Supplementary Material (ESI) for Chemical Communications

Coordination Complex SH2 Domain Proteomimetics: An Alternative Approach to Disrupting Oncogenic Protein–Protein Interactions

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Supporting Information

Chemistry: General Methods. Anhydrous solvents methanol, DMSO, CH_2Cl_2, THF and DMF were purchased from Sigma Aldrich and used directly from Sure-Seal bottles. Molecular sieves were activated by heating to 300 °C under vacuum overnight. All reactions were performed under an atmosphere of dry nitrogen in oven-dried glassware and were monitored for completeness by thin-layer chromatography (TLC) using silica gel (visualized by UV light, or developed by treatment with $KMnO_4$ stain or phosphomolybdic acid stain). ^1H and ^{13}C NMR spectra were recorded on Bruker 400 MHz and a Varian 500 MHz spectrometers in either $CDCl_3$, CD_3OD or d_6-DMSO. Chemical shifts (δ) are reported in parts per million after calibration to residual isotopic solvent. Coupling constants (*J*) are reported in Hz. Before biological testing, inhibitors were subjected to further purification by reversed-phase HPLC (rpHPLC). Analysis and purification by rpHPLC were performed using either Atlantis Prep T3 10 μm C18 (2) 250 x 19 mm column run at 20 mL/min (preparative) or a Microsorb-MV 300 A C18 250 mm x 4.6 mm column run at 1 mL/min (analytical), using gradient mixtures of (A) water with 0.1% TFA and (B) 10:1 acetonitrile/water with 0.1% TFA. Ligand purity was confirmed by analytical rpHPLC using linear gradients from 100% A to 100% B, with changing solvent composition of either (I) 4.5% or (II) 1.5% per minute after an initial 2 minutes of 100% A.

Compound Characterization

Ligand of Complex 1 (L1) oil; δ_H (400 MHz, $CDCl_3$) 2.07 (s, 1H, O<u>H</u>), 2.63 (m, 4H, CHOH<u>CH_2</u>N), 3.86 (m, 8H, N<u>CH_2</u>Pyr), 3.97 (m, 1H, C<u>H</u>OH), 7.11 (t, *J* = 8 Hz, 4H, 4 CH (Ar)), 7.57 (t, *J* = 8 Hz, 4H, 4 CH

(Ar)), 8.49 (d, *J* = 6 Hz, 4H, 4 CH (Ar)); δ_C (400 Hz, $CDCl_3$) 58.91, 60.57, 67.12, 121.84, 122.95, 136.32, 148.69, 159.27; HRMS (ES+) calcd for $C_{27}H_{31}N_6O$ [M+H] 455.2553. Found 455.2567; IR (KBr, cm^{-1}) 3378, 2923, 1640, 1614, 1585, 1474, 1435, 1273.

Proteomimetic 1: HRMS (ES+) calcd for $C_{27}H_{31}N_6OCu_2$ [M^{2+}, Cu(II)] 290.0531. Found 290.0539; IR (KBr, cm^{-1}) 3498, 2959, 1612, 1574, 1484, 1448, 1259, 1172, 1032, 766, 641; mp = 115-120 °C; UV/Vis (D_2O, 10 mM HEPES, pH = 7.5) λ 253, 267.

Ligand of Complex 2 (L2) oil; δ_H (400 MHz, $CDCl_3$) 3.68 (s, 2H, PhC<u>H</u>$_2$N-), 3.69 (s, 2H, PhC<u>H</u>$_2$N-), 3.80 (s, 4H, -NC<u>H</u>$_2$Pyr), 3.85 (s, 4H, -NC<u>H</u>$_2$Pyr), 3.92 (s, 6H, 2 –OC<u>H</u>$_3$), 7.14 (t, *J* = 6.1 Hz, 2H, 2 CH (Ar)), 7.27 (m, 3H, 3 CH (Ar)), 7.45 (s, 1H, CH (Ar)), 7.61 (m, 6H, 6 CH (Ar)), 8.20 (dd, *J* = 8.2 and 2.2 Hz, 2H, 2 CH (Ar)), 8.51 (d, *J* = 4.6 Hz, 2H, 2 CH (Ar)), 9.10 (d, *J* = 1.8 Hz, 2H, 2 CH (Ar)); δ_C (400 MHz, $CDCl_3$) 52.2, 58.3, 58.6, 59.7, 59.8, 121.83, 122.1, 122.6, 124.4, 127.5, 127.8, 128.3, 129.2, 136.3, 137.4, 138.2, 139.0, 148.8, 150.2, 164.1, 165.6, 169.5; HRMS (ES+) calcd for $C_{36}H_{36}N_6O_4$ [M+H] 617.2870. Found 617.2841; IR (KBr, cm^{-1}) 3412, 2951, 2360, 1725, 1637, 1616, 1597, 1567, 1475, 1435.

Proteomimetic 2: HRMS (ES+) calcd for $C_{36}H_{36}N_6O_4Cu_2$ [M^{2+}, Cu(II)] 371.0689. Found 371.0701; IR (KBr, cm^{-1}) 3384, 2963, 1735, 1616, 1490, 1450, 1290, 1030, 963, 760, 637; mp = 110-125 °C; UV/Vis (D_2O, 10 mM HEPES, pH = 7.5) λ 223, 257, 278.

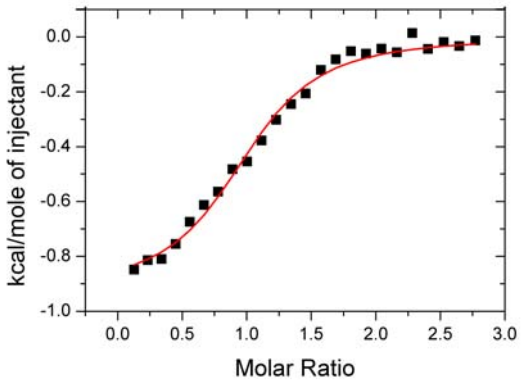
Ligand of Complex 3 (L3) oil; δ_H (400 MHz, $CDCl_3$) 3.64 (s, 4H, -NC<u>H</u>$_2$Pyr), 3.65 (s, 2H, PhC<u>H</u>$_2$N-), 3.67 (s, 2H, PhC<u>H</u>$_2$N-), 3.78 (s, 4H, 2 -NC<u>H</u>$_2$Pyr), 5.33 (s (br), 4H, 2 -NH_2), 6.48 (d, *J* = 8.3 Hz, 2H, 2 CH (Ar)), 6.85 (d, *J* = 7.6 Hz, 2H, 2 CH (Ar)), 7.12 (t, *J* = 6.1 Hz, 2H, 2 CH (Ar)), 7.25 (m, 3H, 3 CH (Ar)), 7.37 (t, *J* = 7.9 Hz, 2H, 2 CH (Ar)), 7.44 (s, 1H, CH (Ar)), 7.60 (m, 4H, 4 CH (Ar)), 8.49 (d, *J* = 4.5 Hz, 2H, 2 CH (Ar)); δ_C (400 MHz, $CDCl_3$) 58.3, 58.5, 59.6, 59.9, 106.3, 112.2, 121.7, 122.6, 127.2, 127.4, 128.0, 128.9, 136.2, 138.0, 138.8, 139.2, 148.7, 157.7, 158.4, 159.8; HRMS (ES+) calcd for $C_{32}H_{34}N_8$ [M+H] 531.2979. Found 531.2953; IR (KBr, cm^{-1}) 3410, 2924, 2851, 2360, 1618, 1570, 1468.

Proteomimetic 3: HRMS (ES+) calcd for $C_{32}H_{34}N_8Cu_2$ [M^{2+}, Cu(II)] 327.0665. Found 327.0680; IR

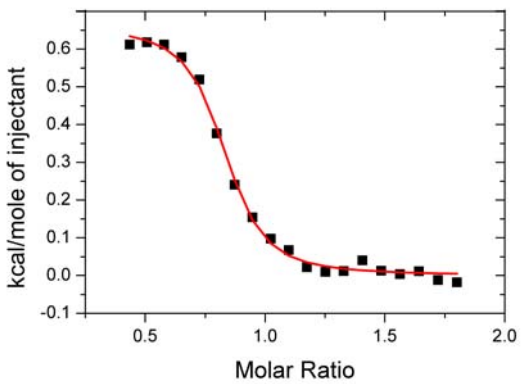
(KBr, cm^{-1}) 3361, 2924, 1641, 1613, 1576, 1489, 1449, 1255, 1175, 1031, 803, 768, 640; mp = 95-114 °C; UV/Vis (D_2O, 10 mM HEPES, pH = 7.5) λ 237, 261, 302.

S1. Isothermal Titration Calorimetry Binding Experiments. Isothermal titration calorimetry (ITC) experiments were used to measure the binding of our metal complexes to various substrates, and were performed at 25 °C (298 K) using Microcal VP-ITC titration microcalorimeter. In order to minimize mixing heat effects caused by differences in solution composition, the substrates and receptor were both dissolved in freshly prepared HEPES buffer (50 mM, pH = 7.2) before each titration experiment. All solutions prior to experiments were degassed before being added to the calorimeter cell. The substrates, at a concentration of approximately 2.0 mM, were injected in 10μL increments into the reaction cell (cell volume 1.49 mL) containing complex at a concentration of *ca* 0.1 mM, until there occurred a saturation of binding sites. A 250 μL injection syringe with 310–400 rpm stirring was used to give a series of 10 μL injections at 3.5-minute intervals. Control experiments for heats of mixing and dilution were performed under identical conditions and used for data correction in subsequent analysis. Data acquisition and subsequent non-linear regression analysis were done in terms of a simple binding model using the Microcal ORIGIN software package.

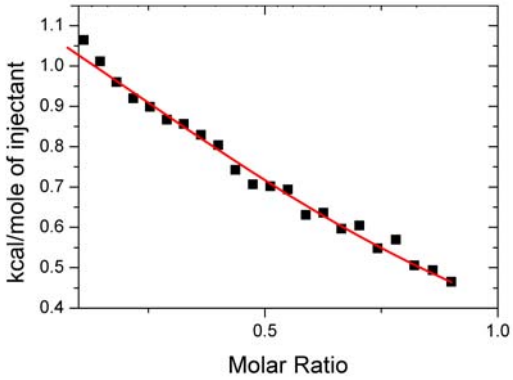
ITC Experiments – representative traces for Proteomimetics 1-3



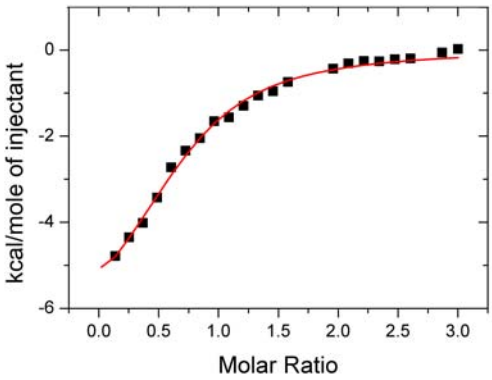
(i) **1** vs pYLKTK (STAT3)



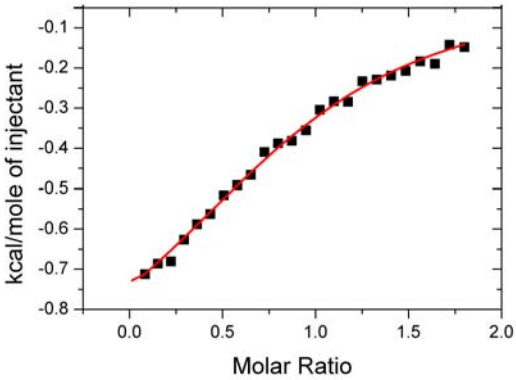
(ii) **1** vs pYLPQTV (gp130)



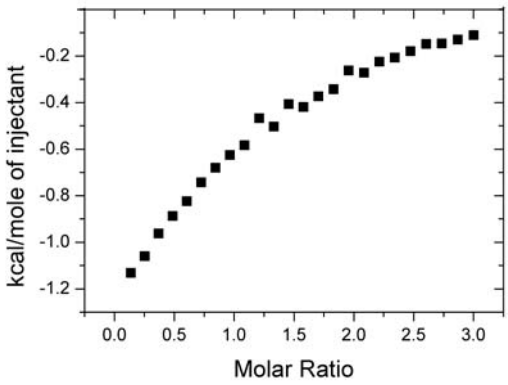
(iii) **2** vs pYLPQTV (gp130)



(iv) **2** vs pYLKTK



(v) **3** vs pYLPQTV (gp130)



(vi) **3** vs pYLKTK

S2. Fluorescence polarization (FP) binding assays.

The fluorescene polarization (FP) measurements were performed on a custom-built multimodal confocal microscope described previously [Baoxu Liu et al, *J. Fluoresc*, A Photostable, pH-Invariant Fluorescein Derivative for Single-Molecule Microscopy]. In brief, a wavelength-tunable femtosecond laser (Tsunami HP, Spectra Physics, Mountain View, CA) is frequencydoubled through a β-BBO crystal (Newlight Photonics, Toronto, Canada) to produce a narrow excitation spectrum centered at 480 nm. The light is linearly polarized, passes through a 1.4 NA/100X plan-apochromat objective (Carl Zeiss Canada) and illuminates the sample at intensities of 50-100 W/cm^2. The emitted fluorescence is collected through the same objective and is spatially and spectrally filtered using a 50-μm pinhole and high-quality long-pass

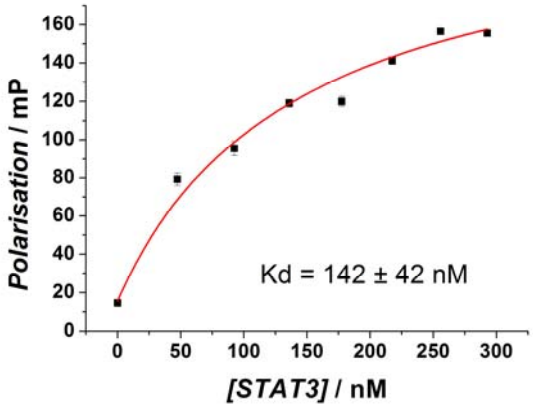
and interference filters (Semrock, Rochester, NY, USA) to remove out-of-focus fluorescence, Rayleigh and Raman scattering. Further, the fluorescence is divided into two components with orthogonal polarization, I_{vv} and I_{vh}, using a broadband polarizing cube beamsplitter (Newport, Irvine, CA). Each beam is then tightly focused onto a 50-µm diameter avalanche photodiode that features a low dark noise and high sensitivity (PD5CTC, Optoelectronic Components, Kirkland, QC, Canada). Each time a photon is detected the detector outputs an electric pulse and these pulses are counted by a PicoHarp300 module (PicoQuant Gmbh, Germany) to provide fluorescence intensity traces.

Prior to each set of experiments, a solution containing 65 nM of the dye Rhodamine110 in water was used to determine the correction factor G for the different detection sensitivity of the two channels. The fluorescence polarization p was then calculated according to the equation:

$$r = \frac{I_{vv} - G \cdot I_{vh}}{I_{vv} + 2 \cdot G \cdot I_{vh}} \quad P = \frac{I_{vv} - G \cdot I_{vh}}{I_{vv} + G \cdot I_{vh}} \cdot 1000 \quad \text{(in mP units)}$$

$$p = \frac{3 \cdot r}{2 + r} \cdot 1000 \text{ in mP}$$

Initially, the formation of the phosphopeptide-STAT3 complex was verified by FP; the overall titration curve and the value of k_d = 142 ± 42nM extracted from the fit concur with previous studies (Berg, Anal. Biochem, **2004**) Note that the free F*-peptide in solution exhibits an FP value of 15 ± 3 mP, much lower than the fitted saturation value of 226 ± 23 mP when virtually all phosphopeptide is bound to the protein.

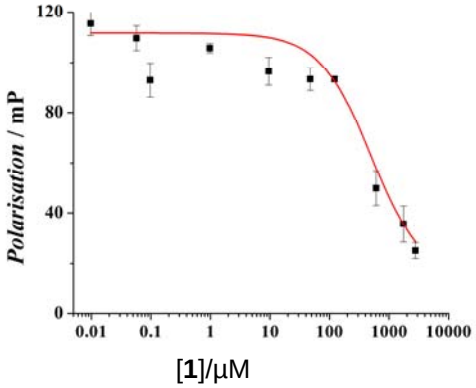


(vii) Stat3-pYLPQTV-NH_2 binding measured by fluorescence polarization assay.

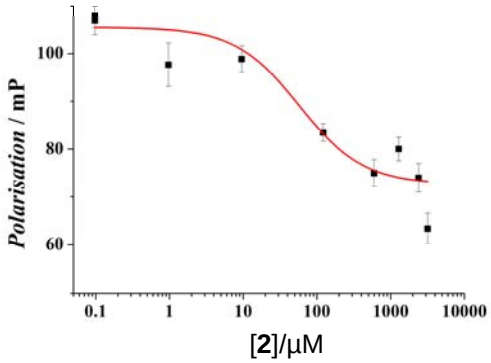
S2.1. FP Binding Experiments Data

All experiments were performed 20 minutes after mixing the starting solution containing 7.2 nM F*-peptide and 292 nM STAT3 with various amounts of inhibitor stock solution (Figure **vii**). The concentration of inhibitor was varied between 0.1 µM and 5 mM and all measurements were perfomed in a buffer (50 mM NaCl, 10 mM Hepes, pH 7.5, 2 mM dithiotreitol, w/w 1 mM EDTA). For statistical purposes, a series of five subsequent one-minute measurements were performed at each concentration of inhibitor.

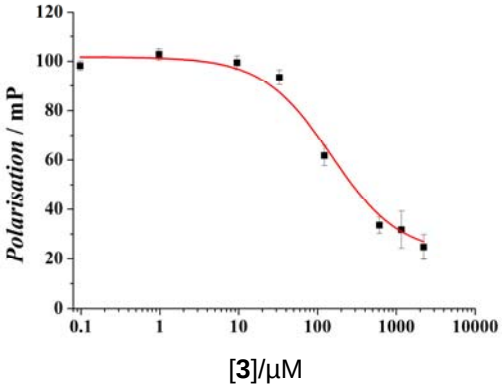
S2.1. FP Binding Experiments Data



(**viii**) Inhibitor **1**



(**ix**) Inhibitor **2**



(**x**) Inhibitor **3**

FP experiments with and without 1 mM EDTA in the buffer solution show that EDTA interferes in the interaction between the metal complex and the phosphopeptide. When EDTA is present, compound **2** has ca. 20% larger IC_{50} value and a significantly steeper transition, which suggests an increase in

cooperativity. This suggests the chelation of copper atoms present in the inhibitor molecules by the EDTA molecules. We recorded similar EDTA mediated drops in affinity for each of the remaining inhibitors (data not shown). Purified Stat3 protein was purchased from SignalChem (CANADA) – 80-90 % purity.

Titration FP data was modelled by the equation:

$$p(X) = \frac{p_1 \cdot IC_{50} + p_2 \cdot X}{IC_{50} + X}$$

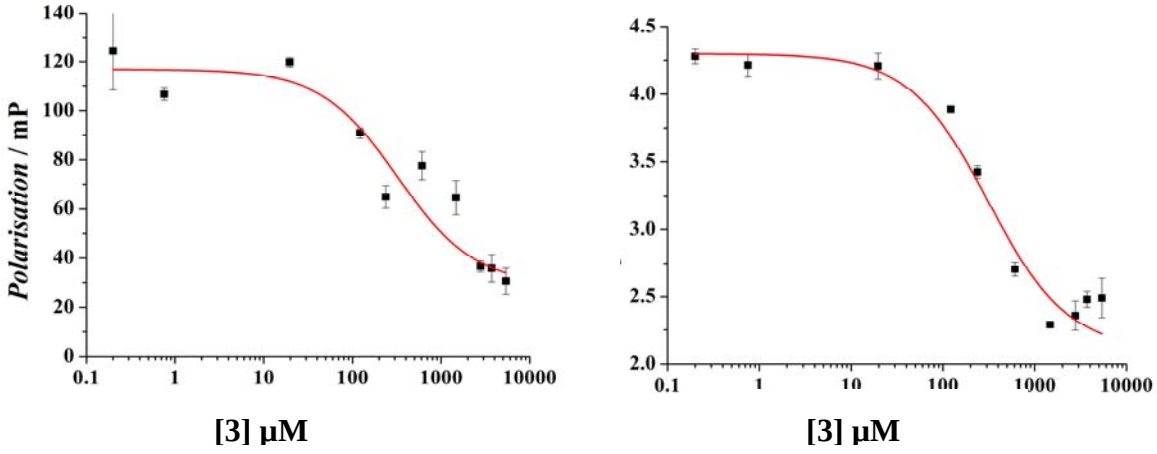
where p is the measured fluorescence polarization and X is the concentration of inhibitor. The free parameter is the half maximal inhibitory concentration (IC_{50}). The limiting values of the polarization (p_1 and p_2) were estimated from the plot and kept constant during the fit. The curve fitting was performed in Origin using the Levenberg-Marquardt algorithm and the reduced chi-square criterion for convergence. The inhibitor dissociation constant K_i was calculated from IC_{50} according to the formula:

$$K_i = \frac{IC_{50}}{1 + \frac{[STAT3]}{K_d}}$$

where [STAT3] = 292 nM and K_d = 100 nM.

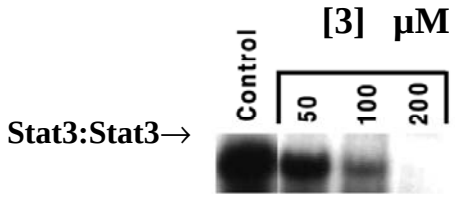
S2.2. Interesting Note: Remarkably, the fluorescence of the fluorescein attached to the model peptide was gradually quenched upon increasing the inhibitor concentration in solution. When the inhibitor reached mM concentrations, the detected fluorescence intensity became ca. 10 times weaker than at low concentrations of inhibitor. The dilution of the peptide upon addition of inhibitor was less than 10%, far smaller than the observed drop in fluorescence intensity, whereas the adsorption of sample to the microscope coverslip was negligible during the time course of the experiments. Our data were acquired using the time-correlated single-photon counting (TCSPC) technique so they include fluorescence decay traces by default. As quenching is expected to influence the rate of fluorescence decay, the time-resolved

curves were fitted to mono-exponential decays and the estimated fluorescence lifetime was plotted as a function of inhibitor concentration (figure **xi**, right). The titration curve closely resembles the FP data and it yields an IC_{50} value that is virtually identical to the FP assay, 315 µM vs. 324 µM. We can conclude that the formation of the peptide-inhibitor complex promotes quenching of the fluorescein probe by bringing charged metal atoms in its close proximity. This finding is significant because it opens up exciting prospects for studying the inhibition potency of novel organo-metallic complexes using fluorescence lifetime, a method that is more sensitive and selective compared to fluorescence polarization.

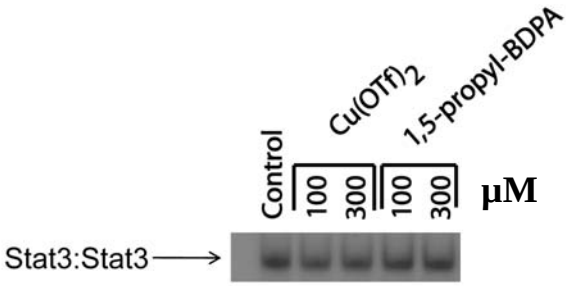


(xi) ***Left:*** Fluorescence polarization measured upon titrating the F*-phosphopeptide (7.2 nM) with compound **3** in the presence of STAT3 protein (292 nM). ***Right***: fluorescence lifetime titration.

S3. Nuclear extract preparation and gel shift assays. Nuclear extract preparation from v-Src-transformed fibroblasts (NIH3T3/v-Src) or mouse fibroblasts overexpressing the human epidermal growth factor receptor (NIH3T3/hEGFR) and electrophoretic mobility shift assay (EMSA) were carried out as previously described (reference 5, 16). Nuclear extracts of equal total protein content were pre-incubated with different concentrations of compounds for 30 min at room temperature prior to incubation with the radiolabeled probe. The ^{32}P-labeled oligonucleotide probe used is hSIE (high affinity sis-inducible element from the *c-fos* gene, m67 variant, 5'-AGCTTCATTTCCCGTAAATCCCTA) that binds STAT1 and STAT3. The EMSA trace for inhibitor 3 is shown below (not shown in manuscript)

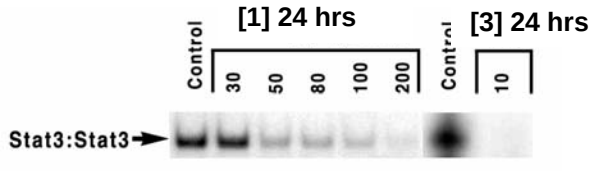


(**xii**) EMSA analysis for NIH3T3/vSrc cells treated with increasing concentrations of compound **3**



(**xiii**) Control EMSA analysis for NIH3T3/vSrc cells treated with increasing concentrations of 1,5-propyl-BDPA and $Cu(OTf)_2$

S3.1 EMSA Whole Cell NIH3T3/vSrc Data. Cells were incubated with varying concentrations of inhibitors for 24 hr, and then the nuclear extract preparations were subjected to the Stat3–Stat3:DNA-binding assay *in vitro* using the hSIE probe followed by EMSA analysis. Encouragingly, our potent *in vitro* activity was mirrored in this whole cell assay, where agents induced significant levels of Stat3 dimer disruption. The three inhibitors were found to significantly reduce the levels of intracellular Stat3–Stat3 homodimer formation. A representative gel from the EMSA experiment for NIH3T3/vSrc fibroblasts treated with increasing concentrations of **1** is illustrated below in (xiii)



(xiv) EMSA analysis of Stat3 DNA-binding activity in nuclear extracts prepared from NIH3T3/vSrc treated with compound **1** for inhibition of Stat3–Stat3 dimerization

S4. Whole cell viability studies in DU145, MDA468 and OCI-AML-2 cell lines (Table 3)

Human OCI-AML2 leukemia, DU145 prostate, and MDA468 breast cancer cells were seeded in 96 well plates. Cells were then treated with increasing concentrations of **1**, **2**, **3** and **4**. Seventy-hours after incubation, cell growth and viability was measured with the CellTiter96 aqueous nonradioactive (MTS) assay according to the manufacturer's instructions (Promega, Madison, WI) and as described previously (Simpson C and Schimmer, *Cancer Res*. **2009**). Data represent the mean $IC_{50} \pm$ SD (n = 5).

S5. WST-1 viability assay procedures

Proliferating cells in 96-well plates were treated with inhibitors for 48 h for WST-1 assay analysis, according to manufacturer's (Roche) instructions.- www.roche-applied-science.com

S6. Whole cell viability studies in healthy NIH3T3 cells

Proteomimetic **2** viability data: NIH3T3 'healthy' cells treated with **2** versus breast cancer cells (MDA-468) and acute myeloid leukemia cells (OCI-AML2) treated with **2**. MDA-468 (Walker, S. R. *Mol. Cancer Res*. **2009**, *7*, 966-976) and OCI-AML2 (Kube, D., *Blood*, **2001**, *98*, 762-770) cells are known to have constitutively activated Stat3 activity. We noted significantly lower cytotoxic effects against healthy NIH3T3 cells *cf.* cancer cells containing Stat3 activity.

S7. Whole cell viability studies of Cu(II)$(OTf)_2$ and Ligand 2 (L2)

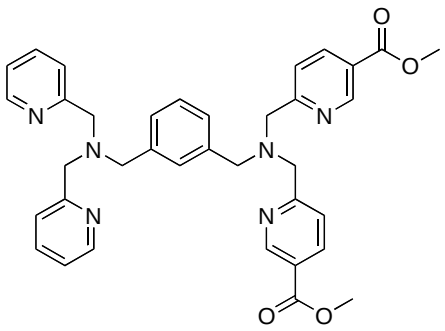
Inhibitory assessment of Cu(II)$(OTf)_2$ on breast cancer cells (MDA-468), prostate cancer (DU145) and acute myeloid leukemia cells (OCI-AML2).

It can be seen that there is no inhibitory affect due to the free metal at the concentrations at which our

compounds are administered.

S8. Whole cell viability studies of Ligand 2 (L2)

Assessment of cytotoxicity of free ligand, **L2** on breast cancer cells (MDA-468), prostate cancer (DU145) and acute myeloid leukemia cells (OCI-AML2).



L2

QuickTime™ and a
decompressor
are needed to see this picture.

The data show negligible cytotoxic effects upon cancer cell viablity by the free ligand L2. These data confirm that cytotoxicity is achieved only when the ligand is complexed with copper (II).

S9. Peptide Characterization

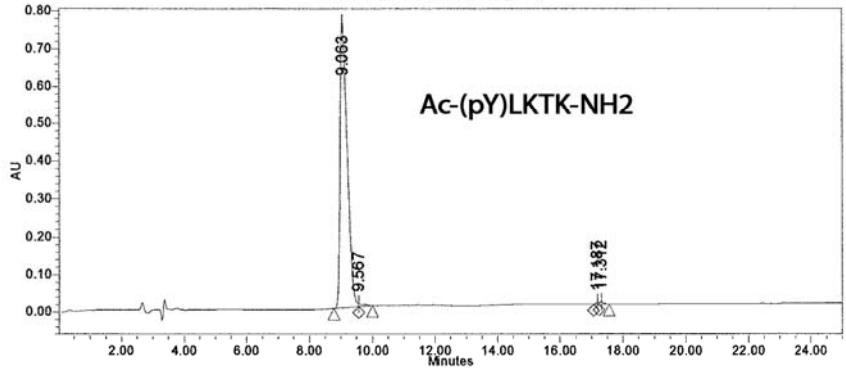
Peptides used in FP and ITC expermients were purchased from CanPeptide (Canada) with purities > 97 % by analytical HPLC analysis. Analytical HPLC traces and ESMS traces of the three peptides, Ac-G(pY)LPQTV-NH_2, 5-FAM-G(pY)LPQTV-NH_2 and Ac-(pY)LKTK-NH_2 are shown below.

(i) Ac-(pY)LKTK-NH_2

CANPEPTIDE HPLC REPORT

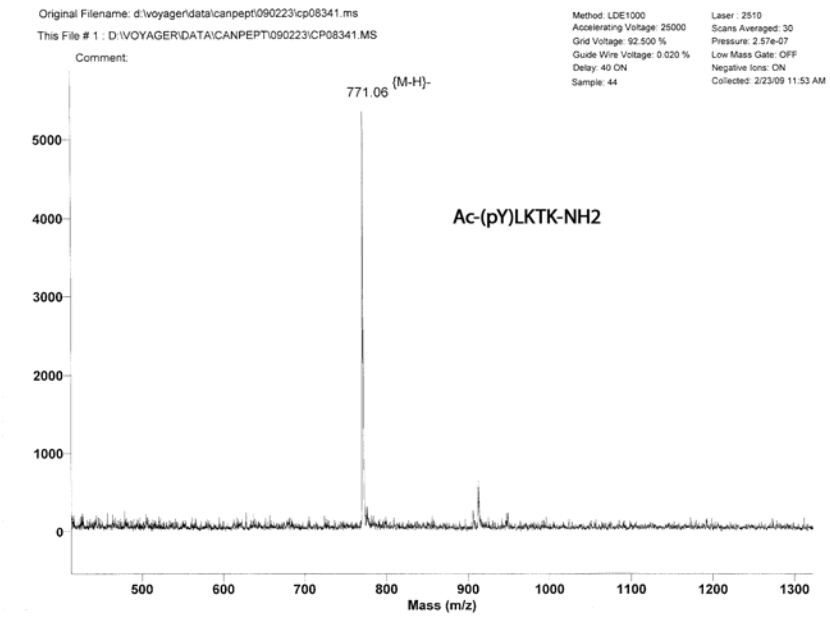
Sample ID	CP08341	Date Acquired	2/24/09 3:28:45 PM
System	Waters 600 Multi-Solvent Delivery System	Date Processed	3/19/09 9:44:02 AM
Injection	1	Acq Method Set	Standard Analysis
Injection Volume	10.00 ul	Processing Method	LC PQ Processing
Channel	2487Channel 1	Solvent	A = 0.1%TFA/ACN B = 0.1%TFA/H2O
Run Time	25.0 Minutes	Flow 1ml/min	5-55% A in 25 min
Column	Vydac Peptide & Protein C-18, 4.6 x250mm	Chemist/Book No	P090312-08341

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	% Area
1		9.063	11921855	756519	98.62
2		9.567	83533	7739	0.69
3		17.187	29603	5009	0.24
4		17.312	53528	7001	0.44

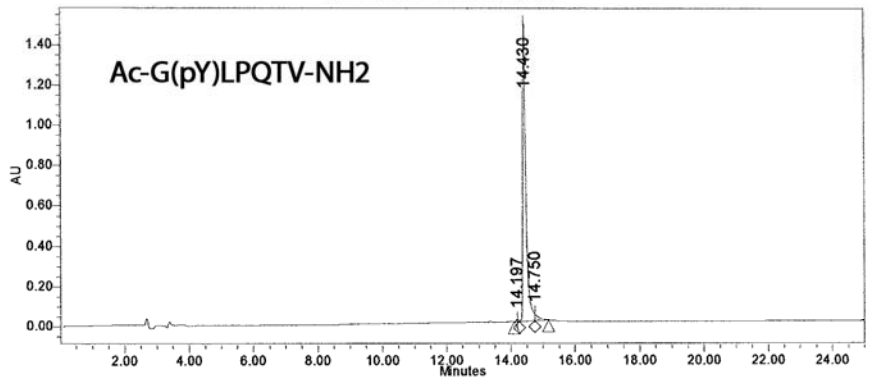


(ii) Ac-G(pY)LPQTV-NH_2

CANPEPTIDE HPLC REPORT

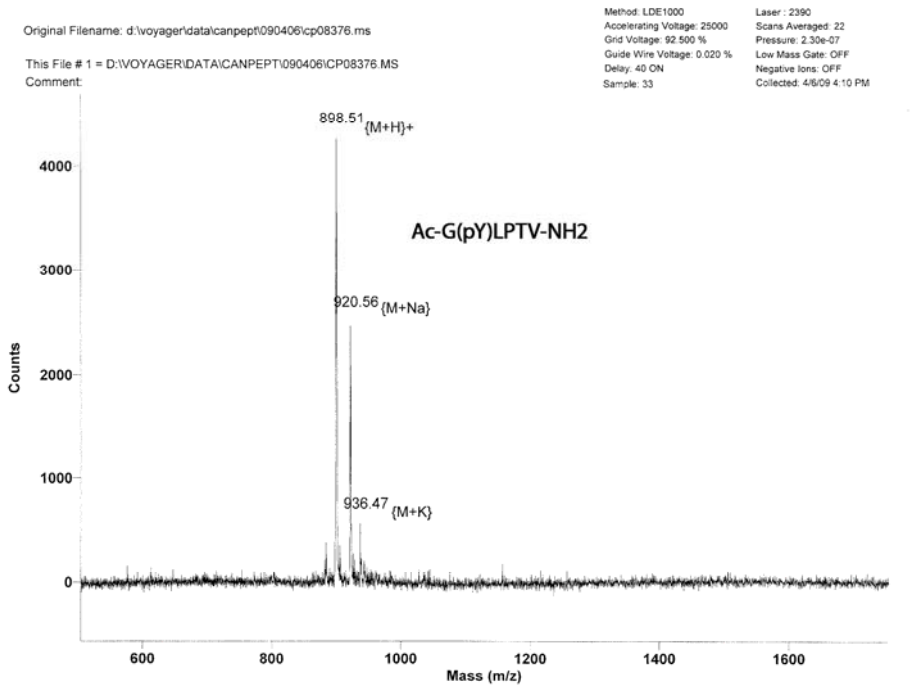
Sample ID	CP08376	Date Acquired	4/8/09 2:19:15 PM
System	Waters 600 Multi-Solvent Delivery System	Date Processed	4/29/09 1:25:12 PM
Injection	1	Acq Method Set	Standard Analysis
Injection Volume	10.00 ul	Processing Method	LC PQ Processing
Channel	2487Channel 1	Solvent	A = 0.1%TFA/ACN B = 0.1%TFA/H2O
Run Time	25.0 Minutes	Flow 1ml/min	5-55% A in 25 min
Column	Vydac Peptide & Protein C-18, 5u, 4.6 x250mm	Chemist/Book No	P090330-08376

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	% Area
1		14.197	54667	9576	0.53
2		14.430	10028021	1480313	96.55
3		14.750	303170	34889	2.92

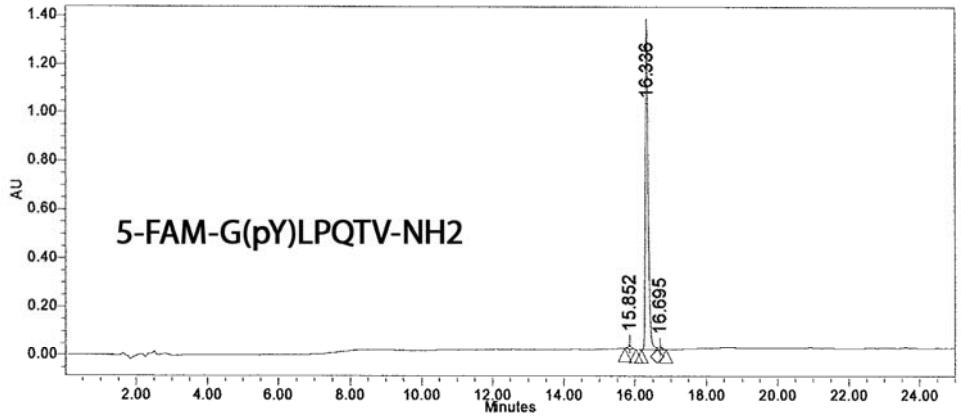


(iii) 5-FAM-G(pY)LPQTV-NH_2

CANPEPTIDE HPLC REPORT

Sample ID	CP08377	Date Acquired	4/27/09 2:42:44 PM
System	Waters 600 Multi-Solvent Delivery System	Date Processed	4/29/09 1:29:35 PM
Injection	1	Acq Method Set	Standard Analysis
Injection Volume	10.00 ul	Processing Method	LC Method
Channel	2487Channel 1	Solvent	A = 0.1%TFA/ACN B = 0.1%TFA/H2O
Run Time	25.0 Minutes	Flow	1ml/min 5-75% A in 25 min
Column	Phenomenex Jupiter Proteo C-12, 5u, 4.6 x150mm	Chemist/Book No	P090418-08377

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	% Area
1		15.852	120254	21609	1.61
2		16.336	7266456	1328880	97.12
3		16.695	95602	12462	1.28