Supporting Information

Oridonin acts as a novel senolytics by targeting Glutathione S-Transferases to activate ROS-p38 signaling axis in senescent cells

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Reagents and Materials

Oridonin (ORI) (purity≥98%) was purchased from Beijing Bethealth People Biomedical Technology (Beijing, China). Cisplatin (HY-17394), Etoposide (HY-13629), SB202190 (HY-10295), BIRB796 (HY-10320), GSTO1-IN-1 (GSTO1-IN-1, HY-11530), N-acetyl-L-cysteine (NAC, HY-B0215) and Z-VAD (OMe)-FMK (Z-VAD-FMK, HY-16658B) were obtained from MedChem Express (USA). Click chemistry reaction and LC-MS/MS reagents: TBTA (1770049), TCEP (C4706), Rhodmine-N3 (83689) and $CuSO_4$ (C1297) were bought from Sigma (USA). TMT 10plex™ reagent (A34808), high capacity neutravidin agarose resin (A53031), and trypsin (90057) were purchased from Thermo Fisher (USA). In addition, specific primary antibodies against p16 (ab51243), p21 (ab109520), p38 (ab60999), GSTK1 (14535-1-AP), MGST1 (ab129175), Phospho-p38 (ab170099) and cleaved Caspase 3 (ab32042) were purchased from Abcam (Shanghai, China).

Cell culture

A549 and HSF cells were purchased from the Chinese Academy of Medical Sciences (Beijing, China). The two cells were cultured in high glucose DMEM (Corning, USA) supplemented with 10% FBS (Corning, USA), and 100 IU penicillin/streptomycin (Thermo Fisher, USA) at 37 ℃ with 5% CO_2. For the experiments of inducing cellular senescence, A549 cells was induced with 15 μM cisplatin for 3 days, while HSF cells were exposed to 5 μM etoposide for 12-18 hours. L929 and HepG2 cells were irradiated with UVA for 20 minutes to induce cellular senescence. Finally, senescent cells were confirmed by SA-β-gal staining or p16 and p21 expression by western blotting after induction.

Screening

To screen out the active ingredients of the traditional Chinese medicinal that can selectively kill senescent cells, we carried the following experiment. Firstly, the

senescent and non-senescent A549 cells were plated at 8×10^3 cells per well and incubated with 20 different compounds at 10 μM for 24 h, respectively. Then, CCK-8 was adopted to evaluate cell survival, and the ratio between senescent and non-senescent cells was calculated. We defined that the compounds with ratio less than 0.6 were considered to have senolytic activities.

SA-β-gal staining

SA-β-gal staining was performed using a staining kit (C0602, Beyotime Biotechnology, Shanghai). Cells were treated with 1 mL of cell fixative for 15 minutes before incubation with β-gal staining solution overnight at 37 °C. At last, the cells were rinsed in PBS and imaged under optical microscope. The relative SA-β-gal positive area was calculated by using ImageJ software to count the total area and SA-β-gal positive area.

Western blotting

Proteins were collected from lysis of senescent and non-senescent A549 cells and transferred to PVDF membranes after separating by SDS-PAGE. Next, PVDF membranes were incubated with primary antibodies and secondary antibodies and visualized on the Azure C400 system with image J software analysis.

Detection of senolytic activity

The senolytic activity was evaluated by detecting cell survival between senescent and non-senescent cells under different concentrations of compound through CCK-8 assay. In brief, senescent and non-senescent cells (A549, HSF, L929 and HepG2) were seeded in 96-well culture plates at 8×10^3 cells per well and cultured for 24 hours. Then, oridonin at concentrations of 0, 6.25, 12.5, 20, 25, 40, 50, 100, 200 μM and 0, 1.5623, 3.125, 6.25, 12.5, 20, 25, 40, 50 μM was added into cells for 24 hours. Finally, the cell viability was detected by CCK-8.

Rescue experiment of NAC, SB202190, BIRB796, and Z-VAD-FMK.

To further test the rescue effect of SB202190, BIRB796, NAC and Z-VAD-FMK, 20 μM SB202190, 10 μM BIRB796, 1.5 mM NAC or 40 μM Z-VAD-FMK were added to the medium with different concentrations of oridonin and cultured at 37 °C for 24 hours, and the cell proliferation was detected by the above method.

Detection of senolytic activity of GSTO1-IN-1

Senescent and non-senescent A549 cells were seeded in 96-well culture plates at 8×10^3 cells per well and cultured for 24 hours. Then, GSTO1-IN-1 at concentrations of 0, 1.875, 3.75, 7.5 and 15 μM was added into cells for 24 hours. Finally, the cell viability was detected by CCK-8 assay.

The competitive in-gel fluorescence labeling of wild-type and mutated GSTK1 protein

In brief, to get cysteine-mutated GSTK1 proteins (Cys176 to Arg176), human *GSTK1* gene with point mutation was constructed into pET28a vector (Sangon, Shanghai, China). Then the *E. coli* BL21 was transformed with plasmids, followed by proteins purification. For labeling competition experiments, purified mutated and wild-type GSTK1 proteins were first pre-treated with oridonin or IAA for 1 h followed by labeling with IAA-yne probe. The click chemistry reaction was further done under conditions described above. At last, samples were separated in a 12% SDS-PAGE gel and scanned for fluorescence. The total proteins were visualized with Coomassie brilliant blue staining.

Cellular Imaging

The accumulation of ROS in senescent A549 cells was detected by the DCFH-DA probe. Briefly, senescent A549 cells (5×10^4 cells/well) were seeded into 4-chamber glass bottom dishes (Cellvis) and treated in 0.5 mL DMEM with 10% FBS in three groups: DMSO group, 50 μM oridonin, 50 μM oridonin+1.5 mM NAC for 6 hours. Subsequently, DCFH-DA probe (C2938, Thermol) and Hoechst (H3569) were

incubated with cells for 30 min before examining by the confocal fluorescence microscope. ImageJ was used to quantify the mean fluorescence intensity.

The regulation of the p38 pathway by oridonin was also captured by fluorescence microscopy. Senescent A549 cells were divided into two groups: DMSO and 50 μM oridonin. Then, cells were fixed with cell fixative for 20 min and permeabilized in 0.2% Triton X-100 for 15 min before incubation with anti-p38 (1:500), p-p38 (1:500) at 4 ℃ overnight and goat anti-rabbit IgG (1:1000) (Alexa Fluor 488) next day for 2 hours. In the end, cells were visualized by laser scanning confocal fluorescence microscopy (Leica TCS SP8).

RNA-seq

Senescent and non-senescent A549 cells were seeded in 15 cm dishes at 2×10^6 cells per well and treated with 0.5 mL DMEM containing 10% FBS in the absence or presence of 25 μM oridonin. After 24 hours, the cells were gently washed with 5 mL PBS, and lysed by 1 mL Trlzol reagent until the whole solution was clear and not viscous. The expression and differential changes of the cell genome were investigated through the Hiseq-PE150 platform.

GSTK1 and MGST1 enzyme activity assay

Enzyme activity was measured by GST activity detection kit (BC0350, Solarbio, China). Different concentrations of oridonin was added to 1 μg GSTK1 and 1 μg MGST1 protein in PBS buffer, respectively. The absorbance at 340 nm was measured to reflect the enzymatic activity. We scanned every 30 minutes until 2 hours through a microplate reader (PerkinElmer EnVision, UK).

The competitive in-gel fluorescence labeling assay

Senescent A549 cells (1×10^6 cells/well) were seeded into 6 cm plates and incubated with different concentrations of oridonin (0, 10, 50, 100, 200 and 400 μM) for 2 hours. Then cells were harvested, and lysed with 200 μL RIPA. After protein concentration determination, cells were then labeled with cysteine-specific probe (IAA-yne) on

shaker at 37 °C for 1 hour. Then, add 13 μL click buffer to each sample, including 9 μL TBTA (10 mM in DMSO), 3 μL TCEP (50 mM in ddH_2O), 3 μL $CuSO_4$ (50 mM in ddH_2O), and 1 μL TAMRA-azide compound (10 mM in DMSO) and incubate for 2 h on a shaker at 37 °C. After adding 1 mL of acetone to the mixture, the labeled protein was precipitated at -80 °C for 30 minutes, centrifuged at 20,000 g for 10 minutes, and the supernatant was discarded to evaporate the acetone. Before separation in a 12% SDS-PAGE gel, the samples were dissolved in 100 μL of 1× loading buffer, denatured by heating in a metal bath at 90 °C for 5 minutes, and finally scanned for fluorescence with an Azure Sapphire RGB NIR scanner (USA). The total proteins were visualized with Coomassie brilliant blue staining.

The pull-down experiment

Pull-down experiments were conducted to identify the interacting protein of oridonin. As previously described, samples were harvested and divided into three groups: DMSO, IAA-yne, and ORI+IAA-yne. Then, 100 μL samples were clicked with 9 μL 10 mM TBTA, 3 μL 50 mM TCEP, 3 μL 50 mM $CuSO_4$, and 1 μL 5 mM TAMRA-N_3 for 2 h at room temperature. Subsequently, labeled proteins were precipitated by acetone, before dissolving in 1 mL 1.2% SDS/PBS, and heating at 95 °C for 15 min. Soluble proteins were diluted to 0.2% SDS with 5 mL 1 × PBS and 50 μL streptavidin beads were added for 4 h at RT to pull down the interacting proteins. After that, beads were collected through centrifuging at 1400 g for 3 min, and washed with 1% SDS, 0.1% SDS, and 6 M Urea three times sequentially. Finally, the enriched proteins were separated by SDS-PAGE gel for immunoblotting through adding 1 × loading buffer for 10 min at 90 ℃.

The streamlined cysteine activity-based protein profiling

To identify the potential protein targets of oridonin in senescent cells, we performed competitive mass spectrometry experiments. A novel desthiobiotin-iodoacetamide (DBIA, ChomiX Biotech Co, Nanjing, China) probe was selected for the following steps. Three groups were set: 500 μM DBIA, 100 μM oridonin+500 μM DBIA, and 200

μM oridonin+500 μM DBIA. Similar to in-gel fluorescent labeling of oridonin in senescent A549 cells as descripted above, the probe labeling process was first performed by adding 500 μM DBIA to 100 μL of oridonin-treated cell lysis for 1 hour. Secondly, 5 mM DTT and 20 mM IAA was added to alkylate the reduced cysteine residue for 30 minutes in the dark. Then, 2 μg of trypsin and Lys-C (Thermo Scientific, USA) was added to digest the protein. For the peptide labeling process, all TMTs were labeled with DBIA-containing cysteines conjugated according to TMT10plex Mass Tag reagent (Thermo Scientific, USA). For peptide enrichment, 100 μL of streptavidin magnetic beads (Thermo Scientific, USA) were added to the TMT-labeled mixed sample dissolved in 1 mL of PBS. To remove nonspecific binding, wash the beads as follows: 3 washes with 1 mL PBS, 1 mL 0.1% SDS, and 1 mL ddH_2O. Finally, peptides were desalted with 0.1% formic acid, and desalted with a commercial C18 column before mass spectrometry detection.

The whole-proteome experiment

Firstly, senescent A549 cells (1×10^6 cells) were seeded into 10-cm plates and treated with 25 μM oridonin or DMSO for 12 hours before collecting cell lysis. Then, BCA kit was used to determine the protein concentration. Next, 5 mM DTT and 10 mM IAA were added for 30 minutes. The samples were digested overnight with trypsin at 37 ℃ and desalted with a commercial C18 column before final use.

Binding site identification

To find the direct binding site of oridonin on GSTK1 protein, 20 μg purified GSTK1 protein was mixed with 2 mM oridonin in PBS system for 2 hours. Then, 5 mM DTT and 20 mM IAA was added to the solution for 30 minutes successively. To exclude the interference of drugs and reagents, 300 μL methanol, 100 μL chloroform together with 300 μL ddH_2O was adopted to precipitate proteins. Finally, 1 μg trypsin was used to digest GSTK1 proteins into peptides, followed by desalting on C18 column, and detected by mass spectrometry.

GO and KEGG enrichment analysis

The abundance changes in the control and oridonin treated groups in the whole proteome experiment were used to identify differential proteins based on absolute fold change ≥ 1.5 and P-value (FDR) < 0.05. Then, we draw the Volcano map through the bioladder website (https://www.bioladder.cn). The biological process and kegg pathway enrichment were selected to visualized functional profiles after analyzing the differential proteins by DAVID+ version (https://david.ncifcrf.gov/).

Statistical analysis

GraphPad Prism 8.0 software (GraphPad Prism, USA) was used to perform Statistical analysis. All data were expressed as mean±standard deviation (SD) and evaluated with Student's *t*-test and ANOVA test. Differences were considered statistically significant at $p < 0.05$, < 0.01, < 0.001 or < 0.0001.

Table. S1 Survival rate of senescent and non-senescent A549 cells under 10 μM natural products treatment

Natural product	Survival rate of sen-A549	Survival rate of non-A549	Ratio
A-1331852	12.78119897	46.04741287	0.25104
Baicalein	114.9713251	99.66339412	1.1536
Tanshinone IIA	69.81587685	63.19775596	1.10472
Salvianic acid A	101.4488379	99.7194951	1.01734
Ferulic acid	106.8819801	108.5273492	0.98484
Ganoderic Acid A	100.1811048	100.5049088	0.99678
Andrographolide	104.8294597	98.20476857	1.06746
Chlorogenic acid	95.41201328	90.18232819	1.05799
Tanshinone I	53.47686936	68.91356067	0.776
Rosmarinic acid	75.99650858	96.03489294	0.79134
Dicoumarin	77.04393366	89.69072165	0.859
Tetrahydroberberine	70.7011929	90.64234734	0.78
Eupalinolide	104.9614396	103.1000319	1.01805
Citric acid	93.23907455	93.19271333	1.0005
Curcumin	89.12596401	98.88143177	0.90134
Isorhynchophylline	89.94858612	111.0898051	0.80969
Sinomenine	66.86375321	95.68552253	0.69879
Oridonin	54.30351815	100.3634948	0.54132
Dihydroartemisinin	74.781491	99.71236818	0.74997
Loganin	99.56357289	109.2783505	0.9111

Table. S2 The protein target list identified by ABPP.

NO.	Protein Accessions	Master Protein Descriptions	Annotated Sequence	Cystinesposition	Ratio
1	P10620	MGST1	VFANPEDCVAFGK	50	12.409
2	Q9Y2Q3	GSTK1	ETTEAACR	176	10.985
3	Q06323	PSME1	GPPCGPVNCNEK	101,106	10.715
4	P00352	ALDH1A1	LECGGGPWGNK	370	10.554
5	P19338	NCL	EALNSCNK	543	10.39
6	Q01082	SPTBN1	AELFTQSCADLDK	1389	9.5302
7	P52272	HNRNPM	GCGVVK	694	9.0083
8	P40261	NNMT	IFCLDGVK	50	8.7642
9	P78527	LGALS1	LPVLAGCLK	223	8.6095
10	P52895	AKR1C3	EEPWVDPNSPVLLEDPVLCALAK	242	8.3229
11	P42330	HNRNPU	WVDPNSPVLLEDPVLCALAK	242	7.7326
12	Q00839	CFL1	APQCLGK	562	7.5926
13	P23528	RRBP1	HELQANCYEEVK	139	7.5814
14	P52272	SFN	GCAVVEFK	114	7.4337
15	P31949	GSTP1	CIESLIAVFQK	13	7.2642
16	P09211	PGRMC1	ASCLYGQLPK	48	7.2392
17	O00264	RPSA	GLATFCLDK	129	7.1766
18	P08865	ACTB	YVDIAIPCNNK	163	6.9683
19	P08670	HSPA2	QVQSLTCEVDALK	328	6.9616
20	P09382	RPS20	FNAHGDANTIVCNSK	61	6.9309
21	P14618	PSME2	GIFPVLCK	474	6.7707
22	P54652	RPL11	GCAGGEK	191	6.704
23	P35221	RAP1B	AAAGEFADDPCSSVK	116	6.6547
24	P14618	MYH9	AEGSDVANAVLDGADCIMLSGETAK	357	6.555
25	P60866	GSTO1	TPCGEGSK	70	6.4841
26	Q9UL46	ECHS1	CGFLPGNEK	91	6.3523
27	P23528	DSTN	CTLAEK	147	6.2596
28	P35580; P35579	RPS3	CIIPNHEK	678; 671	6.2092
29	P78527	PSMC6	APPCEYK	3837	6.1395
30	O60701	IDH1	ASVGFGGSCFQK	276	6.0805

The yellow-labeled proteins are members of the Glutathione S-Transferase family

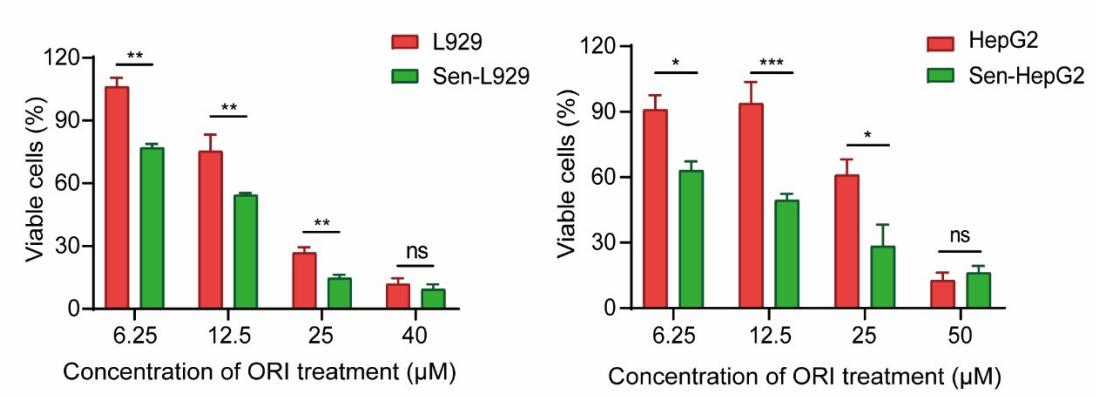

Fig. S1 Detection of cell viability of senescent and non-senescent L929 and HepG2 cells treated with different concentrations of oridonin for 24 h. Data were mean ± s.d., two-way ANOVA test used. *P<0.05, **P<0.01, ***P<0.001, and ns means no significant difference.

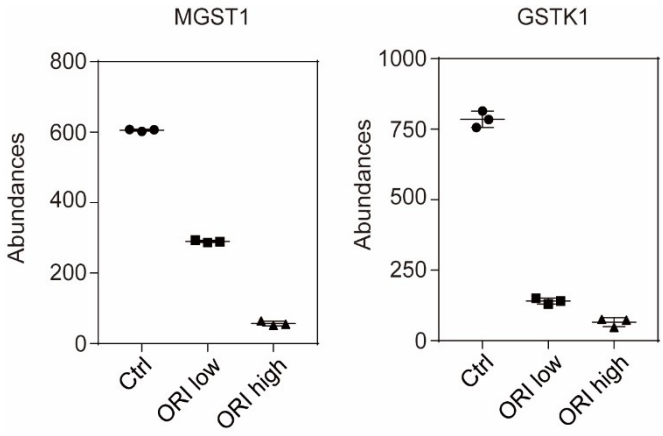

Fig. S2 Protein abundance changes of MGST1 and GSTK1 in control and oridonin-treated samples.

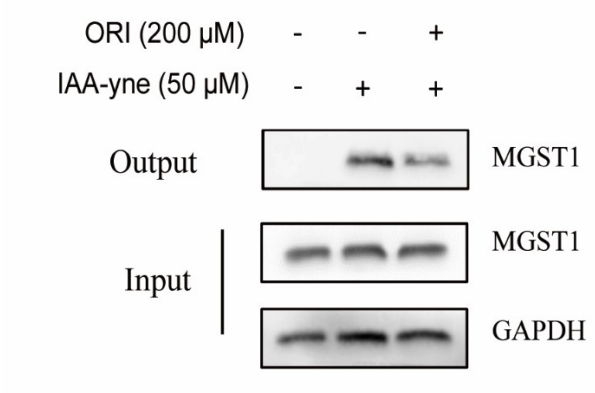

Fig. S3 Oridonin interacted with MGST1 protein in cells *via* the pull-down assay.

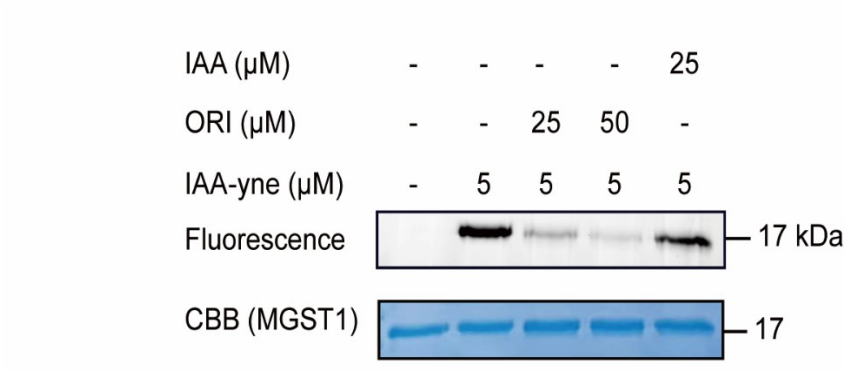

Fig. S4 Oridonin competed with IAA-alkynyl for binding to purified recombinant MGST1 protein *via* gel fluorescence assay.

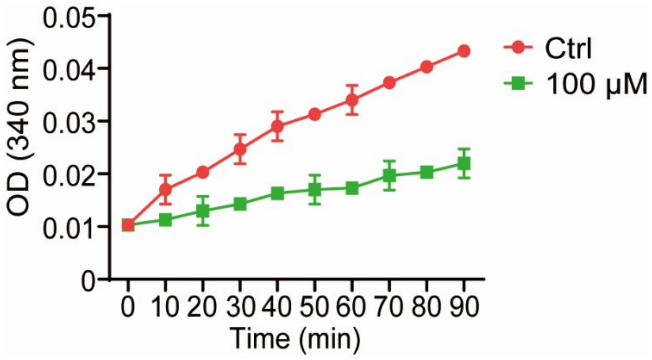

Fig. S5 Oridonin inhibited the enzymatic activity of MGST1 protein *in vitro*.

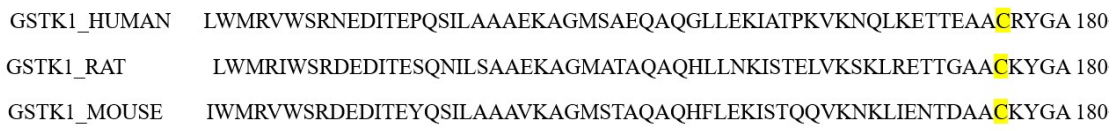

Fig. S6 Alignment of GSTK1 protein sequences from different species.

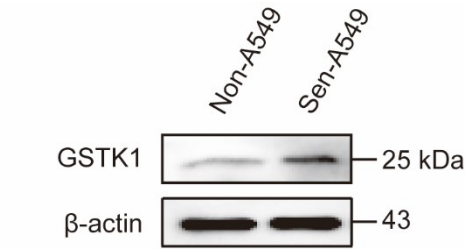

Fig. S7 Detection of the protein level of GSTK1 in senescent A549 cells and non-senescent A549 cells.

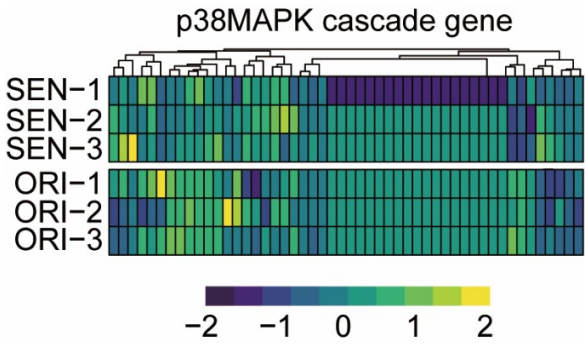

Fig. S8 Expression changes of the genes involved in p38 cascade. SEN-1, SEN-2 and SEN-3 were samples collected from senescent A549 cells treated with DMSO as control. ORI-1, ORI-2 and ORI-3 were samples collected from senescent A549 cells treated with 25 μM oridonin for 24 h.

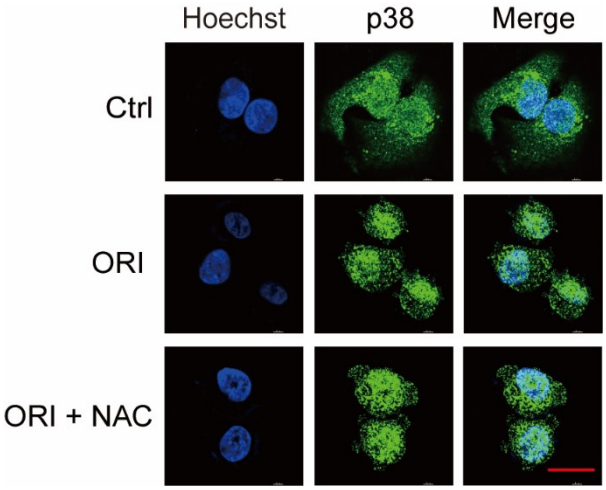

Fig. S9 The effect of oridonin on the level of total p38 in senescent A549 cells was verified by immunofluorescence. Bar=10 μm.