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Covalent chemical co-chaperones of the p300/CBP GACKIX domain

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Abstract

The GACKIX activator-binding domain has been a compelling target for small molecule probe discovery because of the central role activator-GACKIX complexes play in diseases ranging from leukemia to memory disorders. Additionally, GACKIX is an ideal model to dissect the context-dependent function of activator-coactivator complexes. However, the dynamic and transient PPIs formed by GACKIX are difficult targets for small molecules. An additional complication is that activator-binding motifs such as GACKIX are found in multiple coactivators, making specificity difficult to attain. In this study, we demonstrate that the strategy of Tethering can be used to rapidly discover highly specific covalent modulators of the dynamic PPIs between activators and coactivators. These serve as both orthosteric and allosteric modulators, enabling the tunable assembly or disassembly of the activator-coactivator complexes formed between the KIX domain and its cognate activator binding partners MLL and CREB. The molecules maintain their function and selectivity even in human cell lysates and in bacterial cells and thus will ultimately be highly useful probes for cellular studies.

Introduction

GACKIX is one of several conformationally plastic domains found in the master coactivators CBP and p300.[1, 2] It interacts with more than 15 transcriptional activators at two distinct interfaces.[2] NMR studies of GACKIX bound with native transcriptional activation domains (TADs) have shown that the endogenous partners such as MLL and c-Jun interact at a relatively deeper and smaller site (an area of approximately 900 Å²)[3, 4] while the transcriptional activation domains of CREB (pKID) and c-Myb interact with a shallower and larger surface on the opposite side.[5] The two interfaces are allosterically connected such that binding of the transcriptional activation domain of MLL enhances the interaction of c-Myb or pKID at the other binding site by approximately 2-fold.[4, 6] This allosteric enhancement is thought to play a key role in recruitment of p300 or CBP to gene promoters.[4, 6, 7]

The activator-KIX complexes participate in fundamental processes such as hematopoiesis and memory formation.[8-10] The discovery of small molecule

modulators for those processes has been of high priority in the selection, screening and top-down approaches yielding KIX inhibitors.[11-15] One early success identified Naphthol AS-E, a molecule that disrupts oncogenic responses in cancer cell models with the CREB-GACKIX and Myb-GACKIX complexes as its intended targets.[11, 16, 17] As this example illustrates, small molecules that target GACKIX have the potential to delineate activator-coactivator functions on a phenotypic level. However, even the most specific modulators for GACKIX have limited selectivity due to the level of redundancy in the protein interaction network.[14, 18-20] These binding surfaces that coactivators use to interact with activators are often similar. For instance, the activator p53 interacts with GACKIX as well as three other domains within CBP and p300, illustrating similarities in these binding surfaces.[21, 22] Finally the GACKIX motif within CBP has been found in at least four other eukaryotic coactivators, including the closely related p300, but also in unrelated coactivators, such as MED15 and ARC105.[23-25] Therefore, even the unusually specific inhibitor sekikaic acid, a natural product that exhibits high specificity for the GACKIX domain, has the undesirable potential to target other coactivators with this motif in a cellular setting.[14] This severely limits the utility of these probe molecules to dissect individual activator-GACKIX interactions and to understand their role in normal and pathological processes.[24]

Recently we reported that the covalent fragment discovery strategy of Tethering yields small-molecule modulators for GACKIX termed chemical co-chaperones that stabilize distinct conformations of this plastic motif and, in doing so, modulate its ability to form binary and ternary complexes.[26] While useful for structural and *in vitro* biophysical studies, these molecules react with GACKIX via disulfide exchange under reversible conditions and are thus not usable in more complex environments such as those of cells and cell lysates. Here we show, that despite the shallow and poorly defined binding surfaces in GACKIX, irreversible covalent co-chaperones highly selective for their cognate binding site can be readily accessed by replacement of disulfides in the Tethering hits with more reactive moieties (Figure 1). Further, the co-chaperones show high selectivity for their cognate binding site, even in the presence of many potential reactive partners. Finally, changes in the thiophile enable tuning of the

assembly behavior of GACKIX, leading to allosteric enhancement or inhibition of binding with a subset of partners.

Results and Discussion

In a study of the oncogenic K-RAS variant K-RAS(G12C) Shokat and co-workers found that inhibitors discovered through Tethering could be converted to irreversible modulators via replacement of the disulfide moiety with thiophilic moieties (Nature. 2013 Nov 28;503(7477):548-51).[27] Encouraged by these results, we hypothesized that disulfide fragments identified in a screen of the CBP/p300 GACKIX motif could serve as starting points for irreversible covalent co-chaperones.[28-30] However, specificity of the resulting structures was a major concern. The GACKIX motif does not contain attractive small molecule binding pockets but rather binding surfaces that are relatively featureless. These GACKIX binding surfaces adapt upon interacting with their binding partners so the conversion of the disulfide fragments into irreversible co-chaperones can be challenging. The irreversible co-chaperones also need to orient the fragment at the selected point of Tethering and effectively modulate the cysteine-containing GACKIX motif interactions. We chose to start with the GACKIX N627C mutant located in proximity to the MLL-binding site because GACKIX N627C mutant had a negligible effect on the endogenous GACKIX-binding partners.[30,31] Therefore any alterations in the binding of GACKIX to its' partners was attributed to chemical co-chaperones that tethered to this mutant.

We sought a fragment that is well-known to impact the conformations and interactions of the GACKIX motif. The fragment **1-10** tethered to GACKIX N627C stands out as both an effective modulator of GACKIX interactions by both inhibiting MLL binding (IC_{50} 68 μ M)[31] and enhancing pKID binding.[30] Without the tether **1-10** binds to the GACKIX motif at the MLL-binding site with modest affinity.[28]

To initiate the investigation, the disulfide moiety of **1-10** was replaced with three distinct thiophiles with or without a glycine linker to produce **1-10a-f**.[32-37] These molecules were then assessed for dose-dependent alkylation of the GACKIX N627C mutant and, as shown in Table 1, the dose-response (DR_{50}) values varied significantly with the nature and linkage of thiophilic group. The least effective reactive group in this series is the α,β -unsaturated amides including **1-10e** and **1-10f**, with the labeling of the

protein occurring only under forcing conditions. Both **1-10a** and **1-10b** with an α -chloro amide and **1-10c** and **1-10e** from the vinyl sulfonamide series were more effective, with DR₅₀ values ranging from 4.6 - 150 μ M.

The impact of these fragments on GACKIX N627C interactions was considered unique because labeling this mutant with the alkylator, iodoacetamide, had minimal impact on their affinity (SI Figure 1). When covalently attached to GACKIX each of these structures, **1-10a-d**, competitively blocked MLL from interacting with its cognate binding site analogous to the parent disulfide **1-10**. More remarkable are the effects at the distal binding site, with fragments **1-10b** and **1-10c** allosterically *inhibiting* the binding of pKID and **1-10d** *enhancing* the binding nearly 2-fold, comparable to the effects observed with the native ligands MLL, and pKID. The affinity differences can be reasonably associated with how the **1-10** co-chaperones interact with GACKIX. Previously, the **1-10** disulfide was shown to impact the distal site by altering the conformational dynamics of GACKIX.[29] Therefore, we hypothesize that the spacing between the **1-10** fragment and the reactive group influence the dynamics within GACKIX and these structural changes are reflected by how the **1-10** co-chaperones effect MLL and pKID binding.

Assessing target engagement of two 1-10 irreversible probes

A further examination of the reactivity of the covalent modifiers revealed a remarkable degree of selectivity for the MLL-binding site within GACKIX. None of the molecules (**1-10a-f**) react with the KIX domain in the absence of a cysteine residue (native GACKIX). In addition, covalent modification selectively occurs only in the MLL-binding site, even when cysteine residues are introduced around other activator binding sites. As shown in Figure 2A, covalent modifiers **1-10a** and **1-10d**, both of which are reactive electrophiles, only form covalent bonds with GACKIX when a cysteine is present at the cognate (MLL) binding site. In a more complex environment *E. coli* expressing GACKIX N627C protein was dosed with the irreversible probes and the resulting purified protein was qualitatively labeled by the irreversible probes by Q-TOF LC-MS (Figure 2B). The affinity of the core scaffold of **1-10** (no electrophilic moiety) has been measured as approximately 250 μ M; thus, it is unlikely **1-10d** at concentrations under 10 μ M will produce effects due to non-covalent binding interactions.

The concern involving irreversible probes is that they may target critical cellular components in mammalian cells and cause adverse effects due to their reactivity. Preliminary results show that **1-10d** readily labels GACKIX N627C in the presence of excess glutathione with no observed alkylator-glutathione adducts after one hour, suggesting it could function selectively in cells. (SI Figure 2). Thus we decided to visualize the potential targets of these probes in a cellular environment. We ventured in cells by adding the purified GACKIX N627C mutant to HEK 293T lysate and then dosed in a biotinylated variant of the **1-10d** probe. Biotin-containing components were pulled-out of the lysate using NeutrAvidin resin and analyzed by western blot with streptavidin-HRP (Figure 3). Once again, the **1-10d** alkylator displayed significant selectivity for the target.

Conclusion

The purpose of targeting the GACKIX motif is to understand what roles its interactions with activator complexes play in transcriptional events. However, it is difficult to distinguish the role of the GACKIX motif in CBP versus p300 function due to their significant homology (90%).[38]. From our results the irreversible **1-10** derivatives displayed a preference for the cysteine-containing GACKIX N627C mutant. They selectively enhance or disrupt interactions between GACKIX N627C and its activators only when the cognate cysteine residue is present. Since these probes are irreversible, nonspecific targets can drastically weaken their fitness as cellular probes.[36, 43-46] Fortunately, our preliminary evidence shows that **1-10d** engages the KIX N627C target with no prominent, off-targets in different complex environments.

With our strategy one fragment known as **1-10** was taken from the reversible disulfide Tethering screen to provide a framework for a suite of irreversible modulators that target the cysteine-containing GACKIX N627C domain. Since the **1-10** irreversible co-chaperones target GACKIX in cellular environments, they have the potential to examine how GACKIX recognizes different activator binding partners to regulate gene expression. Our chemical co-chaperones were able to change the assembly or disassembly of GACKIX complexes at the distal site. For instance, **1-10c** and **1-10d** both inhibit MLL but they either disrupt or enhance the pKID-GACKIX interaction. Previously our lab showed that the **1-10** tethered to GACKIX N627C prolonged the

residence time of pKID to cause the allosteric enhancement. The **1-10** derivatives exhibit either positive or negative cooperativity by how they alter the conformational dynamics with GACKIX N627C. Finally these co-chaperones, **1-10c** and **1-10d** could also provide novel, contrasting effects on specific pKID (CREB)-dependent genes. Studies in cells bearing cysteine mutants of p300 and CBP are a current focus in order to connect the biophysically-derived model of GACKIX binding and function with cellular function.

Methods

Protein Expression and Purification

As previously described[47], the DNA sequence encoding the GACKIX domain from mouse CBP residues 586-672 was cloned into the bacterial expression pRSETB vector with an additional hexahistidine tag and a short polar linker fused to the N-terminus of GACKIX resulting in protein with the sequence (tag and linker residues are shown in lower case):

mrgshhhhhhgmasGVRKGWHEHVVTQDLRSHLVHKLQVAIFPTPDPAALKDRRMENLVA
YAKKVEGDMYESANSRDEYYHLLAEKIYKIQKELEEKRRSRL

The human MED15 KIX cDNA encoding the amino acids 1 through 78 was synthesized by GenScript USA, Inc. and cloned into the pET-15b plasmid (Novagen, EMD Millipore) using the Nde1 and Xho1 cloning sites. The resulting recombinant wild-type MED15 KIX protein sequence contained the N-terminal hexahistidine tag and a thrombin cleavage site as shown in lower case letters;

mgsshhhhhhssglvprgsHMDVSGQETDWRSTAFRQKLVSQIEDAMRKAGVAHSKSSKD
MESHVFLKAKTRDEYLSLVARLIHFRDIHNKKSQASV

The cysteine mutants at N627, R644, K662, H651, Q609, K606 of CBP GACKIX and at R67 and H70 of MED15 GACKIX were generated using site-directed mutagenesis as previously described.[28]

The GACKIX protein was expressed in Rosetta2(DE3) pLysS *Escherichia coli* (Novagen). Cells were grown to an OD_{600nm} of 0.8 - 1.0 (37 °C, 250 rpm), induced with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours at 25 °C, harvested by centrifugation, and stored at -80 °C. The His₆-tagged GACKIX protein was affinity purified using a batch method with Ni-NTA beads (QIAGEN) following the

manufacturer's instructions and eluted with 400 mM imidazole. GACKIX was polished up with ion-exchange chromatography on a Source S column (GE Healthcare) in 50 mM phosphate buffer, pH 7.2 eluting with NaCl gradient from 0 to 1 M. Purified protein was buffer exchanged into 10 mM sodium phosphate, 100 mM NaCl, pH 6.8 using a PD-10 column (GE Healthcare) and stored at -80°C .

The MED15 KIX (1-78) protein was also expressed in Rosetta2(DE3) pLysS *E. coli* (Novagen). After the cultures reached an $\text{OD}_{600\text{nm}}$ between 0.8 – 1.0 the cultures were cooled to 20°C and MED15 KIX expression was induced with the addition of 125 μM IPTG. After 12 to 18 hours, the cells were harvested by centrifugation for 15 minutes at $7,903 \times g$ in a Sorvall™ LYNX™ Superspeed Centrifuge with a Fiberlight F6-6x1000 LEX Carbon Fiber Rotor (ThermoFisher Scientific), collected in a 50 ml falcon tube, and stored at -80°C .

The MED15 KIX protein was purified by first suspending bacterial cells in approximately 25 ml of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, 1 mM β -ME pH 7.2, cOmplete EDTA-free Protease Inhibitor Cocktail Tablet (Roche)). The cells were lysed by sonication at 50% amplitude on ice using a 6 mm tip with pulsing cycles of 3 seconds on and 6 seconds off for at least 3 minutes of total pulsing time. The soluble lysate was collected by centrifugation for 30 minutes at 9,500 rpm ($9,299 \times g$) in an Allegra X-22R centrifuge (Beckman Coulter) with a C0650 fixed angle rotor, and then incubated with 2 ml of suspended Ni-NTA agarose resin (Qiagen) for 1 to 2 hours rotating at 4°C . The resin was washed with five times with 5 mL of wash buffer (50 mM sodium phosphate, 300 mM NaCl, 30 mM imidazole, 1 mM β -ME, pH 7.2). The nickel-bound protein was eluted with 300 mM imidazole and diluted into 10 mM sodium phosphate, 100 mM NaCl, 10% glycerol, 0.01% NP-40 pH 6.8. The hexahistidine affinity tag was cleaved overnight with Thrombin (Restriction grade, Novagen® 69671) according to the manufacturer's instructions. The cleaved protein was further purified on the AKTA FPLC Purifier (GE Healthcare) with the strong cation exchanger Source 15S media (GE Healthcare) packed to a 17-ml column volume. After loading the sample the column was washed for 1.5 column volumes with buffer A (50 mM sodium phosphate, 1 mM DTT, pH 6.8). The protein was eluted from the column with a gradient of 0% to 60% of buffer B (50 mM sodium phosphate, 1 mM DTT, 1 M

NaCl, pH 6.8) over four column volumes. Using a 3 kDa molecular weight cut-off Amicon Ultra-15 Centrifuge Filter Units (EMD Millipore) the MED15 KIX was concentrated and buffer exchanged into 10 mM sodium phosphate, 100 ml sodium chloride, 10% glycerol, 0.01% NP-40 pH 6.8. Aliquots of protein were flash frozen in liquid nitrogen and stored at -80°C . The concentration of the protein was measured using absorbance at 280 nm on a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Peptide Synthesis and Purification

All peptides were synthesized by standard N-9 Fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis methods[48] as previously described.[47] The peptide sequences written as single letter amino acid abbreviations are as follows:

MLL $\beta\text{A-DAGNILPSDIMDFVLKNTP-CONH}_2$

pKID $\beta\text{A-DSQKRREILSRRPS(Phos)YRKILNDLSSDAPG-CONH}_2$

The abbreviation, βA represents beta-alanine and S(Phos) is phosphoserine. The fluorescent fluorescein isothiocyanate (FITC) tag was added at the amino-terminus of the peptide before the β -alanine residue.

DR₅₀ assessment

For the DR₅₀ values, 5 μM of GACKIX N627C was incubated with varying concentrations (500-0.2 μM) of the compounds (at 1 mM β -ME) for 45 minutes at room temperature (RT). Then the samples were incubated for 15 minutes at 4°C . The percent of protein tethered to fragment molecules were determined by Q-TOF LC-MS (Agilent).[49] The concentration of fragment molecule required for 50% maximum Tethering (DR₅₀) was determined in GraphPad Prism software 4.00, fitting to Equation 1, where x is the log of fragment molecule concentration and y is the normalized response from 1 to 100 (percent of protein tethered to fragment molecule).

$$y = \frac{100}{1+10^{(\text{Log}DR_{50}-x)}} \quad (\text{Eq. 1})$$

Alkylation of GACKIX

The GACKIX N627C mutant was incubated with 5 to 10 equivalents of small molecule in 10 mM phosphate buffer, 100 mM NaCl, pH 6.8 overnight at room temperature. Excess small molecule was removed and small molecule-protein

complexes were concentrated using 10 kDa molecular weight cutoff centrifugal concentrators (Vivascience). The extent of labeling was measured by Q-TOF LC-MS (Agilent). Protein complexes that were at least 95% alkylated were flash frozen in liquid nitrogen and stored at -80°C .

Fluorescent anisotropy assays

The fluorescent anisotropy was measured in triplicate with a final sample volume of $10\ \mu\text{l}$ in a low volume, non-binding, black, 384-well plate (Corning). For each experiment $25\ \text{nM}$ of fluorescently labeled peptide tracers, FITC-MLL and FITC-pKID were incubated with varying concentrations of the small molecule-GACKIX mutant complexes in binding buffer ($10\ \text{mM}$ phosphate, $100\ \text{mM}$ NaCl, pH 6.8) for 30 minutes at RT. The plates were read using a Tecan Genios Pro plate reader with polarized excitation at $485\ \text{nm}$ and emission intensity measured through a parallel and perpendicularly polarized $535\ \text{nm}$ filter. A binding isotherm that accounts for ligand depletion (assuming a 1:1 binding model of peptide to GACKIX) was fit to the observed anisotropy values as a function of GACKIX to obtain the apparent equilibrium dissociation constant, K_D :

$$y = c + (b - c) \times \frac{[(Kd+a+x) - \sqrt{(Kd+a+x)^2 - 4ax}]}{2a} \quad (\text{Eq. 2})$$

where “a” and “x” are the total concentrations of fluorescent peptide and GACKIX, respectively, “y” is the observed anisotropy at any GACKIX concentration, “b” is the maximum observed anisotropy value, and “c” is the minimum observed anisotropy value. Data analysis was performed using GraphPad Prism 7.0 software.

Labeling GACKIX cysteine mutants

$5\ \mu\text{M}$ of cysteine mutants at CBP KIX N627C, N644C, K662C, H651C, Q609C, K606C and MED15 KIX R67C, and H70C were incubated with $100\ \mu\text{M}$ of small molecule in DMSO in the presence of $1\ \text{mM}$ β -ME for 1 hour at RT. The extent of labeling was measured by Q-TOF LC-MS (Agilent) and each labeling reaction was duplicated.

Alkylation in growing E. coli

The protein expression system for the GACKIX N627C protein was initiated as described above. Three hours after induction with $0.25\ \text{mM}$ IPTG the culture was concentrated by centrifugation in which the cell pellet from $50\ \text{mL}$ of culture was

suspended in 1 mL of media. For a 1 mL alkylation reaction 10 μ L of compound in DMSO was added to concentrated cells to obtain concentrations of 1000 μ M, 500 μ M, 250 μ M, 62.5 μ M, and 15.6 μ M. The mixtures were incubated for 1 hour at 25 $^{\circ}$ C (250 rpm). The cell pellets were washed three times with 10 mM phosphate, 100 mM NaCl pH 6.8 and stored at -80 $^{\circ}$ C. Purification was carried out as previously described using Ni-NTA resin.[29] The elutions were buffer exchanged into 10 mM phosphate, 100 mM NaCl, pH 6.8 and concentrated using 5 kilodalton molecular weight cutoff concentrators (Vivascience). The samples were analyzed by Q-TOF LC-MS (Agilent). The extent of labeling was determined by comparing the peak intensity of small molecule-GACKIX N627C complex versus unlabeled GACKIX N627C.

NeutrAvidin pull-down assay with the biotinylated 1-10 probe

HEK 293T cells were maintained in DMEM supplemented with 10% FBS. The cells were lysed in 750 μ L of lysis buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 0.1% Nonidet P-40 (NP-40)) containing halt protease inhibitor cocktail (Thermo Scientific) and soluble lysate was isolated by centrifugation (12,000 rpm, 4 $^{\circ}$ C, 15 min.) HEK 293T lysate (100 μ g total protein) and 25 nM of purified GACKIX N627C were incubated with DMSO or various concentrations of the biotinylated **1-10d** probe (5 μ M, 1 μ M, 200 nM, 40 nM and 8 nM) at RT for 1 hour. Following incubation with NeutrAvidin agarose resin (50 μ L, Thermo Scientific) for 1 hour at 4 $^{\circ}$ C the beads were washed twice with 1 mL of wash buffer (10 mM phosphate, 100 mM sodium chloride, 10% glycerol, 0.1% NP-40, pH 7.2) and the resin-bound complexes were eluted by boiling in NuPAGE[®] LDS Sample Buffer (Invitrogen) containing DTT. The samples (15 μ L) were resolved on a 12% SDS polyacrylamide gel by electrophoresis. The proteins were transferred onto a PVDF membrane and incubated with streptavidin conjugated to hydrogen peroxidase (HRP) enzyme (ab7403, Abcam) at 1:10,000 dilution in 10 mM PBS, 0.2% Tween-20. The membrane was developed using Amersham ECL[™] Prime Western Blotting Detection Reagent (GE Healthcare) and the image was captured on X-ray film.

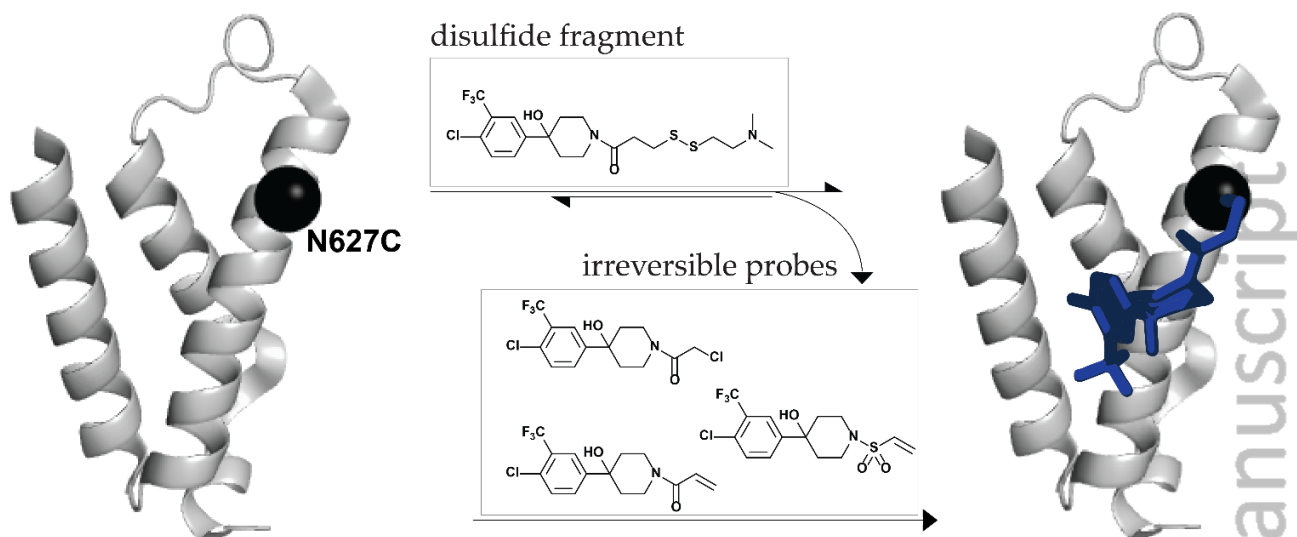
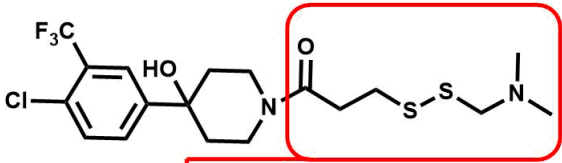


Figure 1

Schematic for the development of irreversible chemical co-chaperones to target the GACKIX domain of CBP. The structure of GACKIX is shown as a cartoon in grey derived from PDB ID 4I9O [29]. The black sphere represents the position of the N627C mutant that was used to target GACKIX with chemical co-chaperones. The standard Tethering scheme involves in the reversible formation of a mixed disulfide with the target when the fragment favorably interacts with the regions surrounding cysteine residue. By replacing the fragment's disulfide with an alkylating moiety, the 1-10 chemical co-chaperones irreversibly bind to the GACKIX N627C represented in the structure at the far right.

Table 1
 Comparing irreversible analogues of 1-10 against GACKIX N627C. The DR₅₀ values were assessed by measuring the extent of GACKIX N627C labeled using Q-TOF LC-MS at various concentrations of compound. The fold inhibition values were obtained by comparing the dissociation constants (K_D) for the unlabeled (DMSO) KIX N627C construct with the K_D for the labeled KIX N627C- 1-10 alkylator complex for both the MLL and pKID tracers. The K_D values were measured from FP experiments that were performed in triplicate and errors reflect the standard deviation (SD) error.



Compound	Dose Response DR ₅₀ (μM)	Fold Inhibition	
		MLL	pKID
1-10a	25	12 ± 1	0.9 ± 0.1
1-10b	150	13 ± 1	1.4 ± 0.2
1-10c	6.8	17 ± 2	1.5 ± 0.2
1-10d	4.6	17 ± 2	0.65 ± 0.08
1-10e	>500	14 ± 2	0.88 ± 0.09
1-10f	>500	5.9 ± 0.6	0.66 ± 0.07

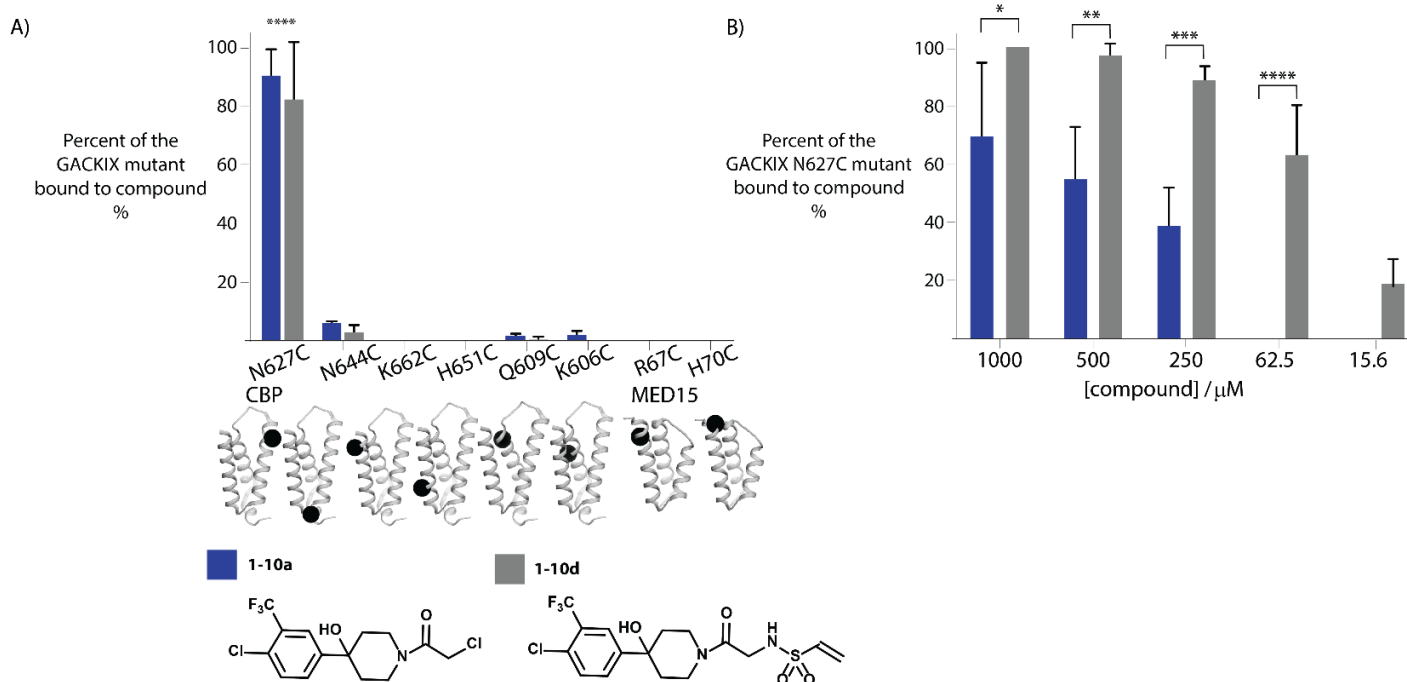
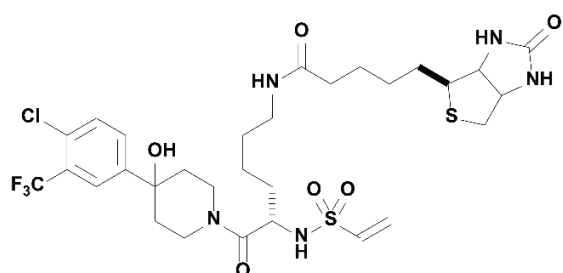


Figure 2

A) 1-10a and 1-10d show preference for the GACKIX N627C mutant located near the MLL-binding site. N644C is located in the loop and the remaining mutants, K662C, H651C, Q609C and K606C are located within the pKID-binding site. The error bars represent standard deviation of the average of two separate experiments. B) The 1-10 chemical co-chaperones were dosed into *E. coli* during the expression of GACKIX N627C and after nickel affinity purification, the extent of labeling was assessed by LC-MS. The error bars represent standard deviation of the average of three values from separate experiments. * $0.01 < P < 0.05$, ** $0.05 < P < 0.001$, *** $0.001 < P < 0.001$, **** $P < 0.001$; P values are calculated by GraphPad Prism 7.0.

Autl



biotinylated 1-10 vinyl sulfonamide probe

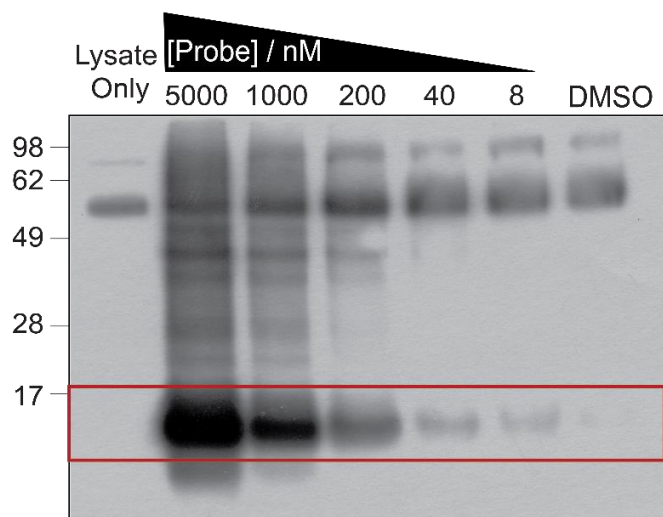
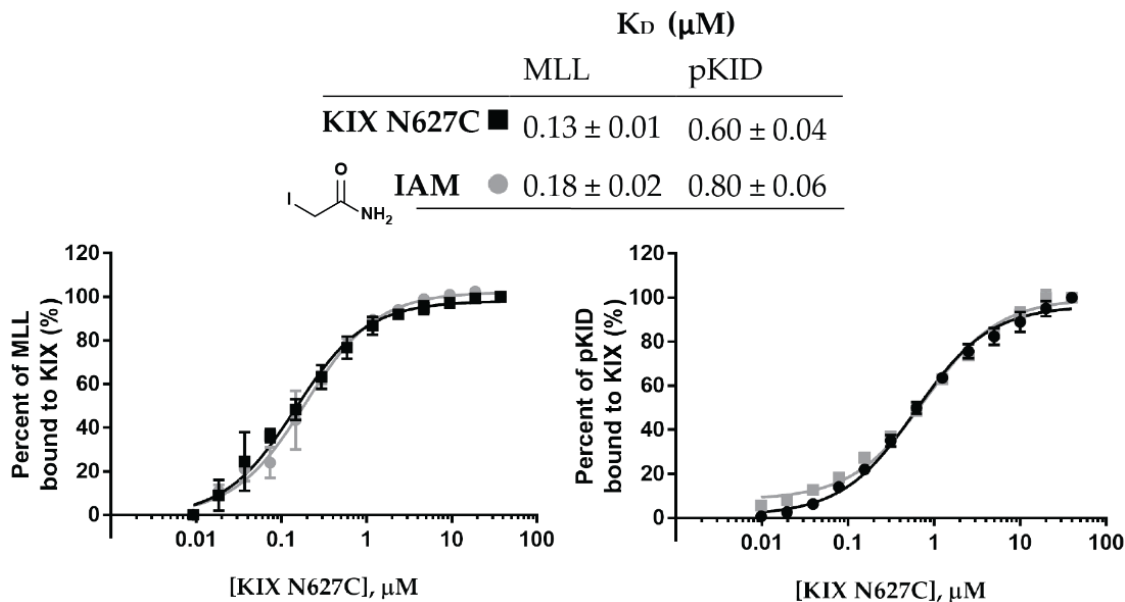


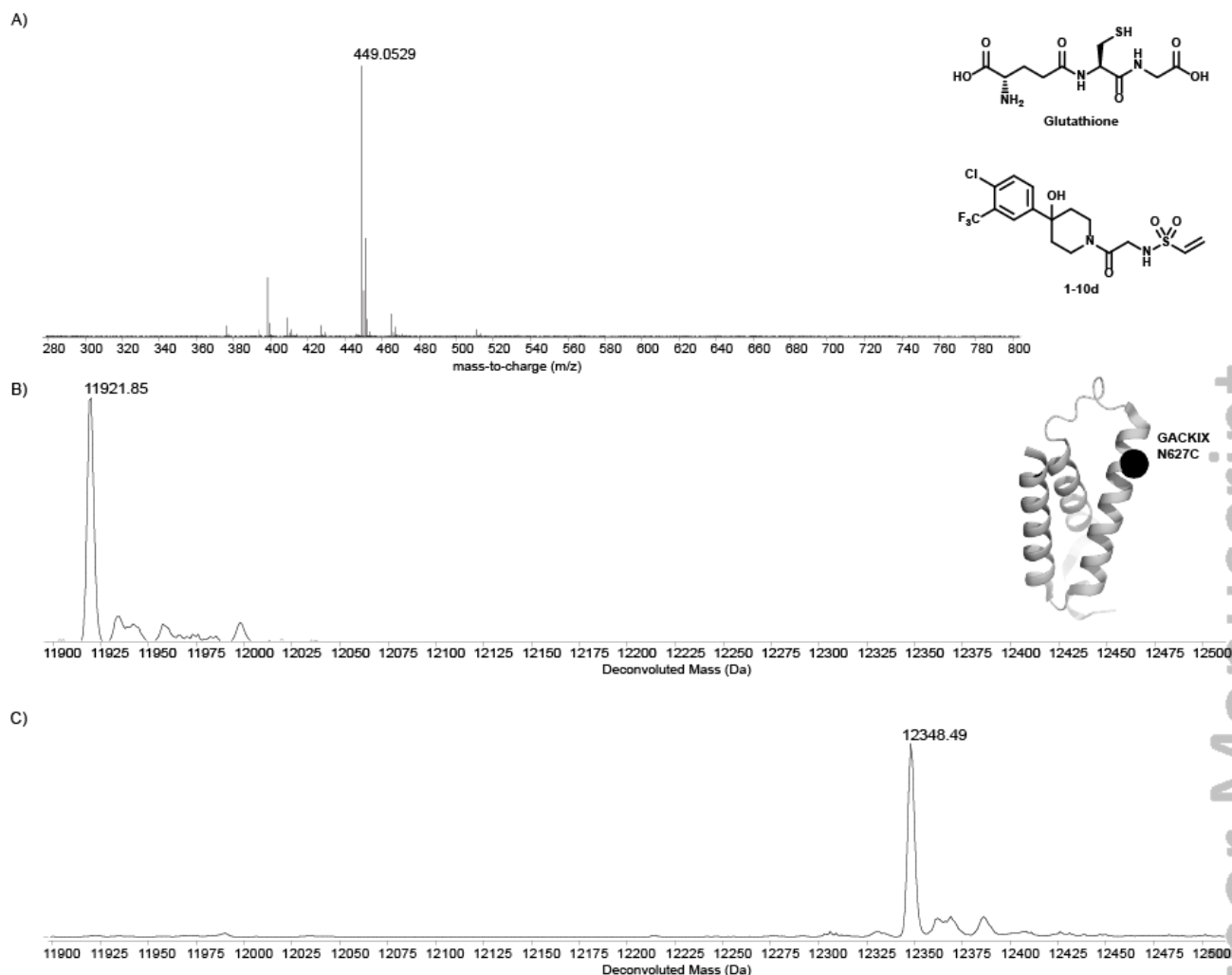
Figure 3

The structure of the biotinylated **1-10d** probe is shown above. This probe was added to 100 μg of HEK293T lysate containing purified GACKIX N627C (25 nM). Any biotin-containing proteins isolated on NeutrAvidin agarose beads were visualized Western Blot using chemiluminescence with Streptavidin- HRP. The bands within the red box represent the expected mass for GACKIX N627C (12 kDa).

Supplementary Information



Supplementary Figure 1 The interactions for unlabeled KIX N627 and KIX N627C labeled by iodoacetamide (IAM) were compared against the binding partners, MLL and pKID using fluorescence polarization (FP) direct binding assays. These interactions were not significantly impacted by the presence of IAM at the MLL-binding site while some distal effects occurred with pKID binding. All measurements were performed in triplicated. The dissociation constants are reported with the standard error (SE). The FP direct binding curves are shown for the MLL and pKID tracers. The values represent the average and the error bars represent the standard deviation (SD). Data analysis was performed in GraphPad Prism 7.0.

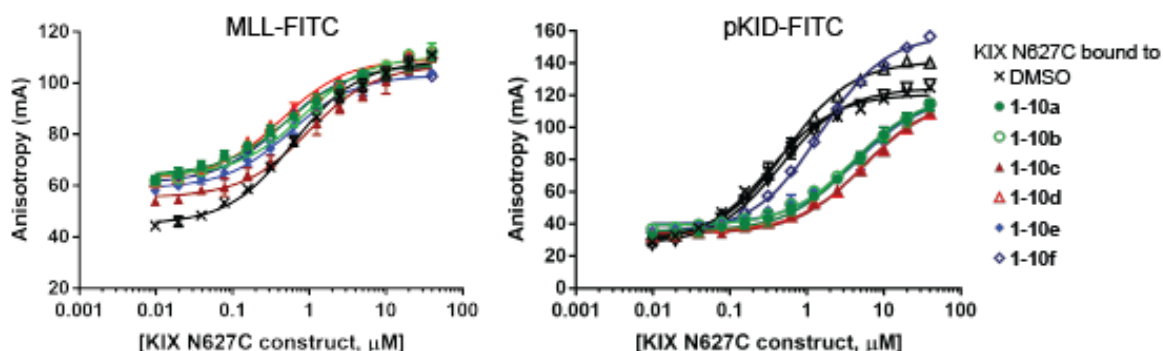


Supplementary Figure 2 A) The reactivity of **1-10d** toward glutathione was tested by incubating 50 μM of **1-10d** with 500 μM of glutathione in 10 mM sodium phosphate buffer, 100 mM NaCl, pH 6.8 for 45 minutes at room temperature. The reaction was injected onto the 1290 Agilent liquid chromatography system equipped with a Proshell 300 SB-C8 column and analyzed with the 6545 Agilent quadrupole time-of-flight (Q-TOF) mass spectrometer. The mass spectrum is shown with a prominent peak at m/z of 449.0529 corresponding to the **1-10d** sodium adduct (calculated $(M+H+Na)^+$ 449.0526 m/z). Free glutathione eluted off the column first with an observed m/z 308.0912 (calculated $(M+H)^+$ 308.0838 m/z) and 330.0731 m/z (calculated $(M+H+Na)^+$ 330.0736). B) Deconvoluted mass spectrum shows the unbound KIX N627C at 11,921.85 Da. C) KIX N627C (5 μM) was mixed with 50 μM of **1-10d** and 500 μM of glutathione for 45 minutes and the reaction was analyzed on the Q-TOF LC-MS (Agilent). The mass spectra were deconvoluted using the Agilent MassHunter Bioconfirm software (Agilent) and maximum entropy algorithm. KIX N627C was fully labeled by **1-10d** with an observed deconvoluted mass of 12348.49 Da (calculated mass 12348.69 Da).

Supplementary Table S1 The K_D values of for MLL and pKID with the unbound (DMSO) and labeled GACKIX N627C complexes. The error reflects the standard error from three FP experiments.

Compound	K_D (μ M)	
	MLL	pKID
DMSO	0.32 ± 0.02	0.60 ± 0.04
1-10a	3.9 ± 0.4	0.52 ± 0.07
1-10b	4.1 ± 0.2	0.8 ± 0.1
1-10c	5.4 ± 0.3	0.9 ± 0.1
1-10d	5.6 ± 0.4	0.40 ± 0.04
1-10e	4.6 ± 0.5	0.52 ± 0.04
1-10f	1.47 ± 0.06	0.40 ± 0.04

Supplementary Figure 3 The anisotropy binding curves of the GACKIX N627C bound and unbound complexes. The K_D values are shown in the supplementary Table 1. All measurements were performed in triplicate and analyzed in GraphPad Prism 7.0. The symbols represent the average and the error bars represent the SD error.



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