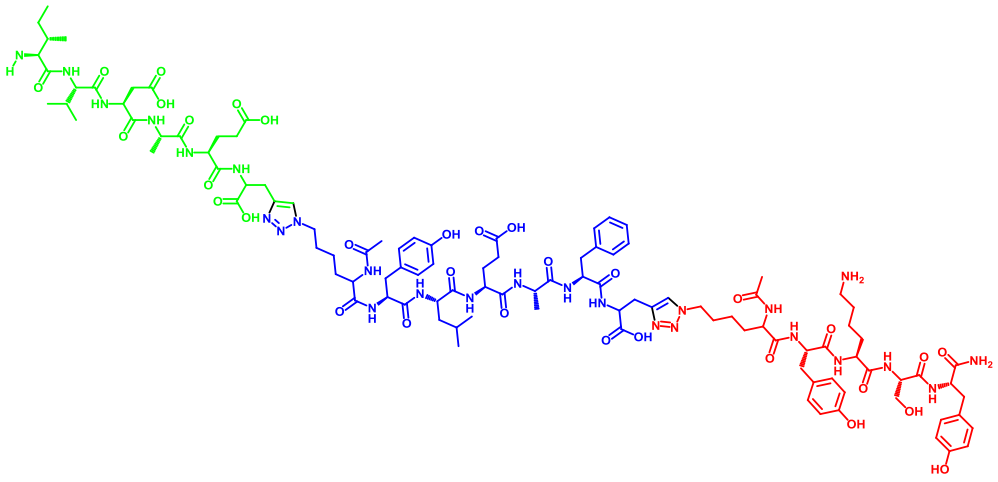
Chapter 4

Expansion of E17K Selective Anchor Ligand into an Inhibitor



4.1 Introduction

As described in previous chapters, there is increased interest in compounds that can selectively inhibit a disease-associated mutant protein target while sparing the wildtype (WT) variant[1]. PHD inhibiting compounds[2,3] with selectivity for the E17K variant of Akt have not been reported. The specificity of the yleaf anchor ligand described in Chapter 3 for the E17K Akt1 in live cells, coupled with the proximity of the E17K mutation to the PIP3 binding site, prompted the consideration of further developing this PCC Agent into a compound capable of blocking the E17K PH Domain interaction with its PIP3 substrate. The yleaf anchor peptide itself did not exhibit evidence of inhibition (**Figure 4-11**). It was reasoned that a similarly targeted, but bulkier PCC Agent might serve as a steric blocker of the PH Domain - PIP3 interaction. To this end, two cycles of iterative *in situ* click chemistry screens, as described in Chapter 2, were executed in order to develop the yleaf ligand into a biligand and then a triligand, which was capable of successfully blocking this binding interaction. This showed that these larger PCC Agents could serve as highly selective inhibitors of E17K Akt1 by blocking binding of the Pleckstrin Homology Domain of Akt1 to the PIP3 substrate.

4.2 Materials and Methods

4.2.1 Screen for Biligand Peptide

The anchor determined above – yleaf – was scaled up with a biotin on the N-terminus for detection, a PEG_5 linker between the biotin and the peptide, and a d-propargylglycine (Pra) on the C-terminus as the *in situ* click handle (Biotin-PEG_5-yleaf-Pra). Screens were performed using a library with 100% Met coupled at the C-terminus for potential MALDI TOF/TOF sequencing. The library consisted of a comprehensive 5-mer containing 18 unnatural D-amino acids, excluding Met and Cys due to stability reasons. The N-terminus of the library was appended with an azide click handle with a 4 carbon chain (Lys(N_3))– for in vivo click with the Pra on the anchor peptide.

Screens were performed with 300mg of dried library beads swelled at least six hours in 1x TBS (25mM Tris-Cl, 150mM NaCl, 10mM $MgCl_2$, pH = 7.5) buffer.

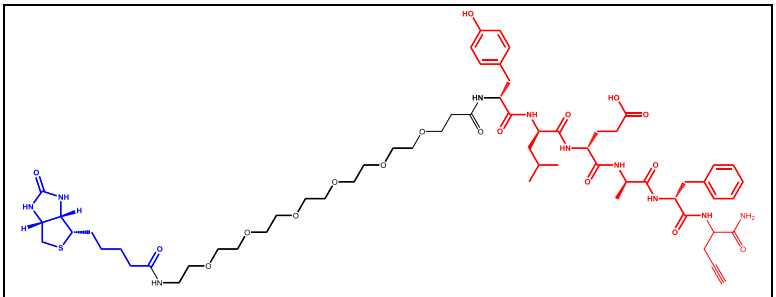


Figure 4-1: Biotin – PEG5 – yleaf – Pra: *Biligand screens were performed using the yleaf anchor (red) with a PEG5 spacer (black) and a biotin tag (blue). MALDI-TOF MS, expected [M+Na] 1319.62, observed 1319.89.*

Preclear (**Figure 5a**):

Swelled library beads were blocked overnight in 5% w/v dried non-fat milk in 1x TBS, then washed with 1x TBS three times. The beads were incubated with a 7.15µM solution of the anchor peptide - Biot-PEG_5-yleaf-Pra for one hour, then washed three times with 1xTBS. Five milliliters of a 1:10,000 dilution of streptavidin-alkaline phosphatase conjugate in 0.5% milk in TBS was added to the beads, and incubated with shaking at room temperature for one hour. The beads were washed with a high-salt TBS buffer (1x TBS plus 600mM NaCl) three times, then let shake in high salt buffer for one hour. The beads were then washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM $MgCl_2$, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13µL BCIP and 26µL NBT to the beads in a 150mm polystyrene tray. After one hour, the purple beads were removed by pipette and discarded. The remaining beads were incubated in NMP for four hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with TBS and blocked overnight in 5% milk.

Target Screen (**Figure 5b**):

The clear beads remaining from the preclear were blocked in 5% milk in 1x TBS for two hours. They were then washed three times with 1x TBS. A pre-incubated solution of E17K mutant

protein (715nM) and anchor ligand (7.15µM) in 3mL of 0.5% milk was added to the blocked library beads and incubated for either five hours or overnight to allow an *in situ* click reaction to occur. In the morning, the beads were washed three times with 1x TBS, then incubated with a 1:4,000 dilution of an anti-His Alkaline Phosphatase conjugated antibody (Abcam) in 0.5% milk for one hour. The beads were then washed three times with a high salt TBS, then incubated on the shaking arm for one hour with the high salt buffer. They were then washed three times with BCIP buffer, and developed as previously. Hit beads turned purple and were removed and washed in NMP for four hours to decolorize, then guanidine-HCl to denature and remove and remaining protein. The beads were then washed ten times with water and blocked in 5% milk overnight.

Off-Target Anti-Screen (**Figure 5c**):

The beads from the target screen were washed three times with 1x TBS, then incubated with the off-target, wildtype PHD protein in 0.5% milk for one hour on the shaking arm at room temperature. The beads were washed three times with 1x TBS, then incubated with a 1:4,000 dilution of Anti-His Alkaline Phosphatase conjugated antibody in 0.5% milk for one hour at room temperature. They were then washed three times with high salt buffer and let shake for one hour in high salt at room temperature before being washed three times with BCIP buffer and developed as previously. The beads that turned purple bind to both mutant and wildtype protein or to the anti-his antibody, and were set aside. The beads that remained clear were picked and washed with guanidine-HCl to remove any bound proteins, and blocked in 5% milk overnight.

Product Screen (**Figure 5d**):

The beads specific for the mutant PH domain were washed three times with 1x TBS. They were then incubated with a 1:10,000 dilution of Streptavidin – Alkaline Phosphatase conjugate in

0.5% milk for one hour. The beads were washed three times with high salt TBS, then let shake for one hour with high salt buffer before being washed three times with BCIP buffer and developed as previously. The beads that turned purple contained the anchor peptide covalently bound to the bead, and had formed a protein-catalyzed in situ click reaction. These beads were collected and stripped with guanidine-HCl for one hour, washed ten times with water, and sequenced via Edman degradation as per the anchor candidate hits. There were 22 total hit beads.

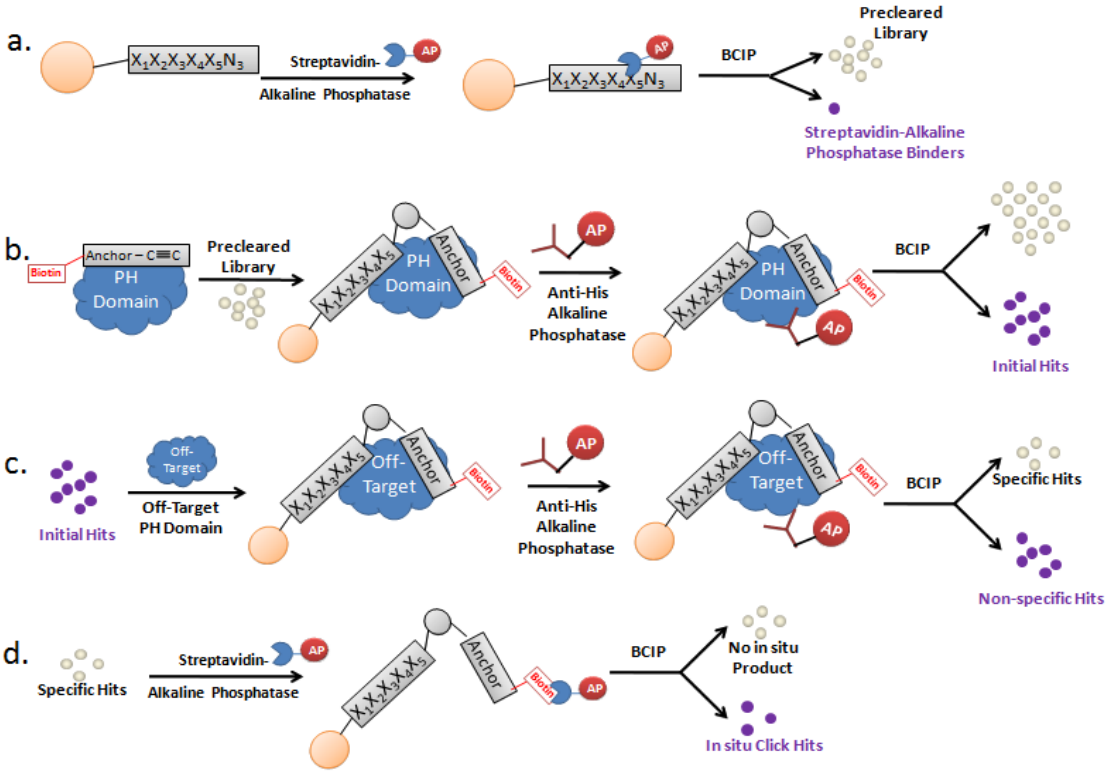


Figure 4-2: Screening Strategy for Biligand Determination: (a) *Preclear: Any library beads that bind to the screening reagents are removed. (b) Target Screen: Precleared beads are incubated with the target and anchor ligand and allowed to "click." The presence of the his-tagged PH Domain is detected via an anti-His antibody. (c) Anti-Screen: Hit beads from the target screen are incubated with the off-target PH Domain and anti-his antibody. These hit beads bind to both the target and off-target (WT and E17K mutant). (d) Product Screen: The beads are probed with streptavidin-alkaline phosphatase to determine which contain the click product and, thereby, have shown biligand formation.*

4.2.2 Streptavidin-Agarose Immunoprecipitation (Pull-down) Assays to Test Biligand Candidates (Figure 4-6)

Four biligand candidates were segregated based on their hydrophobicity and sequence homology using principal component analysis as for the anchor ligands screened in Chapter 3. Biligands were synthesized by coupling the 2° ligand onto Rink Amide Resin on the Titan peptide synthesizer. The amide group on the end of the Lys(N_3) was capped by shaking the resin with 2mL acetic anhydride, 2mL NMP and 0.5mL DIEA for three times for 10 minutes each time, then washed with NMP. FMOC-Propargylglycine-Otbu (Pra) was clicked onto the Lys(N_3) on the 2° ligand by incubating 2 equivalents of the Pra amino acid with 2 equivalents of CuI and 2 equivalents of ascorbic acid with 1 equivalent of azide on the resin in 20% piperidine/NMP for 3

hours. The resin was washed five times with 4 mL of a chelating solution consisting of 1g sodium diethyldithiocarbamate in 20mL NMP and 1mL DIEA. The anchor was then built onto the 2° ligand on bead, and an N-terminal PEG_5-biotin tag were added.

Assays were performed exactly as for the anchor ligands, except for two key differences. The biligand assays were performed using 6ug of GST-tagged PHD protein, instead of the untagged PHD that was used in the anchor pull downs. The pull-downs were also conducted out of 1% serum in 1x TBS, as opposed to just 1x TBS. This is a much more demanding assay that tests not only the affinity, but also the selectivity of the ligands because they need to be able to bind to their target protein in a very complex medium. The performance of the biligand candidates in this challenging assay indicates that they have little off-target interactions with a multitude of other proteins, and would perform well in the condition in which they will need to be detecting these ligands – in cells and out of cell lysates.

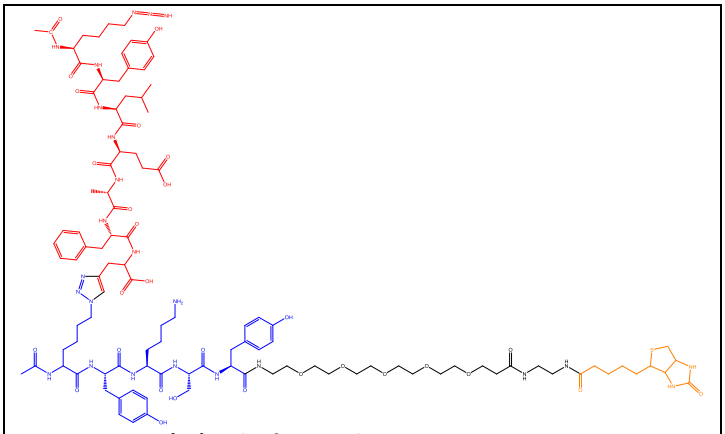


Figure 4-3: Lys(N_3) - yleaf - Tz - yksy - PEG_5 – Biotin: *MALDI-TOF MS expected 2248.1, observed [M+H] 2249.1.*

4.2.3 Screen for Triligand Peptide

The best biligand candidate as determined in immunoprecipitation assays – yleaf–(triazole, Tz)-yksy - was scaled up (**Figure 4-3**) with a C-terminal PEG_5-biotin for detection during the assay by coupling PEG_5 onto NovaTag Biotin resin (EMD). Lys(N_3)-yksy was coupled onto the resin on the Titan peptide synthesizer, and FMOC-Pra-Otbu was clicked on as previously. The remaining "Lys(N_3)-yleaf" portion was then synthesized on the Titan, the Lys(N_3) serving as the click handle for the triligand screen. The screens (**Figure 4-4**) were completed using a random 5 D-amino acid library with a C-terminal D-propargylglycine alkyne click handle, and were otherwise performed exactly as for the biligand, including all concentrations. Three hit beads were discovered in this screen, and the first hit had a nonsensical sequence, so it could not be used. Both of the usable hits were scaled up and tested for binding using ELISA assays.

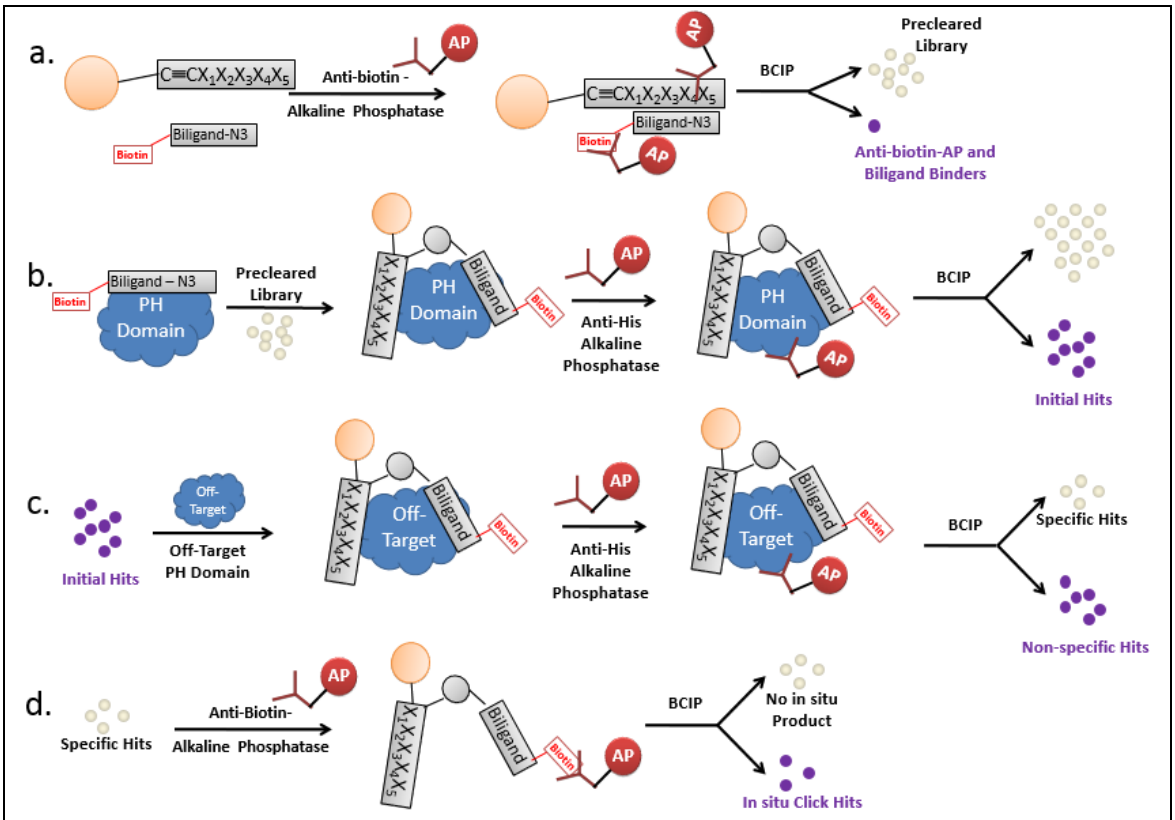


Figure 4-4: Screening Strategy for Triligand Determination: *(a) Preclear: Any library beads that bind to the screening reagents are removed. (b) Target Screen: Precleared beads are incubated with the target and biligand and allowed to "click." The presence of the his-tagged PH Domain is detected via an anti-His antibody. (c) Anti-Screen: Hit beads from the target screen are incubated with the off-target PH Domain and anti-his antibody. These hit beads bind to both the target and off-target (WT and E17K mutant). (d) Product Screen: The remaining beads are probed with streptavidin to determine which contain the click product and, thereby, have shown triligand formation.*

4.2.4 Full ELISA Curves for Ligands

The full curve ELISAs were obtained using streptavidin coated ELISA plates (Pierce). The ligands – anchor, biligand, two triligand candidates (ivdae and iryrn) and "eflya" scrambled anchor peptide blank - were laid down on the plate at a concentration of 1µM for one hour. Two lanes of each ligand were used on the plate for both proteins – WT and E17K GST-PHD. The plates were blocked with 5% BSA for two hours. Dilutions of both WT and E17K GST-PHD proteins were made in 0.5% BSA in 1xTBS ranging from 1µM down to 0.5nM by serially diluting 1:2 down a series of 8 samples. For each ligand, a no protein blank was also used. The proteins were incubated with

the blocked plate for one hour, washed three times with 1xTBST + 0.5% BSA and tapped dry, then detected with a 1:10,000 dilution of an HRP conjugated anti-GST ab. The plate was again washed three times with 1xTBST and tapped dry. It was developed with a 1:1 solution of TMB substrate, and development was stopped with 1M H_2SO_4 and read on a plate reader. The curves were plotted by normalizing the signal by the blank wells, and were fitted by a Hill function in GraphPad using a common saturation and slope (Bmax = 1.466 +/- 0.03, h = 0.7383 +/- 0.025).

4.2.5 Point ELISA Assays for Triligand Binding to Akt1 and Akt2 Wildtype and E17K Mutant Proteins

These assays were conducted to test the binding of the triligand to the off-target Akt2 wildtype and mutant proteins. For this assay, all samples were taken in triplicate for statistical purposes. Triligand peptide was first immobilized onto Neutravidin ELISA plates (Pierce) for one hour. A scrambled anchor peptide, eflya, was used as the no-ligand blank, as the GST proteins have significant background binding to a blank Neutravidin plate. The plates were then blocked with 5% BSA overnight. Protein was laid down on the plate at a concentration of 100nM for samples wells and the blank, scrambled peptide wells. GST protein alone (Abcam) was also incubated with the triligand and scrambled peptide as a control. The proteins were incubated for one hour, then washed three times with 1xTBST. The protein was then detected with 1:10,000 anti-GST mouse mAb (Fisher, #MA4-004) for one hour, washed three times with 1xTBST, and developed with a 1:1 mixture of TMB substrate for ten minutes. The samples were plotted by subtracting the blanks and averaging the sample wells. The highest signal was considered 100% binding, and the other samples were normalized accordingly.

4.2.6 PIP3 Agarose Inhibition Assays

PIP_3 Agarose beads (Echelon) were used to detect for the inhibition of PH Domain binding to its substrate, PIP_3, upon incubation with the anchor candidate peptide ligands. To test the inhibition of each of the ligands, anchor biligand and triligand, 20µL of resin slurry was added to each of four tubes, and washed three times with 1x TBS. Protein, 2µg (234nM) of E17K mutant, was pre-incubated for one hour at room temperature with either DMSO (no peptide ligand blank), anchor, biligand or triligand at 2.38µM (10x in relation to protein) in 200µL of 1x TBS. For the control, mutant PH Domain was incubated with 1x TBS and 1µL DMSO to mimic the ligand conditions. These protein samples were then added to PIP_3 agarose in a Spin-X tube and incubated at room temperature for two hours. The resin was washed three times with 1x TBS with 0.25% IGEPAL CA-630, spun out to dry completely, then denatured with 50µL 3x SDS gel loading buffer for 10 min at 95°C. The gel loading buffer was spun out of the resin and detected via western blot as per the streptavidin – agarose pull downs. Inhibition was indicated by a decrease in the amount of PH Domain that was pulled down by the resin.

Expanded inhibition blots with either wildtype of E17K mutant protein were performed in a similar fashion. Twelve tubes of 20µL of PIP3 agarose were washed three times with 1x TBS. 2µg of either wildtype or mutant PHD-GFP protein (234nM) in 200uL 1xTBS were pre-incubated for 30 minutes with differing concentrations of triligand: 0.1eq (23.4nM), 1eq (234nM), 10eq (2.34µM), 100eq (23.4µM), and 1000eq (234µM). The protein and triligand solutions were then incubated with the PIP3 resin for 2 hours at room temperature. The resins were washed, eluted, and blotted as per all PH Domain western blots.

4.3 Results and Discussion

4.3.1 Biligand Development

It was hypothesized that a similarly targeted, but bulkier PCC Agent might serve as a steric blocker of the PHD-PIP3 interaction. To this end, two cycles of iterative *in situ* click chemistry screens were designed to develop the yleaf ligand into a biligand and then a triligand. To identify the biligand (the first iterative cycle, **Figure 4-2**), the yleaf ligand was modified to present an alkyne at the C-terminus, and a PEG_5-biotin group at the N-terminus (**Figure 4-1**). This modified ligand (called an anchor ligand) was then co-incubated with an alkyne-presenting OBOC library and the (unmodified) E17K PHD. Successful hits are those in which the E17K PHD 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 (**Figure 4-2**). Those hits are candidate 2^o ligands (**Table 4-1**). As with the discovery of the anchor ligands, the biligand hits are clustered according to their hydrophobicity and sequence homology using principle component analysis. These hits cluster into groups, as seen in **Figure 4-5**, and unique clusters are circled. Hits from these different clusters, thus representing the sequence diversity of the screen, were chosen to be scaled up and tested for both affinity and selectivity to the E17K mutant PH Domain protein. For testing, the 2^o ligand candidates are appended to the yleaf anchor ligand via a Cu(I) catalyzed 1,4 triazole, to mimic the triazole formed by the protein target during the screen to form a biligand. The biligand candidates are then subjected to immunoprecipitation assays (pull down) to identify a candidate biligand in a manner that is similar to what was done to identify the original yleaf ligand (**Figure 4-6**). The biligand in lane 6 in **Figure 4-6**, yleaf – yksy, shows the highest affinity for the E17K mutant protein while still maintaining the selectivity over the wildtype protein. This sequence was chosen as the biligand.

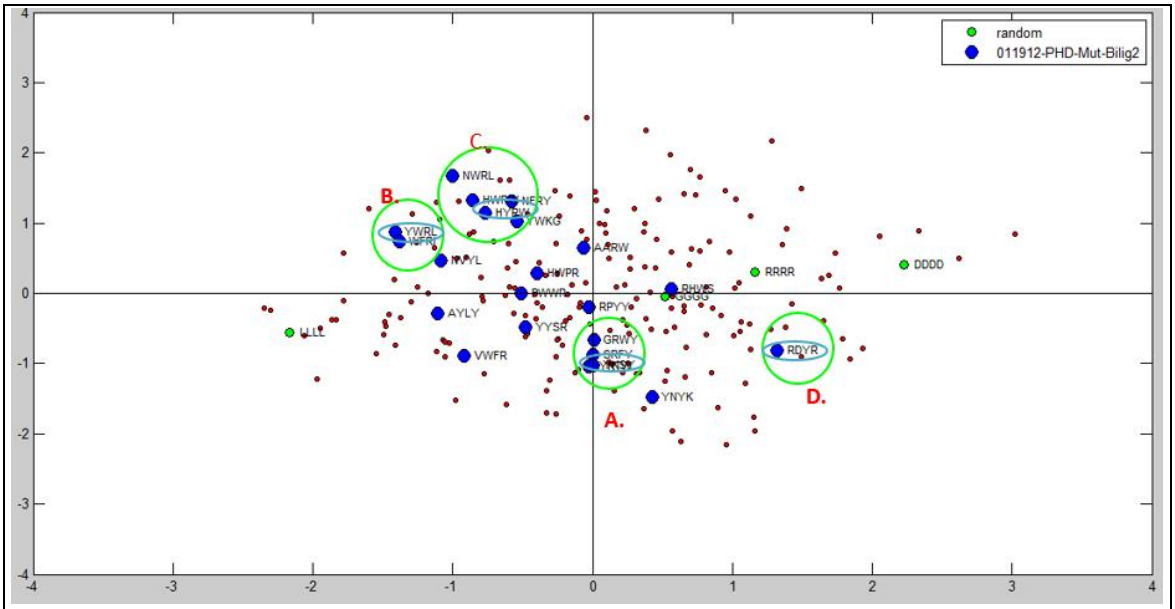


Figure 4-5: Clustering of Biligand Sequence Ligands by AA Similarity: *Hit sequences from the biligand screen were analyzed by their hydrophobicity and sequence homology using principal component analysis. Clusters circled in green indicate clustered regions, and the cyan circles indicate the peptide that was selected and scaled-up as a possible biligand sequence. The potential biligand sequences that were tested are: yleaf-ywrl, yleaf-yksy, yleaf-rdyr, and yleaf-hyrw, where "yleaf" is the anchor ligand and the "-" indicates the location of the triazole linkage.*

Table 4-1: Hit Sequences from Biligand Screen

Az4	h	w	p	r
Az4	n	v	y	l
Az4	h	y	r	w
Az4	r	d	y	r
Az4	y	n	y	k
Az4	y	k	t	w
Az4	s	r	f	y
Az4	y	k	s	y
Az4	y	y	s	r
Az4	r	h	w	s
Az4	p	w	w	r
Az4	n	f	r	y
Az4	y	w	r	l
Az4	y	w	k	G
Az4	a	y	l	y
Az4	h	w	r	w
Az4	n	w	r	l
Az4	a	a	r	w
Az4	G	r	w	y
Az4	w	f	r	i
Az4	r	p	y	y
Az4	v	w	f	r

Figure 4-6: Pull-down Assay Results for Biligand Candidates: *Note that all of the biligand candidates improve upon the binding of the anchor ligand, but yleaf-yksy shows the greatest signal in binding the E17K protein and the lowest in binding the WT protein. This biligand was chosen as the candidate biligand and carried on to triligand screening.*

4.3.2 Triligand Development

Once a candidate biligand has been identified, it is then similarly modified to form a new anchor ligand (**Figure 4-3**), which is then similarly screened (**Figure 4-4**) to identify a triligand. There were only 3 hit sequences from this triligand screen, and one sequence was not able to be called due to low signal and an irregular sequence, seen in **Table 4-2**. Because there were only two valid hits from this screen, both were scaled up and tested in a full-curve ELISA assay (**Figure 4-7**). The iryrn triligand showed significantly improved affinity for the E17K PH Domain, but this benefit was offset by the drastic increase in affinity for the wildtype PH Domain. The ivdae triligand maintains the selectivity seen in the anchor ligand and was chosen as the triligand, whose structure is shown in **Figure 4-8**.

Table 4-2: Hit Sequences from Triligand Screen

G	l	-	-	m	-
i	r	y	r	n	Pra
i	v	d	a	e	Pra

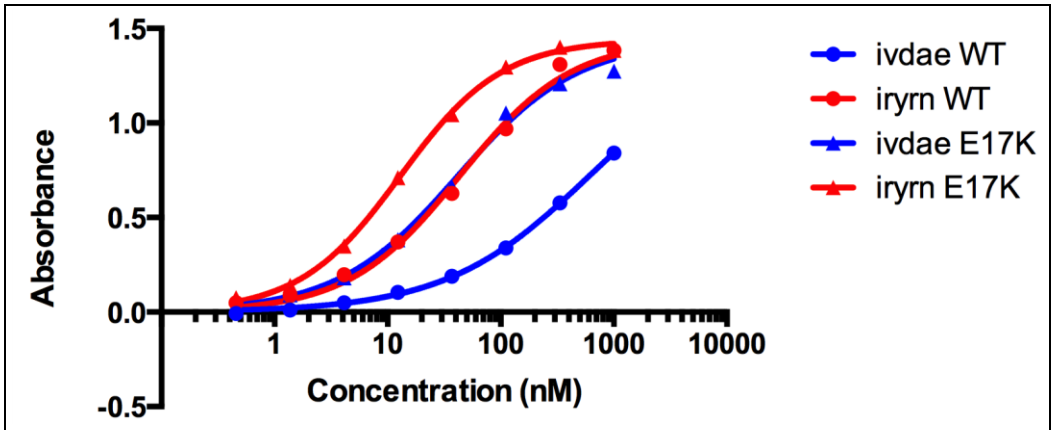


Figure 4-7: ELISA assays for affinity and selectivity of triligand candidates. *The iryrn triligand seen in red shows a significant affinity increase, but loses much of the selectivity for the E17K mutant PH Domain (triangles). The ivdae maintains the selectivity and affinity of the anchor ligand, and was carried forward as the triligand.*

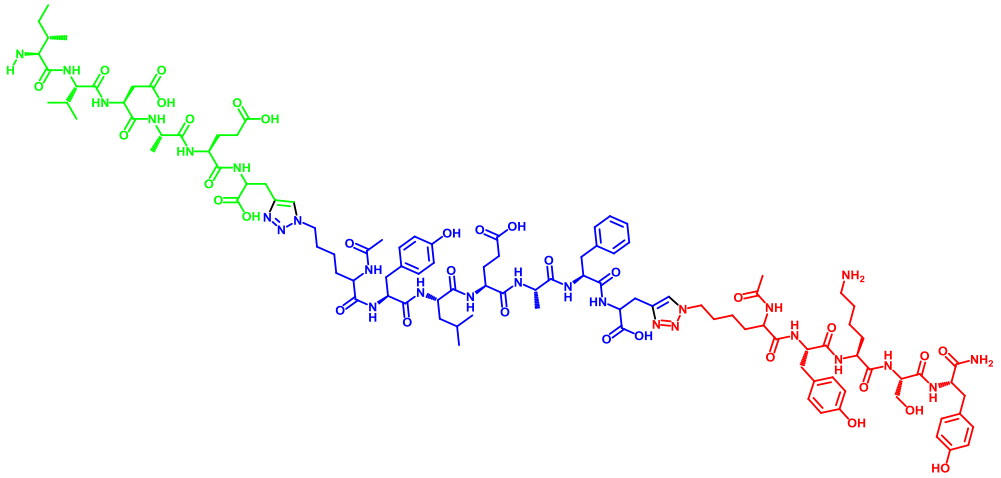


Figure 4-8: Structure of final triligand: ivdae – Pra – Lys (N_3) – yleaf – Pra – Lys (N_3) - yksy

Binding curves that compare the yleaf ligand with the biligand and triligand PCC agents are shown in **Figure 4-9**. Likely because the expanded binding site for these larger PCC agents grows away from the location of the E17K point mutation, increasing the affinity while maintaining the selectivity of the final PCC agent upon the addition of these secondary and tertiary arms proved challenging. For example, the biligand exhibited an increase in affinity for the E17K mutant protein, but this is offset by an even larger increase in affinity for the WT protein. However, at the triligand stage, the selectivity for E17K Akt1 relative to WT Akt1 is largely recovered.

Additionally, there is a slight preference for E17K Akt1 relative to E17K Akt2 (**Figure 4-10**). The homology of the PHD between these isoforms is 79%, as calculated by a pairwise sequence analysis using Blast2Seq between the Akt1 E17K structure (PDB ID: 2UZR) and the Akt2 PH Domain structure (PDB ID: 1P6S). The binding curves of 4B yield EC_{50} values for the E17K Akt1 of 61nM, 19nM, and 45nM for the yleaf ligand, the biligand and the triligand, respectively.

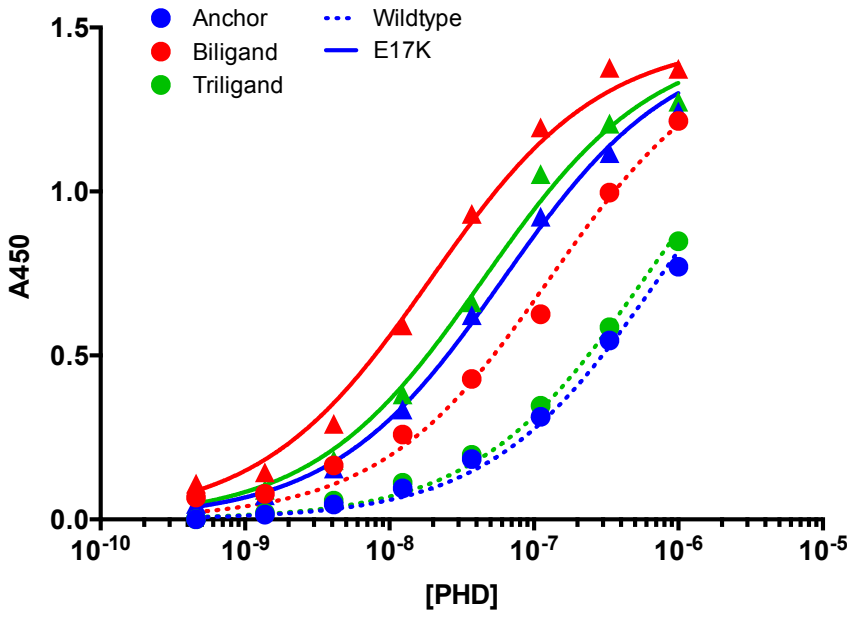


Figure 4-9: Full ELISA curves of Anchor, Biligand, and Triligand. *The ELISAs show that the biligand (red) has an increased affinity for the E17K PH Domain from the anchor ligand (blue), but also for the wildtype protein. The triligand (green) restores the affinity and selectivity of the anchor ligand.*

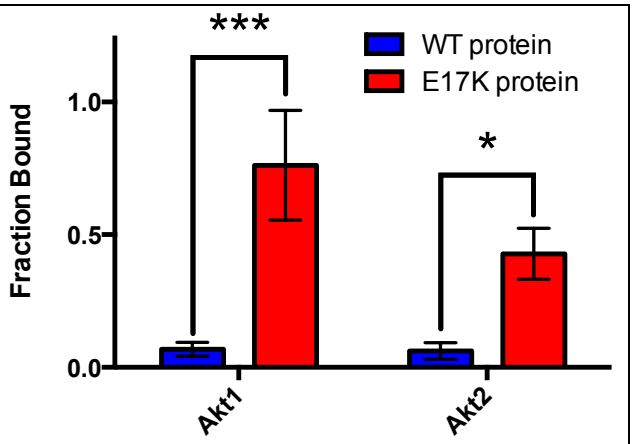


Figure 4-10: Point ELISA of Triligand binding to Akt1 and Akt2. *The binding to both the wildtype and E17K mutant PH Domains was tested for both Akt1 and Akt2. The triligand maintains the selectivity for the E17K in both proteins, and shows only a slight preference for the Akt1 isoform.*

4.3.3 Inhibition Assays

The yleaf ligand, the biligand, and the triligand were all tested for their ability to block the E17K PHD binding with PIP3 (**Figure 4-11**). For this test, PIP3-coated resin (Echelon Biosciences) was used to mimic the PHD interaction with the cell membrane, and could be used to bind the protein as in an immunoprecipitation assay[3]. 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 capture agent was used to show baseline binding of the protein to the PIP3 resin. As mentioned above, the yleaf ligand produced no change in E17K binding ability, but both the biligand and triligand exhibited the ability to block the PHD-PIP3 interaction, with the triligand being the most effective (Fig 4D). In an expanded study, we compared the amount of E17K and WT PHD binding relative to the amount of added triligand. This assay shows significant selective inhibition (by around 10^3) of the E17K mutant relative to the WT.

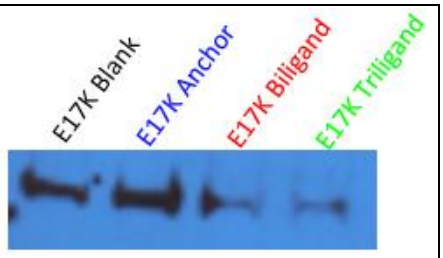


Figure 4-11: PH Domain membrane binding in the presence of each ligand. *The first lane, blank, shows the amount of protein binding to PIP3 with no ligand. Anchor ligand in respect to protein shows no decrease in binding, but binding is starting to be blocked with the biligand and triligands.*

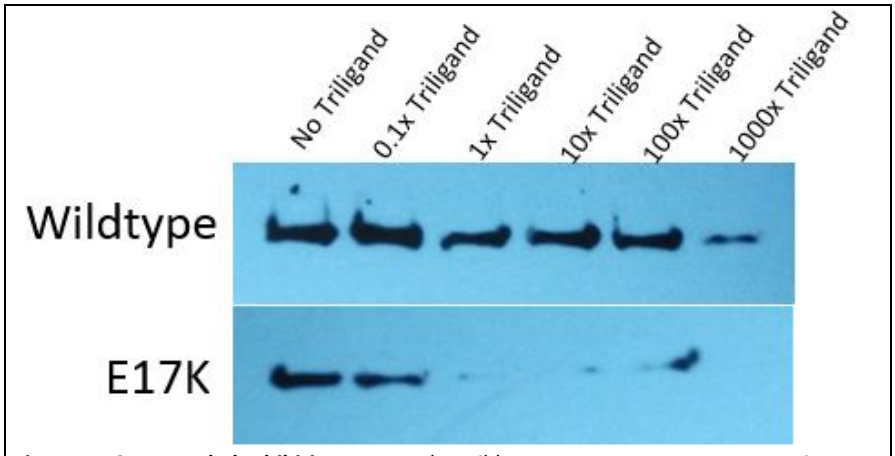


Figure 4-12: Expanded Inhibition Assay. *The wildtype or E17K mutant PH Domains were incubated with varying amounts of triligand. The wildtype protein shows very little reduction in binding until 1000x triligand to protein. The E17K PH Domain, however, shows a significant drop in binding almost immediately.*

4.4 Conclusions

The epitope-targeting strategy allowed for such selective targeting of the E17K mutant PH Domain that selectively blocking the oncogenic activation of this protein was the logical next step. Bulking up the original anchor into a triligand covered more of the PH Domain PIP3 binding pocket, therefore blocking PIP3 binding. By ensuring that the triligand maintained its selectivity for the E17K mutant PH Domain, the ligand demonstrates significantly reduced interference with the wildtype PH Domain protein. The blocking of the E17K protein binding to the cell membrane demonstrates the ability of this click-focused epitope screening technology to produce not only selective binding agents, but also potential therapeutics that could show significantly decreased toxic side-effects due to the reduction in off-target and healthy-cell interactions.

4.5 Acknowledgements

Ying Qiao Hee and Jeremy Work made many of the peptides used in these experiments. Blake Farrow graphed several of the images in GraphPad and fitted the curves.

4.6 References

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