Supporting information for:

Identification of novel inhibitors that disrupt STAT3/DNA interaction from γ-AApeptide OBOC combinatorial library

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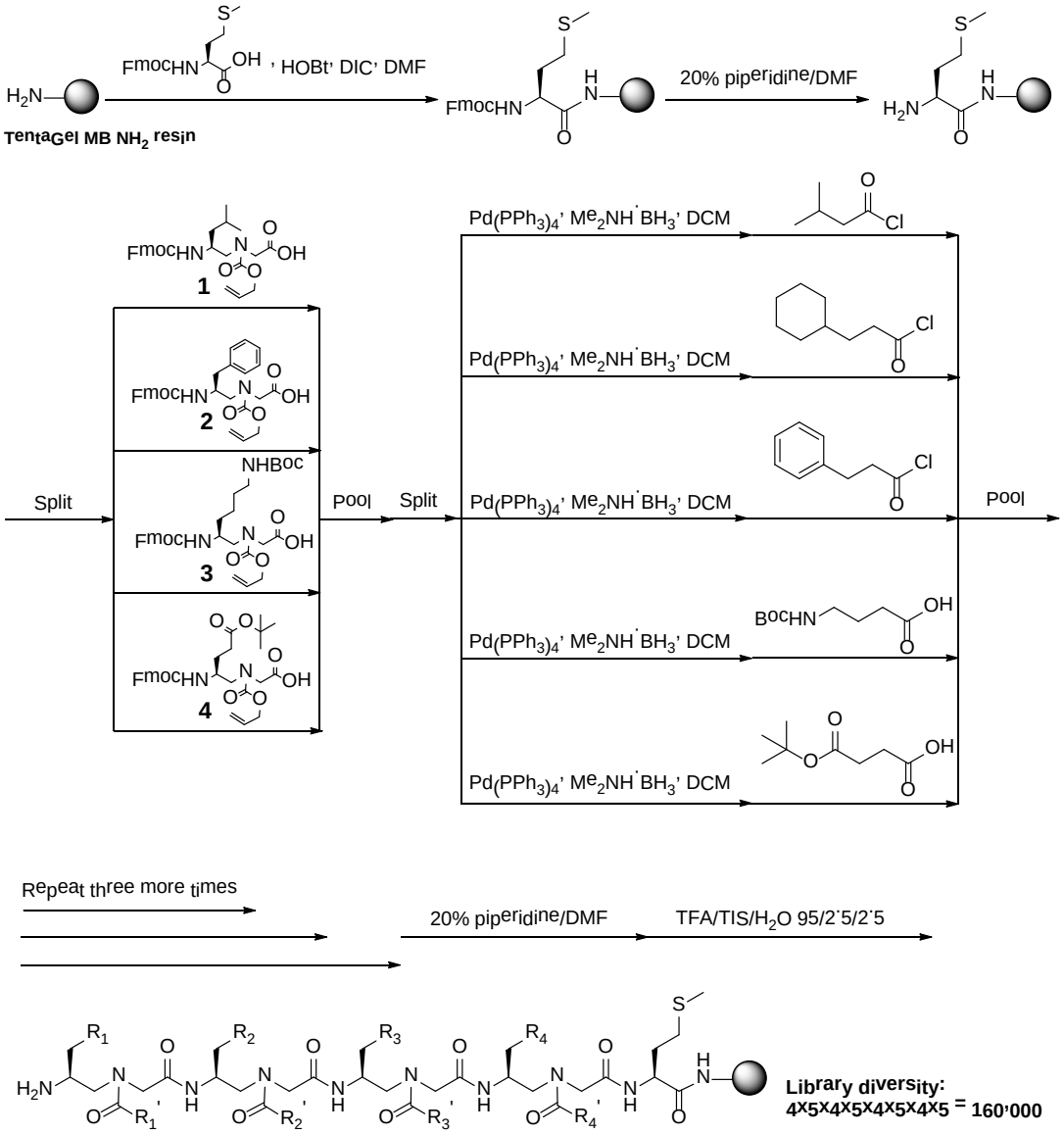
1. General methods and materials.

All Fmoc protected α-amino acids and Rink amide resin (0.7 mmol/g, 200-400 mesh) were purchased from Chem-Impex International, Inc. TentaGel MB NH_2 resin (0.3 mmol/g, 140-170 μm) was purchased from RaPP Polymere GmbH. Masses of γ-AApeptides were obtained on an Applied Biosystems 4700 Proteomics Analyzer. MS/MS analysis was carried out with a Thermo LTQ Orbitrap XL. Solid phase synthesis was conducted in peptide synthesis vessels on a Burrell Wrist-Action shaker. γ-AApeptides were analyzed and purified on a Waters Breeze 2 HPLC system, and then lyophilized on a Labcono lyophilizer. All cell lines were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Human breast carcinoma MDA-MB-468 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 10mM sodium pyruvate, 25mM HEPES, pH 7.5, 1000U/ml penicillin, and 1000μg/ml streptomycin. Primary antibodies against pY705-STAT3, Cyclin D1 and Survivin were purchased from Cell Signaling Technology (Danvers, MA). Primary antibodies against STAT3 (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO)

2. Synthesis of the OBOC *γ*-AApeptide library (Scheme S1) [1, 2 3]

The TentaGel NH_2 resin (1.6 g, 0.48 mmol, 800,000 beads) was swelled in DMF for 1h, then equally distributed into four peptide synthesis vessels, followed by the

treatment with Fmoc-Met-OH (3 equiv.), HOBt (6 equiv.), and DIC (6 equiv.) in DMF. The beads were shaken at room temperature in a peptide synthesis vessel for 4 h. The beads were washed with DCM (×3) and DMF (×3) before De-Fmoc protecting group with 20% piperidine in DMF for 10 min (×2), then washed thoroughly with DCM (×3) and DMF (×3). Each building block (2 equiv.) [2] together with HOBt (4 equiv.) and DIC (4 equiv.) were dissolved in DMF, shaken for 10 min, and then added to each vessel. The coupling reaction was performed at room temperature for 4 h and repeated. The beads in each vessel were then washed, pooled together, and mixed thoroughly by vigorously shaking for 1 h. The beads were equally split into five vessels. The Alloc protecting group was removed by treating beads with $Pd(PPh_3)_4$ (0.1 equiv.) and $Me_2NH{\cdot}BH_3$ (6 equiv.) in DCM for 10 min (×2). After thoroughly washing, each portion was reacted with either acid chloride or carboxylic acid. The reaction with acid chloride (5 equiv.) was carried out in the presence of DIPEA (6 equiv.) in DCM for 30 min (×2). The carboxylic acids (3 equiv.) were pre-activated with DIC (6 equiv.) and HOBt (6 equiv.) in DMF for 10 min before added to beads. The reaction was carried out by shaking the vessel for 6 hours and repeated. After that, all the beads were pooled and washed with DCM (×3) and DMF (×3) before mixed thoroughly. The previous split-and-pool process was repeated four times. At last, all beads were combined in one peptide synthesis vessel and washed thoroughly with DMF and DCM. Beads were treated with 20% piperidine in DMF for 20 min (×2) and then with TFA/TIS/H_2O (95:2.5:2.5) for 2 h to remove all the protecting groups. The beads were washed with DMF and DCM thoroughly and then dried in vacuo.



Scheme S1. Preparation of OBOC γ-AApeptide library.

3. On-bead Screening of γ-AApeptide library

3.1. General information

The STAT3 was used as a target for the combinatorial library screen. The synthesized library compounds were stored in a peptide synthesis vessel, and then washed and incubated in the same container. The beads were screened and picked up manually under Zeiss inverted fluorescence microscope 10x43HE filter. In order to avoid any possible nonspecific binding, both the STAT3 and antibodies solution were made in 1% BSA/TBST blocking buffer.

3.2. Beads screening

The library synthesized on TentaGel beads (1.6 g, 800,000 beads, 160,000 compounds) was swelled in DMF for 1 h, washed with 1×TBST five times and then equilibrated in 1×TBST overnight at room temperature. The beads were blocked in 1% BSA in TBST with a 1000× excess of cleared *E. coli* lysate for 1 hour, washed and equilibrated in 1×PBST before prescreening and screening.

Prescreening: The library was first incubated with mouse 1:1000 diluted STAT3 anti-mouse IgG primary antibodies for 2 hours at room temperature, followed by five times 1×PBST wash and incubation with 1:1000 diluted goat anti-mouse IgG conjugated with Alexa Fluor dylight 594 for 2 hours. The beads were washed with 1×PBST completely and transferred into a 6-well plate, and the bright red beads were removed under bench-top microscope as they had suspicious nonspecific binding. The rest of the beads were pooled together, washed with 1×PBST (5×), and then treated with 1% SDS at 90 ^{o}C for ten minutes to remove any bound proteins. Then the beads were washed with both water (5×) and 1×TBST (5×) to wash away the SDS and

swelled in DMF for 1 hour. After washing (5×) and equilibrating in 1×TBST overnight, the beads were ready for actual STAT3 screening.

Screening: The prescreened beads were equilibrated in 1% BSA/PBST for 1 hour at room temperature. After washing with 1×PBST (3×), the beads were incubated with STAT3 peptide at a concentration of 20 µg/mL for 4 hours at room temperature with a 1000× excess of *E. coli* lysate. After thoroughly washing with 1×PBST, the library beads were incubated in 5 mL of 1% BSA/PBST containing 1:1000 diluted STAT3 anti-mouse IgG primary antibodies for 2 hours at room temperature. The beads were gently washed with 1×PBST (3×) and incubated with 1:1000 diluted goat anti-mouse IgG conjugated with Alexa Fluor dylight 594 for 2 hour at room temperature. The beads were washed with 1×PBST and transferred into the 6-well plate to be observed under Zeiss inverted fluorescence microscope equipped with a 10×43HE filter. The individual bright red beads were picked out manually using pipette tips and used as putative hits for further study.

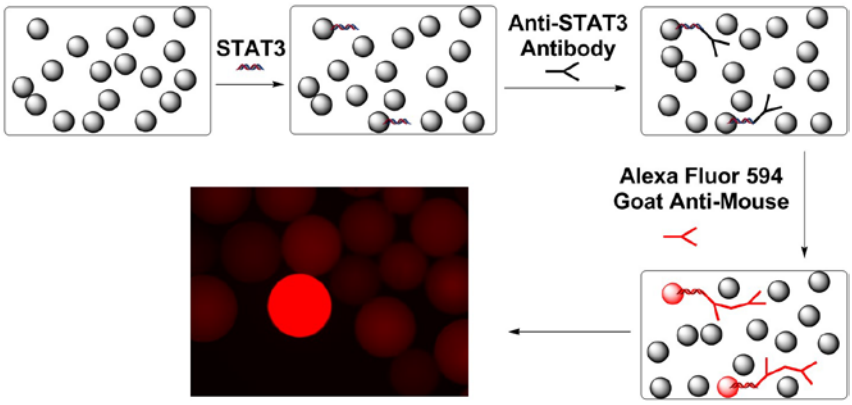
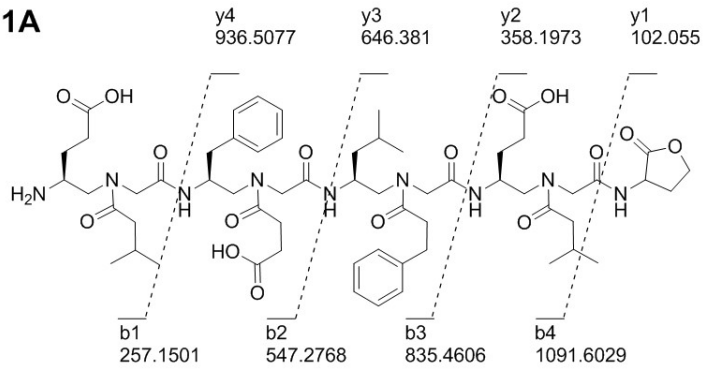


Figure S1. The scheme of OBOC screening for the identification of ligands binding to STAT3.

3.3. Sequence decoding

The putative beads were collected and washed with 1×PBST three times. The bound fluorescent dyes, proteins, and antibodies were removed by treating beads with 1% SDS solution at 90 °C for 10 min. After washing with water (3×), DMSO (3×), and acetonitrile (5×), beads were then subjected to CNBr treatment (50 mg CNBr in 1 mL 5/4/1 $CH_3CN/CH_3COOH/H_2O$) to cleave the compound from the beads for decoding by MS/MS using our previous procedure.[2] The MS/MS was run on a Thermo Scientific LTQ Orbitrap XL mass spectrometer. Higher Energy Collision Dissociation (HCD) was performed at collision energy of 25 or 35 mV. HCD fragmentation of a double charged precursor ion was represented in Figure S2.The structures of the unknown sequences were thus deduced.

1A



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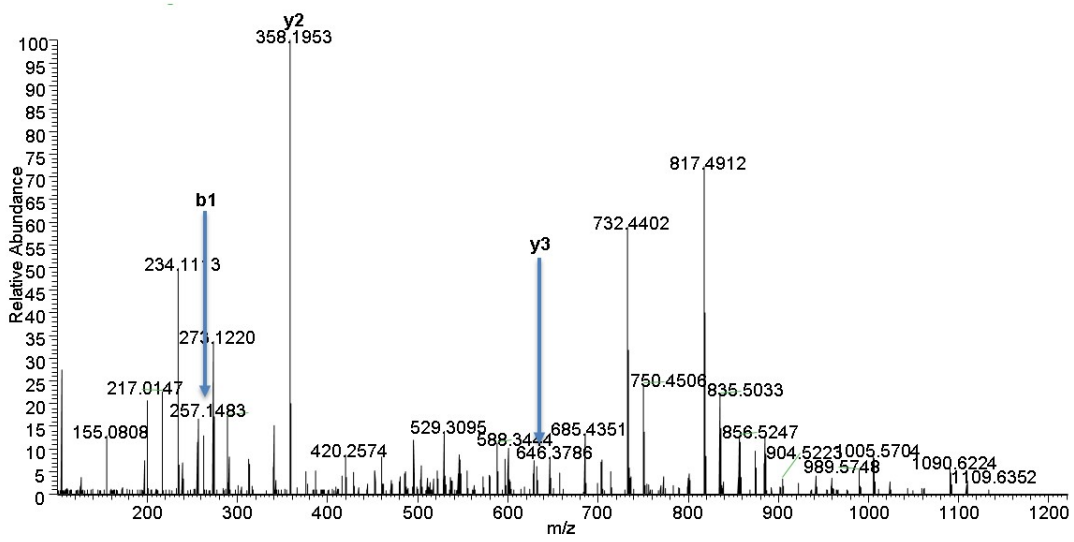
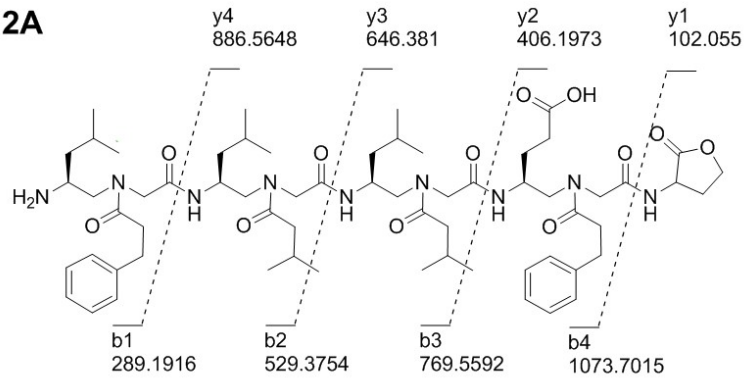


2A



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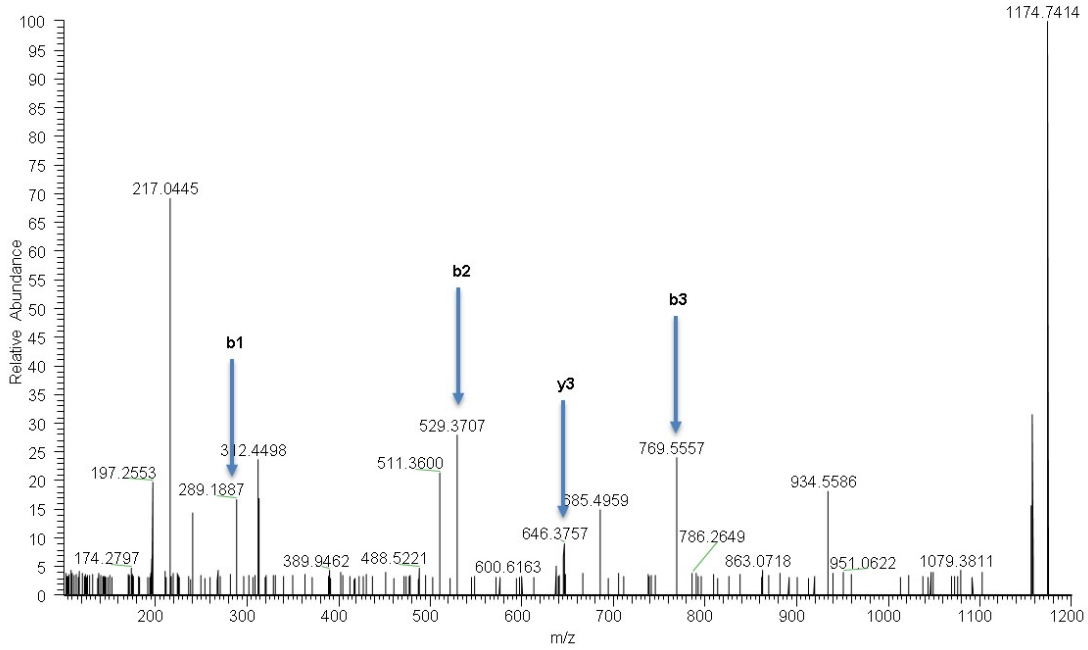
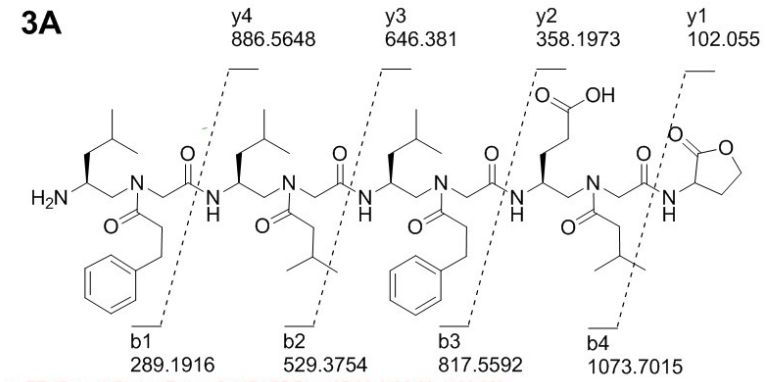


3A



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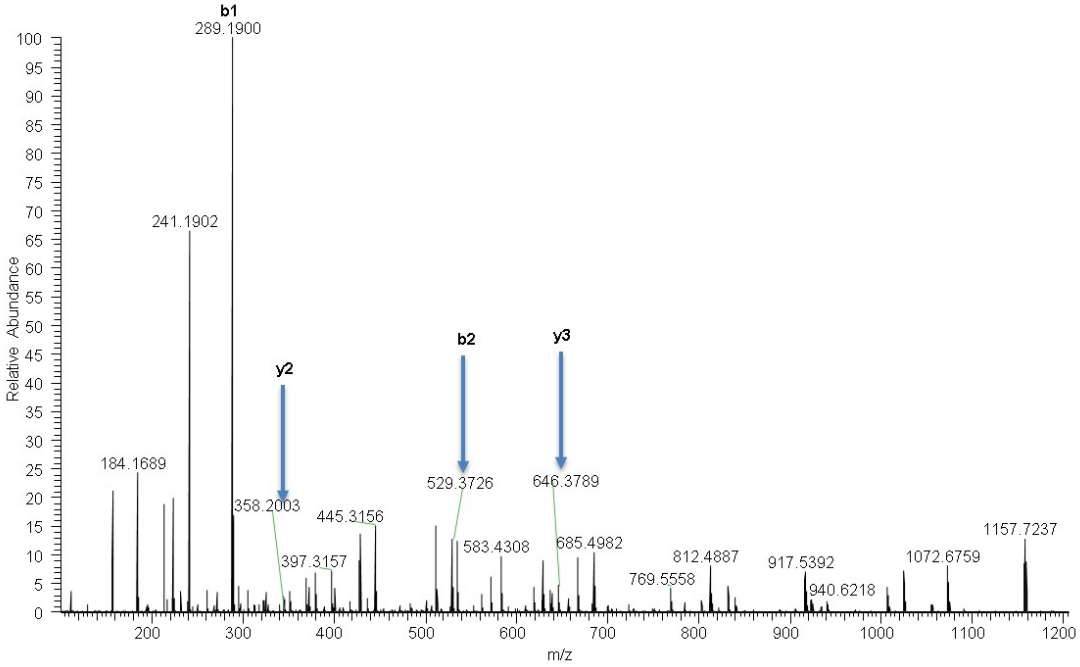


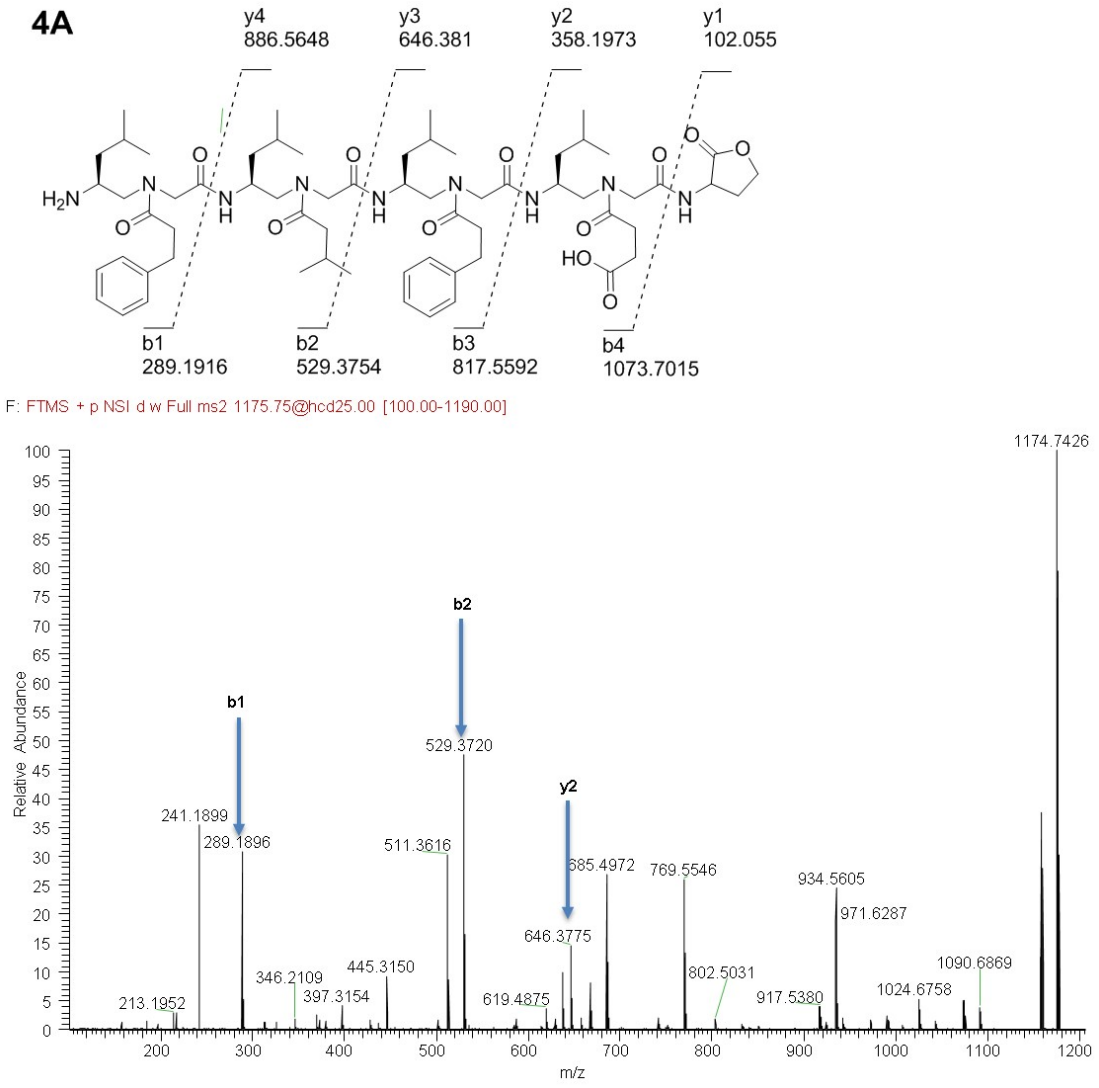


Figure S2. Structural identification of putative hits **1A − 4A** by MS/MS analysis. HCD fragmentation was performed on single or double charged precursor ions and the collision energy was set at 25 or 35.

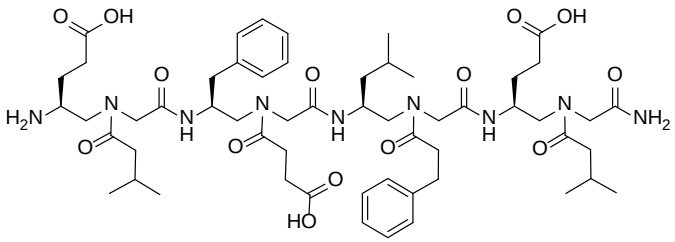
4. Solid phase synthesis of putative hits

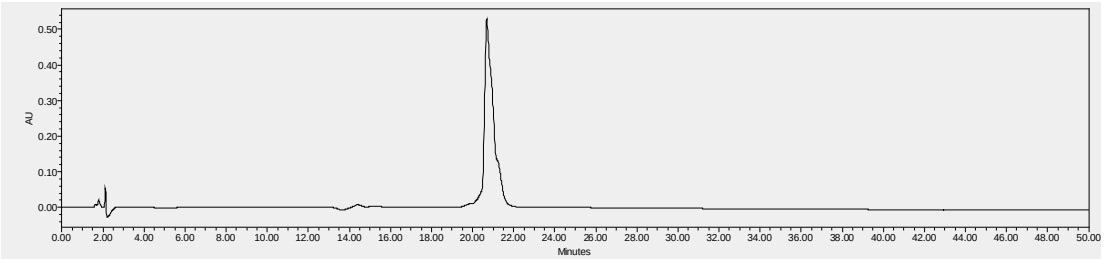
Compounds **1−4** were resynthesized on rink amide resin following our previously reported procedure.[2] Briefly, sequences were assembled on rink amide resin using

HOBt/DIC as coupling reagents. After cleavage with TFA/TIS/H_2O (95:2.5:2.5) for 3 h, TFA was removed under reduced pressure. The peptides were purified and analyzed on a preparative and analytical Waters HPLC system, respectively. The purity was confirmed on an analytical Waters HPLC system with flow rate of 1.0 mL/min and linear gradient from 5% to 100% (CH_3CN in water) in 50 min (Figure **S2**). Their molecular weights were identified by MALDI. [4]

Compound **1**

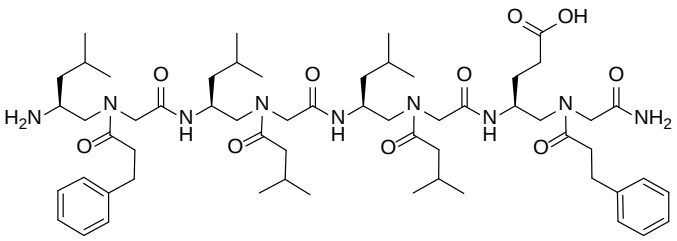
MALDI-TOF:
Theo: 1108.63 $[M+1]^+$
Found: 1108.48

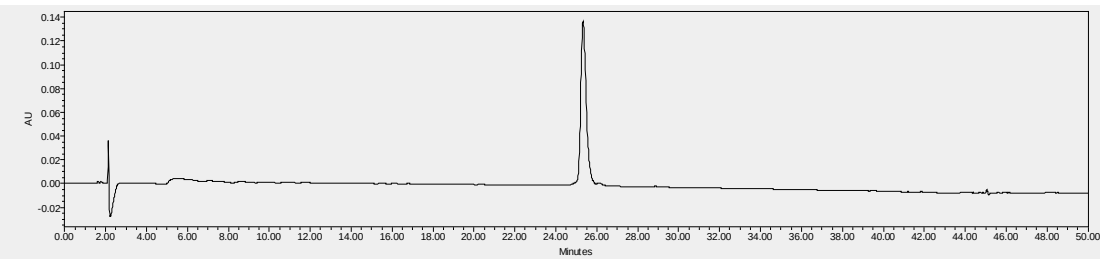




Compound **2**

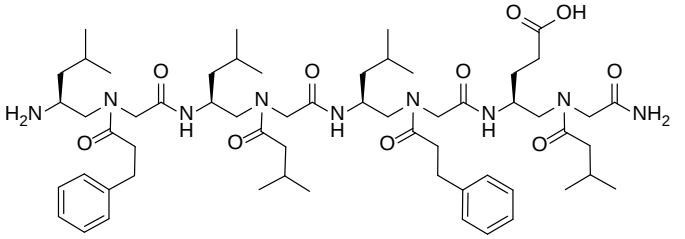
MALDI-TOF:
Theo: 1090.73 $[M+1]^+$
Found: 1090.59

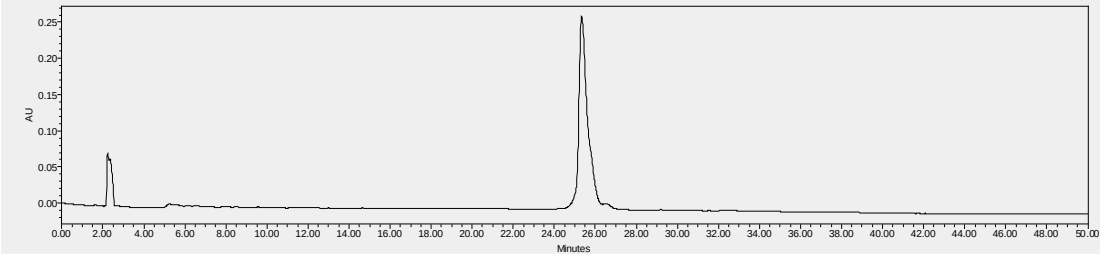




Compound **3**

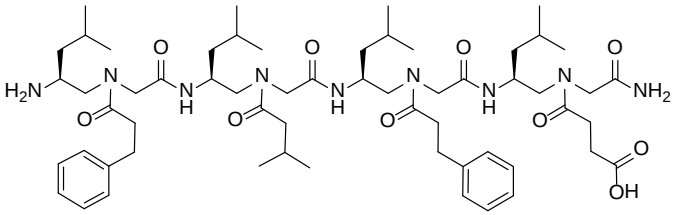
MALDI-TOF:
Theo: 1090.73 $[M+1]^+$
Found: 1090.60





Compound **4**

MALDI-TOF:
Theo: 1090.73 $[M+1]^+$
Found: 1090.62



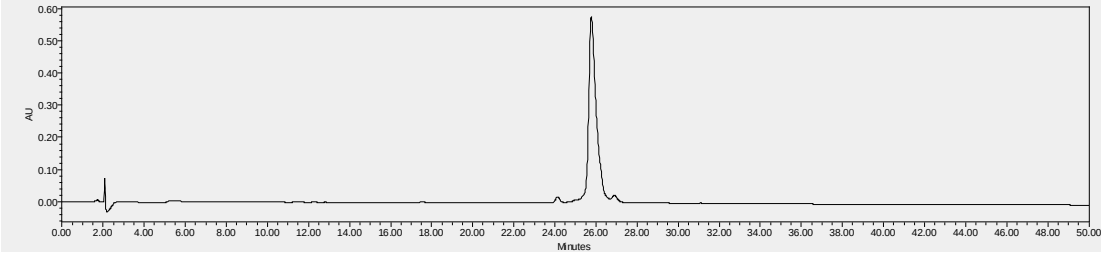


Figure S3. Pure HPLC trace of compounds **1**–**4**.

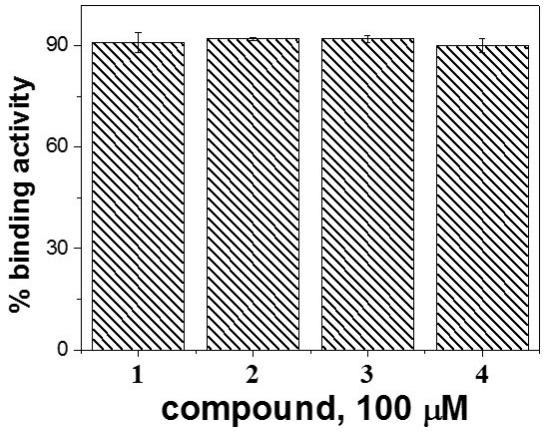


Figure S4. γ-AApeptides **1-4** do not inhibit the binding of STAT3 to fluorescein-labelled GpYLPQTV phosphotyrosine peptide by the fluorescence polarization assay.

5. Nuclear Extract Preparation and STAT3 Filter DNA-Binding Plate Assay

Nuclear extract preparation was carried out as previously described. [5] The STAT3-DNA binding filter plate assay was performed following the manual of the filter plate assay kit (Signosis, Sunnyvale, CA), as described previously. [6] The TF Binding buffer was mixed with the STAT3 probe (biotin labeled STAT3 DNA binding sequence) and nuclear extract and incubated at 16 °C for 30 minutes to form the STAT3-DNA complex. The STAT3-DNA complex was then separated from free probe by using a filter plate. After several steps of binding and washing, bound STAT3 probe was retained on the filter and the free DNA probe was removed. The bound pre-labeled STAT3 probe was then eluted from the filter plate by centrifugation with elution buffer. Eluted probes were hybridized into 96-well hybridization plates for quantitative analysis. The captured STAT3 probe was detected by conjugation with streptavidin-HRP. The chemiluminescence of each well was read using 2104 EnVisionR Multilabel Reader (Perkin Elmer) within 5 minutes after substrates were added. STAT3 filter DNA binding assay on intact cells is similar except the intact cells were incubated with compounds first and then nuclear lysate was incubated with STAT3 probe.

6. Western Blots

Cells were harvested and lysed for 30 min on ice with occasional vortexing in 150mM Hepes, pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40, 10% glycerol, 5mM NaF, 1mM DTT, 1mM PMSF, 2mM Na_3VO_4, and 5μg/ml leupeptin. Proteins readings were obtained using the Bradford protein assay, and equal amounts of protein for each sample were loaded into the wells of SDS-PAGE gels. After separation, proteins were transferred to nitrocellulose and Western blots were performed as we did previously. [6]

7. Computer modeling

The docking of the γ-AApeptide on the STAT3 DNA-binding domain was performed with Glide (Schrodinger) program. The crystal structure (PDB 1BG1) of STAT3 was used for docking. The structure was prepossessed with protein preparation wizard and then the energy minimization was applied to the structure. A box size of 20Å x 20Å x 20Å that covers DNA STAT3 binding interface was used as the grid. The ligands were applied with a conformation search, and these conformations were used to dock the STAT3 DNA binding domain.

1. H. Wu, P. Teng and J. Cai, *Eur. J. Org. Chem*, 2014, 1760-1765.
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6. X. L. Zhang, Y. Sun, R. Pireddu, H. Yang, M. K. Urlam, H. R. Lawrence, W. C. Guida, N. J. Lawrence and S. M. Sebti, *Cancer Research*, 2013, **73**, 1922-1933.