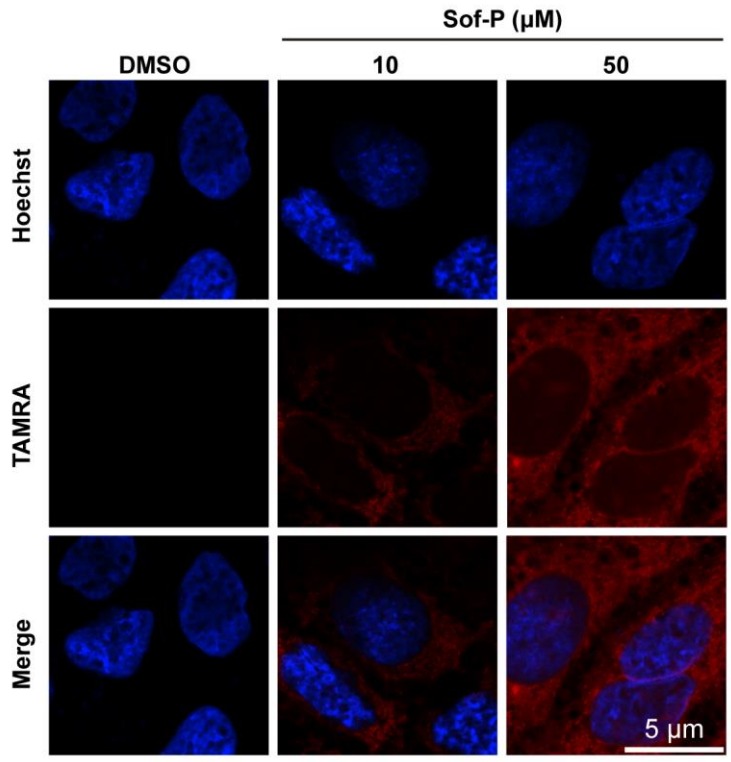

Fig. S7. The distribution of Sof-P in Caco-2 cells by immunofluorescence staining, scale bar = 5 μm.

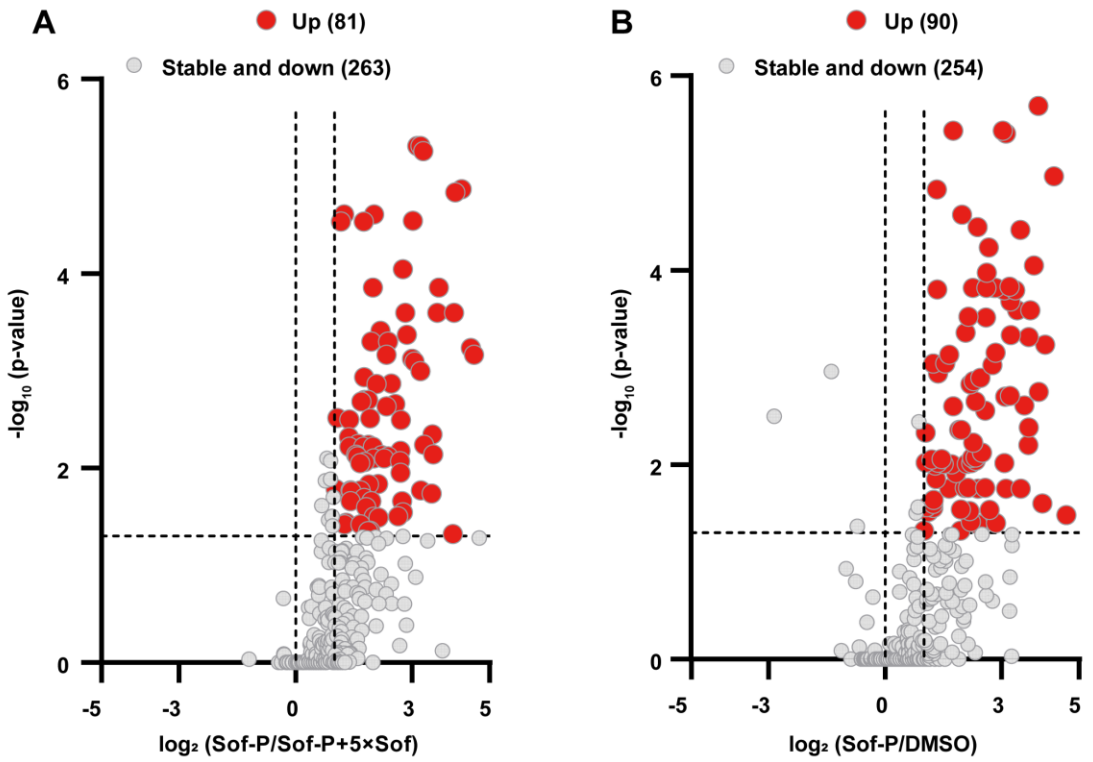

Fig. S8. Volcano plots Sof-targeted proteins. (A) Volcano plot of the targets in the Sof-P group versus the competition group. (B) Volcano plot of the targets in the Sof-P group versus the control group.

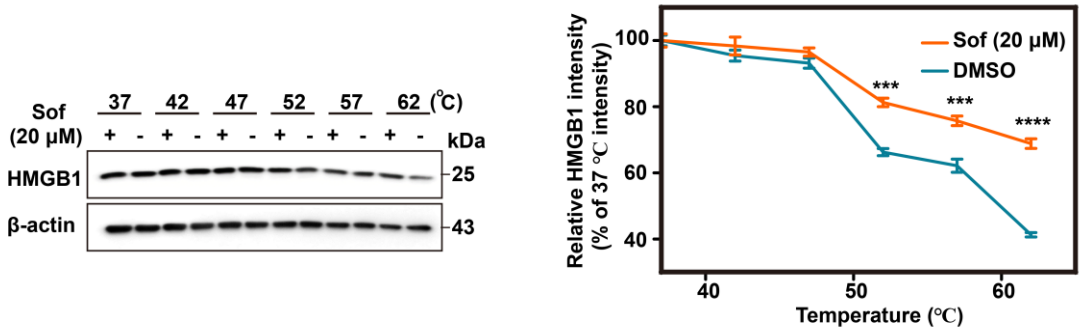

Fig. S9. The interaction of Sof and HMGB1 assessed by CETSA.

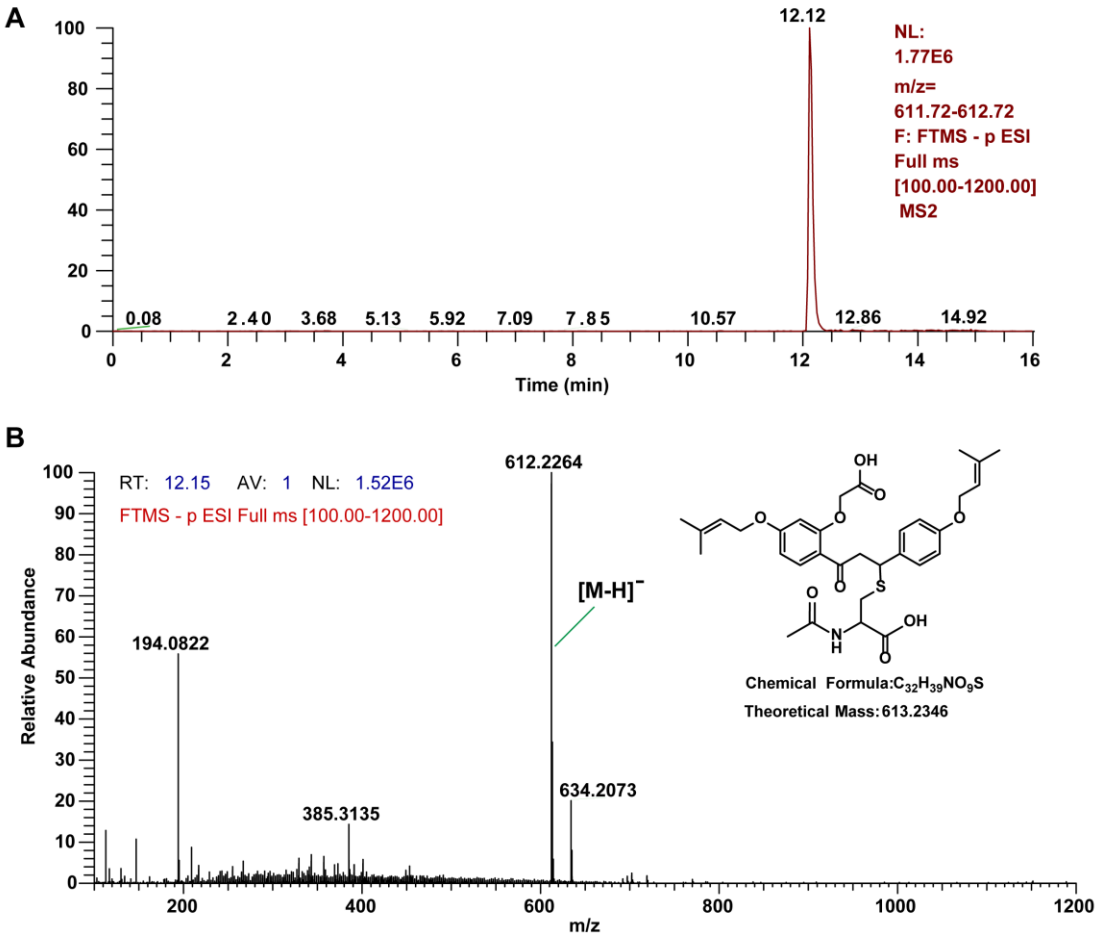

Fig. S10. The reaction of Sofalcone and N-acetylcysteine (NAC) was monitored by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) after Sofalcone (0.2 mM) in 50% (v/v) methanol in PBS was incubated with a 200-fold molar excess of N-acetylcysteine (NAC) for 1 h. Retention time and chromatographic peak (A) and mass peak (B) of the expected adduct were found in negative mode.

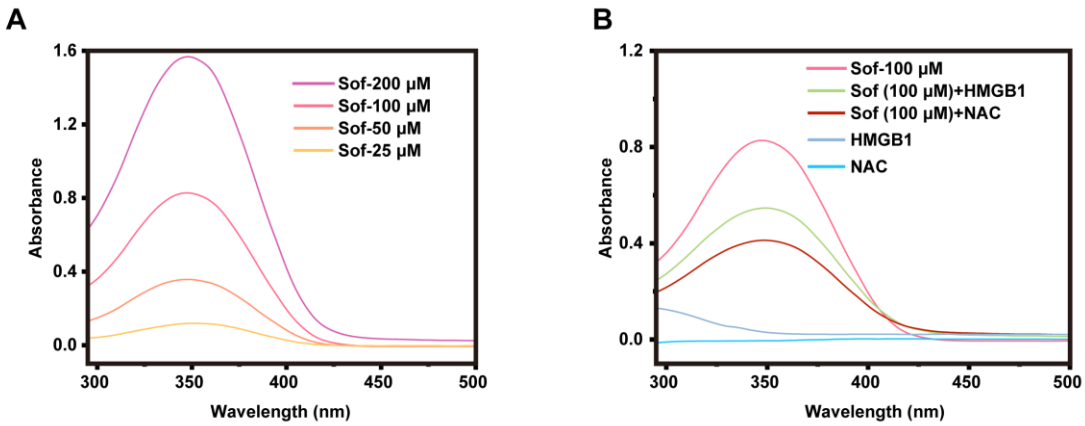

Fig. S11. (A) UV-Vis absorption spectra of Sof at different concentrations. (B) UV-Vis absorption spectra of Sof in the presence or absence of NAC or recombinant human HMGB1 protein.

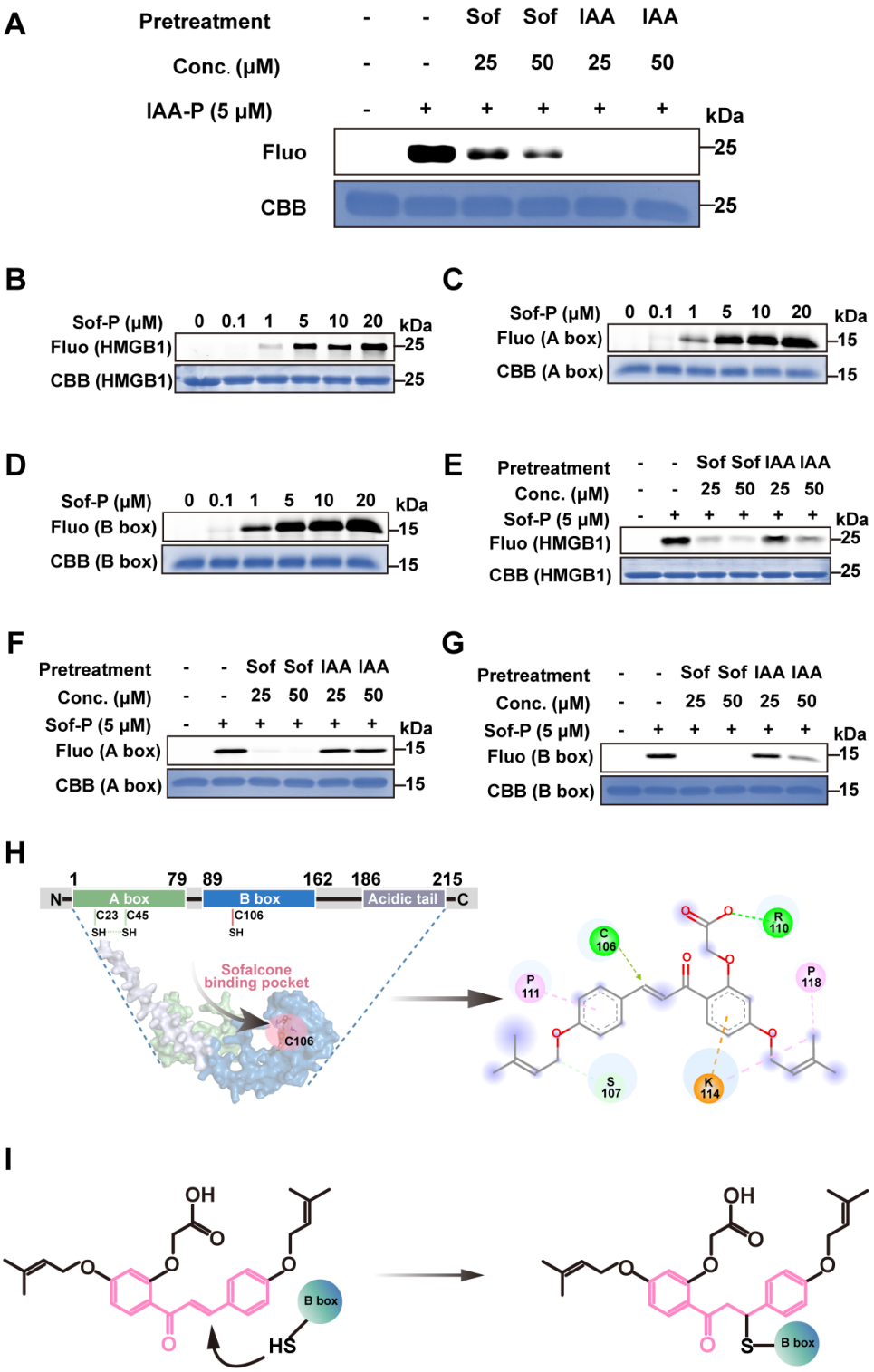

Fig. S12. (A) The labeling of recombinant human HMGB1 by alkyne-tagged IAA (IAA-P) in the presence or absence of the competitors Sof or IAA. (B-D) The labeling of full-length HMGB1 (B), A box (C), and B box (D) by Sof-P (incubation for 1 h). (E-G) The labeling of full-length HMGB1 (E), A box (F), and B box (G) by Sof-P in the presence or absence of IAA and Sof competitors (incubation for 1 h). (H) Binding model of Sof with HMGB1 visualized by molecular docking. (I) Diagram of the binding mechanism between Sof and HMGB1.

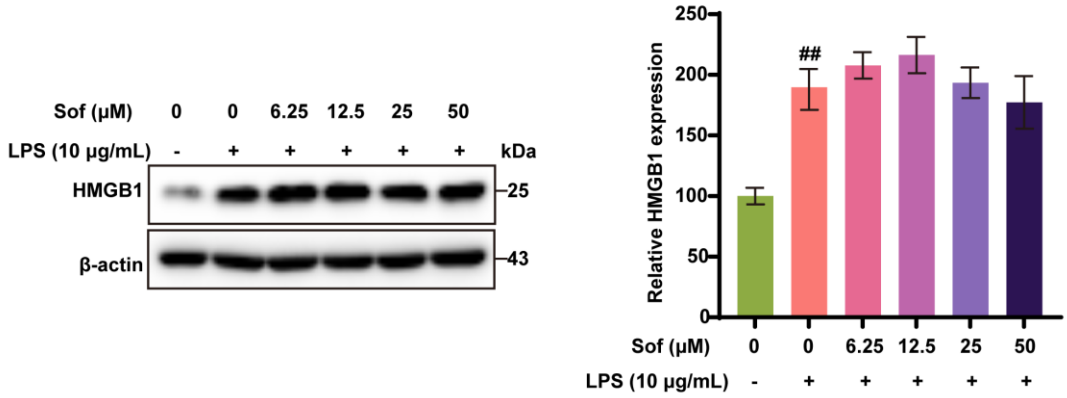

Fig. S13. Sof did not affect HMGB1 expression in LPS-induced Caco-2 cells, $^{\#\#}p < 0.01$ versus control (n=3).

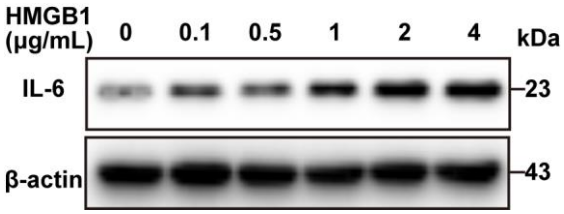

Fig. S14. IL-6 expression in Caco-2 cells induced by different concentrations of recombinant human HMGB1 protein for 24 h.

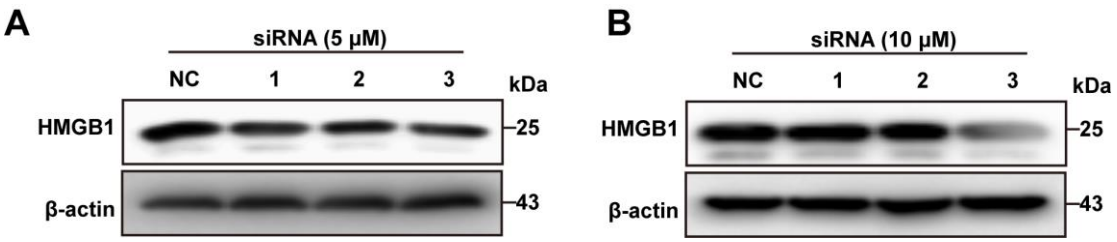

Fig. S15. The knock-down silence effects of HMGB1 protein induced by three siRNA at 5 μM (A) and 10 μM (B) in RNA interference assays.

Table S1. The sequence of siRNA for HMGB1 silence.

siRNA construct	target sequence in mRNA (5′-3′)
HMGB1-1(siRNA-1)	UGACAAGGCUCGUUAUGAAAG
HMGB1-2(siRNA-2)	GAAGAUGAUGAUGAUGAAUAA
HMGB1-3(siRNA-3)	GGGAGGAGCACAAGAAGAA

Materials and methods

Reagents

Sofalcone (Sof, purity ≥97%) was purchased from Bide Pharmatech Co., Ltd. (Shanghai, China). Click chemistry reaction, pull-down, and LC-MS/MS reagents included: $CuSO_4$, TAMRA-azide, Biotin-azide, Tris(2-carboxyethyl) phosphine hydrochloride (TECP), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) were obtained from Sigma-Aldrich (USA); High capacity neutravidin agarose beads, tetraethylammonium bromide (TEAB), sequencing grade modified trypsin, TMT^{10} plex reagent set, and Pierce™ Quantitative Fluorometric Peptide Assay Kit were purchased from Thermo Fisher Scientific (USA). Specific primary antibodies anti-HMGB1 (Servicebio, China), anti-TLR4 (ABclonal Technology Co., Ltd., China), anti-NF-κB p-p65, anti-RAGE (Proteintech, China), and anti-β-actin (Affinity Biosciences, China) were used in the experiments.

The synthesis procedure of sofalcone probe (Sof-P)

According to Scheme S1, Sof (0.2 mmol, 97.45 mg) and 1-amino-2-propargyl (0.4 mmol, 22.03 mg) were dissolved in dry CH_2Cl_2 (2 mL). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 0.6 mmol, 115.02 mg), 1-hydroxybenzotriazole (HOBt, 0.6 mmol, 81.08 mg) and N, N-diisopropylethylamine (DIPEA, 0.6 mmol, 77.55 mg) catalysts were then added into the solution. The mixture was stirred at room temperature for 24 h. When the reaction was over, the mixture was concentrated under reduced pressure, and then the residue was purified by silica gel column chromatography to acquire the desired product Sof-P as a white solid (yield: 83%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.14 (s, 1H), 7.59 (dd, *J* = 16.9, 12.2 Hz, 2H), 7.48 (d, *J* = 8.7 Hz, 2H), 7.11 (d, *J* = 15.7 Hz, 1H), 6.86 (d, *J* = 8.6 Hz, 2H), 6.54 (dd, *J* = 8.6, 1.9 Hz, 1H), 6.40 (d, *J* = 1.9 Hz, 1H), 5.41 (dd, *J* = 3.2, 1.4 Hz, 2H), 4.50 (dd, *J* = 13.3, 5.4 Hz, 6H), 3.94 (dd, *J* = 5.4, 2.4 Hz, 2H), 2.08 (t, *J* = 2.4 Hz, 1H), 1.74 (d, *J* = 4.8 Hz, 6H), 1.69 (d, *J* = 6.4 Hz, 6H). ^{13}C-NMR (126 MHz, CDCl3) δ 190.82, 168.03, 163.30, 161.06, 158.07, 144.22, 139.28, 138.82, 132.62, 130.15, 127.26,

123.47, 121.64, 119.12, 118.71, 115.19, 106.98, 100.83, 79.24, 71.20, 67.72, 65.25, 64.97, 28.72, 25.68, 18.30, 18.26. High-resolution mass spectrometry (HRMS) m/z: $[M+H]^+$ calculated for $C_{30}H_{33}NO_5$ was 488.2437 and found as 488.2437.

Cell culture

The Caco-2 cell line was purchased from the Chinese Academy of Medical Sciences (Beijing, China), and cultured in Dulbecco's MEM with Glutamax-I (Corning, USA) supplemented with 10 mmol/L HEPES, 20% (v/v) heat-inactivated fetal bovine serum (Corning, USA), 1% (v/v) penicillin/streptomycin (ThermoFisher, USA), and 1% (v/v) nonessential amino acids (NEAA) (ThermoFisher, USA) at 37 ℃ with 8% CO_2. In this study, Caco-2 cells were pre-stimulated with LPS (10 μg/mL) for 6 h and then treated with Sof or Sof-P.

Cell viability

Caco-2 cells were seeded in 96-well plates at a density of 1.0×10^4 cells/well for 24 h, and then treated with different concentrations of Sof or Sof-P for 24 h. Cell counting kit-8 (CCK-8) kit (Dojindo, Japan) was used to measure the viability of treated cells as described by the manufacturer. The absorbance at 450 nm of solutions was measured by a multimode plate reader (PerkinElmer, USA).

Intracellular ROS detection

Caco-2 cells were seeded in 6-well plates at 3.0×10^5 cells per well and incubated for 24 h. Then 10 μg/mL LPS was added to stimulate Caco-2 cells. After 6 h of stimulation, the cells were treated with or without serial dilutions of Sof for 12 h. Subsequently, the intracellular reactive oxygen species (ROS) was stained with DCFH-DA (10 μM) for 20 min and quantitative analysis by flow cytometry (Beckman, USA).

Western blotting (WB)

Total proteins were extracted from Caco-2 cells with RIPA lysis buffer (Beyotime, China) supplemented with a 1×protease inhibitor cocktail (ThermoFisher, USA) and separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were further electro-transferred to polyvinylidene fluoride (PVDF)

membranes for 1 h at 120 V. Next, the membranes were blocked in 5% bovine serum albumin (BSA), and incubated overnight at 4 °C with the corresponding primary antibodies and secondary antibody for 2 h at room temperature (RT) in turn. Finally, the protein band was visualized by enzyme-linked chemiluminescence (ECL) (Thermo Fisher, USA). The protein band was semi-quantified by Image J software.

Measurement of NO production in Caco-2 cells

The concentration of nitric oxide (NO) in culture supernatant released from Caco-2 cells was tested by Griess assay. Caco-2 cells were stimulated with LPS (10 μg/mL) for 6 h and then treated with or without serial dilutions of Sof for 24 h. The NO concentration in the collected supernatants was measured by the Griess reagent according to the manufacturer's protocol (Solarbio, China).

In-gel fluorescence labeling experiments

For *in situ* fluorescence labeling experiments, Caco-2 cells were seeded in 6-well plates at a density of 5.0×10^5 cells/well for 24 h. Sof-P (6.25-100 μmol/L) or DMSO was added to the plates for 4 h. Next, the cells were washed three times with PBS to remove the residual drug probes. The harvested cells were lysed with the lysis buffer (0.1% Triton X-100 and 1% protease inhibitor). Soluble proteins were then obtained by centrifugation and protein concentration was determined by BCA assay (Pierce™ BCA protein assay kit). Subsequently, 100 μL of proteins (2 mg/mL) were taken from each group for the click chemistry reaction. The pre-mixed click reaction reagents including 9 μL of TBTA (10 mM in DMSO), 3 μL of TCEP (50 mM in ddH_2O), 3 μL of $CuSO_4$ (50 mM in ddH_2O), and 1 μL of TAMRA-azide (10 mM in DMSO) were added into the lysate samples, and the reaction was conducted in a shaker with 800 rpm at 25 °C for 2 h. The proteins were precipitated using 1 mL of pre-chilled acetone at -20 ℃ for 30 min and purified by centrifugation. The samples were then dissolved in 50 μL of 1× loading buffer and then denatured for 5 min at 95°C followed by separation in 12% SDS-PAGE gels. Finally, the labeling proteins were fluorescently imaged by a laser scanner (Azure Sapphire RGBNIR, USA) and stained with coomassie brilliant blue (CBB).

For *in situ* fluorescent labeling competition experiments, Caco-2 cells were initially pretreated with Sof for 4 h and then incubated with Sof-P for another 4 h. Protein extraction, click chemical reaction and electrophoretic separation were performed as described above. For fluorescence labeling of recombinant proteins, the protein was pretreated with or without different concentrations of Sof or iodoacetamide (IAA) in PBS with vigorous shaking at 37 ℃ for 1 h, followed by probe treatment for another 1 h. And then the samples were clicked by chemical reaction, separated in SDS-PAGE gel, and visualized as described above.

Confocal fluorescence imaging

A cellular imaging experiment was first performed to visualize the distribution of Sof-P in Caco-2 cells. Caco-2 cells were seeded in 4-chamber dishes for 24 h and pre-stimulated with LPS (10 μg/mL) for 6 h. Next, the samples were incubated with 0.5 mL of complete culture medium containing Sof-P (10 μM or 50 μM) for 4 h. The cells were then fixed with 4% paraformaldehyde at RT for 15 min after PBS washing and penetrated with 0.2% Triton X-100 in PBS for 15 min. Subsequently, the click chemistry reaction was carried out for 2 h at RT under the protection from light, followed by PBS washing to remove residual reagents. The cells were stained with Hoechst (1:5000) for 10 min and imaged with confocal fluorescence microscopy.

For co-localization of Sof-P and HMGB1, after being stimulated or not by LPS (10 μg/mL) for 6 h, Caco-2 cells were treated with Sof-P (50 μM) in the absence or presence of Sof (250 μM) for 4 h. The cells were fixed, permeated, and labeled by click chemistry reaction in turn as mentioned above, and finally blocked with 5% BSA for 1 h. After that, the samples were incubated overnight at 4 °C with an anti-HMGB1 antibody and the secondary fluorescence antibody (goat anti-rabbit, 1:500, Abcam) in sequence for 1 h at RT. The samples were stained with Hoechst (1:5000) for 10 min and imaged with confocal fluorescence microscopy.

For co-localization observation of TLR4 and NF-κB p-p65, after being stimulated or not by LPS for 6 h, Caco-2 cells were further treated with Sof at a concentration of

50 μM for 24 h. The cells were fixed, permeated, and blocked as mentioned above and the samples were co-incubated overnight at 4 °C with anti-TLR4 and anti- NF-κB p-p65 antibodies, followed by the same operations mentioned above.

Target identification based on ABPP

Caco-2 cells were first stimulated with LPS for 6 h and then treated with the Sof-P (50 μM) for 4 h in the presence or absence of a competitor (Sof, 250 μM). The proteins were extracted according to the aforementioned method and conjugated with biotin-azide by a clicked chemistry reaction. Subsequently, the samples were precipitated using pre-chilled acetone and re-dissolved in 1.2 % SDS. Streptavidin beads (50 μL) were added to the samples and incubated for 4 h at RT. After that, the beads were successively washed with 1 % SDS, 0.1 % SDS, and 6 M urea in PBS. Next, the samples were reduced and alkylated with dithiothreitol (DTT) and iodoacetamide (IAA). The proteins captured by beads were then digested into peptides using trypsin. The peptides in supernatants were collected by centrifugation and desalted using C18 columns. Then, the peptides were labeled using the TMT^{10} plex reagent according to the MS/MS quantification instructions. Finally, the samples were analyzed by LC-MS/MS (Orbitrap Fusion Lumos, Thermo, USA).

Targeted protein analysis and KEGG pathway enrichment

Differential analysis of target proteins was conducted based on changes in TMT signals among the DMSO (control) group, Sof + Sof-P (compete), and Sof-P (treatment) group, and the target proteins were selected based on absolute fold changes >2 and *p*-values (FDR) <0.05. According to previous literature [1], KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was performed to visualize functional profiles and pathway enrichment.

Cellular thermal shift assay (CETSA)

CETSA was used to validate the binding of small molecules and target proteins [2]. Proteins were extracted from Caco-2 cells, and equal amounts of proteins were incubated with Sof (20 μM) or DMSO respectively for 1 h at 37 °C, followed by heating

at the corresponding temperatures for 3 min using a thermocycler (Applied Biosystems, USA). After centrifugation and denaturation, the samples were detected by WB.

Expression and purification of HMGB1 full-length protein and its C106G mutant, A box and B box

Recombinant human HMGB1 full-length protein and its C106G mutant, A box, and B box gene were subcloned into a pET28a vector with a 6 × His-tag fusion (Sangon, Shanghai, China). The E. coli BL21 was transformed with the expression plasmid and then cultured in LB medium containing kanamycin (50 μg/mL) with shaking at 37 °C until OD_{600} reached 0.6-0.8, and the protein expression was induced with isopropyl-D-1-thiogalactopyranoside (IPTG-0.4 mmol/L) at 17 °C for 14 h. After harvesting the bacteria by centrifugation and resuspension, the proteins were extracted by lysis buffer (200 mmol/L NaCl, 20 mmol/L Tris-HCl, 1 mmol/L PMSF, pH=8.0) combined with sonication. The lysis mixture was centrifuged and the reserved supernatant was further incubated with Ni-NTA beads column (Qiagen, USA) at 4°C for 4 h. Subsequently, the proteins bound to the beads were eluted by the elution buffer (20 mM Tris-HCl, pH=8.0, 200 mM NaCl and 200 mM Imidazole). The samples were washed with PBS and concentrated in a centrifugal filtration tube. Protein concentrations were determined by the BCA assay and the purity and integrity of purified proteins were verified by SDS-PAGE gel electrophoresis.

Microscale thermophoresis

Microscale thermophoresis (MST) was utilized to quantitatively analyze the interactions between Sof and its target proteins according to the literature [3]. The binding affinity was detected with a Monolith NT.115 (NanoTemper Technologies, Munich, Germany). In brief, recombinant human HMGB1 full-length protein was fluorescently labeled by Monolith His-Tag Labeling Kit (NanoTemper Technologies, Munich, Germany). Sof (1 mM) was then diluted into a series of concentrations and mixed with HMGB1 solution (50% v/v). Afterward, the series of mixtures were loaded into capillaries separately and detected by MST with 40% power. The direct binding of

Sof to HMGB1 was observed as a change in the thermophoresis of the fluorescently labeled proteins upon the complex formation. The decrease in the fluorescence counts of the bound complex compared to the unbound proteins with increasing Sof concentration indicated a positive thermophoretic effect. The data was analyzed by MO Affinity Analysis Software v2.3.

UV-visible absorption spectrum measurement

The covalent binding of Sof to HMGB1 was tested by the UV-Vis absorption spectrum of Sof at 350 nm [4]. Briefly, the intensity of the absorption peak of Sof in the presence or absence of HMGB1 protein was measured by a multimode 96-well plate reader (PerkinElmer, USA). An excess of N-acetylcysteine (sulfhydryl donor) was used to react with Sof as a positive control. All recorded values were displayed by subtracting the blank value of PBS.

The reaction of Sof with N-acetylcysteine (NAC)

Sofalcone (0.2 mM) was first dissolved in phosphate buffered saline (PBS, pH=7.4): ACN (1:1, v/v, 1 mL), and then treated with a 200-M excess of t N-acetylcysteine (NAC) at 37 °C for 1 h. The reaction mixture was then subjected to high-performance liquid chromatography (Ultimate 3000, Thermo Fisher) coupled with mass spectrometry (LTQ Orbitrap velos pro, Thermo Fisher) analysis (HPLC-MS). A symmetry C18 column (100 × 2.1 mm, 1.8 μm) with a guard column (20 × 4.6 mm) was used for chromatographic separation. Samples from each experiment were filtered through membrane filters (0.45 μm). HPLC analysis was conducted at a flow rate of 0.3 mL/min using a mobile phase comprised of acetonitrile and 0.1% aqueous acetic acid.

Molecular docking of Sof with HMGB1

The structure of Sof was downloaded from PubChem, and the 3D structure of HMGB1 (AlphaFold: AF-P09429-F1) was obtained from the AlphaFold Protein Structure Database. The protein and chemical structure were first hydrogenated and dehydrated by Autodock soft (version 4.2.6), and then molecular docking was followed.

Discovery Studio 2019 client (version 2.5) and Pymol software (version 2.5) were respectively utilized to analyze and map intermolecular interactions.

Measurement of HMGB1 bioactivity

The effect of Sof on the pro-inflammatory activity of recombinant human HMGB1 protein was evaluated by detecting the content of IL-6 and TNF-α in Caco-2 cells. The WB assay was used to measure the levels of IL-6 and TNF-α after the cells were treated with HMGB1 (2 μg/mL) alone or Sof-HMGB1 complex for 24 h. To assess the effect of Sof on the NF-κB signaling pathway in LPS-stimulated Caco-2 cells, the content of NF-κB p-p65 in Caco-2 cells was determined by the WB assay after the treatment above mentioned.

To verify whether Sof blocked the binding site of HMGB1 protein to the corresponding receptors, the interactions of HMGB1 protein with Toll-like receptor 4 (TLR4) and advanced glycation endproducts (RAGE) were investigated by the co-precipitation experiment. To be specific, HMGB1 protein (10 nmol) was first incubated with or without an equivalent amount of Sof (10 nmol) or a five-fold amount of Sof (50 nmol) at 37 °C for 1 h, followed by the addition of freshly extracted Caco-2 cell lysate for another 1 h of shaking incubation. Afterward, the Ni-beads column was added to the mixture and the reaction was carried out at 4°C for 2 h. Then, the supernatant was discarded to remove unbound proteins by centrifugation. Subsequently, elution was performed to separate and purify HMGB1 binding proteins. Finally, the contents of TLR4 and RAGE in denatured samples were analyzed by the WB assay.

RNA interference assay

Three small interfering RNA (siRNA) targeting HMGB1 designed with different sequences (Table S1) and negative control (NC) vectors were synthesized by Sangon (Shanghai, China). According to the manufacturer's instructions, these siRNAs or NC lipofectamine transfected into Caco-2 cells by lipofectamine 2000 (Invitrogen). Briefly, Caco-2 cells were seeded in 6-well plates to achieve confluency of 80% and then starved with Opti-MEM treatment for 1 h. The transfection mixtures of lipofectamine

2000 and siRNA were incubated with cells for 6 h. After that, the medium containing transfection complexes was replaced with a medium containing 20% FBS and the cells were further cultured for 48 h before being harvested. The concentration of siRNA in the RNA interference assay was optimized as shown in Fig. S11, and siRNA-3 was selected for the subsequent experiments (Fig. 4D) based on the silencing effect.

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM) of three independent experiments. A one-way ANOVA test was used for the statistical analysis of data by GraphPad Prism 8.0 software (San Diego, CA, USA). p-values < 0.05 were considered statistically significant.

Reference

[1] P. Luo, D.D. Liu, Q. Zhang, et al., Acta Pharm Sin B 12 (2022) 2300-2314.

[2] R. Jafari, H. Almqvist, H. Axelsson, et al., Nat. Protoc. 9 (2014) 2100-2122.

[3] H. Tang, T. Yang, L. Chen, et al., J. Colloid Interface Sci. 630(Pt B) (2023) 179-192.

[4] P. Luo, Q. Zhang, T.Y. Zhong, et al., Mil. Med. Res. 9 (2022) 22.