Screening Bicyclic Peptide Libraries for Protein-Protein Interaction Inhibitors: Discovery of a Tumor Necrosis Factor-alpha Antagonist

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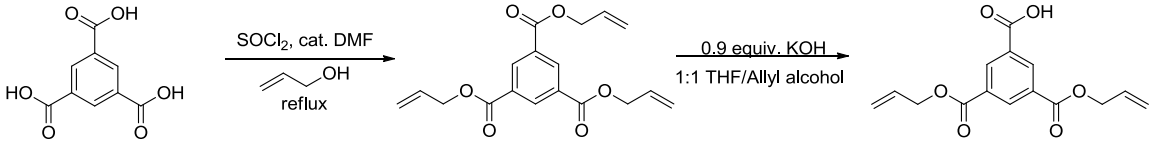
Supporting Information

Materials. Fmoc-protected L-amino acids were purchased from Advanced ChemTech (Louisville, KY), Peptides International (Louisville, KY), or Aapptec (Louisville, KY). O-Benzotriazole-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole hydrate (HOBt) were from Aapptec. Tetramethylrhodamine azide was prepared as previously described.[1] All solvents and other chemical reagents were obtained from Sigma-Aldrich, Fisher Scientific (Pittsburgh, PA), or VWR (West Chester, PA) and were used without further purification unless noted otherwise. N-(9-Fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu) was from Advanced ChemTech. Phenyl isothiocyanate was purchased in 1-mL sealed ampoules from Sigma-Aldrich, and a freshly opened ampoule was used in each experiment.

Synthesis of diallyl trimesic acid. Trimesic acid (2 g, 9.52 mmol) was dissolved in 20 ml of allyl alcohol and cooled to 0 °C. Five equiv. of thionyl chloride and 0.1 equiv of DMF were slowly added to the above solution and the reaction mixture was allowed to warm to room temperature. The reaction was refluxed overnight and stopped by evaporation to dryness under reduced pressure. The residue was dissolved in DCM and washed with saturated $NaHCO_3$ solution and brine. The organic layer was dried over $MgSO_4$ and evaporated. The resulting product was dissolved in 1:1 THF/allyl alcohol and 0.9 equiv. of KOH was added. The solution was stirred for 1 h. The reaction mixture was evaporated and the residue was dissolved in DCM and extracted with 0.1 M NaOH. The organic layer was discarded. Concentrated HCl was added to the aqueous layer until the product completely precipitated out of the solution. The precipitate was collected by vacuum filtration and dried under vacuum to afford the desired product (80% yield). ^{1}H-NMR (250 MHz, DMSO-d6): δ 8.67-8.69 (m, 3H), 6.02-6.18 (m, 2H), 5.47 (d, J_{trans} = 17.5 Hz, 2H), 5.35 (d, J_{cis} = 10 Hz, 2H), and 4.88 (d, 4H).



Protein expression and purification. The gene coding for the extracellular domain of human TNFα (aa 77-233) was amplified by the polymerase chain reaction using the full-length TNFα cDNA (Open Biosystem) as template and oligonucleotides 5'-catctcgagtcacagggcaatgatcccaaagt-3' and 5'-caccgcaagcttgtcagatcatcttctcgaacc-3' as primers. The resulting DNA fragment was digested with endonucleases *Hind* III and *Xho* I and inserted into prokaryotic vector pET-22b(+)-ybbR.[2] This cloning procedure resulted in the addition of a ybbR tag (MVLDSLEFIASKL) to the N-terminus of TNFα. *E. coli* BL21(DE3) cells transformed with the pET22b-ybbR-TNFα plasmid were grown at 37 °C in Luria broth (LB) supplemented with 0.05 mg/ml ampicillin to an OD_{600} of 0.50, when protein expression was induced by the addition

of isopropyl β-D-1-thiogalactopyranoside (150 μM final concentration). After 5 h at 30 °C, the cells (1 L) were harvested by centrifugation. The cell pellet was suspended in 20 mL of lysis buffer (40 mM Tris-HCl, 150 mM NaCl, pH 8.0) plus 0.5% protamine sulfate, 20 μg/mL trypsin inhibitor, 100 μg/ml phenylmethylsulfonyl fluorideand 100 μg/mL lysozyme. The mixture was stirred at 4 °C for 30 min and briefly sonicated (2 x 10 s pulses). The crude lysate was centrifuged to yield a clear supernatant, which was diluted 10 times in running buffer (20 mM Tris-HCl, 1 mM EDTA, 0.5% triton X-100, pH 8.0) and passed through Q-Sepherase column. The column was eluted with 50 mL of running buffer with a gradient of 0-1000 mM NaCl. The ybbR tagged TNFα fractions were pooled and concentrated to ~1 mL using an Amicon Ultra-15 cellulose membrane filter. The resulting solution was passed through a Mono-Q 10/100 GL anion-exchange column equilibrated in the running buffer. The column was eluted with the running buffer plus a linear gradient of 0-1000 mM NaCl. Fractions containing TNFα were pooled and concentrated in an Amicon Ultra-15 cellulose filter. Protein concentration was determined by Bradford assay using bovine serum albumin as the standard.

Protein labeling. Biotinylation of TNFα was carried out by treating the ybbR-tagged TNFα protein (80 μM) in 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$ with Sfp phosphopantetheinyl transferase (1 μM) and biotin-CoA[2] (100 μM) for 30 min at room temperature. Texas-Red labeling of TNFα was similarly carried out except that a Texas Red-CoA adduct[2] was used instead of biotin-CoA. The reaction mixture was passed through a G-25 Fast-Desalting column equilibrated in 30 mM HEPES, pH 7.4, 150 mM NaCl to remove any free biotin or dye molecules.

Library synthesis. The bicyclic peptide library was synthesized on 2.0 g of TentaGel S NH_2 resin (90 μm, 0.2 mmol/g). All of the manipulations were performed at room temperature unless otherwise noted. The linker sequence (BBFM) was synthesized with 4 equiv of Fmoc-amino acids, using HBTU/HOBt/DIPEA as the coupling reagents. The coupling reaction was typically allowed to proceed for 1 h, and the beads were washed with DMF (3x) and DCM (3x). The Fmoc group was removed by treatment twice with 20% piperidine in DMF (5 + 15 min), and the beads were exhaustively washed with DMF (6x). To spatially segregate the beads into outer and inner layers, the resin (after removal of N-terminal Fmoc group) was washed with DMF and water, and soaked in water overnight. The resin was quickly drained and suspended in a solution of Fmoc-OSu (0.26 mmol, 0.50 equiv) and diisopropylethylamine (1.2 mmol or 2.0 equiv) in 30 mL of 55:45 (v/v) DCM/diethyl ether. The mixture was incubated on a carousel shaker for 30 min. The beads were washed with 55:45 DCM/diethyl ether (3x) and DMF (8x) to remove water from the beads and then treated with 5 equiv of di-*t*-butyl dicarbonate in DMF. Next, the Fmoc group was removed by piperidine treatment and 2 equiv of 4-hydroxymethylbenzoic acid and HBTU/HOBt/DIEA (2:2:4 equiv) were added to the resin. Fmoc-β-Ala-OH (5 equiv) was coupled to the Hmb linker by using DIC/DMAP (5.5:0.1 equiv), and the coupling was repeated twice to drive the reaction to completion. Then, Fmoc-L-Pra-OH, two Fmoc-β-Ala-OH, and Fmoc-L-Dap(Alloc)-OH were sequentially coupled by standard Fmoc/HBTU chemistry. The Boc protecting group on the encoding peptide was removed by treatment with TFA/water/ triisopropylsilane (95:2.5:2.5), and the exposed amine was coupled with Fmoc-Arg(Pbf)-OH. The random region was synthesized by the split-and-pool method using 5 equiv of Fmoc-amino acids and HATU as the coupling agent. The coupling reaction was repeated once to ensure complete reaction at each step. To differentiate isobaric amino acids during PED-MS analysis,

4% (mol/mol) of CD_3CO_2D was added to the coupling reactions of D-Ala, D-Leu, D-Lys, and Orn, while 4% $CH_3CD_2CO_2D$ was added to the Nle reactions.[3] Fmoc-Lys(Mmt)-OH was placed in the middle of the random positions using HATU/DIPEA (4 and 8 equiv) to facilitate the formation of bicyclic compounds. After the entire sequence was synthesized, the Alloc group on the C-terminal Dap residue was removed by treatment with a DCM solution containing tetrakis(triphenylphosphine)palladium (0.25 equiv) and phenylsilane (5 equiv) for 15 min (3x). The beads were sequentially washed with 0.5% diisopropylethylamine in DMF, 0.5% sodium dimethyldithiocarbamate hydrate in DMF, DMF (3x), DCM (3x), and DMF (3x). The resulting free amine was coupled to diallyl protected trimesic acid using HATU/DIPEA (5 equiv, 10 equiv) for 2 h. The allyl protecting groups on trimesic acid scaffold was removed using the same procedure as described for the Alloc group. The lysine Mmt group was removed using 2% TFA/5% triisopropylsilane in DCM for 40 min. The N-terminal Fmoc group was then removed with 20% piperidine in DMF. The beads were washed with DMF (6x), 1 M HOBt in DMF (3x), DMF (3x), and DCM (3x). For peptide cyclization, a solution of PyBOP/HOBt/NMM (5, 5, 10 equiv, respectively) in DMF was mixed with the resin and the mixture was incubated on a carousel shaker for 3 h. The resin was washed with DMF (3x) and DCM (3x) and dried under vacuum for >1 h. Sidechain deprotection was carried out with modified reagent K (7.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, 1% anisole, and 1% triisopropylsilane in TFA) for 1 h. The resin was washed with TFA and DCM and dried under vacuum before storage at -20 °C.

Library screening. Library resin (100 mg) was swollen in DCM, washed extensively with DMF, doubly distilled H_2O, and incubated in 1 mL of blocking buffer (PBS, pH 7.4, 150 mM NaCl, 0.05% Tween 20 and 0.1% gelatin) containing 800 nM biotinylated TNFα overnight at 4 °C. The beads were washed with the blocking buffer, suspended in 1 mL of the blocking buffer supplemented with 10 μL of M280 streptavidin-coated Dynabeads (Invitrogen), and incubated for 1 h at 4 °C. The magnetic beads were separated from the rest of the resin using a TA Dynal MPC-1 magnetic particle concentrator (Invitrogen). The hits from magnetic screening were transferred into a BioSpin column (0.8 mL, BioRad) and washed exhaustively with 6 M guanidine hydrochloride, water, and the blocking buffer to remove the bound proteins. The second round of screening was performed by incubating the initial hits with 1.5 μM biotinylated TNFα as described above. After washing with the blocking buffer, the beads were suspended in 1 mL of the blocking buffer containing streptavidin-alkaline phosphatase (1 μg/mL final concentration) at 4 °C for 10 min. The beads were quickly washed with 1 mL of the blocking buffer (3x) and 1 mL of a staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM $MgCl_2$, 20 μM $ZnCl_2$) (3x). Next, 1 mL of the staining buffer and 100 μL of a BCIP stock solution (5 mg/mL) were added to the beads and intense turquoise color developed on positive beads in 25 min. The turquoise colored beads were manually removed under a dissecting microscope, and subjected to a third round of screening after extensive washing with PBS, ddH_2O, and 8 M guanidine hydrochloride. The resulting beads were incubated overnight at 4 °C with 300 nM Texas-red labeled TNFα in the blocking buffer. The beads were viewed under an Olympus SZX12 microscope equipped with a fluorescence illuminator (Olympus America, Center Valley, PA) and the intensely fluorescent beads were manually collected as positive hits.

On-bead labeling and peptide release: The positive beads derived from on-bead screening were pooled, washed with water and DMF, and soaked in 60 μL of 1:1 (v/v) water/DMF mixture. The labeling reaction was initiated by the addition of 20 μL of freshly prepared ascorbic acid and

copper sulfate solutions (each at 5 mg/mL in water) and 5 μL of tetramethylrhodamine azide in DMSO (10 mM). The reaction was allowed to proceed at room temperature overnight in the dark. The reaction was terminated by extensive washing of the beads with water/DMF, and the beads were transferred into individual microcentrifuge tubes (one bead/tube) and each treated with 5 μL of 0.1 M NaOH solution for 4 h at room temperature in the dark. The solution was neutralized by the addition of 5.5 μL of 0.1 M HCl, transferred to a new tube, evaporated to dryness in a vacuum concentrator, and redissolved in 26 μL of double distilled water to generate a stock solution of ~1 μM bicyclic peptide. The beads containing the linear coding peptides were kept in the original tubes and stored for later PED-MS analysis.

Fluorescence anisotropy. A primary FA experiment was performed by incubating 100 nM TMR-labeled bicyclic peptide with 2 μM TNFα in the blocking buffer. The full FA titration experiment was similarly performed by incubating 50 nM labeled bicyclic peptide with varying concentrations (0-6 μM) of TNFα. The FA values were measured on a Molecular Devices Spectramax M5 spectrofluorimeter, with excitation and emission wavelengths at 545 and 585 nm, respectively. Equilibrium dissociation constants (K_D) were determined by plotting the fluorescence anisotropy values as a function of TNFα concentration. The titration curves were fitted to the following equation

$$Y = \frac{\left(A_{min} + \left(A_{max} \times \frac{Q_b}{Q_f} - A_{min}\right)\left(\frac{(L + x + K_d) - \sqrt{((L + x + K_d)^2 - 4Lx)}}{2L}\right)\right)}{\left(1 + \left(\frac{Q_b}{Q_f} - 1\right)\left(\frac{(L + x + K_d) - \sqrt{((L + x + K_d)^2 - 4Lx)}}{2L}\right)\right)}$$

where Y is the measured anisotropy at a given TNFα concentration x; L is the bicyclic peptide concentration; Qb/Qf is the correction fact for dye-protein interaction; A_{max} is the maximum anisotropy when all the peptides are bound to TNFα, while A_{min} is the minimum anisotropy.

Peptide sequencing by PED-MS. Beads containing the encoding linear peptides were placed into individual wells of an AcroPrep 96-well filter plate (Pall Corporation, PN5030) with one bead per well. To each well was added a freshly mixed solution containing 25 μL of pyridine/water (v/v 2:1) plus 0.1% triethylamine and 25 μL of Fmoc-OSu (2 μmol) and phenyl isothiocyanate (100 μmol) in dry pyridine. The reaction was allowed to proceed for 6 min and drained by a universal vacuum manifold system designed for 96-well plates (United Chemical Technologies, Inc.). The bead was washed five times with DCM and once with TFA, and incubated with 100 μL of TFA (2 x 6 min). The bead was washed with DCM and pyridine and PED cycle was repeated for 11 times. After the last PED cycle, the N-terminal Fmoc group was removed by treatment with 20% piperidine in DMF. For MALDI-TOF analysis, each bead was treated with 100 μL of TFA containing ammonium iodide (1.0 mg) and dimethylsulfide (10 μL) for 20 min to reduce any oxidized Met. The bead was washed with water and transferred into a microcentrifuge tube and treated overnight with 20 μL of CNBr in 70% TFA (40 mg/mL) in the dark. The solvents were evaporated under vacuum to dryness and the peptides released from the bead were dissolved in 5 μL of 0.1% TFA in water. One μL of the peptide solution was mixed with 2 μL of saturated 4-hydroxy-α-cyanocinnamic acid in acetonitrile/ 0.1% TFA (1:1) and 1 μL of the mixture was spotted onto a MALDI sample plate. Mass spectrometry was performed on a

Bruker Microflex MALDI-TOF instrument. The data obtained were analyzed by Moverz software (Proteometrics LLC, Winnipeg, Canada).

Effect of bicyclic peptide on TNFα-TNRF1 interaction. Recombinant TNFR1 was purchased from R&D Systems. EZ-Link Plus activated peroxidase, an amine-reactive form of HRP was purchased from Thermo Scientific. TNFR1 was labeled with HRP by combining 50 μL of TNFR1 (1.0 μM) and 4 μL of HRP (2.7 μM) in 950 μL of Na_2CO_3 buffer (0.2 M Na_2CO_3, 0.15 M NaCl, pH 9.0) for 1 h. The resulting TNFR1-HRP conjugate was treated with $NaCNBH_3$ to reduce the resulting Schiff base and quenched with 20 μl of ethanolamine. A Nunc 96F Maxisorp plate was coated overnight with 100 μL of 5 μg/mL Neutravidin (in 50 mM Na_2CO_3, pH 9.0) at 4 °C. The solution was removed and each well was washed with 100 μL of the blocking buffer containing 3% BSA. Next, 100 μL of 7.5 nM biotinylated TNFα in PBS was added to each well and incubated at 4 °C for 1 h. The wells were quickly washed twice with a washing buffer (0.01% Tween 20 in PBS buffer). Peptides of varying concentrations (50 μL) were added to the wells, followed by the addition of 50 μL of 0.5 nM TNFR1-HRP. After incubation at 4 °C for 1.5 h, the plate was washed twice with the washing buffer and incubated with 100 μL of 3,3′,5,5′-tetramethylbenzidine (Sigma) for 30 min. The reaction was quenched by the addition of 100 μL of 1 M phosphoric acid. The absorbance at 450 nm was measured and plotted against the peptide concentration and the IC_{50} value was obtained by curve fitting.

MTT assay. WEHI-13VAR fibroblasts (American Type Culture Collection) were seeded at a density of 5 X 10^4 cells/well in 100 μL of culture medium (10% FBS in RPMI 1640) and allowed to grow overnight at 37 °C and 5% CO_2. TNFα (0.04 ng/mL final concentration), varying concentrations of peptide (0-25 μM), and actinomycin D (1 μg/mL) were mixed and incubated for 1 h in the CO_2 incubator. Next, 50 μl of the resulting mixture was added into each well and the plate was incubated overnight. Ten μL of the MTT labeling reagent (final concentration 0.5 mg/ml) was added to each well and incubated for 4 h. One hundred μL of the MTT solubilization solution (Roche) was added to each well and the plate was let stand in the incubator overnight and the absorbance at 550 nm was measured and plotted as a function of the peptide concentration.

1. Liu, T., Qian, Z., Xiao, Q. & Pei, D. High-throughput screening of one-bead-one-compound libraries: identification of cyclic peptidyl inhibitors against calcineurin/NFAT interaction. *ACS Comb. Sci*. **13,** 537-546 (2011).
2. Yin, J. *et al*. Genetically encoded short peptide tag for versatile protein labeling by Sfp phosphopantetheinyl transferase. *Proc. Natl. Acad. Sci. USA* **102,** 15815–15820 (2005).
3. Thakkar, A., Wavreille, A-S. & Pei, D. Traceless capping agent for peptide sequencing by partial Edman degradation and mass spectrometry. *Anal. Chem.* **78,** 5935–5939 (2006).

Figure S1. FA analysis of TNFα Binding by bicyclic peptides released from single beads. For each bead, the released TMR-labeled bicyclic peptide (~50 nM) was incubated with varying concentrations of TNFα (0-18 μM) and the FA values are plotted against TNFα concentration. Curve fitting (as described in main text) gave the K_D values.

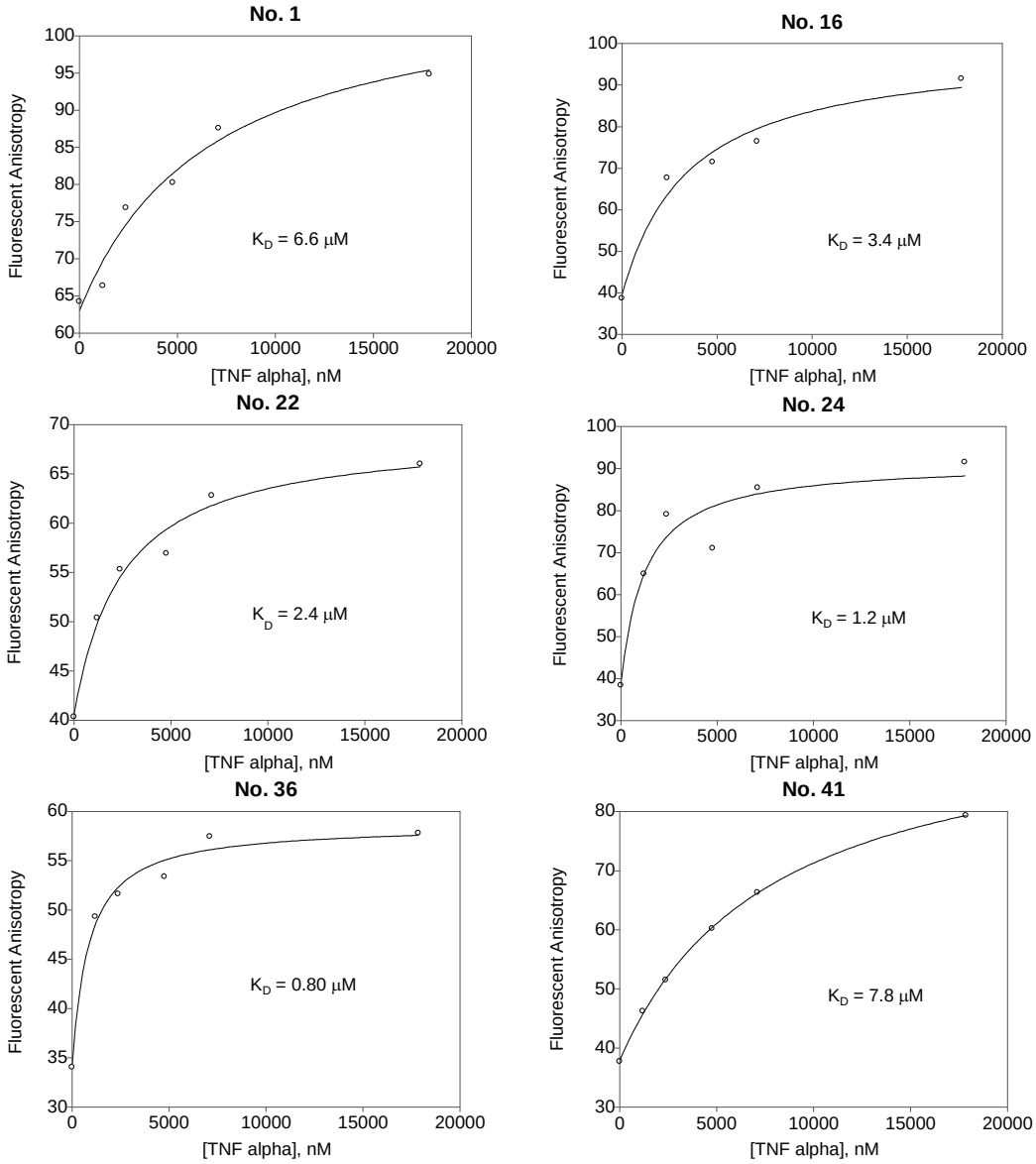
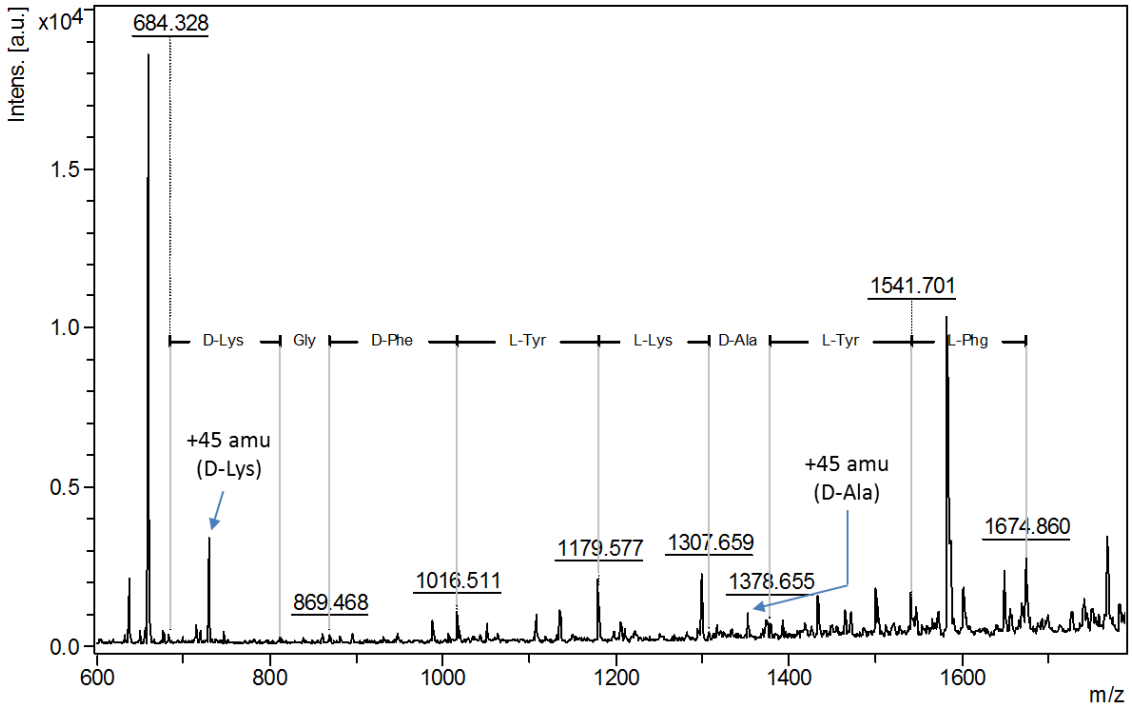


Figure S2. Sequence determination of positive hits by PED-MS. Positive beads after the solution-phase screening (4th round) were subjected to 11 cycles of PED, and the peptides were released from each bead by CNBr, and analyzed by MALDI-TOF MS. M*, homoserine lactone.

(a) Anticachexin C1: Phg-Tyr-D-Ala-Lys-Tyr-D-Phe-Gly-D-Lys-His-RBBFM*



(b) Anticachexin C2: Ala-D-Phe-Trp-D-Thr-Gln-Lys-Nle-D-Leu-Ala-His-RBBFM*

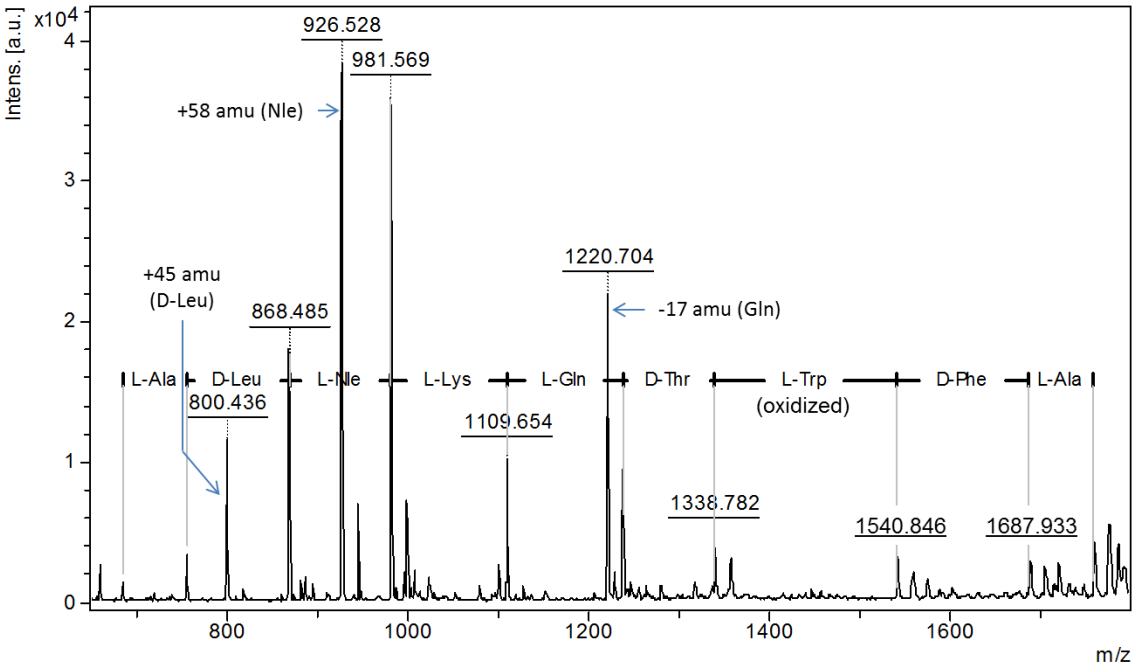
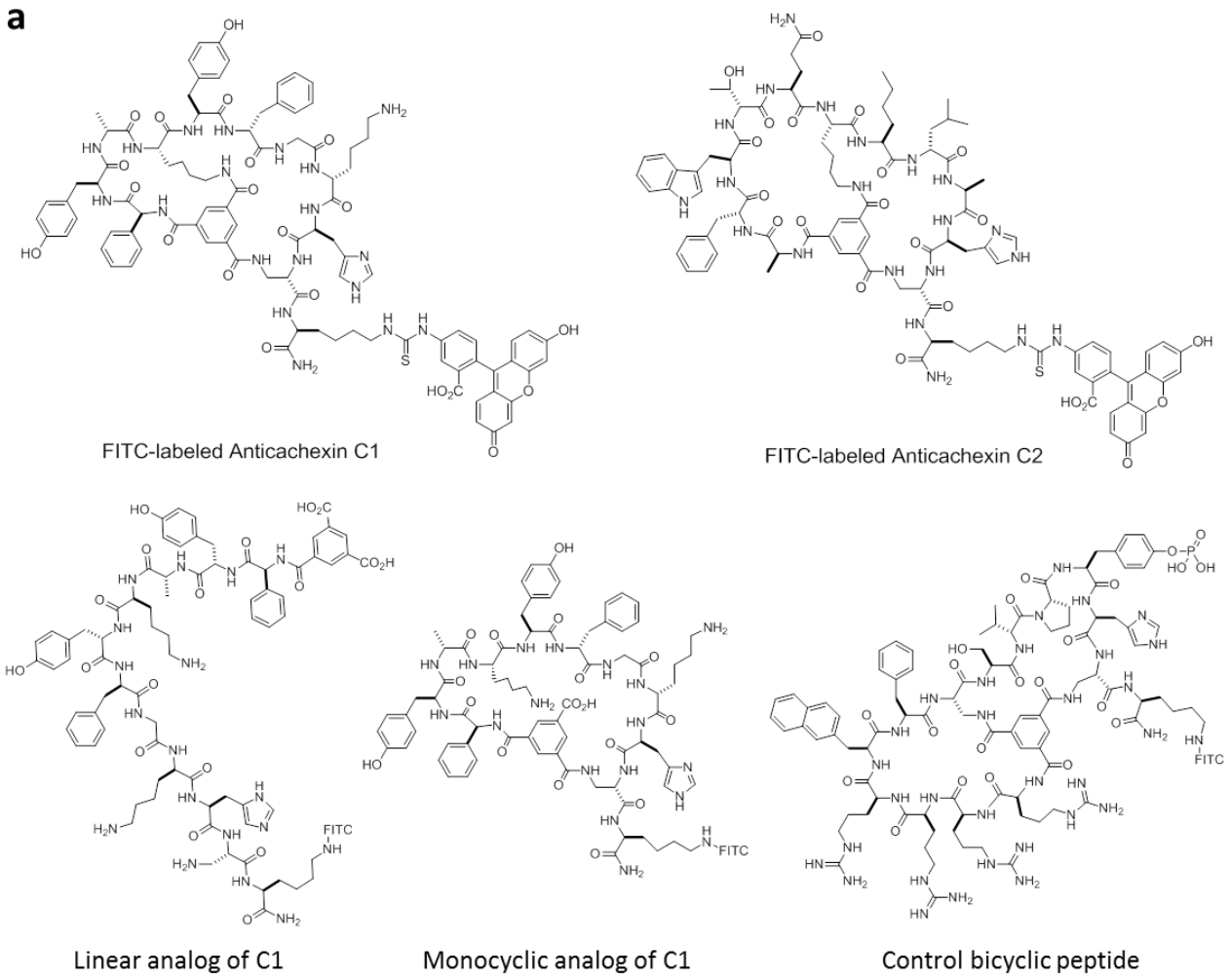


Figure S3. a, Structures of FITC-labeled Anticachexin C1 and C2, the linear and monocyclic analogs of C1, and a control bicyclic peptide. **b,** Binding of C1, C2, and C1 analogs and control bicyclic peptide to TNFα as determined by FA.



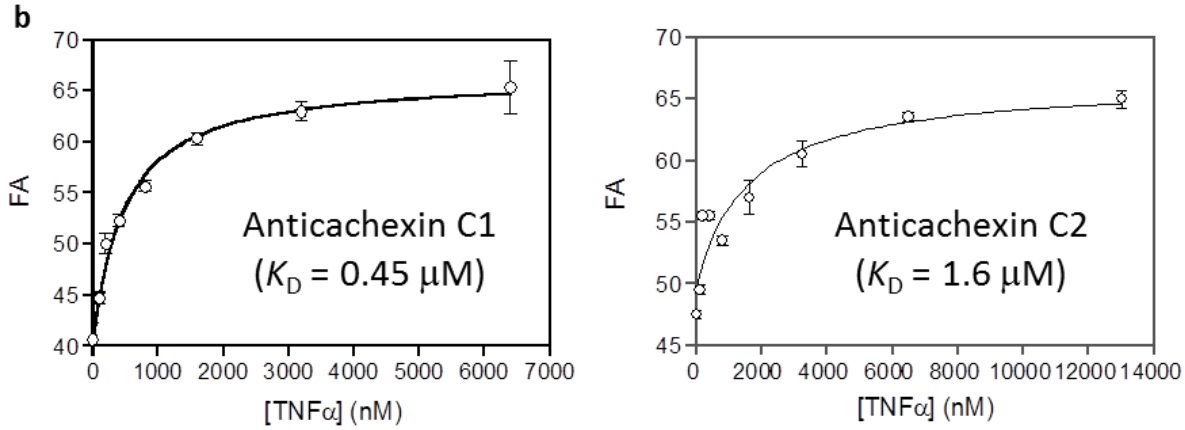


Figure S3 cont'd

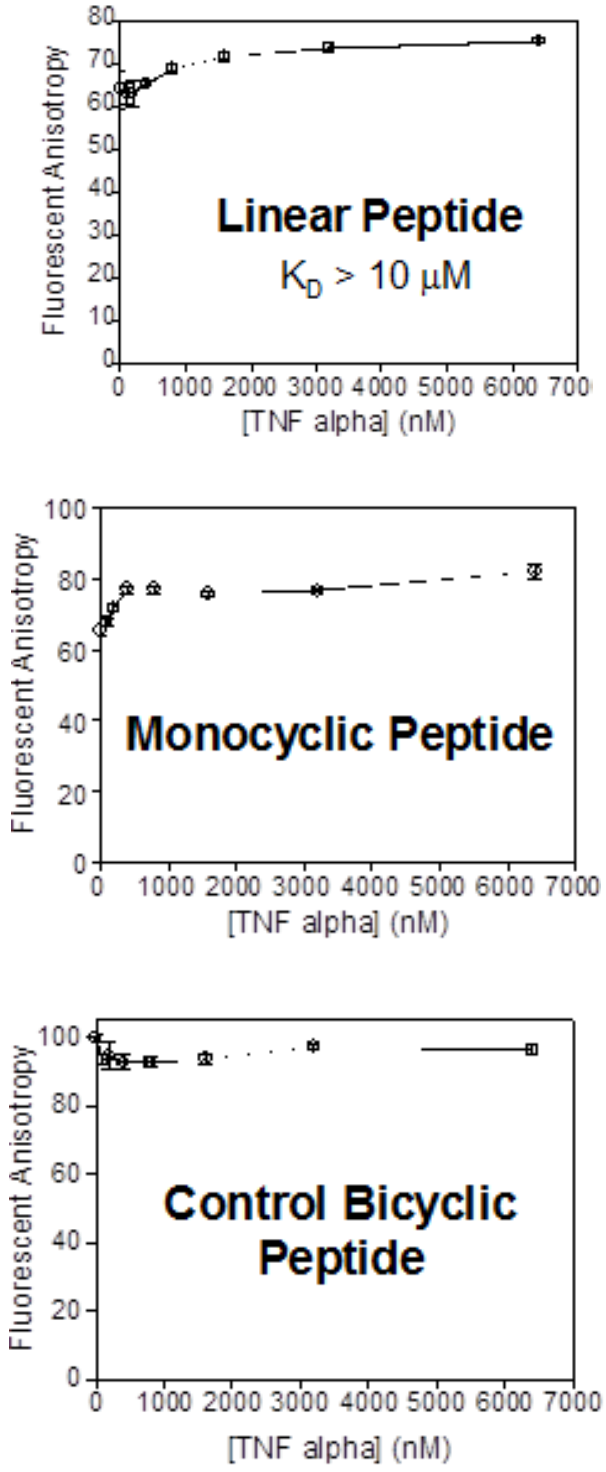


Figure S4. FA analysis of binding of FITC-labeled Anticachexin C1 and C2 to control proteins. BSA, bovine serum albumin; BRCT, GST fusion with the BRCT domain of TopBP1; CA, HIV-1 capsid protein; PLCγ2N, GST fusion with the N-SH2 domain of SH2 of PLCγ; and PTP1B, protein tyrosine phosphatase 1B.

(a) Anticachexin C1

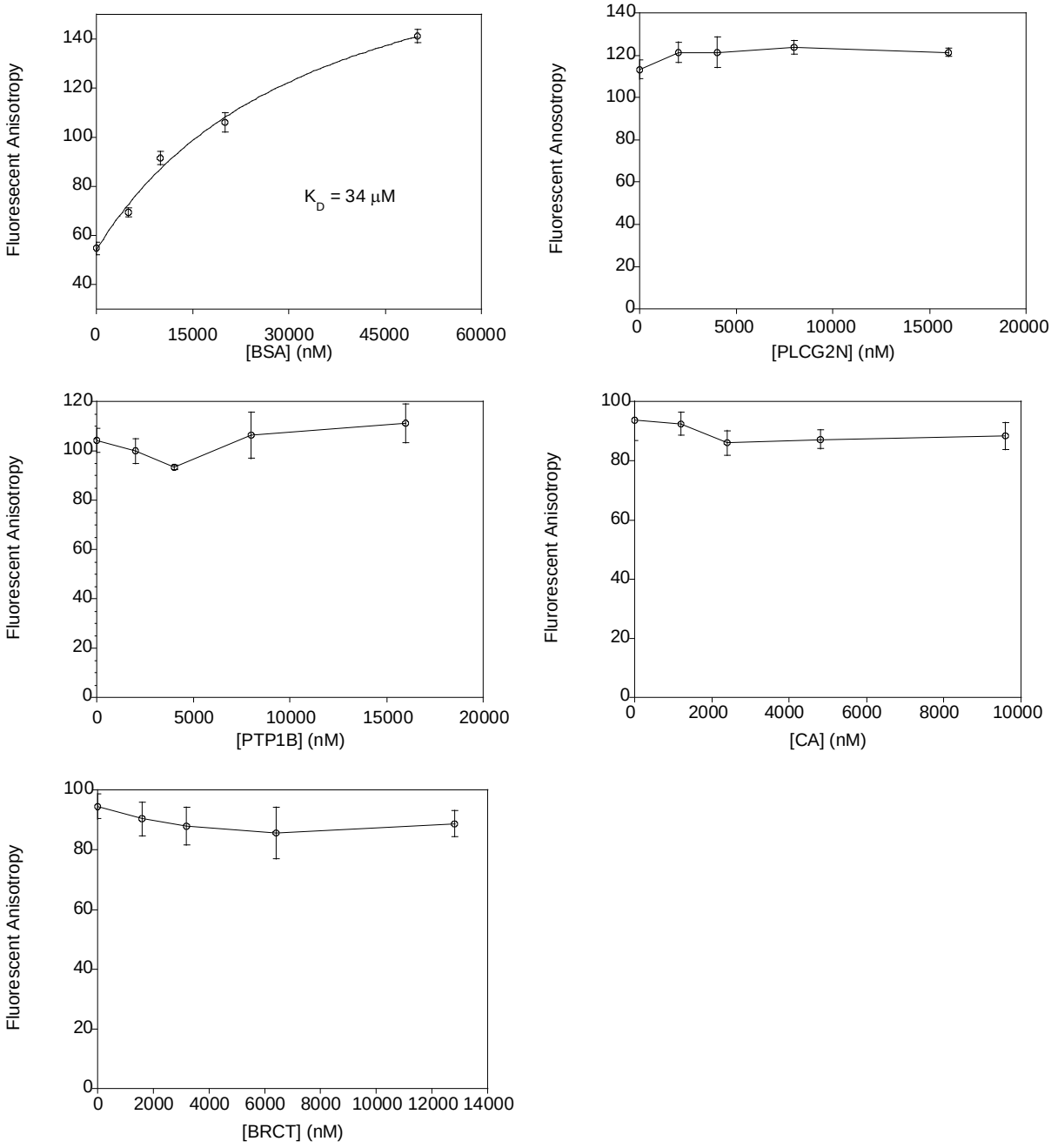
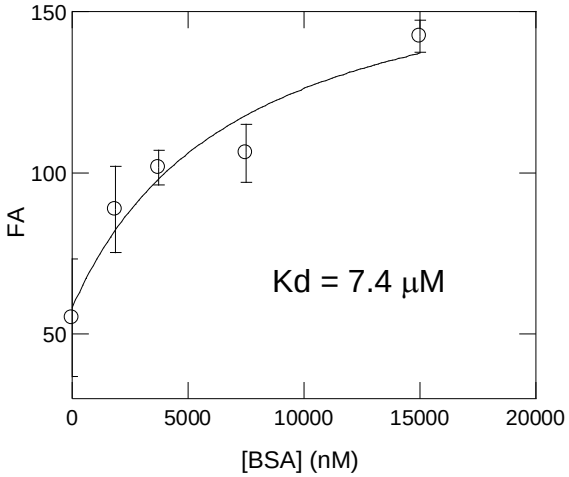
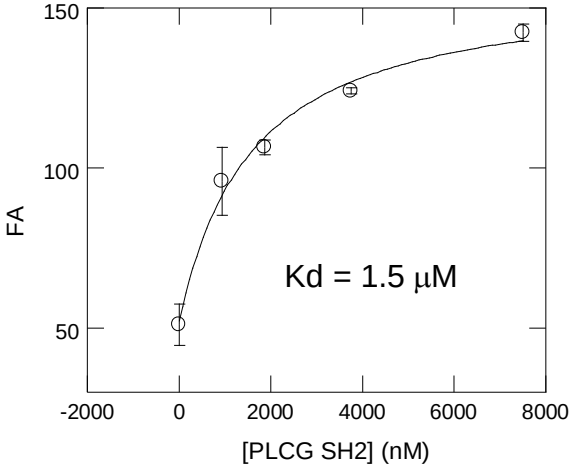
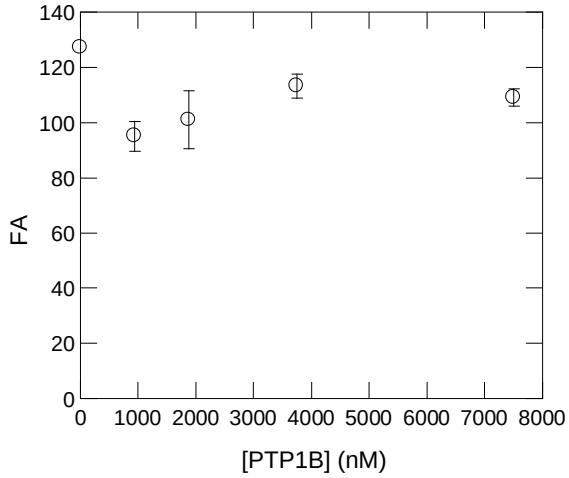


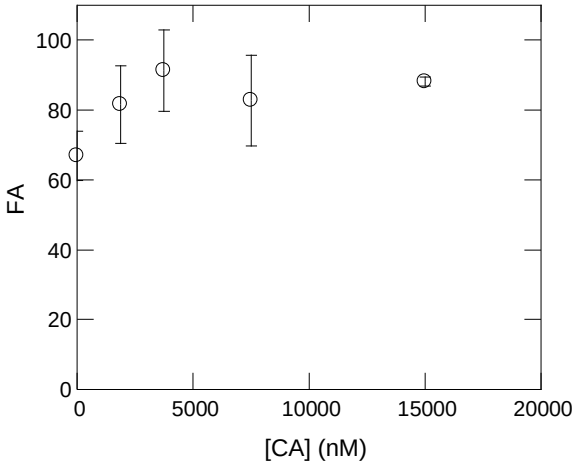
Figure S4 cont'd

(b) Anticachexin C2









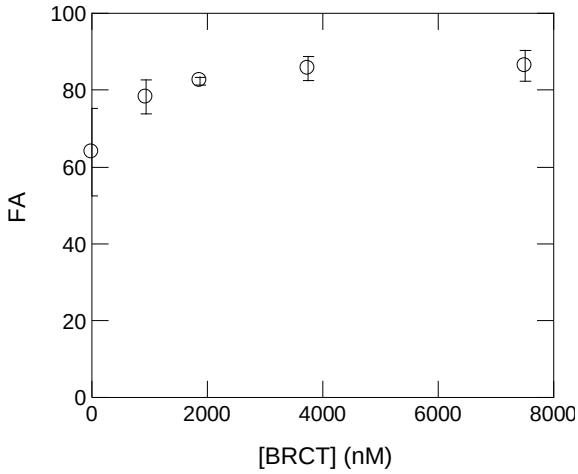


Figure S5. Competition between Anticachexin C1 and C2 for binding to TNFα. TNFα (1600 nM), FITC-labeled Anticachexin C1 or C2 (100 nM), and varying concentrations of unlabeled Anticachexin C2 (0-16 μM) were incubated for 1 h at 37 °C and the FA values were measured and plotted as a function of Anticachexin C2 concentration.

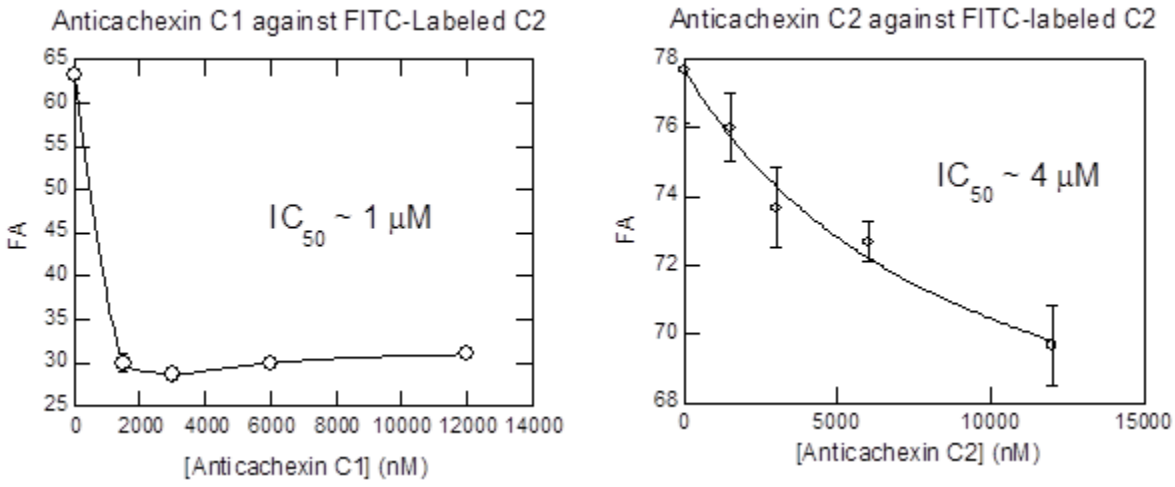


Figure S6. Protection of TNFα-induced cell death by Anticachexin C1. WEHI-13VAR cells were treated with increasing concentrations of TNFα (0-250 ng/mL) in the absence and presence of 50 μM Anticachexin C1. After incubation at 37 °C overnight, the number of live cells was determined by the MTT assay and plotted as a function of TNFα concentration. All y axis values are relative to that in the absence of TNFα or Anticachexin C1 (100%).