Electronic Supplementary Material (ESI) for MedChemComm.

Electronic Supplementary Information

Structure-guided design of a potent peptide-inhibitor targeting the interaction between CRK and ABL kinase

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Experimental Section

Protein and peptides: All protein samples for crystallization, fluorescence and NMR experiments, were prepared as described elsewhere.[1] Wild type and T315I ABL kinase were purchased from ProQinase. Recombinant human CRK-II was expressed in E.coli and purified as described elsewhere.[2] Synthetic peptides were purchased in a crude form, and further purified using reverse-phase high performance liquid chromatography in our laboratory. The N- and C-termini of peptides were acetylated and amidated, respectively. The peptide concentration was determined by measuring the UV absorption at 280 nm of a single tyrosine at the C-terminal end of the peptides.

Crystallization and structure determination: Crystallization conditions were screened using several commercial (Hampton Research) and *in house* screens. The protein as well as the PRM-2 were prepared in buffer G. The best crystals were obtained from 0.2 M lithium sulfate monohydrate, 0.1 M BIS-TRIS pH 5.5, 35% w/v PEG 3350. These crystals diffracted to 1.8 Å resolution in the space group $P2_12_12_1$ and had one nSH3-ligand complex in the asymmetric unit. A 1.8 Å resolution data set was collected at 113 K using an R-AXIS IV imaging-plate detector and Cu Kα radiation generated by a rotating-anode X-ray generator (Rigaku–MSC). The data were processed using HKL2000 [3] in the same space group with cell dimensions of a = 27.6 Å, b = 41.3 Å and c = 56.8 Å. Molecular replacement in Phaser [4] using 1cka as the search model [5] could output a unique solution in the same space group with one copy of the protein in the asymmetric unit. The solution was subjected to an initial cycle of model building using AutoBuild module of PHENIX[6] following which several iterations of manual model building in COOT[7] and refinement in REFMAC[8] were carried out. PRM-2 was added to the model guided by the Fo-Fc map. Water molecules were added after one more round of real and reciprocal space refinement. The final structure has R/R_{free} = 17%/21%. The electrostatic potential surface of nSH3 domain was calculated using APBS and PDB2PQR.[9-11]

NMR spectroscopy: NMR HSQC spectrum was acquired using a protein sample in 20 mM sodium phosphate (pH 6.1), 80 mM NaCl, 0.02% sodium azide, 1 mM EDTA, 10 μM DSS (4,4-dimethyl-4-silapentane-sulfonate), and 10% D_2O at 25 °C. The NMR experiment was performed on Bruker Avance 600 MHz spectrometers equipped with a cryogenic probe. NMR spectra were processed with NMRPipe[12] and analyzed with NMRViewJ (One Moon Scientific, Inc.). The temperatures of NMR sample were calibrated using the deuterated methanol-d_4.[13]

Fluorescence binding assay: The dissociation constants (K_d) of nSH3:PRM complexes were measured by monitoring the change of tryptophan fluorescence signal. Excitation wavelength was

295 nm. All binding assays were performed in a stirred 1-cm path length cuvette using a PTI QM-400 fluorimeter. Protein concentration used for the fluorescence-based binding assays was 0.1 μM. The measurements were done in 20 mM sodium phosphate (pH 6.1) and 80 mM NaCl at 25 °C. The K_d was calculated by assuming a 1:1 complex, and by the global fitting of the repeatedly measured fluorescence intensities to the eq. 1:

$$\Delta F = \Delta F_{\max}\left(\frac{[P_t]+[L_t]+K_d \pm \sqrt{\left([P_t]+[L_t]+K_d\right)^2 - 4[P_t][L_t]}}{2[P_t]}\right) \quad (1)$$

where ΔF and ΔF_{max} are the change and the maximum amplitude of signal change, respectively. P_t is the total protein concentration and L_t is the total ligand concentration at each titration point. The reported K_d values are the average of 2 repeated measurements. The K_d values of nSH3:PRM-3 were measured at 30, 35, and 37 °C. The binding free energy, ΔG_{bind}, was calculated using $\Delta G_{bind} = -RT\ln(1/K_d)$. The ΔG_{bind} at 298 K was calculated by a linear fitting of the data. The uncertainty of the ΔG_{bind} (298 K) was estimated by 95% confidence intervals of the linear regression curve.

CRK-phosphorylation assay: 2 μM of CRK-II (residues 1 – 304) was incubated with 5 nM recombinant human wild type ABL kinase (ProQinase, residues 118 – 535) in 20 m M Tris (pH 7.6), 50mM NaCl, 2 mM DTT, 2 mM $MgCl_2$, 100 μM ATP at 25 °C. The reaction conditions for T315I ABL kinase were the same except that the concentration of T315I ABL kinase was 25 nM. The specific activity of T315I ABL was 10-fold lower than that of wild type ABL. The phosphorylation reaction was stopped by adding 15 mM EDTA. The phosphorylated and non-phosphorylated CRK-II were separated by running a native polyacrylamide gel electrophoresis (12%). Thermo Scientific Pierce Silver Statin kit was used for detection of protein bands. The band intensity was measured by ImageJ.[14] It should also be noted that the inhibitory activity of PRM-3 depends on the concentration of CRK-II used in the assay since PRM-3 binds to CRK-II, which is a substrate of ABL kinase. This rules out using IC_{50} as an indicator for inhibitor activity because the IC_{50} value changes depending on the concentration of CRK-II used in the *in vitro* assay. Instead, comparison with imatinib provides an alternative way to assess the inhibitory activity of PRM-3 on ABL-dependent CRK phosphorylation.

Table S1. Data collection and refinement statistics of the crystal structure of the nSH3:PRM-2 complex

PDB ID: 5L23	
Data Collection Statistics	
source	Cu Kα
space group	$P2_12_12_1$
unit cell parameters	
a, b, c (Å)	27.6, 41.3, 56.8
α, β, γ (deg)	90.0, 94.3, 90.0
temperature (K)	113.15
wavelength (Å)	1.5
resolution (Å)	24.83-1.77 (1.87-1.77)[a]
R_{merge}[b]	0.16 (0.20)
completeness (%)	100 (99.9)
$\langle I/\sigma(I) \rangle$	11.7 (8.4)
total number of reflections	50023
Average redundancy	7.4 (7.2)
Refinement Statistics	
resolution (Å)	24.83-1.77
number of reflections	6462
R_{factor}[c]	0.17
R_{free}[c]	0.21
number of protein atoms	509
number of water atoms	99
number of ligand atoms	126
rms deviation from ideal values for bond distances (Å)[d]	0.029
rms deviation from ideal values for bond angles (deg)[d]	2.61
average B-factors	
protein chain (Å^2)	12.60
ligand chain(Å^2)	13.43
water molecules (Å^2)	25.30
Ramachandran plot[e]	
favored (%)	98.44
disallowed (%)	0

[a]The numbers in parentheses refer to the highest resolution shell.

[b]$R_{merge} = \sum_{hkl} \sum_j |I_{hkl,j} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_j \langle I_{hkl} \rangle$

[c]$R_{factor} = \sum |F_{obs} - F_{calc}| / \sum |F|_{obs}$, where R_{free} refers to the R_{factor} for 3% of the data that were excluded from the refinement

Figure S1

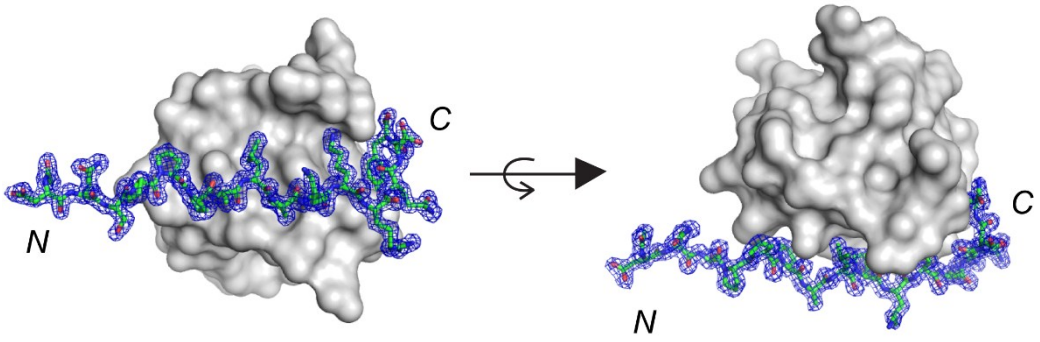

Figure S1. Crystal structure of the nSH3:PRM-2 complex (PDB ID: 5L23). Representation of the $2F_O$-F_C electron map of PRM-2 contoured at 1σ.

Figure S2

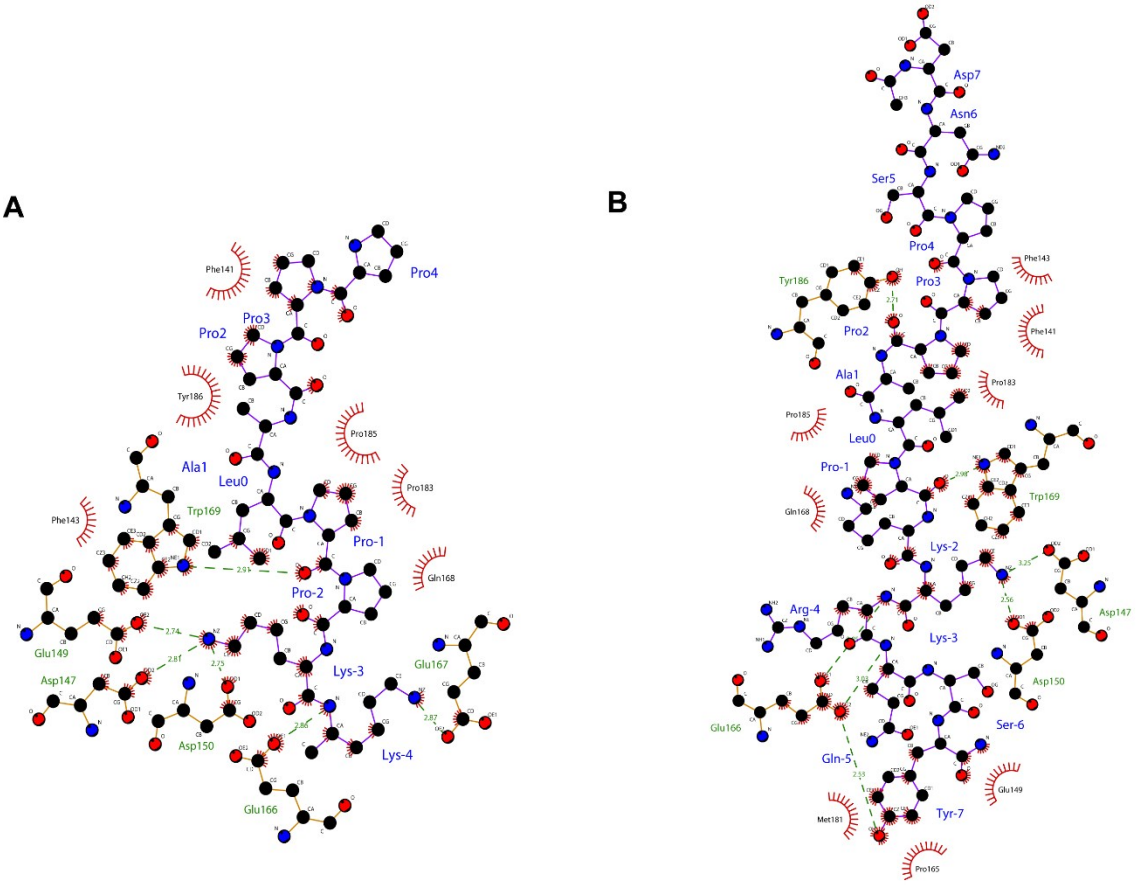

Figure S2. Schematic diagrams of the intermolecular interactions between the nSH3 domain and (A) PRM-1 and (B) PRM-2. Polar interactions are depicted as dotted lines. These figures were prepared using LIGPLOT+.[15]

Figure S3.

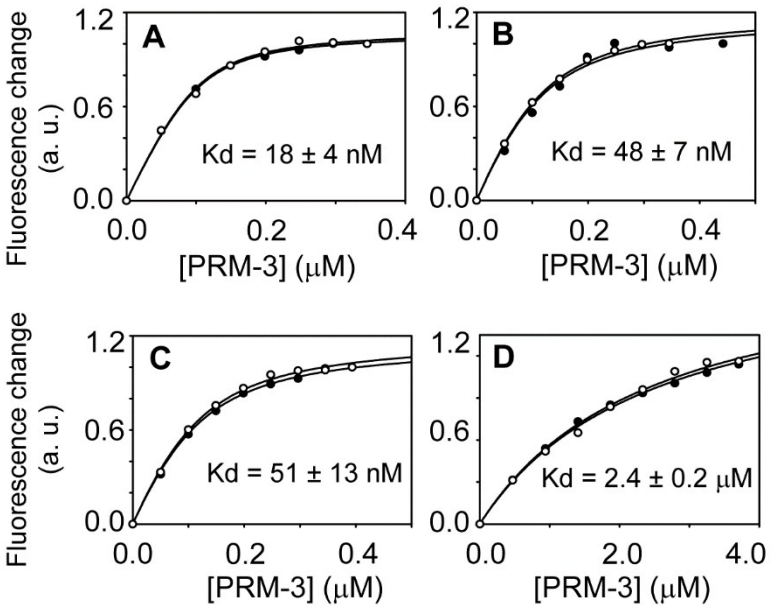

Figure S3. Binding isotherms of the nSH3 and PRM-3 at (A) 30 °C, (B) 35 °C, and (C) 37 °C in the presence of 80 mM NaCl, and (D) 35 °C in the presence of 1 M NaCl. The K_d values are average of two repeated measurements at each temperature.

Figure S4.

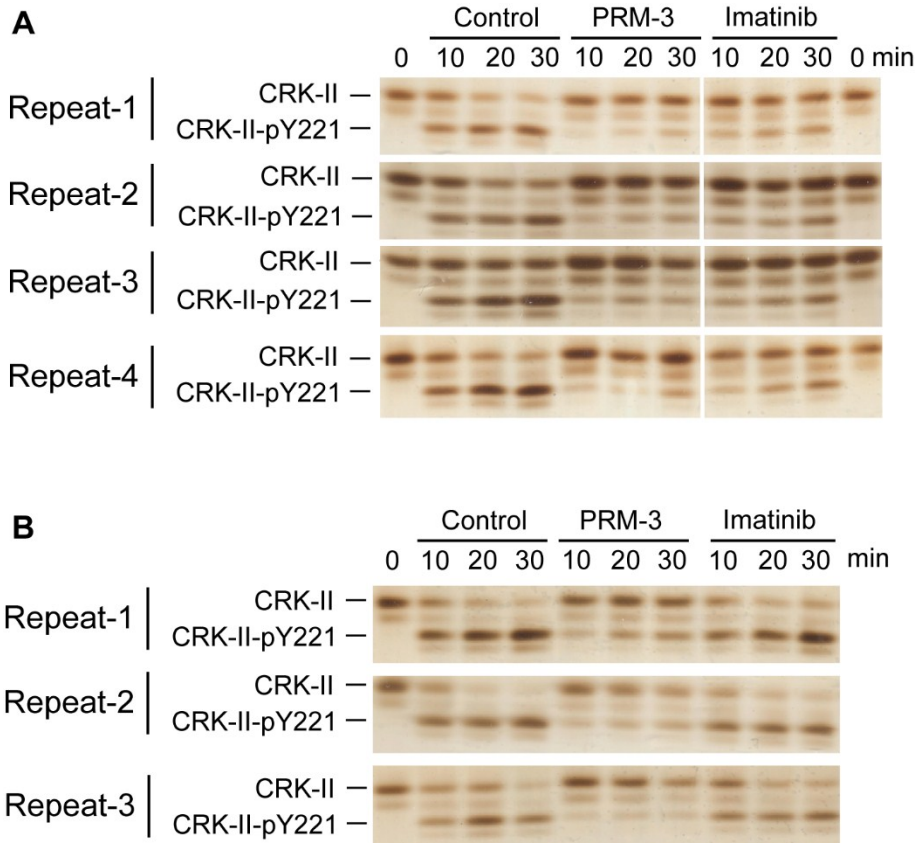

Figure S4. Time-dependent phosphorylation of CRK-II using (A) wild type and (B) T315I ABL kinase in the absence (control) and presence of PRM-3 or imatinib. The minor band below a major band is not an impurity but an alternative conformation separated during native PAGE. CRK-II undergoes structural exchange between alternative conformations.[16] The reported phosphorylation ratio is the average and standard deviation of four repeated assays.

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