Supplementary information

Inhibition of cIAP1 as a strategy for targeting c-MYC driven oncogenic activity

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Supplementary Methods

Chemistry

All reagents and starting materials were obtained from commercial suppliers and used without further purification unless otherwise stated. Reaction progress was monitored by thin layer chromatography (TLC) on preloaded silica gel 60 F254 plates. Visualization was achieved with UV light. Yields were of purified product and were not optimized. ^{1}H NMR and ^{13}C NMR were recorded on Bruker AM-400 in the corresponding solvent. LCMS was carried out on Agilent G6100 LC/MSD (ESI) single Quand mass spectrometer. High-resolution mass spectra were recorded on Thermo Fisher Scientific LTQ FTICR-MS. The purity was determined by high-performance liquid chromatography (HPLC). Purity of all final compounds was 95% or higher. The instrument was an Agilent Technologies 6120 LC/MS system. The column was a Phenomenex Luna C18, 100A, 2.0 50 mm, 5 μm.

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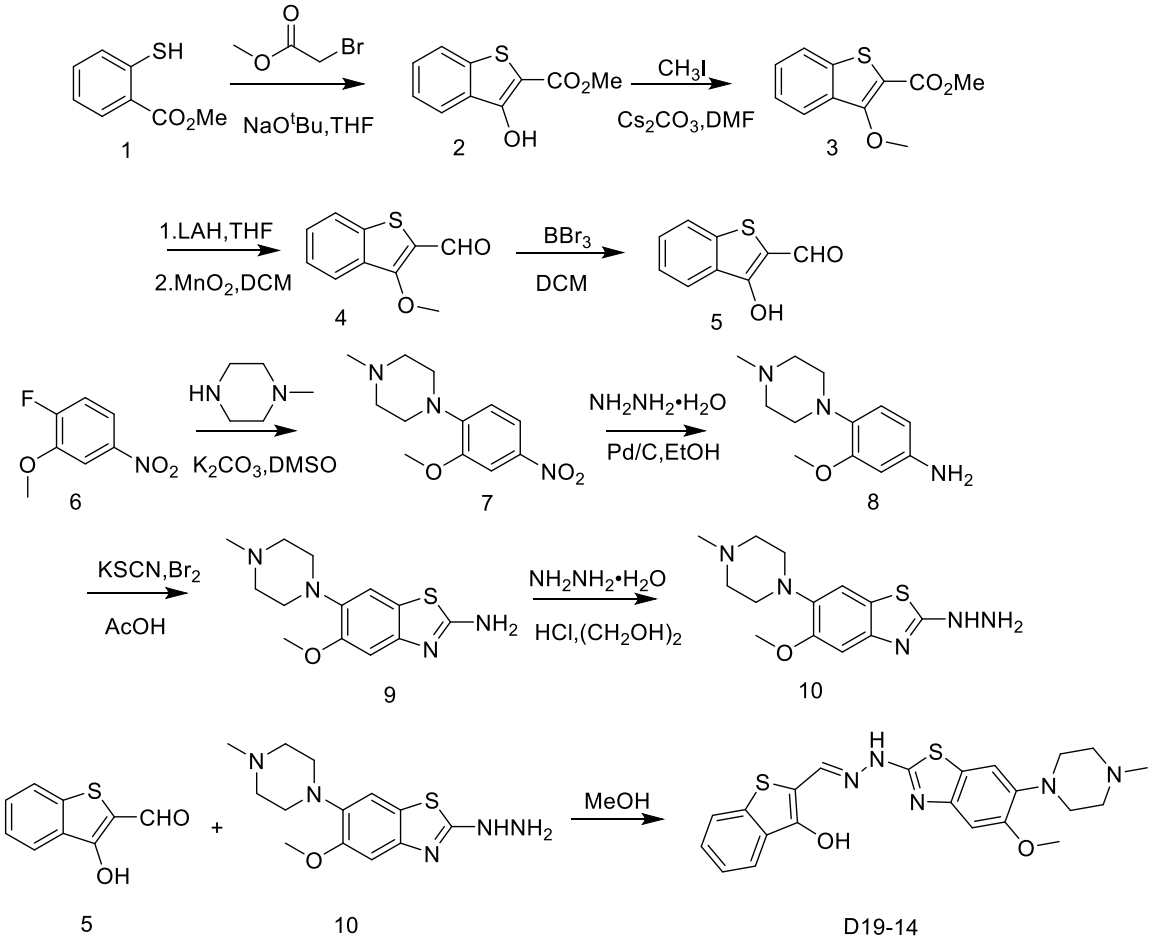
D19-14 was synthesized as shown in Scheme 1



Scheme 1. Synthesis of D19-14

Synthesis of methyl 3-hydroxybenzo[b]thiophene-2-carboxylate (2).

Sodium tert-butoxide (0.577 g, 6.0 mmol) was added slowly to a solution of methyl thiosalicylate (0.138 mL,1.0 mmol) and methyl bromoacetate (0.094 mL, 1.0 mmol) dissolved in anhydrous THF (4.0 mL) at 0°C. Then, the resultant yellow suspension was stirred at room temperature for 15 min. 2M aqueous HCl was added until pH=2, followed by three extractions with EtOAc. The combined organic phases were washed with brine, dried over anhydrous sodium sulfate and evaporated in vacuo. The crude material was purified via column chromatography on silica gel (solvent gradient: 0%-2% EtOAc in petroleum ether) to afford the compound 2 (0.187 g, yield 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 7.94 (d, J = 8.1 Hz, 2H), 7.57 (t, J = 8.2 Hz, 1H), 7.46 (t, J = 7.5 Hz, 1H), 3.86 (s, 3H). HRMS-ESI: calcd. for $C_{10}H_9O_3S$ $[M + H]^+$, 209.0267; found, 209.0265

Synthesis of methyl 3-methoxybenzo[b]thiophene-2-carboxylate (3)

To a solution of cesium carbonate (0.489 g,1.5 mmol) and compound 2 (0.208 g,1.0 mmol) dissolved in anhydrous DMF (4.0 mL) was added Iodomethane (0.093 mL, 1.5 mmol). The reaction was stirred at room temperature for 2 hrs.The mixture was diluted with water, followed by three extractions with EtOAc. The combined organic phases were washed with brine, dried over anhydrous sodium sulfate and evaporated in vacuo. The crude material was purified via column chromatography on silica gel (solvent gradient: 0%-2% EtOAc in petroleum ether) to afford the compound 3 (0.211g, yield 95%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.99 (dt, *J* = 8.2, 0.9 Hz, 1H), 7.90 (dt, *J* = 8.1, 1.0 Hz, 1H), 7.58 (ddd, *J* = 8.2, 7.0, 1.3 Hz, 1H), 7.49 (ddd, *J* = 8.1, 7.1, 1.1 Hz, 1H), 4.09 (s, 3H), 3.86 (s, 3H). HRMS-ESI: calcd. for $C_{11}H_{11}O_3S$ $[M + H]^+$, 223.0423; found, 223.0422.

Synthesis of 3-methoxybenzo[b]thiophene-2-carbaldehyde (4)

To a solution of compound 3 (0.222 g, 1.0 mmol) in anhydrous THF (4.0 mL) was added Lithium aluminum hydride (2.5 M in THF, 1.2 mL, 3.0 mmol) at 0°C under nitrogen atmosphere, the reaction was stirred at 0°C for 30 min, quenched with ice water until no precipitate formed. Then, 2 M aqueous HCl was added until the precipate disappeared, followed by three extractions with EtOAc. The combined organic phases were washed with brine, dried over anhydrous sodium sulfate and evaporated in vacuo. Without further purification, the crude material was dissolved in dichloromethane (5.0 mL) and activated manganese dioxide (0.869 g, 10.0 mmol) was added to the solution. The reaction was stirred at room temperature overnight, the mixture was filtered with diatomite and concentrated in vacuo. The crude material was purified via column chromatography on silica gel (solvent gradient: 5%-10% EtOAc in petroleum ether) to afford the compound 4 (0.138 g, yield 72%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.37 (s, 1H), 7.91 (d, *J* = 8.1 Hz, 3H), 7.76 (d, *J* = 8.2 Hz, 3H), 7.50 (ddd, *J* = 8.2, 7.2, 1.2 Hz, 3H), 7.39(ddd, 3H), 4.34 (s, 8H). HRMS-ESI: calcd. for $C_{10}H_9O_2S$ $[M + H]^+$, 193.0318; found, 193.0316.

Synthesis of 3-hydroxybenzo[b]thiophene-2-carbaldehyde (5)

To a solution of compound 4 (0.192 g, 1.0 mmol) in anhydrous dichloromethane (4.0 mL) was added boron tribromide (1.0 M in dichloromethane, 3.0 mL, 3.0 mmol) at -78°C under nitrogen atmosphere. After 20 min, the reaction was stirred at room temperature overnight. The mixture was poured into ice water, followed by three extractions with dichloromethane. The combined organic phases were washed with brine, dried over anhydrous sodium sulfate and evaporated in vacuo. The crude material was purified via column chromatography on silica gel (solvent gradient: 0%-10% EtOAc in petroleum ether) to afford the compound compound 5 (0.144 g, yield 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.76 (s, 1H), 8.03 (d, *J* = 8.0 Hz, 1H), 7.78 (d, *J*

= 8.2 Hz, 1H), 7.57 (ddd, *J* = 8.3, 7.1, 1.2 Hz, 1H), 7.47-7.42 (m, 1H). HRMS-ESI: calcd. for $C_9H_7O_2S$ $[M + H]^+$,179.0161; found, 179.0160.

Synthesis of 1-(2-methoxy-4-nitrophenyl)-4-methylpiperazine (7)

Potassium carbonate (0.276 g, 2.0 mmol) was added to a solution of 2-fluoro-5-nitroanisole (0.171 g, 1.0 mmol) and 1-methylpiperazine (0.100 g, 1.0 mmol) dissolved in dimethyl sulfoxide (4.0 mL),the reaction was heated to 120°C thermally. After 18 hrs, the reaction was cooled to room temperature, diluted with water, and extracted with EtOAc for three times. The combined organic phases were washed with brine, dried over anhydrous sodium sulfate and evaporated in vacuo. The crude material was purified via column chromatography on silica gel (solvent gradient: 0%-2% MeOH in dichloromethane) to afford the compound 7 (0.234g,yield 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (dd, *J* = 8.8, 2.5 Hz, 1H), 7.71 (d, *J* = 2.5 Hz, 1H), 6.90 (d, *J* = 8.8 Hz, 1H), 3.95 (s, 3H), 3.33-3.25 (m, 4H), 2.69-2.64 (m, 4H), 2.40 (s, 3H). HRMS-ESI : calcd. for $C_{12}H_{18}O_3N_3$ $[M + H]^+$, 252.1343; found, 252.1342.

Synthesis of 3-methoxy-4-(4-methylpiperazin-1-yl)aniline (8)

To a solution of compound 7 (0.251 g,1.0 mmol) dissolved in anhydrous ethanol (10.0 mL) was added 5% Pd/C (0.05 g), the mixture was heated to 85°C. Then, 80% hydrazine hydrate (2.34 mL) was added slowly and the reaction was stirred at 85°C for 3 hrs. The mixture was cooled to room temperature, filtered with diatomite and concentrated in vacuo. The crude material was purified via column chromatography on silica gel (solvent gradient: 2%-5% MeOH in dichloromethane) to afford the compound 8 (0.184 mg,yield 83%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.60 (d, *J* = 8.3 Hz, 1H), 6.22 (d, *J* = 2.3 Hz, 1H), 6.07 (dd, *J* = 8.3, 2.4 Hz, 1H), 4.70 (s, 2H), 3.67 (s, 3H), 2.79 (s, 4H), 2.42 (s, 3H), 2.20 (s, 3H). HRMS-ESI: calcd. for $C_{12}H_{20}ON_3$ $[M + H]^+$, 222.1601; found, 222.1599.

Synthesis of 5-methoxy-6-(4-methylpiperazin-1-yl)benzo[d]thiazol-2-amine (9)

A mixture of compound 9 (0.278 g, 1.0 mmol) and potassium thiocyanate (0.389 g, 4.0 mmol) was dissolved in 2.0 mL acetic acid and the reaction mixture was stirred at 35°C for 1 h. Then Bromine (0.051 mL, 1.0 mmol) was added slowly, and the reaction was stirred at room temperature for 3 hrs. After completion of the reaction, the mixture was cooled to 0°C and ammonium hydroxide was added until pH=10. The resulting solid was filtered, washed with water and dried. Then the crude material was purified via column chromatography on silica gel (solvent gradient: 0%-5% MeOH in dichloromethane) to afford the compound 9 (0.159 g, yield 57%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.18 (s, 3H), 6.93 (s, 1H), 3.77 (s, 3H), 2.90 (s, 4H), 2.47 (s,

4H), 2.23 (s, 3H) HRMS-ESI: calcd. for $C_{13}H_{19}ON_4S$ $[M + H]^+$, 279.1274; found, 279.1272.

Synthesis of 2-hydrazinyl-5-methoxy-6-(4-methylpiperazin-1-yl)benzo [d]thiazole (10)

To a solution of hydrazine hydrate (0.250 mL, 4.0 mmol) at 0°C, conc. HCl (182 mL, 2.0 mmol) was added drop wise with stirring. After 15min, compound 9 (0.278 g, 1.0 mmol) and ethylene glycol (2.0 mL) were added. The reaction was heated at 150°C for 4 hrs and cooled to the room temperature. Water was added to the reaction mixture, the resulting solid formed was filtered, washed with water and recrystallized from ethanol to afford compound 10 (0.223 g, yield 76%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72 (s, 1H), 7.21 (s, 1H), 6.93 (s, 1H), 4.90 (s, 2H), 3.77 (s, 3H), 2.90 (s, 4H), 2.44 (s, 4H), 2.21 (s, 3H). HRMS-ESI: calcd. for $C_{13}H_{20}ON_5S$ $[M + H]^+$, 294.1383; found, 294.1381.

Synthesis of 2-((2-(5-methoxy-6-(4-methylpiperazin-1-yl)benzo[d]thiazol-2-yl)hydrazono)methyl)benzo[b]thiophen-3-ol (D19-14)

A mixture of compound 5 (0.178 g, 1.0 mmol) and compound 10 (0.293 g, 1.0 mmol) was dissolved in 5.0 mL methanol. Then the reaction mixture was heated at 40°C for 5 hrs. After completion of reaction, the mixture was filtered and the filtrate was purified via column chromatography on silica gel (solvent gradient：2%-5% MeOH in dichloromethane) to afford the compound D19-14 (0.341 g,yield 75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.99 (s, 1H), 8.51 (s, 1H), 7.91 - 7.72 (m, 2H), 7.43 - 7.29 (m, 3H), 7.01 (s, 1H), 3.81 (d, J = 5.5 Hz, 3H), 2.95 (s, 4H), 2.26 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.74, 152.25, 149.77, 137.48, 137.08, 133.42, 126.88, 124.62, 123.48, 121.75, 113.81, 111.43, 102.10, 56.11, 55.36, 50.90, 46.14. HRMS-ESI: calcd. for $C_{22}H_{24}O_2N_5S_2$ $[M + H]^+$,454.1366; found, 454.1366.

Cell culture and transfection. NCI-H1299, HEK293T, NCI-H1838, MCF7, HL60, NB4, K562, NCI-H1975, A549, Hela, EOL1, MOLM13, MOLT4, MV4;11, SKOV3, BT20, DU145, KATOIII, BT474, NCI-H1650, BT474, THP1, PC3, RKO, NUGC3, SEM, REH, JEKO1, KOPN8, RS411, MDA-MB-468, and MDA-MB-231 cell lines were purchased from the Cell Bank, Chinese Academy of Sciences. Wild-type and cIAP1/2 DKO MEFs were cultured as described previously (1). The cells tested negative for *Mycoplasma* and were maintained accordingly to the guidance from American Type Culture Collection supplemented with 10% (v/v) FBS (Gibco), 100 units/mL penicillin and 100 mg/mL streptomycin (Gibco). All cells were cultured at 37ºC in a humidified atmosphere of 95% air and 5% CO2. Stably-transfected NCI-H1299 and MCF7 cells were selected using blasticidin (Invitrogen) (for pl-MCS-based vectors) or puromycin (Invitrogen) (for pLKO.1-

or pMSCV-based vectors). All lentiviruses were generated by co-trasfecting HEK-293T cells with packing plasmid and expression plasmid using Lipofectamine™ 2000 Transfection Reagent (11668019, Invitrogen). Transient transfection was performed using Lipofectamine™ 2000 Transfection Reagent (11668019, Invitrogen) or Lipofectamine™ RNAiMAX Transfection Reagent (13778150, Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated at 37ºC for 48 hrs to allow plasmid-driven protein or siRNA expression before further experimental procedures.

Western blot and Antibodies. Whole cell lysates were prepared in RIPA lysis buffer (P0013B, Beyotime), then centrifuged and separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and subsequently transferred onto a PVDF membrane (Millipore). Membrane was blocked with 5% (m/v) nonfat milk for 1 hr at room temperature and then incubated with primary antibodies at 4ºC overnight. Antibodies were used against MAD1 (ab80259, Abcam), cIAP1 (AF8181, R&D), cIAP2 (552782, BD biosciences), c-MYC (sc-40, Santa Cruz), MAX (sc-197, Santa Cruz), Flag (F1804, Sigma-Aldrich), GST (#2622, Cell Signaling), Ubiquitin (#3933, Cell Signaling), HA (#3724, Cell Signaling), α-tubulin (ab4074, Abcam), β-actin (60008-1-Ig, Proteintech) and GAPDH (60004-1-Ig, Proteintech).

Immunoprecipitation. IP was performed as described in the protocols Dynabeads™ Protein G for Immunoprecipitation Kit (10003D, Invitrogen). For detecting the level of dimeric c-MYC/MAX and MAD1/MAX, whole cell lysates were prepared in cell lysis buffer (P0013J, Beyotime) and supernatants were collected by centrifugation, and incubated with anti-HA Ab (ab9110, Abcam) coupled to Dynabeads™ Protein G. The beads were washed in lysis buffer and samples were examined by immunoblotting.
For dectecting the ubiquitination level of c-MYC or MAD1, whole cell lysates were prepared in cell lysis buffer (P0013J, Beyotime) and supernatants were collected by centrifugation, and incubated with anti-c-MYC (ab56, Abcam) or Flag (F1804, Sigma-Aldrich) coupled to Dynabeads™ Protein G. The beads were washed in lysis buffer, then were boiled in PBS with 1% SDS for 5 min. SDS was then diluted to 0.1% by addition of cell lysis buffer (P0013J, Beyotime) to the eluate, followed by re-IP with a anti-c-MYC (sc-40, Santa Cruz) or Flag (F1804, Sigma-Aldrich) coupled to Dynabeads™ Protein G. The beads were washed in lysis buffer and samples were examined by immunoblotting.

GST pulldown assay. For the GST pulldown assay to detect cIAP1 dimerization and binding between cIAP1 and UbcH5b, Flag-cIAP1 was purified with ANTI-FLAG® M2 Affinity Gel (A2220, Sigma-Aldrich) from HEK293T cells overexpressing Flag-cIAP1. Purified Flag-cIAP1 was incubated with recombinant GST-tagged cIAP1, GST-tagged UbcH5b or GST alone immobilized on glutathione agarose in the presence of DMSO or

compounds. GST pulldown complexes were washed in lysis buffer and proteins were eluted using SDS loading buffer.

Cell proliferation assay. Cell proliferation was measured using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) according to the manufacturer's instruction. Luminescence was detected by a spectrophotometer (Molecular Device). The percentage of proliferation was normalized to readouts of control (DMSO) cells. Data is representative of independent experiments and shows the average value of triplicates.The error bars indicate standard deviation (SD).

Flow cytometry. DMSO- or D19-treated H1299 cells were collected, washed with cold PBS and fixed in 70% ethanol at -20ºC overnight. Cells were then washed with PBS and re-suspended in 200 µl staining buffer with propidium iodide (P4170, Sigma-Aldrich) for 1 hr at 37ºC in the dark. Flow cytometric analysis was immediately performed using a FACS Calibur Instrument (BD Biosciences).

Colony formation assay. 5×10^3 cells were suspended in media containing 0.35% agar and plated on a bottom layer of media containing 1% agar in a 6-well plate. Cells were then treated with DMSO or the indicated concentrations of D19. Plates were incubated at 37°C for 2 weeks to enable colony formation. Growth medium was removed and colonies were stained with 0.1% crystal violet. Colonies were visualized by light microscopy or Gel Imager (Bio-Rad).

Protein expression and purification. cIAP1 and its variants, UbcH5b, ubiquitin, E1 proteins were expressed as GST fusions, His fusions, or MBP fusions in *Escherichia coli* BL21 (DE3). Cells were grown to an OD of 0.8, then isopropyl 1-thio-β-d-galactopyranoside (IPTG) (ST079, Beyotime) was added to induce protein expression, followed by incubation overnight at 16°C. Cell pellets were collected and sonicated in lysis buffer (50 mM Tris,160 mM NaCl, 2 mM DTT for GST--tagged fusion proteins; 20 mM Tris, 300 mM NaCl,10 mM imidazole for His-tagged fusion protein; 20 mM Tris, 200 mM NaCl, 1 mM EDTA, 1 mM DTT for MBP-tagged fusion protein) in the presence of 1 mM PMSF (ST506, Beyotime). Then supernatants were centrifuged and incubated with Glutathione Sepharose 4 Fast Flow (17-5132-01, GE Healthcare), Ni Sepharose 6 Fast Flow (17-5318-01, GE Healthcare), or Dextrin Sepharose High Performance (28-9367-24, GE Healthcare). The resin was washed, and the protein was eluted using lysis buffer supplemented with 20 mM glutathione, 50 mM imidazole, or 10 mM maltose.

E2 discharge assay. Charged UbcH5a-Ub (E2-800) was purchased from Boston Biochem. GST-cIAP1 (5 µM) was pre-incubated with DMSO or D19 (100 µM) at room temperature for 30 min, then UbcH5a-Ub (5 µM) was added and further incubated at 37°C

for 30 min and 60 min, Reactions were stopped by adding loading buffer, and were analyzed by western blotting.

In vitro ubiquitination assay. E1 (100 ng), UbcH5b (250 ng), ubiquitin (6 μg), E3 (2 μg) and substrate (50 ng) were incubated in reaction buffer (50 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 10 nM Okadaic acid, and 0.6 mM DTT) at 37°C for 1 hr. The reactions were analyzed by western blotting (2).

Compound library screen. Briefly, purified GST-cIAP1 protein (2 μg) in a reaction buffer (50 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 2 mM NaF, 100 μM ATP, and 0.6 mM DTT) was pre-incubated with compound (20 μM) in 384-well plate for 30 min, followed by addition of recombinant E1 (50 ng), UbcH5b (250 ng), ub (6 μg) and pyrophosphatase (0.01 U, Sigma-Aldrich). The plates were sealed and incubated at 37°C for 5 hrs. 5 μL of malachite green solution (10 mM in 1N HCl) was added to the reaction and incubated at room temperature for 10 min. The ubiquitination was then quantified by measuring the absorbance at 630 nM.

DSF assay. Assay was performed in a 96-well PCR plate using RT-PCR machine. Proteins (5 μM), 10 X SYPRO orange Protein Gel Stain (S6650, Invitrogen) and indicated concentrations of compound were diluted in analysis buffer (150 mM NaCl, 100 mM Tris, pH 7.5). Reaction was performed at 25°C for 2 min and then temperature was increased to 95°C at a rate of 1°C/min, and held at 95°C for 10 min. The Tm of protein was calculated using the Protein Thermal Shift software (Thermo Fisher).

BLI Assay. The binding between D19 and cIAP1 RING was quantified using BLI Octet RED96 (ForteБio Inc., Menlo Park, CA). The purified GST-cIAP1 RING protein was cleaved with thrombin (T4648, Sigma-Aldrich), followed by incubation with glutathione-sepharose beads to remove free GST and uncleaved GST-c-IAP1 RING. The RING protein was concentrated using a centrifugal filter (UFC900324, Millipore). Streptavidin biosensors (ForteБioInc.) were pre-soaked in assay buffer (PBS and 0.1% DMSO), then coated in an assay buffer containing 50 μg/mL of biotinylated protein for 6 min. As a control, a duplicate set of sensors were incubated in an assay buffer. Biotinylated protein coated and control sensors were dipped into D19 (48, 24, 12, 6, 3, and 1.5 μM) for 200 sec, followed by a 240 sec dissociation. Data was analyzed using OctetRED analysis software.

RT-PCR. Cells were treated with compound for 24 hrs, then total RNAs were extracted with TRIzol™ Reagent (15596018, Invitrogen) and reverse transcribed using the M-MuLV First Strand cDNA Synthesis Kit (B532435, Sangon Biotech). Quantitative RT-PCR was performed with SYBR® Premix Ex Taq™ (RR420, Takara). All RT-PCR primers are summarized in *SI Appendix*, Table S4.

EdU incorporation assay. MCF7 and H1299 cell were treated with DMSO or D19 (20 μM or 40 μM) for 24 hrs, then stained with Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit (C10425, Invitrogen).

Senescence staining. H1299 cells were treated with DMSO or D19 (20 μM) for 24 hrs, then stained with Senescence β-Galactosidase Staining Kit (#9860, Cell Signaling) to detect SA-β-galactosidase activity.

Patient-derived tumor organoids (PDTOs) culture and viability assay. All human breast cancer samples were obtained from female patients (46, 51 and 59 years old), with their informed consent, at the Cancer Hospital Chinese Academy of Medical Sciences. Tumors were minced and digested to get single tumor cells. The organoids KOBR040, KOBR050 and KOBR056 were maintained in 3D culture system with matrigel and GAS medium, and were seeded in 24 well plate (3473, Corning), then were treated with D19-14 at indicated concentrations for 6 days. Images were taken with Leica DMi1.

Animal experiments. 6-8 week old female Balb/c nude mice were obtained from Shanghai SLAC Laboratory Animal Co.,Ltd. 8-10 week old male ICR mouse were obtained from Sino-British SIPPR/B&K Lab Animal Ltd. EOL1 cells were inoculated subcutaneously (s.c.) into female Balb/c nude mice at 5×10^6 cells/mouse in 0.1 ml matrigel basement membrane matrix (BD Biosciences) / PBS (1:1). D19-14 was dissolved in assay buffer (PEG300:Solutol HS 15:pH 4.65 acetate buffer (10:10:80)). Once the tumor size reached 195mm^3, mice were randomized and intraperitoneally injected with D19-14 (50 mg/kg, 10 ml/kg body weight) (n = 8) or vehicle (n = 8). For single dose PD assay, tumors (800-1000mm^3) were collected at 4 or 8 hrs after injection. For multiple dose PD assay, mice were injected intraperitoneally once daily with D19-14. On the 13th day of treatment, tumors were collected at 2 or 4 hrs following the last dose. The protein levels of c-MYC, MAD1, cIAP1 were analyzed by immunoblotting. For the pharmacokinetic study, mice were administered D19-14 (5 mg/kg) by intraperitoneal injection. Tumor biopsies and plasma were collected at the indicated time periods following treatment. EOL1 cells were inoculated subcutaneously (s.c.) into male ICR mouse at 5×10^6 cells/mouse in 0.1 ml matrigel basement membrane matrix (BD Biosciences) / PBS (1:1). D19-14 was dissolved in assay buffer (PEG300:Solutol HS 15:pH4.65 acetate buffer (10:10:80)). Once the tumor size reached 195mm^3, mice were randomized and oral administrated with D19-14 (50 mg/kg, 10 ml/kg body weight) (n = 9) or vehicle (n = 9). The concentration of D19-14 was measured for the PK study. In the efficacy study body weight and tumor volume were measured twice per week and the tumor volume was calculated using the formula: tumor volume = (length ×width ×width)/2.

Animal use. The animal protocol (IACUC#IRCBC-2017-003) was approved by The Institutional Animal Care and Use Committee of Interdisciplinary Research Center on

Biology and Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences.

References

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2. Xu L, et al. (2007) c-IAP1 cooperates with Myc by acting as a ubiquitin ligase for Mad1. *Mol Cell* 28:914-922

Supplementary Figure Legends

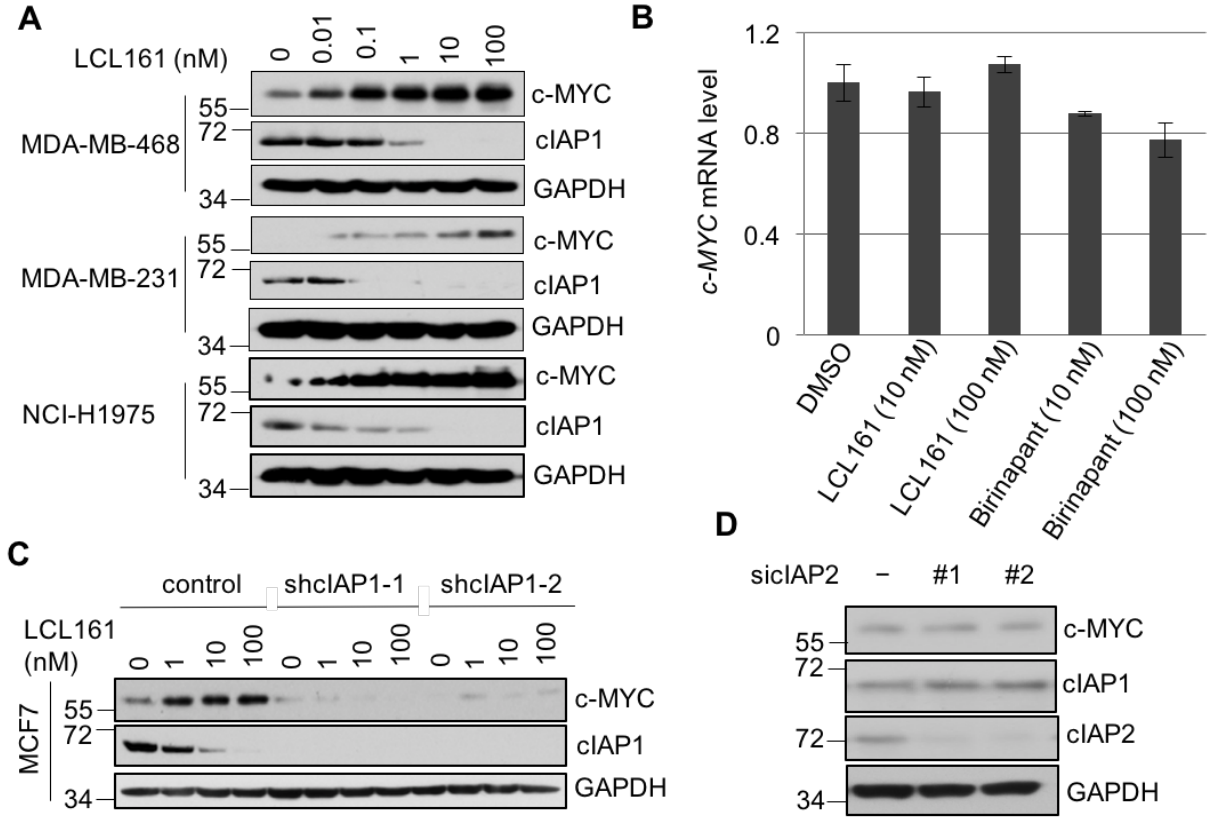

Fig. S1. Smac mimetics stabilize c-MYC by promoting E3 ubiquitin ligase activity of cIAP1. (*A*) LCL161 downregulates c-MYC protein levels in cells. The cells were treated with LCL161 at the indicated concentrations for 4 hrs. (*B*) LCL161 or Birinapant has no effect on *c-MYC* mRNA levels. H1299 cells were treated with LCL161 or Birinapant at the indicated concentrations for 24 hrs. *GAPDH* mRNA was used as an internal control and the data was normalized to the DMSO control. The data represent the mean ± s.d. of 3 independent experiments. (*C*) The upregulation of c-MYC protein level by Smac mimetics is dependent on cIAP1. MCF7 cells stably expressing control shRNA or cIAP1 shRNAs were treated with LCL161 at the indicated concentrations for 4 hrs. (*D*) H1299 cells expressing non-target siRNA or cIAP2 siRNAs were harvested for western blotting using the indicated antibodies.

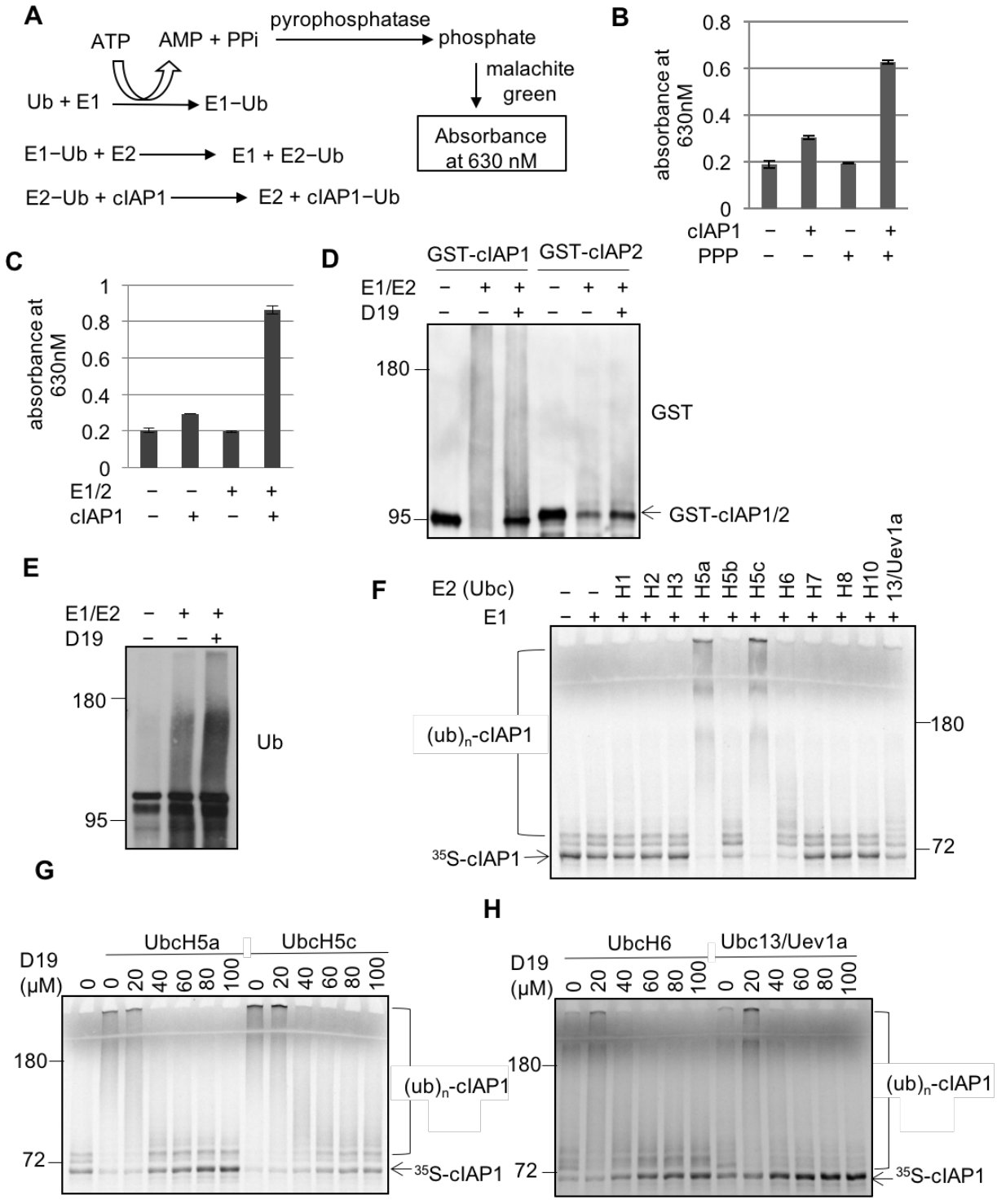

Fig. S2. Identification of a small molecule D19 as an inhibitor of cIAP1 E3 ligase. (*A*) The schematic of the coupled colorimetric assay. (*B*, *C*) Pyrophosphate production is a surrogate marker for E3 ubiquitin ligase activity. PPP: pyrophosphate. The data represent the mean ± s.d. of 3 independent experiments. (*D*) The auto-ubiquitination of both GST-

cIAP1 and -cIAP2 is inhibited by D19 (100 μM). (*E*) D19 (100 μM) has no effect on auto-ubiquitination of GST-BRCA1/BARD1. (*F*) cIAP1 auto-ubiquitination in the presence of various E2s. (*G,H*) D19 inhibits cIAP1 auto-ubiquitination mediated by UbcH5a, UbcH5c, UbcH6 or Ubc13/Uev1a.

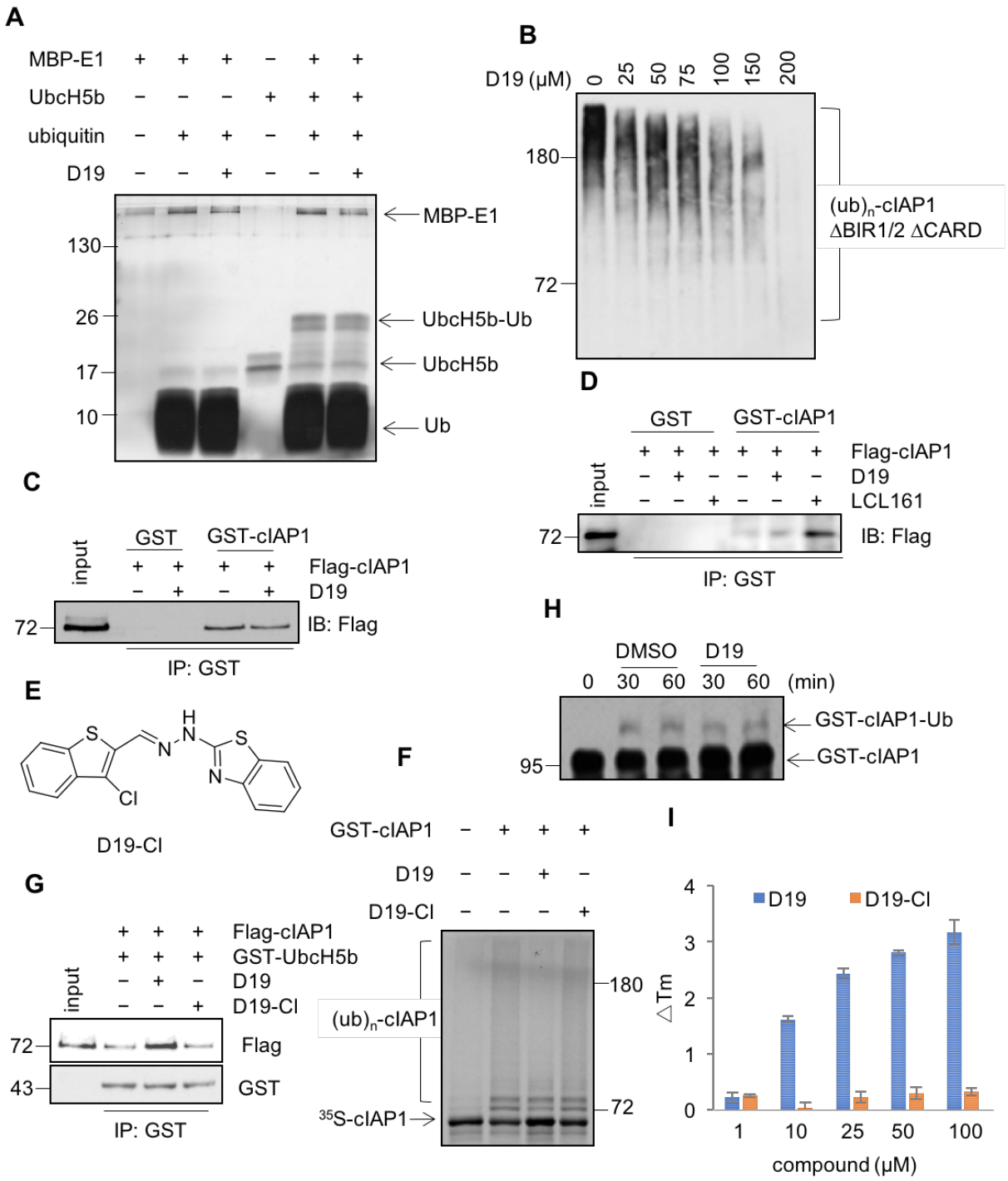

Fig. S3. D19 changes the dynamics of cIAP1 and E2 interaction. (*A*) D19 has no effect on the thioester formation of UbcH5b-ubiquitin. E1-mediated UbcH5b-ubiquitin charging assay was performed in the presence or absence of D19 (100 μM). (*B*) D19 inhibits the auto-ubiquitination of cIAP1ΔBIR1/2ΔCARD in a dose-dependent manner. (*C*, *D*) LCL161, but not D19, promotes the dimerization of cIAP1. Flag-tagged cIAP1 protein was co-precipitated with recombinant GST-tagged cIAP1 immobilized on glutathione agarose in the presence or absence of D19 (100 μM) or LCL161 (10 μM). (*E*) Chemical structure of D19-Cl. (*F*) D19-Cl cannot inhibit cIAP1 auto-ubiquitination. *In vitro* cIAP1 auto-ubiquitination assay utilizing ^{35}S-labeled cIAP1 in the presence of DMSO, D19 (100 μM) or D19-Cl (100 μM). (*G*) D19-Cl has no effect on the binding of cIAP1 and UbcH5b. Flag-tagged c-IAP1 was co-precipitated with recombinant GST-tagged UbcH5b immobilized on glutathione agarose in the presence of DMSO, D19 (100 μM) or D19-Cl (100 μM). (*H*) D19 does not inhibit cIAP1-Ub conjugate formation. E2-Ub conjugate was incubated with recombinant GST-cIAP1ΔBIR1/2 protein for the indicated time periods in the absence or presence of D19 (100 μM). (*I*) D19, but not D19-Cl, binds to the cIAP1 RING domain. Purified GST-cIAP1-RING protein was subjected to the DSF assay in the presence of the indicated concentrations of D19 or D19-Cl. The thermal shift (ΔTm) was calculated and plotted. The data represent the mean ± s.d. of 3 independent experiments.

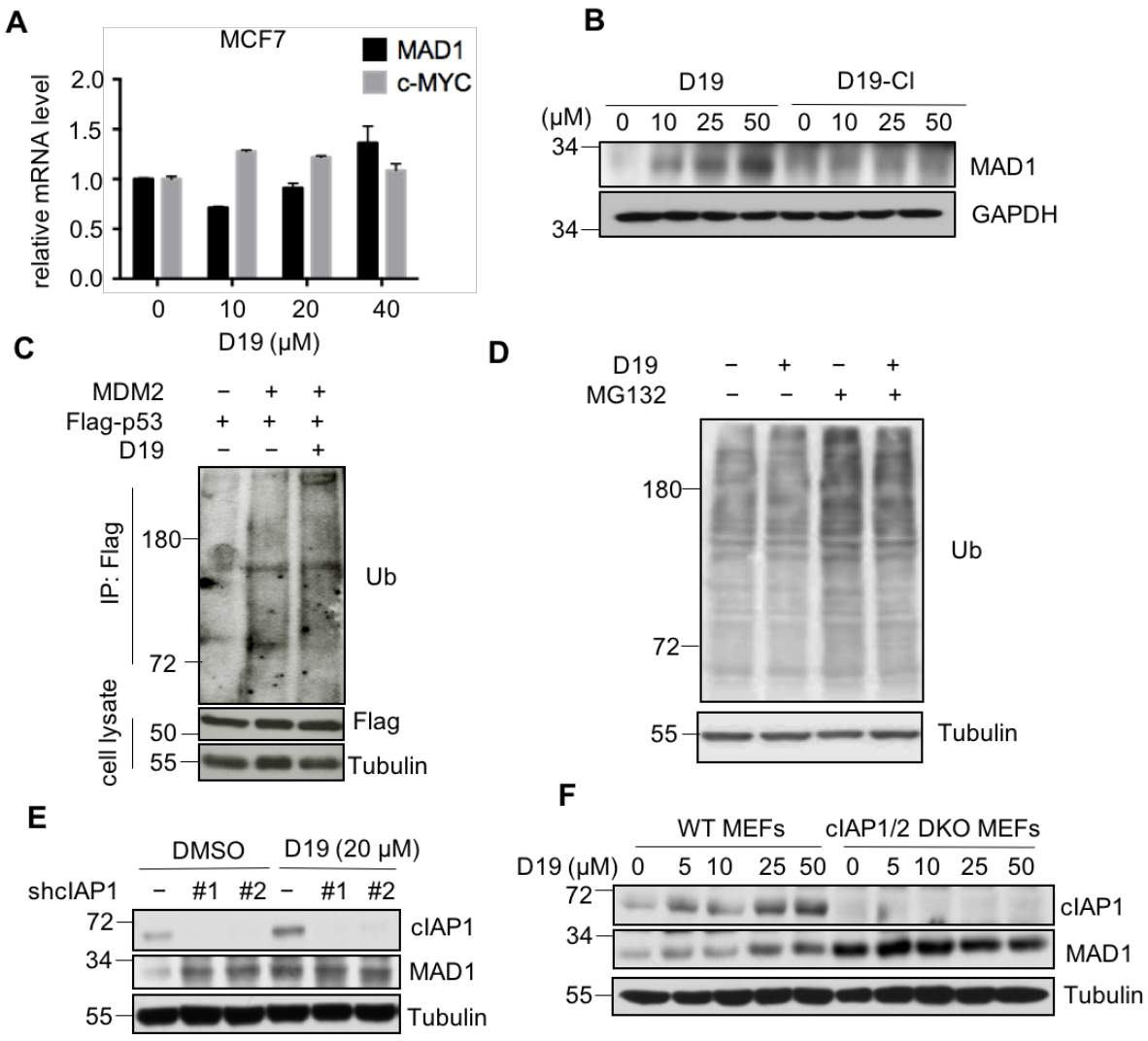

Fig. S4. D19 promotes the proteasomal degradation of c-MYC. (*A*) D19 has no effect on the mRNA levels of c-MYC and MAD1. MCF7 cells were treated with D19 at the indicated concentrations for 24 hrs. *GAPDH* mRNA was used as an internal control and the data was normalized to the DMSO control. The data represent the mean ± s.d. of 3 independent experiments. (*B*) D19 upregulates Mad1 protein levels while D19-Cl is much less effective. H1299 cells were treated with D19 or D19-Cl at the indicated concentration for 4 hrs. (*C*) D19 has no effect on p53 ubiquitination by MDM2. HEK293T cells were transfected with Flag-p53 and MDM2 for 20 hrs and then treated with D19 (20 μM) or DMSO for 12 hrs, followed by treatment with MG132 (10 μM) for 4 hrs. (*D*) D19 has no effect on total protein ubiquitination. H1299 cells were treated with or without D19 (20 μM) for 12 hrs, followed by treatment with DMSO or MG132 (10 μM) for 4 hrs. (*E*, *F*) The upregulation of Mad1 protein levels by D19 is dependent on cIAP1. H1299 cells stably expressing control shRNA or c-IAP1 shRNAs were treated with D19 (20 μM) for 4 hrs (*E*).

Wild-type or cIAP1/2 DKO MEFs were treated with the indicated concentrations of D19 for 4 hrs (*F*).

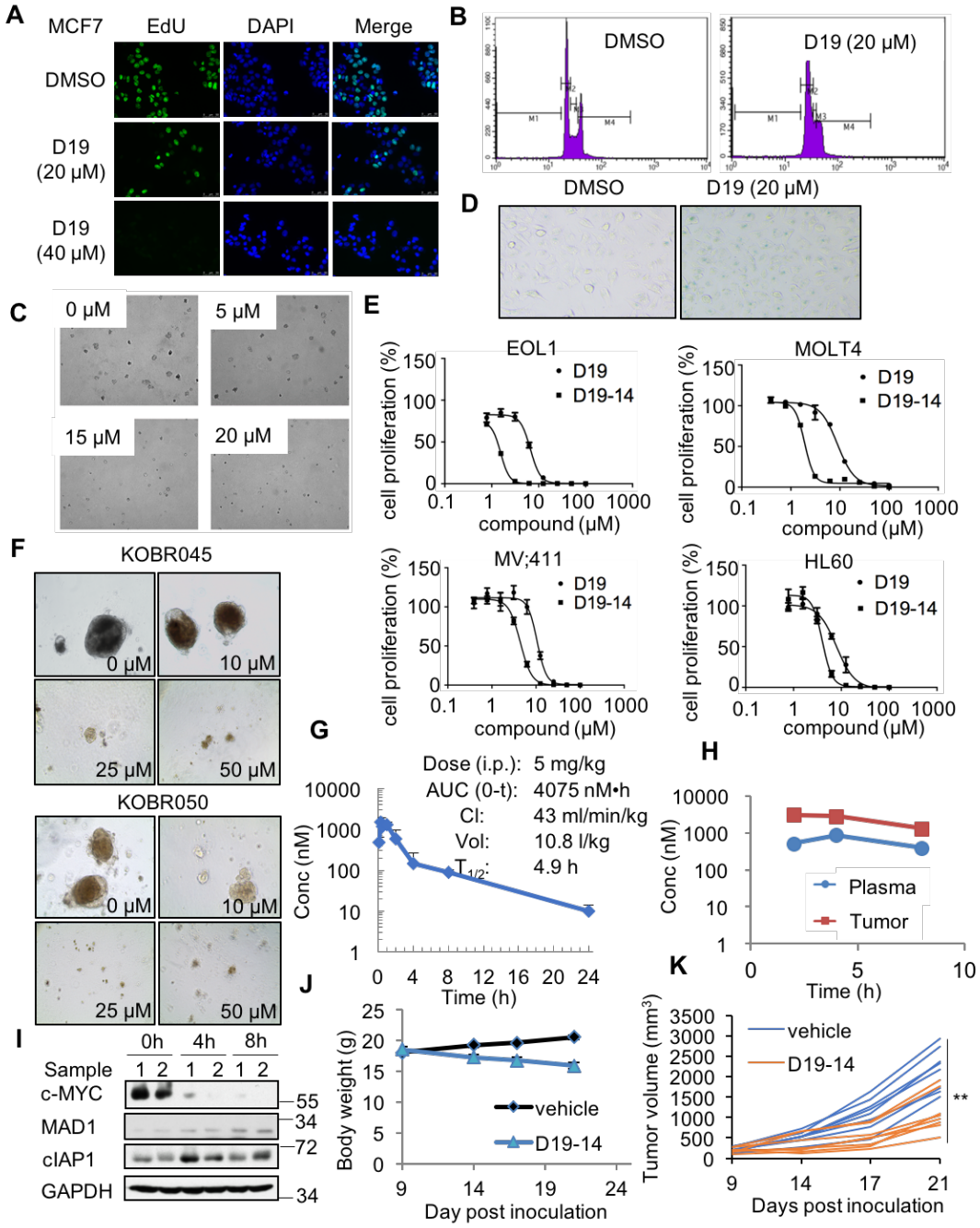

Fig. S5. Developing cIAP1 E3 ligase inhibitors to antagonize c-MYC for cancer treatment. (*A*) D19 inhibits DNA synthesis. EdU incorporation was measured using imaging analysis in MCF7 cells followed D19 treatment for 24 hrs. (*B*) D19 induces cell cycle arrest in G1 phase. Flow cytometric evaluation of propidium iodide (PI) staining for cell cycle analysis

in H1299 cells treated with D19 (20 μM) for 48 hrs. (*C*) D19 inhibits the formation of foci in MCF7 cells in a dose-dependent manner. The colony formation of MCF7 cells treated with DMSO or the indicated concentrations of D19 for 2 weeks. (*D*) D19 induces cellular senescence. H1299 cells were treated with DMSO or D19 (20 μM) for 24 hrs, then stained for SA-β-galactosidase activity (pH 6.0). (*E*) D19-14 is a more potent inhibitor of proliferation in cancer cells than D19. EOL1, MOLT4, HL60 and MV4;11 cells were treated with D19 or D19-14 at the indicated concentrations for 48 hrs. Cell proliferation was measured by CellTiterGlo. Data were presented as mean percentage of DMSO-treated cells ± SD. Experiments were performed in triplicate. (*F*) D19-14 inhibits PDTO proliferation. The PDTOs KOBR045 and KOBR050 were treated with D19-14 at the indicated concentrations for 6 days. (*G*) Pharmacokinetic study of D19-14 *in vivo*. The mice were administered D19-14 (5 mg/kg) by intraperitoneal injection. The concentration of D19-14 in plasma was measured (8 mice per group; mean ± s.e.m). (*H*) D19-14 is enriched in tumors. Tumor biopsies and plasma were collected at the indicated time periods following D19-14 treatment (50 mg/kg). The concentrations of D19-14 were measured for the PK study (9 mice per group; mean ± s.e.m). (*I*) The protein levels of c-MYC, MAD1, cIAP1 and GADPH were analyzed by western blotting for PD study. (*J*) Body weight measurement of the mice treated with vehicle and D19-14 (50 mg/kg) (8 mice per group; mean ± s.e.m). (*K*) The growth curves of individual subcutaneous EOL1 xenograft tumors in mice treated with D19-14 (50 mg/kg) or vehicle by intraperitoneal injection once daily (8 mice per group). Tumor volume was calculated using the formula: tumor volume = [length × width × width]/2.

Supplementary Tables

Table S1. c-MYC expression level and D19 IC_{50}s in 25 cancer cell lines

Cancer cell line	c-MYC expression	D19 IC_{50} (μM)
BT20_BREAST	10.98616	31.43
DU145_PROSTATE	11.10875	30.08
KATOIII_STOMACH	11.54524	26.18
BT474_BREAST	10.84007	24.89
NCIH1650_LUNG	9.659314	24.34
SKOV3_OVARY	10.5234	22.72
NCIH1838_LUNG	12.07907	22.27
THP1_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	12.34791	21.82
MDAMB468_BREAST	11.25006	21.4
PC3_PROSTATE	11.26923	19.71
MDAMB231_BREAST	11.05092	18.88
RKO_LARGE_INTESTINE	12.11267	15.98
NCIH1299_LUNG	11.8054	15.93
MCF7_BREAST	10.7374	14.23
NUGC3_STOMACH	11.76034	12.92
SEM_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	12.49937	12.15
HL60_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	13.53562	11.68
MV;411_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	12.70719	11.07
MOLT4_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	12.64064	8.58
MOLM13_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	13.18919	8.25
RS4;11_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	12.58498	8.05
REH_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	12.4784	7.21
JEKO1_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	13.56554	6.16
KOPN8_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	12.74737	5.29
EOL1_HAEMATOPOIETIC_AND_LYMPHOID_TISSUE	13.8362	4.16

Table S2. shRNA primers

Gene	Primer sequences
sh*cIAP1*-1 forward	CCGGACAACTGGAGAGAACTATAAACTCGAGTTTATAGTTCTCTCCAGTTGTTTTTTG
sh*cIAP1*-1 reverse	AATTCAAAAAACAACTGGAGAGAACTATAAACTCGAGTTTATAGTTCTCTCCAGTTGT
sh*cIAP1*-2 forward	CCGGATGCTGACCCACCAATTATTCCTCGAGGAATAATTGGTGGGTCAGCATTTTTTG
sh*cIAP1*-2 reverse	AATTCAAAAAATGCTGACCCACCAATTATTCCTCGAGGAATAATTGGTGGGTCAGCAT
sh*MAD1*-1 forward	CCGGCGAGACCATCAACGCACTGAACTCGAGTTCAGTGCGTTGATGGTCTCGTTTTTG
sh*MAD1*-1 reverse	AATTCAAAAACGAGACCATCAACGCACTGAACTCGAGTTCAGTGCGTTGATGGTCTCG
sh*MAD1*-2 forward	CCGGAGCAAGAGGCTGCGTGAGAAACTCGAGTTTCTCACGCAGCCTCTTGCTTTTTTG
sh*MAD1*-2 reverse	AATTCAAAAAAGCAAGAGGCTGCGTGAGAAACTCGAGTTTCTCACGCAGCCTCTTGCT

Table S3. The sequences of the siRNA oligonucleotides were as follows:

Gene	sequences
non-targeting	GGAUCCUUGACAAUACCAAtt
si-cIAP1-1	GGCCAAGAGUUUGUUGAUtt
si-cIAP1-2	AAAGAGAGCCAUUCUGUUCUUtt
si-cIAP2-1	UCUAACACAAGAUCAUUGAUUtt
si-cIAP2-2	UCUAACACAAGAUCAUUGAUUtt

Table S4. RT-PCR primers

Gene	**Primer sequences**
GAPDH forward	GGTCATCCCTGAGCTGAACG
GAPDH reverse	TTCGTTGTCATACCAGGAATTG
CDCA7 forward	CGCGTGCCGCAGAAAGA
CDCA7 reverse	GCAAAGCTGTCACAACTGTCAT
p21 forward	GGCAGACCAGCATGACAGATT
p21 reverse	GCGGATTAGGGCTTCCTCT
BRCA1 forward	TTAAAGAAAGAAAAATGCTGAATGAG
BRCA1 reverse	GGTGGTTTCTTCCATTGACC
MSH2 forward	CACTGTCTGCGGTAATCAAGT
MSH2 reverse	CTCTGACTGCTGCAATATCCAAT
Bcl2 forward	CATGTGTGTGGAGAGCGTCAA
Bcl2 reverse	GCCGGTTCAGGTACTCAGTCA
ODC forward	GAGCACATCCCAAAGCAAAGT
ODC reverse	TCCAGAGTCTGACGGAAAGTA
c-MYC forward	CCTGGTGCTCCATGAGGAGAC
c-MYC reverse	CAGACTCTGACCTTTTGCCAGG
TERT forward	CGAGAGCAGACACCAGCAG
TERT reverse	TTTTACTCCCACAGCACCTC
MAD1 forward	CGACTCCGACAGGGAAGAAA
MAD1 reverse	CTCTTGATGCTGGTGCTGGA