Supplementary Material (ESI) for Chemical Communications

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Supporting Information

Profiling human Src homology 2 (SH2) domain proteins and ligand discovery using a peptide-hybrid small molecule microarray

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1. Materials and methods

1.1. General information

All chemicals were purchased from company vendors and used without further purification. Fmoc-acid, HBTU, HOBt, TIS, PyBrOP and Rink Amide resin were purchased from GL Biochem (China). PL-FMP 4-Formyl-3-methoxyphenoxy Resin (PL-FMP Resin, 0.9 mmol/g, 75~150 μ m, Part no:1465-799) was purchased from Polymer Laboratories (USA). HPLC grade solvents were used for peptide synthesis. The reaction was carried out at room temperature and monitored by ninhydrin or chloranil test. HPLC and Mass spectra profiling were recorded on Shimadzu LC-IT-TOP system using reverse-phase Phenomenex Luna 2.6 μ m C18 100 Å 50 × 3.0 mm columns. Preparative HPLC was carried out on Gilson preparative HPLC system using Trilution software and reverse-phase Phenomenex Luna 5 μ m C18(2) 100 Å 50 × 30.00 mm column, to purify selected hits. 0.1% TFA/H2O and 0.1% TFA/CH3CN were used as eluents for both systems. The flow rates were 0.6 ml/min for HPLC and 8ml/min for preparative HPLC.

SH2 domain were expressed, purified and labelled on beads. Gels were scanned on a Typhoon fluorescence gel scanner (GE Healthcare, USA). Plain glass slides were purchased from Sigma Aldrich (USA) and the generation of avidin slides were performed as described previously.¹ Microarray slides were scanned using a Tecan Launch LS Reloaded Microarray Scanner (TecanTrading AG, Switzerland) installed with suitable lasers: Cy3: $\lambda_{Ex/Em}$ = 532/575 nm; Cy5: $\lambda_{Ex/Em}$ = 633/692 nm.

1.2. Expression and labelling of protein

The mammalian SH2 domain collection was cloned into modified pET28 bacterial expression vectors with His tagged was obtained from Open Biosystems. All expression constructs were transformed into *E.coli* BL21 (DE3) competent cells.

The transformed cells were then plated onto the LB-kanamycin agar plates and placed in the incubator at 37°C for 16 hours. Overnight cultures were diluted 1:100 in LB media supplemented with 50µg/ml of kanamycin and grown at 37°C with shaking. When OD_{600} was about 0.6-0.8, expression was induced with 0.1mM of isopropyl β -D-1-thiogalactopyranoside (IPTG) and cultures were grown further at 18°C for 18 hours. After cell harvest (4°C, 10000g, 30mins), lysis buffer (pH 7.4, 50mM Tris-HCl, 300mM NaCl) and 1mg/ml lysozyme were added. After incubation on ice for 20min, lysis was performed by sonication ($12 \times$ pulses of 10s each at half maximal power, on ice), the cell debris was pelleted by centrifugation at 10000g, 20min, 4°C, which the supernatant was loaded into a column containing 50-80µl of Ni-NTA resin preequilibrated with wash buffer (pH 7.4, 50mM Tris-HCl, 300mM NaCl, 20mM imidazole). Following incubation for 2 hours at 4°C, the resin was washed 3 times with the wash buffer and the protein was finally eluted with elution buffer (pH 7.4, 50mM Tris-HCl, 300mM NaCl, 200mM imidazole). Fractions containing the desired proteins were pooled and dialysed onto Microcon® centrifugal filter device (3kDacutoff) and stored at -20°C inPBS containing 20% glycerol. Protein concentration was determined from a standard curve using the Bradford protein assay (Bio-Rad) with BSA protein. Protein purity was determined by separation on a 15% SDS-PAGE gel by Coomassie® brilliant blue staining.

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1.2.1. Solution-phase protein labelling

Protein samples were labelled with Cy5 dye (Amersham, G.E. Healthcare, USA) for 1 hour on ice, following manufacturer's protocol.¹ A 50µl protein sample at 1mg/ml was quenched with 5µl quench solution (3M hydroxylamine, 2.5M NaOH) for further 30min. The excess dye was removed with a Microcon® centrifugal filter device (3kD cutoff). The labelled protein was determined by separation on a 15% SDS-PAGE gel by Coomassie® brilliant blue staining.

1.2.2. Protein labelling while on solid-support

Following washing the Ni-NTA resin with wash buffer, the resin was washed twice with PBS. To the resulting protein-bound resin, a 200µl Cy5-NHS ester solution(Amersham, G.E. Healthcare, USA) was added to label the protein. Following incubation on ice for 1 hour, the unreacted dye was quenched with 20µl quench solution for a further 30 min. 15ml PBS was used to wash the labelled-resin thrice to remove the excess dye. The protein was finally eluted with elution buffer (pH 7.4, 50mM Tris-HCl, 300mM NaCl, 200mM imidazole). Fractions containing the desired protein were pooled and dialysed onto Microcon® centrifugal filter device (for 3000Da) and stored at -20°C with PBS containing 20% glycerol. The labelled protein was determined by separation on a 15% SDS-PAGE gel by fluorescence scan on Typhoon.



Figure S1.Florescent gel of SH2 domainspurified and labelled by "one-step" labelling

1.3. Synthesis of peptide-small molecule hybrid library

The library was synthesized using the standard Fmoc strategy combined with IRORITM technology (Scheme S1).

Briefly, the 396-member library was synthesized on PL-FMP resin 50mg resin was used for each compound. The resin was first swelled in DCE for 2h and then coupled the amine building blocks (X_{1-44}) onto the PL-FMP resin by reductive animation. Four molar equivalents of the amine building block was added in DCE and the incubated with shaking for 2h. Next, HOAc(1%) and Na(OAc)₃ (6.0eq.) in DCE was added and the reaction was further shaken for 16h. Subsequently, the resin was washed thoroughly with THF, MeOH and DCM and dried in high vacuo. To confirm completion, the reaction was monitored using chlorinal test. Next, the resin was with Fmoc-Tyr[PO(OBzl)OH]-OH(4.0eq.), PyBrOP(4.0eq.) coupling and DIEA(8.0eq.) in DMF. After reacting for overnight at room temperature, the result resin was extensive washed with DMF and DCM and dried. To confirm completion, the reaction was also monitored by chlorinal test. Subsequently, the Fmoc group was

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removed by 20% piperidine and ready for next step. Repeat the cycle using HOBt/HBTU/DIEA coupling method until the Biotin-GG linker was coupled.

Cleavage was performed with TFA/H₂O/TIS (95:2.5:2.5, total 2ml) for each micro-reactor. After continuous shaking for 6h at room temperature, the resin was separated and the filtrate was collected and concentrated under reduced pressure. Cold ether (chilled to -20°C, ~5ml) was added to the liquid concentrates to precipitate the peptide-small molecule compounds. The mixture was stored at -20 °C overnight. Upon centrifugation, the ether layer was decanted off and washed with ether twice. The precipitates were then dried thoroughly *in vacuo*, dissolved in DMSO (0.5ml) and stored at -20°C for future use.

The 14 individual hits were also re-synthesised using the procedure above. Each micro-reactor reactor contained 200mg of PL-FMP resin. Unique R_F tags were used to facilitate sorting. The peptide-small molecule compounds were further purified using preparative HPLC (Gilson) and lyophilised. LC-MS was performed to ensure the peptide-small molecules were of correct mass and pure for subsequent microarray experiments, the spectrums and MS results are shown in **Section 2.1**.



Scheme S1. Synthesis of the peptide-small molecule library

1.4. Pro-Q staining and detection

The spotted slide was washed with distilled water and stained with $Pro-Q^{TM}$ Diamond dye for 1h at room temperature in a humidified chamber. The slide was then de-stained with a solution of 20% acetonitrile in sodium acetate (*p*H 4) for 25 mins, rinsed with water, dried and scanned under the Cy3 channel ($\lambda_{ex/em}$: 532/575 nm).

1.5. Microarray preparation

The peptide-small molecular hybrid library stock solutions were prepared to approximately 1.0mM in 50% DMSO and 50% PBS and were distributed in Genetix384 well plates. Stock spotting plates were not recycled more than 5 times, to minimize variability. Avidin coated glass slides (75x25mm) were spotted on an OmniGrid® Accent (DigiLab, USA) microarray spotter with the print head installed with four Stealth SMP8B Micro-spotting pins (TeleChem USA). Spots generated were approximately 295 μ m diameter and were printed with a spot-spot spacing of 450 μ m. The pins were rinsed in between samples using two cycles of wash (for 5s) and sonication (for 5s) in reservoirs containing 70% ethanol followed by drying under reduced pressure (for 5s). The slides were allowed to stand for overnight on the printer platform and stored at 4°C until use (spotted slides were stable for 6 months under these storage conditions). Before incubation with the labelled protein, the slides were rinsed with PBS (*p*H 7.4) for 20 min and blocked with PBS containing 1% BSA

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for 1h. For studies with the 396-member library, all were spotted on the slide in duplicate. For the K_D experiments, a new microarray was fabrication where up to 8 identical subarrays were created on the same slide using the same set of 14 selected hits. ProQ assays were performed to ensure batch-batch variability.

1.6. Data extraction and analysis

Microarray data was extracted using the Array-Pro \mathbb{R} software. Values from duplicated points and was background subtracted and averaged (Duplicated spots with a standard deviation >0.8 were rejected) and were depicted in grey in heatmaps, to indicate data not obtained.

1.7. K_D analysis of selected high binders

14 hits including the previous synthesized hits and the newly synthesized were spotted onto the same slide were up 8 identical subarray were generated on the same platform, allowing consistent/uniform screening and binding of Lck SH2 domain. By using dose-dependent experiments, as previous described,² we extracted the binding data of Lck SH2 domain. The corresponding K_D was generated by fitting the data to the following equation, under the assumption that equilibrium was achieved during the incubation period:

Observed fluorescence of
$$x = \frac{(Maxium Fluorescence, X) \times [Protein Concentration]}{K_D + [Protein Concentration]}$$

Saturation dynamics observed when plotting Observed Fluorescence against the applied Protein Concentration were fitted the above equation using the Graph pad Prism software ver 4.03 (GraphPad, San Diego, USA) revealing the binding dissociation constant, $K_{D.}^{2}$

1.8. Thermfluor based melting point shift assay

Thermal shift assay was determined by the Thermal Shift Assay Dye kit (Applied Biosystem, #4461146) followed by the manufacturer's guidelines. The Lck and Grb2 SH2 domain protein was added to a final concentration of 40 μ M in buffer. (5 μ l Protein Thermal ShiftTM buffer, 2.5 μ l Protein Thermal ShiftTM dye). The final volume was 20 μ l/well in RT-PCR 96-well white microplates. The temperature gradient was performed in the range of 30-80°C, using a 7500 real-time PCR instrument from Applied Biosystems (Carlsbad, CA) with a standard ramp of 1% over the course of 60min. The compound screening was performed at the 400 μ M (in 4% DMSO) concentration. The detection of protein unfolding was performed with an excitation wavelength of 567 nm and an emission of 591nm. The Prism software ver 4.03 (GraphPad, San Diego, USA) was used to analysed the result.³

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2. Supporting Results:

2.1. HPLC-MS profiles of 14 potential hits

All compounds were purified and characterized by LCMS and were shown to be of correct molecular weight and sufficient purity to be used. Below are the LCMS profiles of the 14 potential hits.

PP-pY-X₅



500

750

1000

1250

m/z

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Figure S2.LC-MS profiling of resynthesized 14 hits

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2.2. Screening of Cy5-labeled SH₂ domain against the peptide-small molecule hybrid microarray K_D Analysis of Selected Compounds

	nybriu microarray N _D Analysis (
((A)	-					
	PI-pY-X		В РР-рҮ-Х				
	VV-pY-X		ЖЖЖЖЖ ЖЖЖЖК NP-рY-X				
	VE-pY-X		QP-pY-X				
	PI-pY-X		Же-рҮ-Х				
	KK-pY-X		Жеру-х				



(B)

(C)

SrcLckAbl1Image: Abl2Image: Abl2Image: Abl2Abl2Sh2d1aShip2Image: Abl2Image: Abl2Image: Abl2

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(D)



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Figure S3 (A) Print code of the 396 library (B) ProQ image of 396 member library. (C) Microarray image of 15 SH2 proteins (D)The heat map of 15 SH2 domains against 14 hits. The relative scale is shown inset, results were undetermined if differences between duplicated spots were >50%



Figure S4. Cladograms of SH2 domains based on binding fingerprints

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Figure S5. Average binding contributions across N-terminal peptide sequences (Left) and 44 C-terminal small molecule building blocks (Right). Each bar represents averaged binding across in the library presenting the relevant peptide sequences. The error bar denotes the standard deviation across each group.



Table S1. Structures of 14 potential binders identified

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(A)

)				
PP-pY-X₅	PP- <i>p</i> Y-X ₆	PP- <i>p</i> Y-X ₄₁	VV-pY-X ₆	PI- <i>p</i> Y-X ₁₆
РР- <i>р</i> Ү-Х ₃₃	PP- <i>p</i> Y-X ₃₇	PP- <i>p</i> Y-X ₂₅	VV- <i>p</i> Y-X ₁₂	VV- <i>p</i> Y-X ₃₆
VV- <i>p</i> Y-X ₁₀	РІ- <i>р</i> Ү-Х ₂₉	РІ- <i>р</i> Ү-Х ₁	РІ- <i>р</i> Ү-Х ₂₅	PI- <i>p</i> Y-X ₂₈
PI- <i>p</i> Y-X ₄₁	PI- <i>p</i> Y-X ₁₃	РІ- <i>р</i> Ү-Х ₃₆	TMR	



(B)

Figure S6. Concentration dependent application and apparent K_D results for selected hits.(A) Grid arrangement. (B) Pro-Q image of the potential binding hits indicating successful immobilization (top grid in green). Concentration-dependent screening of the microarray against Cy5-labeled Lck(bottom grids in red). (C) K_D analysis of selected compounds. (D) Summary of K_D values obtained from the microarray experiment. TMR - tetramethylrhodamine (spotted dye reference).



Figure S7. (A) Representative unfolding curves of Lck-SH2 domain obtained from protein thermal shift assay. (B) Representative unfolding curves of Grb2-SH2 domain obtained from protein thermal shift assay.

ID	Lck(40µM)		Grb2(40µM)	
	T _m (∘C)	ΔT _m (°C)	T _m (∘C)	ΔT _m (°C)
Control	55.4		67.6	
PP-pY-X ₅	58.4	3.0±0.14	69.2	1.5±0.40
PP-pY-X ₆	58.2	2.8±0.11	N.D.	N.D.
PP- <i>p</i> Y-X ₂₅	58.6	3.2±0.12	68.5	0.9±0.10
PI-pY-X ₁	55.7	0.3±0.10	69.4	1.8±0.13

Table S2. Result of protein thermal shift assay

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References

- 1. C. H. S. Lu, M. Uttamchandani and S. Q. Yao, Angew. Chem. Int. Ed., 2008, 47, 7438-7441.
- 2. A. Gordus and G. MacBeath, J. Am. Chem. Soc., 2006, 128, 13668-13669.
- 3. W. A. Lea and A. Simeonov, PLoS One, 2012, 7, e36219.