Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supplementary information

Stapling proteins in the RELA complex inhibits TNFα-induced nuclear translocation of RELA

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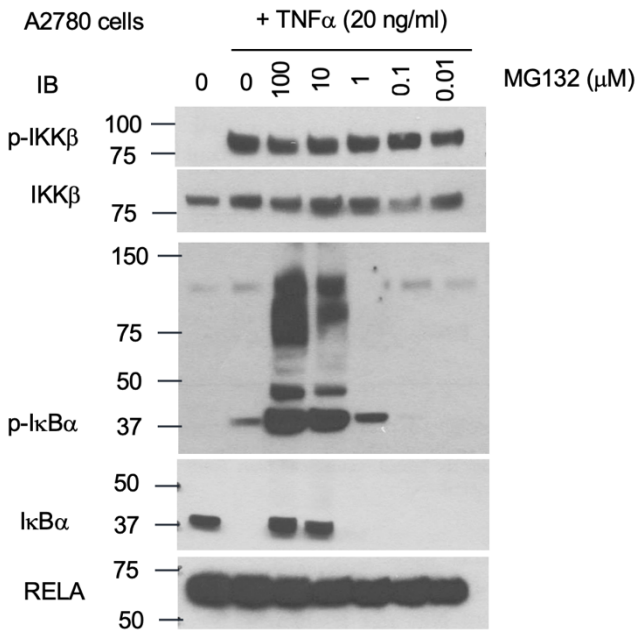


Figure S1. TNFα induced IKKβ mediated phosphorylation of IκBα and ubiquitination-dependent degradation of IκBα

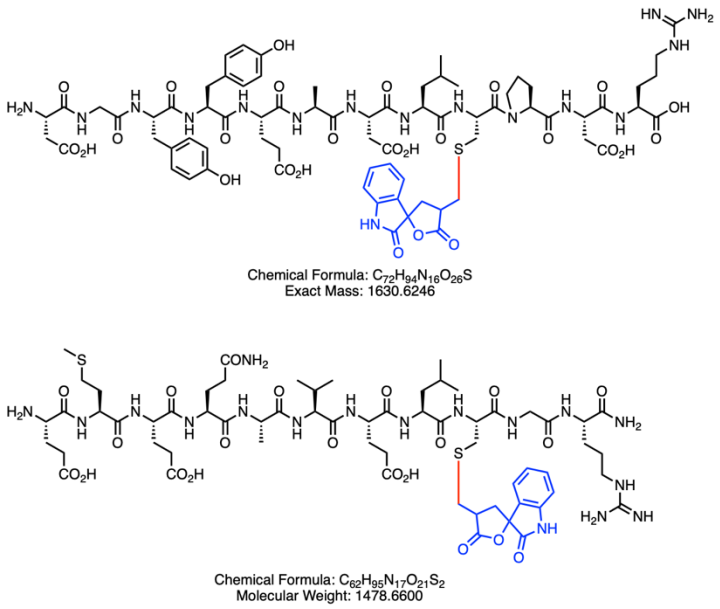


Figure S2. Structures of the peptide adduct shown in Fig 1F and 1G

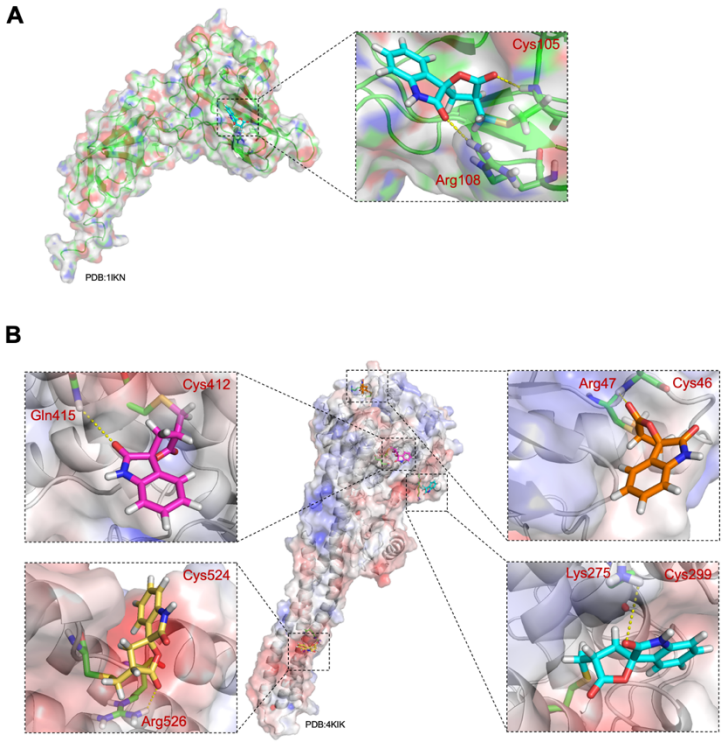


Figure S3. Docked structures (**A**) RELA covalently bound to 19 (**B**) IKKβ covalently bound to 19

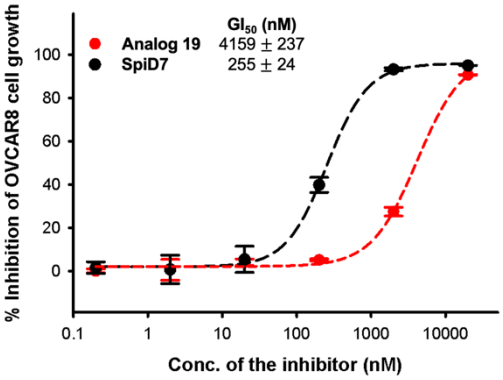


Figure S4. Growth inhibition studies with OVCAR8 cells

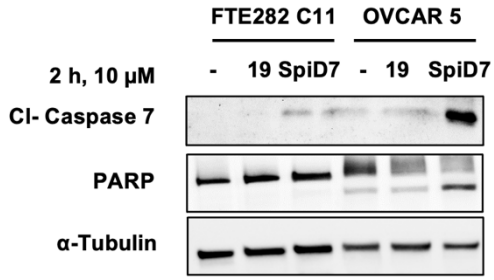


Figure S5. Induction of apoptosis in FTE282C11 and OVCAR5 cells

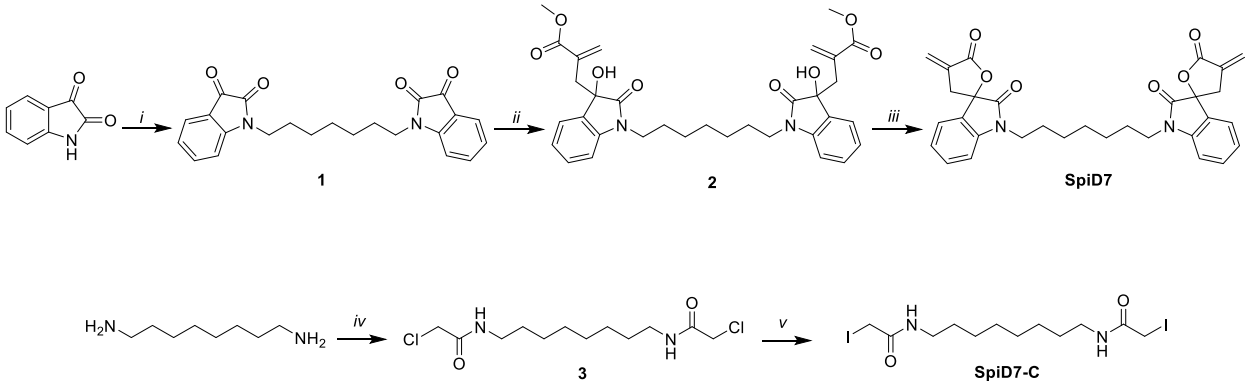
Table 1. Docking energies

Protein	**AA#**	**Docking score** (kcal/mol)	**XP G Score** (kcal/mol)
RELA	Cys105	-4.267	-2.136
IKKβ	Cys46	-1.060	-1.967
IKKβ	Cys299	-1.139	-1.817
IKKβ	Cys412	-2.416	-1.220
IKKβ	Cys524	-1.668	-0.786

Chemistry Experimental:

All reagents were purchased from commercial sources and were used without further purification. Flash chromatography was carried out on silica gel (200–400 mesh). Thin layer chromatography (TLC) were run on pre-coated EMD silica gel 60 F254 plates and observed under UV light at 254 nm and with basic potassium permanganate dip. Column chromatography was performed with silica gel (230-400 mesh, grade 60, Fisher scientific, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded in chloroform-d_3 or DMSO-d_6 on a Varian-500, Varian-600 and Bruker-500 spectrometer (DMSO-d_6 2.50 ppm for ^{1}H and 39.00 ppm for ^{13}C and $CDCl_3$ was 7.26 ppm for ^{1}H and 77.00 ppm for ^{13}C. Proton and carbon chemical shifts were reported in ppm relative to the signal from residual solvent proton and carbon. The purity of all final compounds was ≥95% as determined by analytical HPLC on a reverse-phase column (Zorbax 300SB C18, 2.1 × 150 mm, 5 µm particle size) using an Agilent 1200 series system with UV detector (214nm and 254nm) with the binary system water/acetonitrile containing 0.1% trifluoracetic acid (TFA) as eluent or Analytical HPLC was carried out on 250 x 4.60 mm C-18 column using gradient conditions (10 – 100% B, flow rate = 1.0 mL/min, 15 min). The eluents used were: solvent A (H2O with 0.1% Formic acid) and solvent B (CH_3CN with 0.1% Formic acid).

Synthesis of spirocyclic dimer 7 (SpiD7) and iodoacetamide dimer (SpiD7-C)



Scheme 1. Reagents and conditions: (i). 1,7-dibromoalkane, NaH, DMF, rt, 16h; 80%; (ii). Methyl 2- (bromomethyl)acrylate, In, THF: water, rt, 24h, 75%; (iii). TsOH, DCM, rt, 24h, 80%; (iv) 2-chloroacetyl chloride, K_2CO_3, DCM, 90%; (v) NaI, acetone, reflux, 24h, 55%.

Step i: In a dry round bottom flask isatin (1 eq.) was dissolved in dry DMF and the reaction mixture was cooled to 0°C. NaH (0.45 eq.) was added and the reaction mixture was stirred for 15 minutes followed by the addition of 1,7-dibromoalkane (0.45 eq). The reaction was warmed to room temperature and stirred at room temperature for 24h. The crude mixture was diluted in ethyl acetate and washed with ammonium chloride and brine. The organic layer was separated and dried over dried over Na_2SO_4 and evaporated under reduced pressure. Then crude was purified on silica gel chromatography using hexane/EtOAc to yield **1** as orange solid, Yield= 80%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.70 – 7.61 (m, 2H), 7.54 (d, *J* = 7.3 Hz, 2H), 7.21 – 7.08 (m, 4H), 3.65 (t, *J* = 7.1 Hz, 4H), 1.60 (t, *J* = 6.9 Hz, 4H), 1.33 (d, *J* = 5.9 Hz, 6H). HRMS (ESI-MS) calcd for $C_{23}H_{23}N_2O_4^+$m/z $(M+H)^+$ 391.1652, found: 391.1662.

Step ii: In a dry round bottom flask Indium (3.5 eq.) was taken in THF: Water (6:4) and stirred at 50°C for 10 minutes followed by addition of methyl 2-(bromomethyl) acrylate (3 eq.) and stirring continued for another 30 minutes. To this mixture isatin (1 eq.) was added and the reaction was stirred for 24h. Progress of the reaction was monitored by TLC. Upon completion, the reaction mixture was diluted with ethyl acetate and washed with 0.1% HCl followed by brine. The organic layer was separated and dried using magnesium sulfate. The crude mixture was purified on a column using a hexane-ethyl acetate gradient to obtain the acyclic compound as white solid, Yield= 75%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.30 – 7.13 (m, 4H), 6.96 (m, 4H), 6.12 (s, 2H), 5.91 (s, 2H), 5.42 (s, 2H), 3.70 – 3.63 (m, 2H), 3.56 – 3.42 (m, 2H), 3.42 (s, 6H), 2.97 (d, *J* = 12.9 Hz, 2H), 2.75 (d, *J* = 13.0 Hz, 2H), 1.57 – 1.47 (m, 4H), 1.29 – 1.21 (m, 6H). HRMS (ESI-MS) calcd for $C_{33}H_{39}N_2O_8^+$m/z $(M+H)^+$ 591.2701, found: 591.2701.

Step iii: The acyclic compound (1 eq.) from step ii was taken in round bottom flask, dissolved in dry dichloromethane and the reaction mixture was cooled to 0°C. *p*-Toluene sulfonic acid monohydrate salt (2.2 eq.) was added and the reaction mixture was stirred at room temperature for 12 h under inert atmosphere. Progress of the reaction was monitored by TLC. The crude mixture was washed with brine and extracted using dichloromethane and dried over magnesium sulfate. It was purified on a column using a hexane-ethyl acetate gradient to obtain the desired product as white solid, Yield= 80%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (td, J = 7.9, 3.1 Hz, 2H),

7.31 (d, J = 7.4 Hz, 2H), 7.10 (t, J = 7.6 Hz, 2H), 6.87 (dd, J = 7.9, 3.5 Hz, 2H), 6.40 (q, J = 2.9 Hz, 2H), 5.80 (dt, J = 5.3, 2.6 Hz, 2H), 3.75 – 3.58 (m, 4H), 3.29 (ddt, J = 17.2, 5.0, 2.5 Hz, 2H), 1.67 (t, J = 7.4 Hz, 5H), 3.10 (dt, J = 17.3, 2.9 Hz, 2H), 1.35 (d, J = 9.8 Hz, 7H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.43, 169.02, 143.32, 132.85, 131.26, 126.81, 124.30, 123.44, 123.07, 109.24, 79.30, 77.26, 77.00, 76.75, 40.16, 36.29, 28.64, 27.02, 26.52. HRMS (ESI-MS) calcd for $C_{31}H_{31}N_2O_6^+$m/z $(M+H)^+$ 527.2177, found: 527.2180.

Step iv: Potassium carbonate (2.5 eq.) was added to a cooled (0 ºC) stirring solution of octane-1,8-diamine (1 eq.) in a mixture of dichloromethane (15 mL) and water (10 mL). Chloroacetyl chloride (2 eq.) in DCM (10 mL) was added dropwise to the above mixture with constant stirring. The reaction mixture was stirred overnight at room temperature and evaporated to dryness under vacuum. Cold water was added to the above and the reaction mixture was filtered to yield compound **3** as white solid, Yield =90%. ^{1}H NMR (499 MHz, DMSO-d_6) δ 8.17 (t, J = 5.7 Hz, 2H), 4.02 (s, 4H), 3.06 (q, J = 6.6 Hz, 4H), 1.40 (p, J = 6.7 Hz, 5H), 1.25 (broad s, 9H). ^{13}C NMR (126 MHz, DMSO-d6) δ 165.12, 42.12, 39.48, 39.31, 39.14, 38.98, 38.81, 38.64, 38.48, 38.34, 28.25, 28.06, 25.68.

Step v: Compound **3** (1 eq.) was dissolved in acetone (7 mL) followed by addition of sodium iodide (3 eq.). Reaction mixture was refluxed overnight and solvent was evaporated to dryness. Dichloromethane was added to crude mixture and washed with 5% sodium bisulfite and brine. Solvent was removed under vacuum to yield **SpiD7-C**, Yield =55%.^{1}H NMR (499 MHz, DMSO-d_6) δ 8.27 (s, 2H), 4.03 (s, 4H), 3.05 (q, J = 6.6 Hz, 4H), 2.53 – 2.47 (m, 3H), 1.39 (p, J = 6.6 Hz, 5H), 1.24 (s, 10H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 165.18, 42.15, 39.50, 39.33, 39.16, 39.00, 38.83, 38.66, 38.49, 38.34, 28.25, 28.06, 25.70.

Modeling and docking analysis

Molecular modeling was performed using the Schrödinger small molecule drug discovery suite 2020-1. The crystal structures of RELA (PDB: 1IKN) and IKKβ (PDB: 4KIK) were retrieved from the protein data. The crystal structures were analyzed using Maestro version 12.3.013 (Schrödinger Inc.) and subjected to docking protocol which involves several steps including preparing protein of interest, grid generation, ligand preparation and docking. The crystal structure

was refined using protein preparation wizard in which missing hydrogen atoms and side chains were added and minimized using OPLS3e force field to optimize hydrogen bonding network and converge the heavy atoms to a rmsd of 0.3 Å.

The structure of analog **19** was drawn in Maestro and subjected to Lig Prep to generate conformers, possible protonation at pH of 7 ± 2 that serves as an input for docking process. The receptor grid generation tool in Maestro (Schrödinger Inc.) was used to define an active site around the ligand (analog **19**) to cover all the residues within 15 Å. All the dockings were performed using GLIDE XP with the van der Waals radii of nonpolar atoms for each of the ligands were scaled by a factor of 0.8 and partial charge cutoff 0.15. The docked poses from GLIDE XP were used to perform covalent docking of analog **19** using CovDock (Schrödinger Inc.) for covalent C-S bond formation in RELA and IKKβ. The reactive residue was used Cys105 in RELA, Cys46, Cys299, Cys 412 and Cys524 in IKKβ and selected Michael Addition as reaction type. The binding mode of the ligand in the covalent complex was analyzed using Maestro version 12.3.013 (Schrödinger Inc.).