Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Electronic Supporting Information

FP Tethering: a screening technique to rapidly identify compounds that disrupt protein-protein interactions.

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Experimental Section

Expression and purification of the KIX domain

The KIX domain (residues 586-672) of mouse CBP protein fused to a hexahistadine tag and short polar linker was cloned into the bacterial expression pRSETB vector. All cysteine mutants were generated by using site-direct mutagenesis as previously described.[1,2] The KIX domain protein was overexpressed from *Escherichia coli* Rosetta2(DE3) pLysS (Novagen) cells in Terrific Broth (37° C, 250 rpm). When the cell density reached an OD_{600nm} of ~0.8, protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for approximately 12 hours at 25° C. The cells were harvested by centrifugation and stored at -80° C. The hexahistadine-tagged KIX protein was affinity purified using Ni-NTA beads (Qiagen) followed by ion-exchange chromatography (Source S column, GE Healthcare) using an FPLC system. The purified protein was stored in 10 mM phosphate, 100 mM NaCl, pH 6.8 at -80^{0}C.

Synthesis of fluorescent peptides

Peptides were synthesized using standard Fmoc solid phase synthesis as previously described.[3]

Fluorescent polarization (FP) binding assay

In 384-well microplates varying concentrations of KIX domain protein were mixed in binding buffer (10 mM phosphate, 100 mM NaCl, pH 6.8) with a constant concentration of fluorescein isothiocyanate (FITC)-labeled peptides (25 nM) and β-mercaptoethanol (BME) to a final volume of 10 μL in each well. The samples were incubated at room temperature for 1 hour to reach equilibrium. The anisotropy and fluorescence intensity were monitored using the Tecan Genios Pro microplate reader at an excitation wavelength of 485 nm and an emission wavelength at 535 nm. The equilibrium dissociation constant (K_d) were calculated by fitting the observed FP or anisotropy values as a function of KIX protein concentration to the ligand depletion model assuming single site binding of peptide to protein,

$$F = F_f + \left(F_b - F_f\right) \times \frac{\left[([L_T]+K_d+[P_T]) - \sqrt{([L_T]-K_d-[P_T])^2 - 4[L_T][P_T]}\right]}{2[L_T]},$$

where $[L_T]$ and $[P_T]$ are the total concentrations of fluorescent peptide and KIX domain protein, respectively, F is the observed polarization, F_b is the maximum polarization value for the bound form of the fluorescent peptide, and F_f is the minimum polarization value for the free form of the

fluorescent peptide. All nonlinear data analysis was performed with Prism 4 software (GraphPad Software).

Fluorescence polarization Tethering screen

KIX N627C (0.5 μM concentration) was mixed in binding buffer with FITC-labeled MLL peptide (25 nM), β-mercaptoethanol (1 mM or 5 mM), and one of 80 fragment disulphides (160 μM) in a final volume of 30 μL. For the screen with the KIX H602C mutant, the protein at a concentration of 4 μM was mixed in binding buffer with FITC-labeled pKID peptide (25 nM), β-mercaptoethanol (0.2 mM, 1 mM or 5 mM), and one of 960 fragment disulphides (125 μM) in a final volume of 20 μL. Additional information regarding the fragment library can be found in reference (5).

All plates were shaken at room temperature for 1h. The fluorescence polarization (FP) and total fluorescence intensity (TFI) were measured using the Analyst HT Multi-Plate Reader (Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength at 530 nm. Fragments that yielded TFI greater than 150% of the DMSO control were flagged as artifacts and excluded from further analysis. FP readings were transformed to percent inhibition, using the formula $[P_{Bound}-P_{Sample}]/[P_{Bound}-P_{Free}]*100$.

Mass spectrometric validation of fragment hits

KIX H602C (4 μM) was mixed in binding buffer with β-mercaptoethanol (0.2 mM, 1 mM or 5 mM) and each fragment disulfide (125 μM) in a total volume of 25 μL. The plate was shaken for 1 hour at room temperature to reach equilibrium. Each sample was subjected to LC-MS using an LCT Premier ESI-MS (Waters) with an inline C4 protein desalting column (Microtrap). Protein masses were deconvoluted using the Max-Ent algorithm within the MassLynx software. Percent labeling was measured by comparing the peak areas for the labeled versus unlabeled protein.

Determination of dose response (DR_{50})

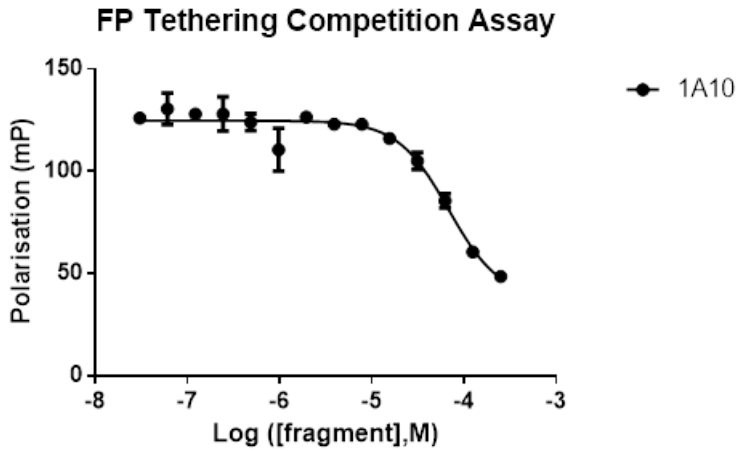
A constant concentration of the KIX H602C mutant and fresh β-ME in 10 mM phosphate buffer (100 mM NaCl, pH 6.8) were incubated with varying concentrations of compound (0.2 μM -125 μM) at room temperature for one hour. The samples were centrifuged for 1 minute at 10,000 rpm and analyzed by mass spectrometry on an Agilent Q-TOF HPLC-MS instrument. Protein masses were deconvoluted using the Max-Ent algorithm within the Agilent MassHunter Workstation Bioconfirm software. The dose-response (DR_{50}) values were determined with a normalized response from 1 to 100 using the equation:

$$y = \frac{100}{1+10^{(LogDR_{50}-x)}}$$

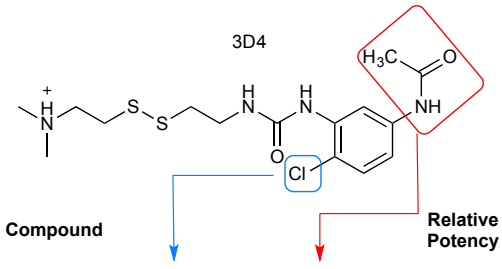
where x is the logarithm of the concentration of the fragment and y is the percent of protein bound to the fragment.[4]

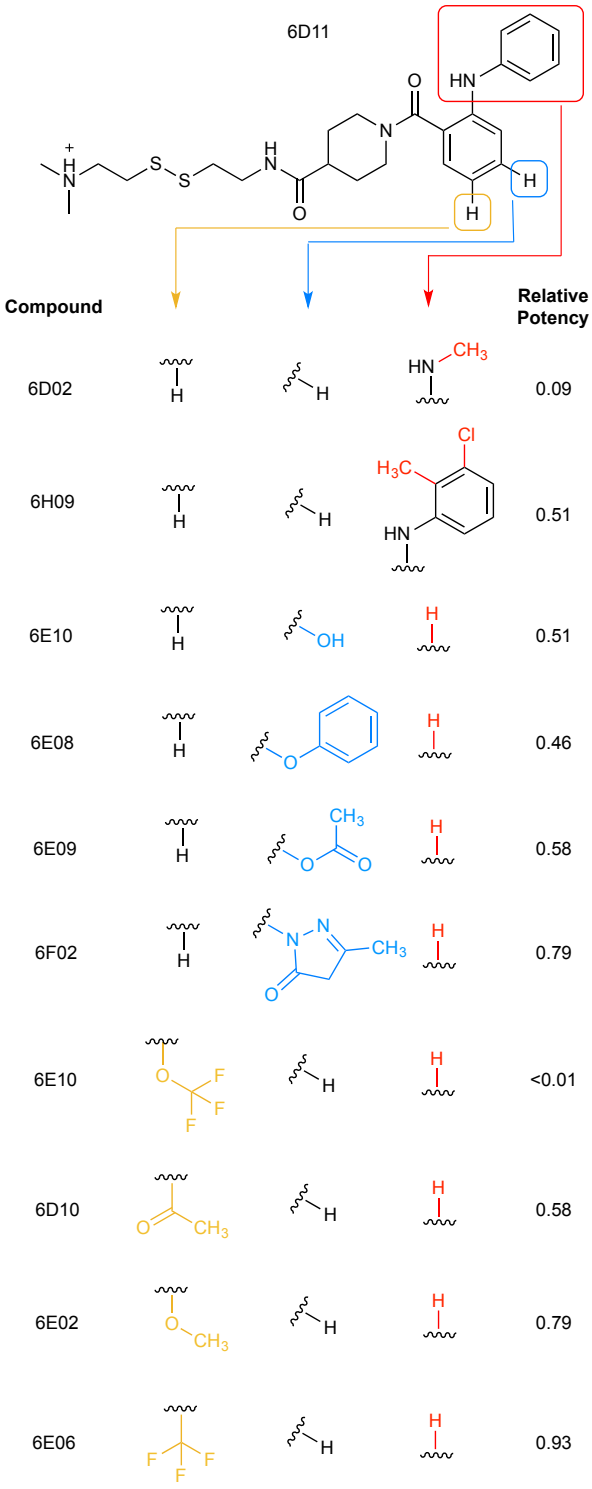
Fragment Tethering

The KIX H602C mutant was incubated with 8 - 10 equivalents of small molecule and fresh β-ME in binding buffer (10 mM phosphate buffer, 100 mM NaCl, pH 6.8) and incubated overnight. Excess small molecule was removed and small molecule-protein complexes were concentrated using 10 KD molecular weight cutoff concentrators (Vivascience). The extent of labeling was measured by Q-TOF LC-MS (Aligent). Protein complexes that were at least 90% alkylated were used for the FP binding assays.



SI Figure S1: A FP competition assay was tested using a constant concentration of KIX N627C mutant complexed with fluorescently-labeled MLL and varying the concentrations of the fragment **1A10**, a known binder and inhibitor of the KIX domain and MLL.[2,3] The polarization values were plotted against the varying concentrations of the fragment **1A10**. Each data point represents an average of three independent experiments with the standard deviation. The IC_{50} value was determined from the nonlinear regression equation for "log(inhibitor) vs response -- variable slope (four parameters)" using GraphPad Prism 4.00 software.





SI Figure S2: A comparison of the inhibition values of 3D4 and 6D11 and related fragments reveals that both scaffolds are sensitive to substituent placement and identity. Relative potency values are the inhibition value of each fragment measured under stringent (5 mM BME) conditions divided by the inhibition value for the parent fragment (3D4 or 6D11) and normalized to 1.

SI Table S1: The equilibrium dissociation constants (K_d) for the two tracers, pKID, and c-Myb were compared between the KIX H602C mutant and wild-type KIX using the FP direct binding assay described above. Here a constant concentration of tracer (25 nM) was mixed with increasing concentrations of the wild-type KIX domain or KIX H602C mutant (from 0 to 20 μM). The average anisotropy values from three independent experiments were used to determine the K_d with the indicated error (standard deviation).

K_d (μM)	**pKID**	**c-Myb**
Wild-type KIX	0.48 ± 0.03	1.5 ± 0.2
KIX H602C mutant	0.67 ± 0.06	1.8 ± 0.4

References

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