Protein-targeting strategy used to develop a selective inhibitor of the E17K point mutation in the PH Domain of Akt1

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Abstract

Ligands that can selectively bind to proteins with single amino acid point mutations offer the potential to detect or treat an abnormal protein in the presence of the wildtype. However, it is difficult to develop a selective ligand if the point mutation is not associated with an addressable location, such as a binding pocket. Here we report an all-chemical, synthetic epitope-targeting strategy which we used to discover a 5-mer peptide with selectivity for the E17K transforming point mutation in the Pleckstrin Homology Domain of the Akt1 oncoprotein. A fragment of Akt1 containing the E17K mutation and a I19[Propargylglycine] substitution was synthesized to form an addressable synthetic epitope. Azide-presenting peptides that covalently clicked onto this alkyne-presenting epitope were selected from a library using *in situ* screening. One peptide exhibits a 10:1 *in vitro* selectivity for the oncoprotein relative to wildtype, with a similar selectivity in cells. This 5-mer peptide was expanded into a larger ligand that selectively blocks the E17K Akt1 interaction with its PIP3 substrate.

Ligands that selectively bind to proteins with single amino acid point mutations are becoming increasingly important for both diagnostics and therapeutics. In a diagnostic setting, such binders can be used to assay for the mutant protein within diseased tissues, and

thus potentially provide clinical guidance for treatment decisions[1]. A more ambitious application is the development of drugs that can selectively inhibit mutant proteins and thus avoid the toxic side-effects that stem from the inhibition of the wild-type (WT) variants[2] that reside in non-diseased tissues. A relevant example is compound CO-1686, which is an a epidermal growth factor receptor (EGFR) inhibitor specific for the T790M point mutation associated with certain non-small cell lung carcinomas. That small molecule drug, which is currently in trials, is designed to minimize the toxicities (such as skin rash) that can appear when WT EGFR is targeted, since WT EGFR is expressed throughout the healthy tissues in the body[3].

A challenge of developing a drug targeting a single point mutation is that the mutation may not be directly associated with a binding pocket. The presence of a binding pocket is traditionally required for small molecule inhibitor development. This requirement does not hold for antibodies and, in fact, several examples of monoclonal antibodies directed against epitopes containing single amino acid mutations do exist[4,5,6]. However, antibodies do not readily enter the living cells that can harbor the mutated proteins[7,8], and so mutation-selective antibodies are typically only used as diagnostic reagents for staining fixed cells or tissues. Thus, there is a need for an approach to identify molecules that can be targeted against epitopes containing single amino acid point mutations, and which can potentially be developed into cell-penetrant inhibitors[9]. Here we report on an all-chemical epitope targeting strategy that focuses ligand development specifically to the E17K oncogenic point mutation of Akt1 (Akt1^{E17K}). We use this technique to develop a short-chain peptide to bind to Akt1^{E17K}, and we take the first steps towards developing that binder into a cell-penetrant, mutant-selective inhibitor.

Akt1 kinase plays a critical role in the PI3K signaling pathway[10] – the activation of which is closely linked to tumor development and cancer cell survival[11]. The recently discovered E17K mutation in the Pleckstrin Homology Domain (PH Domain) of Akt1 results in an increased affinity for the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) substrate at the cell membrane[12]. Consequently, this deregulated recruitment of Akt1 to the cell membrane causes constitutive activation of the PI3K pathway, which has been shown to induce leukemia in mice[12]. The oncogenic properties of Akt1^{E17K} make it a target for both detection and inhibition.

We recently reported on a strategy for focusing peptide ligand development near phosphorylation sites[13]. For that approach, a protein fragment representing the phosphorylated epitope of interest was synthesized, and a metal organic Zn-chelator was utilized to bind to the phosphate group and present an azide near that site. The azide-containing Zn-chelator was used as an anchor to focus the *in situ* click screening of a one-bead, one-compound (OBOC) library of 5-mer alkyne-presenting peptides to the phospho-site of the protein fragment. This basic approach was restricted to the targeting of naturally addressable residues, and required a separate anchor ligand. Using this technique, peptide ligands with high selectivity for the Akt2 isoform, and with affinities as low as 19 nM were developed. We have called the class of peptide ligands identified through *in situ* click screening Protein Catalyzed Capture agents, or PCC agents.[13–18] A technical goal for the

present work was to develop a method for synthetically introducing this addressable residue, potentially extending the generality of the technique.

Our epitope-targeting, small molecule approach, although 100% synthetic, is inspired by the biological technique for developing an epitope-targeted monoclonal antibody (mAb). Such mAbs are often developed by injecting a small fragment of the protein of interest containing the mutation (the epitope), into an animal, and screening for an immune response that has the desired selectivity[5,6,4]. We report here on a targeting strategy that directly substitutes an alkyne click handle into a chemically-synthesized peptide epitope which makes an easily addressable residue for selectively targeting a region of interest in a protein. This technique potentially eliminates the need for a binding pocket or a naturally addressable residue such as a phosphorylation site. For this work, the peptide represents the epitope of Akt1 containing the E17K point mutation. The epitope target is subjected to an *in situ* click screen against an OBOC peptide library of 5-mers (comprehensive in 18 amino acids), each terminated in an azide-presenting amino acid. For such a screen, the protein fragment provides a highly-selective scaffold that replaces the Cu(I) catalyst typically used in promoting the cyclo-addition between the alkyne and azide groups to form a triazole linkage (the Huisgen click reaction)[14]. Hits are defined as those compounds that are covalently coupled to the synthetic epitope through a triazole linkage. The juxtaposition of the chemically-substituted alkyne click handle to the E17K point mutation should mean that any hit peptide that has been covalently linked to the target sequence should bind in close proximity to the mutation.

This technique allowed us to focus our PCC agent development to a location on the PH Domain that was adjacent to the E17K oncogenic mutation. We identified a 5-mer peptide that exhibited a 10:1 selectivity for Akt1^{E17K} relative to WT. We exploited the chemical flexibility and modularity of the PCC agent to append a dye and a cell penetrating peptide. The resultant ligand could preferentially localize in live cells expressing Akt1^{E17K}, again with high selectivity relative to WT. Finally, we developed the PCC agent into a biligand and then a triligand through the use of iterative *in situ* click chemistry[14]. We showed that these larger PCC agents could serve as highly selective inhibitors of Akt1^{E17K} by blocking binding of the Pleckstrin Homology Domain of Akt1 to the PIP3 substrate.

Results

In situ click epitope-targeted screening strategy for E17K PH Domain-specific ligand

Using FMOC solid-phase peptide synthesis (SPPS) techniques[19] a polypeptide representing residues 1–32 of the E17K PH Domain of Akt1 was synthesized. From the crystal structure (2UZR) (Fig. 1), these residues form a β-sheet around the E17K mutation (blue). The epitope fragment was appended with an N-terminal PEG$_5$-biotin to serve as a detection handle when screening. This manual synthesis of the epitope allowed for an I19Pra substitution (Pra = Propargylglycine) to provide an alkyne click handle on the most proximal side-chain residue to the E17K mutation. Following chromatographic purification, and characterization via circular dichroism (Supplemental Fig. S1), the modified epitope was ready for screening.

A single generation *in situ* click screen can yield ligands with a high selectivity for the target. The *in situ* click reaction itself is low yielding[14], but the biotin handle on the synthetic epitope permits the delineation of those beads that contain the covalently coupled epitope (the hits). This is done using a colorimetric streptavidin-linked alkaline phosphatase assay. The basic screening strategy is shown in Supplemental Figure S2. Out of the theoretical 1.5 million library members that were screened against the alkyne-containing 33-mer E17K PH Domain fragment, only 21 beads (0.0015%) showed the presence of the covalently coupled epitope. These beads were sequenced using Edman degradation (Supplemental Tables S1 and S2). The hits were segregated based on their hydrophobicity and sequence homology using principal component analysis (Supplemental Fig. S3). Five ligands that represented the diversity of hits (**circled in** Supplemental Fig. S3) were scaled-up and tested for binding to both E17K and WT full-length PH Domain proteins. Immunoprecipitation assays (Supplemental Fig. S4) were used to probe for differential binding to the proteins in buffer. One ligand candidate showed a distinctively stronger binding to the E17K PH Domain relative to the WT. This peptide, sequence "yleaf", (Fig. 2a) was carried forward for additional investigations. Here the lower case sequence letters indicate that the amino acids that comprise the peptide are (non-natural) D-stereoisomers.

Verification of epitope targeting strategy

The label-modified yleaf peptide (Fig. 2a) was subjected to a variety of binding assays against the synthesized WT and E17K 33-mer PH Domain fragments prepared without the biotin label and alkyne click handle. First, the yleaf peptide was used in immunoprecipitation pull-down assays to pull from solution either the WT or E17K mutant 6-His-tagged 33-mer peptide fragments, as opposed to the full-length proteins that were used to initially validate the candidates. Typical immunoprecipitation assays involve western blotting to estimate the amount of protein binding, but small peptide fragments are not easily quantified on a blot. Because of this, the amount of peptide epitope precipitated in these assays was quantified via injection on an analytical HPLC. These assays further confirmed preferential yleaf ligand binding to the E17K 33-mer epitope relative to the WT epitope (Supplemental Fig. S5). As an assay control, another candidate ligand that, in initial testing, did not exhibit preferential E17K binding to the full protein, was tested, and yielded consistent results.

The selectivity of the yleaf peptide for the E17K 33-mer epitope was also tested in an ELISA assay format. For these assays, the WT or E17K 33-mer peptide fragments were captured using the PEG-biotin-modified yleaf ligand immobilized on a Neutravidin-coated plate. The yleaf ligand exhibited significant selectivity for the E17K fragment over the WT across a 100 nM – 1 μM concentration range (Fig. 2b) This selectivity was quantified by measuring the K_D values for the binding of the yleaf peptide to both epitope fragments (Fig 2d), as well as full length Akt1 and Akt1^{E17K} (Fig 2c, Supplemental Fig. S6) via fluorescence polarization (FP). The yleaf peptide exhibited K_D values of 328 ± 96 nM and 54 ± 7.0 nM for the mutant epitope and for full length Akt1^{E17K}, respectively. These compare to K_D values of 2.8 ± 0.84 μM and > 1 μM shown for the WT epitopes and Akt1, respectively. These results confirm the highly selective nature of the epitope targeting

strategy, and demonstrate that high selectivity achieved through that strategy is retained for the full length protein.

Ligand-directed labeling experiment to confirm epitope targeting

The binding of the yleaf ligand onto Akt1[E17K] was further explored using the directed labeling technique reported by Tsukiji *et al*[20]. The approach yields information relative to the binding location of the ligand on the protein target. For this method, a payload is attached to the N-terminus of the targeting yleaf ligand through an electrophilic phenyl sulfonate linker. Upon ligand binding to the protein target, the payload is transferred onto the protein through an S_N2 reaction with proximal nucleophilic amino acid side chains (Fig. 3a). The protein can then be trypsin digested and the identity of the fragments containing the payload can be mapped on the protein surface using mass spectrometry (MS). Thus, the site of ligand binding can be estimated. The assay also serves as an independent validation of the binding assays discussed above.

For the assay, yleaf was modified at the N-terminus to contain a tosylate linker attached to a Cy5 dye molecule to enable easy identification of the labeled and digested protein fragments (Fig. 2a). A Glutathione S-Transferase (GST)-Akt1[E17K] protein (SignalChem) was incubated with the Cy5-appended yleaf. The labeling of protein target was initially confirmed by visualization on a fluorescent gel reader (Fig. 3b), as well as a Western blotting visualization of an experiment in which a biotin-label was substituted for the fluorescent tag (Supplemental Fig. S7). The labeled protein and an unlabeled control were then trypsin digested from the gel and were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (Supplemental Figs. S8 and S9). Five peaks appeared in the MS of the labeled protein that were not present in the unlabeled protein digests. Those peaks all corresponded to an expected trypsin fragment plus the weight of the linker and dye. These peaks were then analyzed by MALDI-TOF/TOF MS to extract sequence information for the labeled regions of the protein. All but one of the dye labeled peptides were difficult to fragment, as is characteristic of cationic peptide labels[21]. The labeled digest YFLLK could be fragmented (Supplemental Fig. S10) and indicated the presence of the dye on the Y amino acid. The labeling can be seen clearly when compared to the unlabeled YFLLK upon fragmentation (Supplemental Fig. S11). This is consistent with the original literature on the labeling technique[20] which showed that Y, E and H amino acids are the nucleophiles that can be labeled. The other labeled Akt1[E17K] fragments that were identified contain at least one of these amino acids. One fragment contains two such amino acids and, in fact, there were MALDI-TOF peaks corresponding to the masses of both the singly and doubly labeled fragment. Supplemental Figure S12 shows the location of the labeled fragments in the PH Domain sequence, as well as the amino acids that should contain the label.

The labeling sites were then mapped on a composite crystal structure of GST (PDB: 1UA5) and Akt1[E17K] (Akt PDB: 3096, E17K PDB: 2UZR) (Supplemental Fig. S12, Fig. 3c). All labeled sites surround the epitope originally used in screening, which is the anticipated binding site of the yleaf ligand. A thorough search of the entire MALDI spectra was conducted to identify any other labeled fragments anywhere on the large protein, but none

were found. Thus, this experiment demonstrates that only sites around the expected N-terminal binding site of the yleaf ligand are labeled, confirming the very specific binding of the peptide ligand at the site directed by the epitope-targeted in situ click screening process.

Cell Imaging

Cell based assays can provide a demanding environment for demonstrating the selectivity of the yleaf PCC agent to Akt1^{E17K}. To demonstrate target binding in a complex cellular milieu, HEK-293T cells were transfected to express GFP-tagged E17K or GFP-tagged WT PH Domain proteins. The yleaf ligand was then labeled with both a Tat cell-penetrating peptide[22] and a Cy5 dye (Fig. 2a). The combination of the GFP label on the protein and the Cy5 label on the dye permitted the use of multi-color fluorescence microscopy for interrogating any spatial registry between the two fluorescent labels, as well as tracking the efficiency of the protein expression in the cells. Live HEK-293T cells expressing these GFP-tagged proteins were exposed to varying concentrations of the modified yleaf anchor ligand for one hour. The cells were then incubated in fresh media for one hour before being thoroughly washed in PBS to remove weakly and nonspecifically bound PCC and fixed for fluorescence microscopy measurements (see Supplemental Fig. S13 for a comparison of the anchor ligand retention before and after the buffer incubation step).

Confocal microscopy images of the two differentially expressing Akt1 PH Domain cells showed a consistent level of expression between the GFP-WT PH Domain and GFP-E17K PH Domain. However, the level of the PCC agent retained by the cells was substantially different (Fig. 4, for control images, see Supplemental Figs. S14–S15). Nearly all of the cells expressing the mutant protein show PCC agent retention and demonstrate some level of co-localization of PCC agent and GFP-PH Domain protein. The GFP-PH Domain WT cells, however, show very low levels of PCC agent retention. Pearson's correlation coefficient was used to quantify the co-localization of the two different dyes[23] by calculating over four representative images for each of the WT or E17K mutant cells and averaging the results. In a perfect correlation event the Pearson coefficient would be equal to 1, while no correlation equals 0, and a perfect negative correlation would produce a value of −1. The cells expressing GFP-PH Domain WT protein have an average correlation coefficient of r = 0.14 ± 0.06, implying little correlation, whereas the cells expressing the GFP-PH Domain E17K mutant protein have an average coefficient of r = 0.47 ± 0.13. These Pearson coefficients show a statistically significant difference between the two sets of images (p = 0.0045). The Cy5 fluorescence intensity on a per cell basis has also been calculated for the cells expressing the WT and E17K proteins (Supplemental Fig. S13), and this difference is again statistically significant (p = 0.00018). These assays demonstrate the selectivity of the E17K PCC agent for its target within live cells.

Biligand and triligand development

There is an interest in compounds that can selectively inhibit a disease-associated mutated protein target while sparing the WT variant[2]. PH Domain inhibiting compounds[24,25] with selectivity for Akt1^{E17K} have not been reported. The proximity of the E17K mutation to the PIP3 binding site prompted us to consider further developing this PCC agent into a compound capable of selectively blocking the E17K PH Domain interaction with its PIP3

substrate. The yleaf peptide itself did not exhibit evidence of inhibition (Fig. 5d). We reasoned that a similarly targeted, but bulkier PCC agent might serve as a steric blocker of the PH Domain-PIP3 interaction. To this end, we executed two cycles of iterative *in situ* click chemistry screens (Fig. 5a)[26] to develop the yleaf ligand into a biligand, and then a triligand. To identify the biligand (the first cycle), the yleaf ligand was modified to present an alkyne at the C-terminus and a PEG$_5$-biotin group at the N-terminus. This modified yleaf anchor ligand was then co-incubated with an azide-presenting OBOC library and the (unmodified) E17K PH Domain protein. Successful hits are those in which the E17K PH Domain promotes the click coupling of the anchor ligand onto a library peptide, and those hits are detected by screening for the formation of this clicked product. Those hits are candidate 2° ligands. For testing, the 2° ligand candidates are appended to the yleaf anchor ligand via a Cu catalyzed 1,4 triazole linkage to form a biligand. The biligand candidates are then tested for affinity and specificity in a manner that is similar to what was done to identify the original yleaf ligand. Once a biligand is identified, it is modified to form a new anchor ligand, which is then similarly screened to identify a triligand. Screening details, including candidate hit sequences that were tested at both the biligand and triligand stage, are provided in the supplementary materials (Fig. S16 – S21 and Tables S4, S5). Several examples of PCC agent biligands or triligands and related screening techniques have been reported[16]. The triligand structure is shown in Figure 5b.

The final biligand and triligand sequences were chosen to retain a high selectivity of binding to Akt1^{E17K} relative to WT. This was challenging, considering that Akt1^{E17K} and the WT are sequence identical away from the E17K mutation. A comparison of the yleaf ligand, the biligand, and the triligand in binding assays against both full length proteins is provided in Supplementary Figure S21. In those assays, as well as in fluorescence polarization measurements of K_D values, the triligand exhibited a selectivity for Akt1^{E17K} (K_D=115 ±8.7 nM) relative to WT (K_D=1.2 ±0.180 μM) that was similar to the yleaf anchor. Furthermore, there is a slight preference for Akt1^{E17K} relative to the Akt2^{E17K} (Supplementary Fig. S22). The homology of the PH Domain between these isoforms is 79%. In addition to the specificity for homologous proteins, the anchor and triligand were tested for their performance in 1% and 2% human serum (Supplemental Fig. S23). This assay shows very little reduction in binding efficiency in increasing amounts of serum, indicating that there are not significant off-target interactions with any proteins found in human serum.

Inhibition assays

The yleaf ligand, the biligand, and the triligand were all tested for their ability to block the E17K PH Domain binding with PIP3. For this test, PIP3-coated resin (Echelon Biosciences) was used to mimic the PH Domain interaction with the cell membrane, and could be used to bind the protein as in an immunoprecipitation assay[25]. The presence of an effective blocking compound would reduce the ability of the resin to capture the protein, and would thus appear as a diminished signal in the corresponding western blot assay. A control lane containing no PCC agent was used to show baseline binding of the protein to the PIP3 resin. Although the yleaf ligand produced no change in E17K binding ability, both the biligand and triligand did block the PH Domain-PIP3 interaction, with the triligand being the most effective (Fig. 5d). In an expanded study, we compared the amount of E17K and WT PH

domain binding relative to the amount of added triligand (Fig. 5e). This assay shows significant selective inhibition of the E17K mutant relative to the WT.

Discussion

The all-synthetic epitope targeting strategy described here provided an approach for developing a PCC agent peptide ligand that could be used to specifically detect an oncoprotein possessing a single transforming point mutation. The approach is conceptually simple and may be broadly applicable: a fragment of the target protein that contains the point mutation is chemically synthesized so that it presents a click handle near that mutation. That fragment (the epitope) is then subjected to an *in situ* click screen against a large library of peptides that present the complementary click chemistry handle. Viable hit candidates are those library elements that are covalently coupled to the fragment via a triazole linkage catalyzed by the strong and specific binding of the candidate ligand to the epitope. In this work, a polypeptide fragment that represented residues 1–32 of the PH Domain of Akt1[E17K] was prepared with an I19Pra substitution that displayed an alkyne functionality near the E17K mutation. A single generation *in situ* click screen yielded a 0.0015% hit rate and a peptide sequence that exhibited an approximately 10:1 selectivity for the E17K PH Domain relative to WT, with binding constant (K_D) of 54 ± 7.0 nM.

PCC agents are peptides, which allows for the straightforward incorporation of strategic chemical modifications so as to permit several experimental illustrations of the *in vitro* binding specificity of the peptide ligand to the E17K PH Domain of Akt1 as well as full-length Akt1[E17K]. In particular, ligand-directed labeling was used to confirm that the approximate location of the ligand binding on the target protein was consistent with the epitope-targeting strategy. The yleaf ligand, when modified with a cell-penetrating peptide and a dye label, could also be used to deliver the E17K PH domain into live cells. Upon fixing and washing the cells, those cells containing the E17K PH domain preferentially retained the ligand relative to WT, and that retained ligand exhibited statistically significant spatial correlation with the GFP-labeled E17K PH domain.

The epitope-targeting approach was demonstrated through the very demanding application of identifying a ligand specific for a single amino acid point mutation. With the synthetically-included alkyne amino acid residue to focus the library screening, there are no apparent protein structural requirements such as the need for binding pockets or other naturally addressable residues. Extending this approach towards the development of ligands specific to traditionally undruggable proteins, to post-translational modifications (e.g. phosphorylated or glycosylated epitopes), or to macrocyclic library architectures known to increase cell permeability[27] should be possible, and we are currently pursuing such routes. A working concept here is that the initial PCC agent ligand (the yleaf peptide in this current example) provides for the ability to bring different chemical interactions to a specific region of a specific protein. In this paper, we used the yleaf ligand to direct the covalent attachment of a payload to proximal amino acid side chains, or to bring a dye label to the protein target, or to bring a larger peptide framework that could disrupt the PH Domain - PIP3 interaction. The implication is that this epitope targeting strategy may permit the exploration of non-

traditional drugging approaches that can open up interesting targets, such as Akt[E17K], for selective inhibition.

Methods

Peptide Synthesis and Characterization

All peptides described were synthesized using standard FMOC SPPS peptide chemistry, described in more detail in the supplemental information. Structures and characterizations of all peptides can be found in Supplemental Figures S24 – S34.

Design of Epitope-Targeting Anchor/Target Peptide

The 33-mer epitope fragment sequence used in these studies corresponds to the peptide sequence: MSDVAIVKEGWLKKRGKY[Pra]KTWRPRYFLLKNDG. This 33-mer fragment was capped with an N-terminal biotin label for detection in the screen.

In Situ Click Screen for Initial Anchor Peptide (Supplemental Fig. S2)

Anchor peptide screens were performed using a library[28] containing an N-terminal azide click handle with varying carbon chain lengths – 2 carbon, 4 carbon and 8 carbon – for in vivo click with the Pra on the target 33-mer. The resin was first "precleared" by incubating it with the streptavidin-alkaline phosphatase and BCIP developer. It was then incubated with the epitope target for either 5 hours or overnight for the click reaction to occur, washed extensively, and developed using streptavidin-alkaline phosphatase and BCIP. Beads that turned purple were considered hits, picked, and sequenced via Edman degradation. More details on the screening process are available in the supplemental information.

Ligand-Directed Tosylate Labeling Experiments

For these assays, the yleaf anchor was appended with an N-terminal FMOC-piperidine-4-carboxylic acid as a linker on 300mg of rink amide resin in NMP using standard FMOC amino acid coupling techniques. The resin was equilibrated in anhydrous DCM and 250μL of 3-(chlorosulfonyl)benzylchloride was added with 450μL of DIEA for 30 minutes at room temperature. Then 250μL of 2-(2-(2-aminoethoxy)ethoxy)ethanol, 450μL of DIEA and 19mg DMAP in anhydrous DMC were added and incubated overnight, then 2eq Cy5 carboxylic acid (Lumiprobe) was coupled at 37°C overnight using standard FMOC coupling techniques.

In order to label the protein, 50μL of full-length GST-E17K Akt1 (SignalChem) was treated with $10\times$ molar excess of the anchor ligand with the tosylate dye label and incubated for two days at room temperature. The mixture was lyophilized after two days and then denatured by boiling in SDS-PAGE loading buffer. The labeled protein was run alongside an unlabeled control on an Any-KD gel from Biorad, then imaged on an Odyssey fluorescent gel reader at 700nm emission (Fig. 3b). After confirming that labeling had occurred, the gel was stained with BioSafe Coomassie blue stain (BioRad) and the blue protein bands were cut out. The gel pieces were trypsin digested using the Pierce In-gel Digest Kit. The tryptic fragments from both the unlabeled and labeled protein digests were lyophilized to concentrate them, taken up in 2μL of 50% H_2O/50% Acetonitrile and were analyzed by MALDI TOF MS

(Supplemental Figs. S8 and S9), then MALDI TOF/TOF MS to determine the exact labeling sites (Supplemental Figs. S10 and S11).

Images of anchor ligand in HEK-293T cells expressing PH Domains

The yleaf anchor ligand was synthesized with an N-terminal PEG_5, TAT (YGRKKRRQRR), and Cy5 dye (Fig. 2a). HEK-293T cells were grown in DMEM media supplemented with 10% FBS (both Invitrogen), 100× non-essential amino acid solution (Sigma), and PenStrep antibiotic (Invitrogen). Once the cells reached ~80% confluency, they were treated with trypsin to remove from the plate and split into small wells with a D-poly-lysine (BD) coverslip at approximately a 50% confluency in 1mL total volume. The cells were allowed to attach to the coverslips for approximately 24 hours, then were transfected to express either wildtype GFP-PH domain or E17K mutant GFP-PH domain proteins using XtremeGene HD transfection agent at a ratio of 3:1 transfection agent to DNA. Several wells were left untreated as no protein blanks (Figure S14). The cells were given 24 hours to express protein. They were then serum starved for one hour in DMEM media prepared as above, but without the FBS. After one hour, the Cy5-labeled anchor was added to the wells to a final concentration of 50nM. As the HEK-293T cells are expressing endogenous Akt1 protein, this level was adjusted to give the lowest background signal possible. After a one hour incubation with the peptide, the cells were washed once in serum starved media, then incubated one hour in serum starved media to wash out any excess peptide (see Figure S13 for a comparison of peptide with and without the one hour wash step). During this time, the cells were also treated with 10μg of Hoescht 33342 dye to stain the nuclei. The cells were then washed twice with cold PBS buffer, fixed with 10% Neutral Buffered Formalin Solution (Sigma) and glued onto microscope slides. Images were taken on a Zeiss LSM 510 Meta NLO with Coherent Chameleon confocal microscope. The laser intensity and gain were fixed for all pairs of images between wildtype and mutant samples to ensure that the differences seen were not artificially created.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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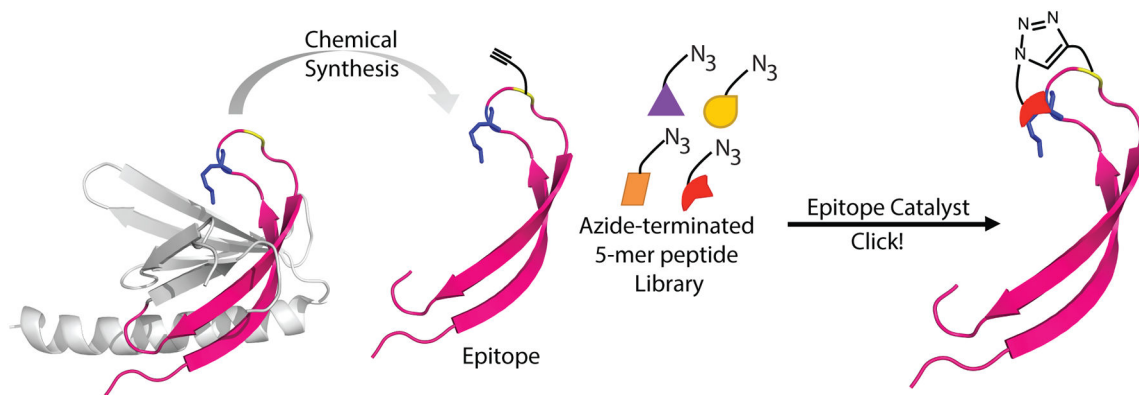
Figure 1. Epitope design strategy for *in situ* click screen

The full Akt1 PH Domain protein (grey) contains an E17K mutation (blue). To focus a chemical library screen on the region surrounding this mutation, only a portion of the PH Domain (pink) containing the E17K mutation was synthesized as a separate peptide epitope. Chemical synthesis of the epitope allowed for the substitution of the isoleucine at residue 19 (yellow), the most proximal side-chain to the E17K mutation, with an alkyne-containing propargylglycine amino acid. This substitution focused the azide library screening directly to the site of the mutation. This *in situ* click screen format allows for the determination of peptides that bind in close proximity to the E17K mutation. Triazoles formed between an azide-containing library peptide and the alkyne-substituted epitope must be catalyzed by a molecular recognition event, indicating that the library peptide binds strongly near the mutation.
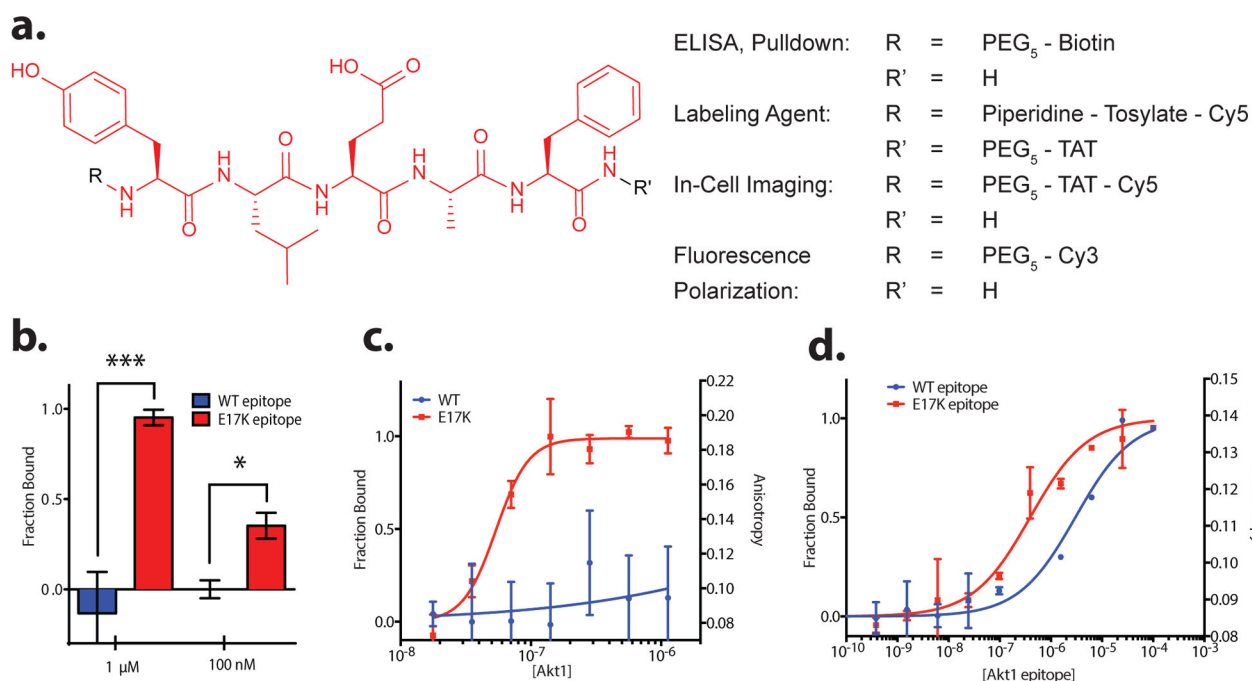
Figure 2. Anchor ligand structure and characterization

(a.) The peptide anchor ligand, yleaf, which was discovered using the epitope-targeting technique. The different sets of R groups indicate the modifications that were made to perform the various experiments described. (b.) Point ELISA for specificity obtained by immobilizing biotinylated yleaf anchor ligand onto a Neutravidin plate, then incubating with a 6 His-tagged epitope. This assay demonstrates the preference of the biotinylated yleaf anchor ligand for the E17K mutant epitope while immobilized on a surface. (c.) Binding curves of yleaf anchor ligand to the full-length WT or E17K mutant Akt1 proteins obtained in solution by fluorescence polarization. A saturation point for the WT ligand was not reached due to the prohibitively high protein concentration required. (d.) Full binding curves of yleaf anchor ligand to the WT or E17K mutant epitopes. These fluorescence polarization curves demonstrate the significant preference of the yleaf ligand for the E17K mutation in both the full protein assay (c) and the epitope peptide assay (d). All assays were performed in triplicate and averaged. Error bars indicate standard deviation.
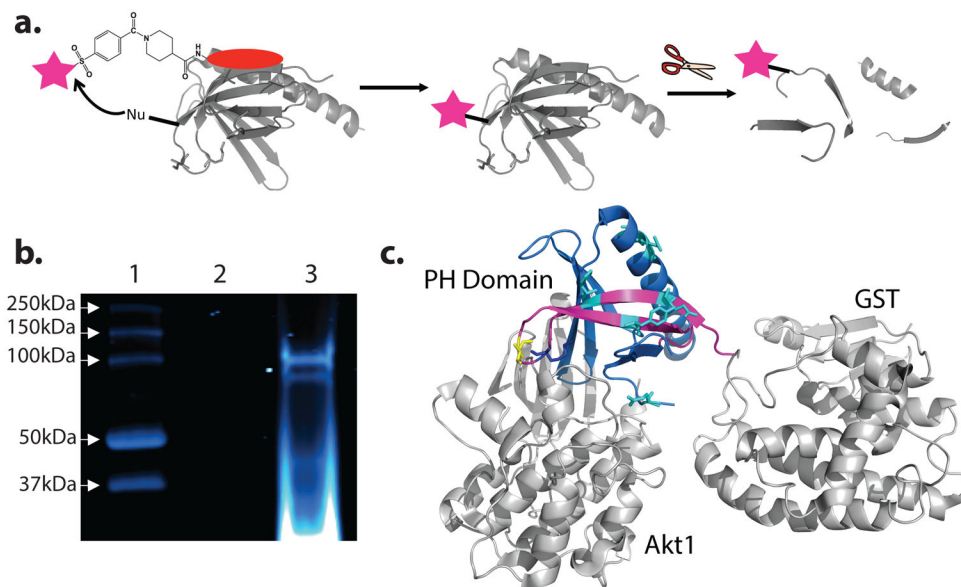
Figure 3. Ligand-Directed Labeling Experiments

(a.) Schematic of the ligand-directed labeling reaction. The yleaf anchor ligand (red) binds to the PH Domain (grey), and a nucleophilic amino acid (Nu) on the protein surface attacks the tosyl group, transferring the dye (pink star) onto the protein near the anchor binding site. The protein was then digested and analyzed via MALDI-TOF MS to discover fragments that have increased in mass by the weight of the dye. This experiment was designed to locate areas of the protein that are in close proximity to the bound yleaf anchor. (b.) Fluorescent gel image of GST-Akt1-PH Domain prior to trypsin digestion. Lane 1 is ladder, lane 2 is unlabeled protein, and lane 3 shows fluorescently-labeled protein (and excess fluorescent ligand at the bottom), confirming that the reaction has taken place. (c.) This Pymol-assembled fusion protein highlights the PH Domain (blue) and the 33-mer epitope fragment used for screening (pink). Sites containing a label on the GST-Akt1-PH Domain, as identified by MS analysis, are indicated in cyan. These results indicate that the yleaf anchor is binding only in the area of the epitope, and does not have off-target binding interactions with any other part of this large protein.
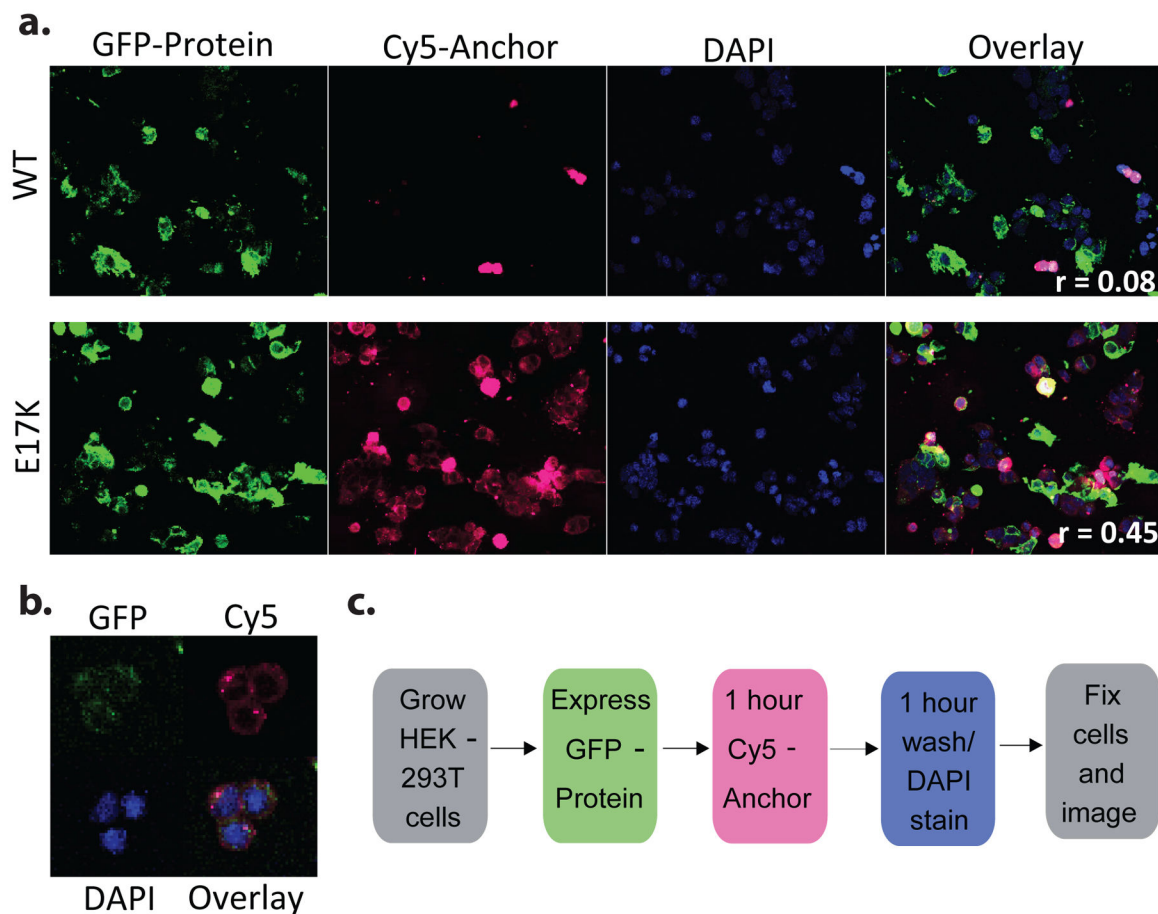
Figure 4. Images of the Cy5-yleaf-PEG$_5$-TAT peptide ligand in cells

(a.) The PH Domain was fused to green fluorescent protein (GFP) in order to visualize expression. The Cy5-yleaf-PEG$_5$-TAT was added to live cells expressing the protein, washed extensively, and imaged. The panel shows the spatial map of GFP expression in green. The second panel shows the spatial map of the dye-labeled yleaf ligand, and the third panel shows DAPI-stained nuclei. The r values indicate the Pearson correlation coefficient between the GFP protein and the Cy5 anchor. As demonstrated by the difference in both the Cy5 signal and the Pearson correlation coefficients, the anchor ligand is retained in E17K mutant-expressing cells to a significant degree more than in the wildtype cells, indicating ligand binding and selectivity even in a complex cellular environment. (b.) Zoomed in image of cells expressing E17K mutant protein with anchor ligand showing significant Cy5 signal in the areas of the cells containing GFP-tagged protein. (c.) Schematic of the experimental design for the imaging experiments. The HEK-293T cells were transfected to express GST-tagged WT or E17K mutant protein, then incubated with the CPP – anchor – Cy5 peptide. These cells were then incubated for one hour in media, then fixed and imaged.
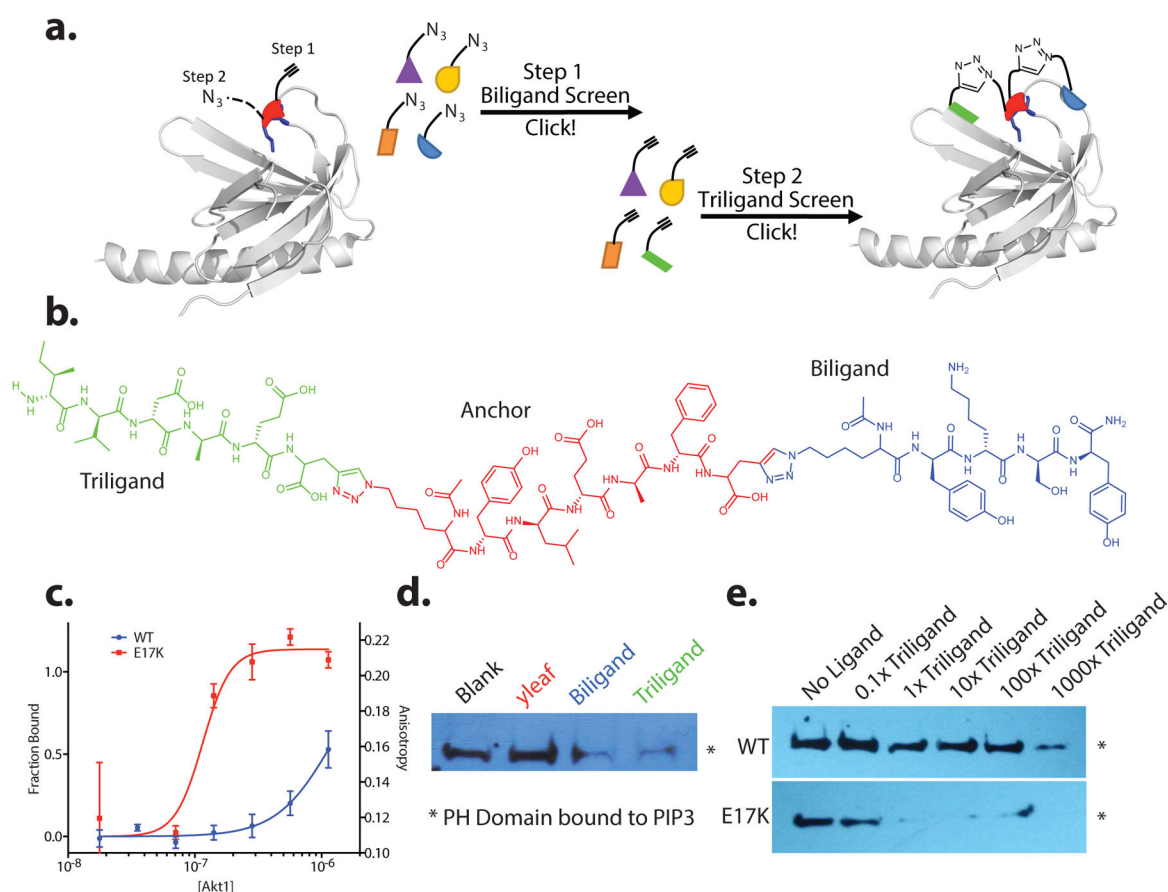
Figure 5. The PCC agent triligand development strategy, structure, and inhibition data
(a.) The yleaf anchor ligand appended with a C-terminal alkyne was screened against an azide-terminated library in the presence of the full-length PH Domain to identify a biligand. The biligand was then appended with an N-terminal azide and screened against an alkyne library to identify a triligand. (b.) The final triligand structure, which is color-coded to highlight each segment. (c.) Fluorescence polarization assays demonstrating triligand affinity for the WT (blue) and E17K (red) full-length proteins, indicating that the E17K mutant selectivity is retained by the triligand. All conditions were performed in triplicate and averaged. Error bars indicate standard deviation. (d.) Inhibition assays were performed with PIP3-coated resin that was incubated with constant concentrations of E17K mutant protein and each ligand. The blank measures the binding of the E17K PH Domain to PIP3 with no ligand present. The decreased binding of the protein in the presence of the biligand and triligand indicates inhibition. (e.) Expanded inhibition assay with varying concentrations of triligand (indicated here as the molar ratio with respect to the protein) incubated with WT or E17K proteins. The only slight drop-off in WT binding indicates little inhibition of this protein, while significant inhibition is shown against the E17K variant.