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Quantitative Affinity-Based Chemical Proteomics of TrkA Inhibitors

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General. A 4000 Qtrap mass spectrometer (Applied Biosystems, Warrington, UK) was operated using a Nanospray II Interface (MDS Sciex). A stainless steel emitter (50 mm x 30 μm i.d., Proxeon, Odense, Denmark) was used together with the following instrumental parameters: ion spray voltage, 4200 V; nebulizing gas, 10; curtain gas, 10; interface heater temperature, 180°C. The dwell time was 50 ms, collision energy was set to 29 V for P1, 40V for P2 and 33.5 for P3 SRM transitions, and Q1 and Q3 were operated in unit resolution. Other mass spectrometer parameters were set as follows: declustering potential, 100 V; entrance potential, 11 V; collision cell exit potential, 13 V.

Abbreviations are used in conjunction with standard chemical practice. Additionally: AmBic = ammonium bicarbonate; BCIP = 5-Bromo-4-chloro-3-indolyl phosphate disodium salt; DTT = dithiothreitol; EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; EDTA = ethylene diaminetetraacetic acid; MRM = multiple reaction monitoring; NBT = nitro blue tetrazolium; PBS = phosphate buffered saline; PBST = phosphate buffered saline Tween20; RIPA = radio-immunoprecipitation buffer; SDS = sodium dodecyl sulfate; SIL = stable isotopic label; SRM = selected reaction monitoring.

Unless otherwise stated, reagents and buffers, including purvalanol B and staurosporine, were purchased from Sigma Aldrich.

Purvalanol B beads

A procedure similar to that described by Drewes *et al*[8,15] was used. Four aliquots of EAH Sepharose™ 4B bead suspension (GE Healthcare Life Sciences) (500 μL, 20% ethanol) were washed with dilute HCl aq. (1M, 3x1 mL), then NaCl aq. (0.5M, 1x1 mL), then DMSO aq. (30%, 1x1 mL then 60%, 1x1 mL). Purvalanol B (4.75 mg, 11 μmol) in DMSO (250 μL) was added per aliquot (19 mg total) followed by EDC (10.5 mg, 55 μmol) per aliquot (42 mg total) and the suspensions were placed on a roller for 16 hours. The beads were spun down in a centrifuge, the supernatant removed and the beads washed with DMSO aq. (65%, 1x1 mL then 30% 1x1 mL) and stored in the fridge at 3°C. Before use, the beads were further washed with PBS (3x1 mL) and returned to the original suspension concentration.

Recombinant protein pull-down and competition experiments

Three aliquots of a solution of TrkA kinase domain (commercially available) in PBS (0.1 mg/mL, 3x100 μL) were treated with DMSO (1 μL – negative control) or DMSO solutions of purvalanol B or staurosporine at the concentrations shown in Figure 3. The samples were placed on a roller for 30 minutes followed by addition of the

purvalanol bead suspension (50 μL) and then placed back on the roller for 5 minutes. The samples were then spun down in a centrifuge, the supernatant removed and the beads washed five times with PBS. The beads in each experiment were split into two aliquots, with one aliquot being used for mass spectrometry analysis. The remaining bead suspension aliquots were treated with SDS loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris HCl) to elute the protein and resolved using 4-12% tris-tricinegel electrophoresis, and the gel visualized using Coomassie stain.

TrkA enrichment from PC12 cells and competitive proteomics

PC12 cells (approximately 125 x 10^6) were suspended in Tris/NP40 lysis buffer (2.5 mL) at 0°C for 45 minutes. [Lysis buffer: 50 mM Tris pH 7.4; 5% glycerol; 150 mM NaCl aq.; 1.5 mM $MgCl_2$; 20 mM NaF; 1 mM sodium vanadate; 1 mM DTT (added fresh); 0.8% Igepal; Protease inhibitor cocktail (SigmaFast™ tablets, EDTA free - added fresh)]. Other lysis conditions provided poor recovery of TrkA (CelLytic™, RIPA, hypotonic cell lysis buffer – see Pierce Cell Lysis Handbook for details). The suspension was spun down in a centrifuge for 10 minutes (14,500 rpm) to maximise lysis and the soluble fraction transferred.

The lysate (650 μL, 4 mg/mL, 2.6 mg total protein) was treated with DMSO (6.5 μL – negative control) or DMSO solutions of purvalanol B or staurosporine at the concentrations shown in Figure 4. A lower percentage of DMSO in the final solution caused compound precipitation, and higher percentages resulted in poor TrkA recovery. Samples were then placed on a roller for 30 minutes followed by the addition of purvalanol bead suspension (100 μL) and placed back on the roller for 5 minutes. The amount of beads used was essential to maximize TrkA enrichment. Samples were then spun down in a centrifuge and washed 5 times with PBS. The beads in each experiment were split into three aliquots, with one aliquot being used for mass spectrometry analysis.

The second bead suspension aliquot was treated with SDS loading buffer to elute the protein and resolved using 4-12% tris-tricinegel electrophoresis, and the gel visualized using Coomassie stain. The third aliquot was used for Western blot: the beads were boiled for 5 minutes at 90°C and the samples resolved on 6% tris glycine gel electrophoresis. The SNAP i.d.® system (Millipore) was used for blocking (with 30 ml 0.5% ECL™ advance blocking solution, Amersham), followed by treatment with primary antibody (Epitomics TrkA rabbit mAb #2244-1, 4 μL in 3.3 mL, 10 minutes incubation), then washed with phosphate buffered saline Tween 20 (PBST: 0.1M PBS; 0.5% Tween20; pH7.4; 3 x 10 mL) and treatment with anti-rabbit secondary (Sigma A3687 alkaline phosphatase) (0.5 μL in 3.5 mL, 10 minute incubation). The gel was then washed with PBST (3 x 10 mL), removed from SNAP i.d. and developed using the BCIP®/NBT premixed solution (Sigma).

Sample processing for MS analysis

Beads were washed further using HPLC water (Millipore) (5 times). SDS page standard solution (40 μL) was added to the beads and boiled for 30 minutes. Samples were spun down and the supernatant was loaded on to a NuPAGE® Novex® Bis-Tris gel (Invitrogen). Gels were stained using Staining NuPAGE® kit (Invitrogen). Desired bands were cut out of the gel, and cut into small pieces (only band at around 35 kDa for the recombinant kinase, and 2 bands from 110 to 80 kDa for the cell lysate samples) and washed in 500 μL of 20 mM ammonium bicarbonate (AmBic) for

around 30 minutes. The supernatant was discarded and 150 µL of AmBic was added to the gel pieces, and DTT was added so the final concentration was 20 mM DTT, this was incubated for 30 minutes at 65°C. After cooling to room temperature, 100 mM iodoacetamide was added and the samples were incubated in the dark for 30 minutes. The supernatant was discarded and the samples were washed again in 500 µL of 60% MeCN 20 mM AmBic. The supernatant was discarded and 50 µL of MeCN was added to the samples and they were dried under reduced vacuum. After the samples were dried, 20 µL of a 0.5 µg/µL of trypsin in 20 mM Ambic was added, 10 minutes later 20 µL of 20 mM AmBic was added. Samples were left to digest at 37°C overnight. The volume was collected and the samples were washed twice with 50% MeCN, and the volume from the washes was also collected. The samples were dried under vacuum to a volume of approximately 20 µL. 200 fmol of each of the heavy internal standards peptide was added to the mixture.

Selection of TrkA derived peptides for MRM-based quantification of TrkA

Three peptides from different areas of the TrkA sequence were selected for developing the MRM assay. Two of the peptides correspond to the kinase domain and were selected based on mass spectrometry data generated from a tryptic digest of the over-expressed kinase domain of TrkA. Peptides that showed high signal intensity in both MS and MS/MS were initially chosen, and from this list, peptides that did not meet the criteria described in Pan *et al*[18] were eliminated. In addition, any peptides that corresponded to known TrkA phosphorylation sites were excluded.

In addition, a third peptide which is derived from the extracellular domain of TrkA was chosen from an *in-silico* digest using MRMPilot™ Software (AB Sciex). To this end, several candidate peptides were selected and their performances were validated using MS data generated from a tryptic digest of TrkA enriched from PC12 cells. Again, the peptide showing the highest signal intensity in both MS and MS/MS spectra was chosen. The sequences of the 3 peptides are as follows: WELGEGAFGK (P1), ACPPEVYAIMR(P2) and WMPPESILYR(P3).

Isotopically labeled versions of the 3 peptides were obtained from Chinese Peptide Company in > 95% purity. The C-terminal residue was heavy labeled for each of the peptides, using $^{13}C_6{}^{15}N_4$ labeled arginine and $^{13}C_6{}^{15}N_2$ labeled lysine respectively.

The peptides were detected as doubly charged ions and the following transitions were used for MRM-based quantitation, four selected reaction monitoring (SRM) transitions were recorded for the doubly charged ion of the native and stable isotopically labeled (SIL) peptides, respectively:

For P1: Q1 547.3 to Q3 907.5 ($y9^+$), 778.4($y8^+$), 665.3($y7^+$), 608.3($y6^+$)
For P2: Q1 625.3 to Q3 978.5 ($y8^+$), 881.5 ($y7^+$), 752.4 ($y6^+$), 653.3 ($y5^+$)
For P3: Q1 646.3 to Q3 974.5 ($y8^+$), 877.5 ($y7^+$), 780.4 ($y6^+$), 651.4($y5^+$)

LC configuration

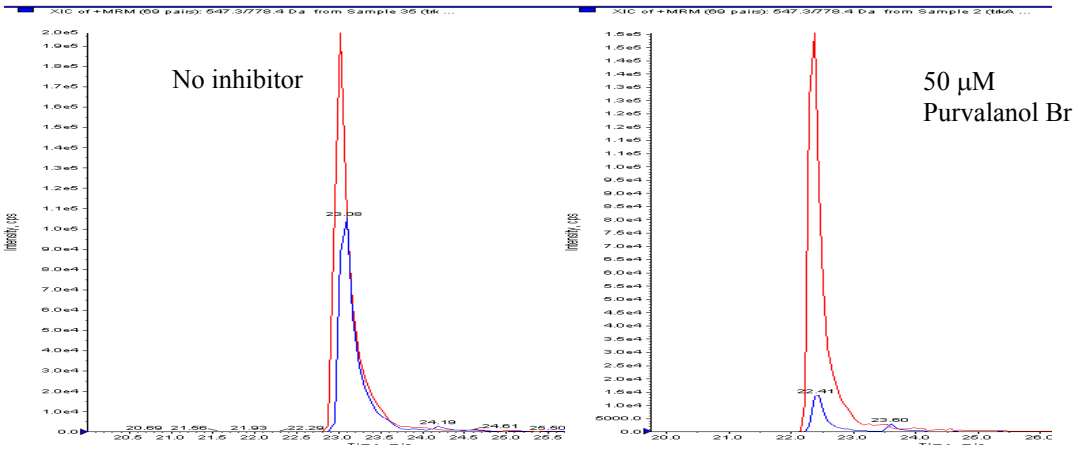
The protein digests were separated using an Ultimate 3000 capillary HPLC system (LC Packings, Dionex, Amsterdam, Netherlands) consisting of an Ultimate 3000 solvent rack with integrated vacuum degasser, an Ultimate 3000 quaternary low-pressure gradient pump, two Ultimate 3000 flow managers with thermostatted column compartment set to 50°C, two dual low-pressure gradient pumps housed in an Ultimate 3000 Pump model, and an Ultimate 3000 thermostatted well plate autosampler. The system was controlled by Chromeleon® software 6.8 (Dionex). An

on-line C18 reverse-phase trap column was used to concentrate and desalt the target peptides prior to nanoflow chromatography.

A sample volume of 200 µL was loaded onto an in-line C18 pre-column (PepMap100™ C18 trap cartridge (5 x 0.3 mm, 5 µm, 120 Å, Dionex)) using a flow rate of 50 µL/min using 0.1% formic acid in 2% MeCN. The target peptides were back-eluted from the C18 trap onto a PepMap100™ C18 analytical nanocolumn (15 cm x 75 µm, 3 µm, 100 Å, Dionex) at a flow rate of 300 nL/min. The solvents were 0.1% formic acid in 2% MeCN (solvent A) and 0.1% formic acid in 90% MeCN (solvent B), and the gradient started from 5% B at 8 minutes to 35% B at 17 minutes.

Data processing for quantitation

Four transitions per peptide were summed, and signal area ratios between native and SIL peptides were established using the Quantitate feature of Analyst™. Absolute amounts of detected native P1, P2 and P3 peptides (fmol) were calculated by multiplication of the area ratio by 200 fmol (amount of SIL standard added). Calibration curves were obtained by plotting total quantities of detected P1 peptide (fmol) against the TrkA concentration (nM). A weighted ($1/y^2$) unconstrained sigmoidal curve-fit was calculated to accommodate the ligand binding component of this assay. The quality of the curve fit was evaluated by back-calculating the TrkA concentration of calibrants. Measurements of TrkA concentration are based on molar equivalency between the surrogate peptide and TrkA. Sample spectra showing quantitation of WELGEGAFGK (SIL peptide in red) using purvalanol B competition are shown:



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