

Chapter 4: Characterization of protein capture agents developed against Akt2

4.1 Introduction

In chapter 3, we discussed the chemical epitope targeting strategy and the development of multivalent ligands against the Akt2 protein through consecutive in situ target catalyzed click reaction. During the development of the various peptide arms from the 1° ligand to the 3° ligand, the resulting ligand is validated in at least one assay and the peptide arm showing the best affinity and specificity is further used in the next step of multivalent ligand development. In this chapter we describe the assays used to choose the peptide arms, and characterize the final developed ligands in additional assays. We also explore the effect of the ligands on the kinase activity of the protein. The relative binding affinities of the ligands for Akt2 were determined through ELISA assays. The triligand candidates had low nanomolar binding affinities. The values are in agreement with the absolute values obtained through SPR experiments. Selectivity or specificity of each ligand were determined against the epitope and against the full protein. We demonstrate that the ligands bind selectively to the target C-terminal 32-mer polypeptide fragment of Akt2. Finally, we explored if the developed ligands have any effects on the kinase activity of the Akt2 protein. Remarkably, the two triligands, sharing the same biligand fragment, were found to have opposite effects on the kinase activity of the Akt2 protein. While one of the ligands enhances the kinase activity of the protein, the other ligand inhibits the kinase activity.

4.2 Materials and Methods

4.2.1 Materials

Mouse anti biotin monoclonal antibody- Horse Radish Peroxide conjugate was purchased from Cell Signaling Technology. Anti His₆ mouse monoclonal antibody, Akt2 phospho S474 antibody and goat anti mouse IgG-Horse Radish Peroxide conjugate were bought from Abcam. Anti Akt (pan) rabbit antibody (11E7) and anti rabbit antibody- Horse Radish Peroxide conjugate,

used in Western blot and dot blot experiments, were purchased from Cell Signalling Technology. Biacore chip, reagents and buffers used in the SPR experiment were bought from GE Healthcare. High capacity Streptavidin coated 96 well plates were obtained from Thermo Scientific. 96 well plates were bought from Nunc. TMB substrate for HRP, used in ELISA assays, was bought from KPL.

4.2.2 Methods

4.2.2.1 Peptide synthesis

Synthesis of His₆ tagged target phosphopeptide and off target peptide sequence:

The phosphorylated version of the target sequence, amino acids 450-481 of Akt2, with pS474, and the 33mer off target peptide (Akt2 amino acids 346-378) were synthesized on Rink Amide MBHA resin, using standard Fmoc based SPPS protocols. Fmoc-NH-(PEG)₂-OH was then coupled with each peptide. Then six successive couplings were done with Fmoc-L-His(Trt)-OH. The peptides were cleaved by TFA/TES/ddH₂O, precipitated in cold ether and purified using a gradient of water and acetonitrile and 0.1% TFA on the RP-HPLC. Target phospho-peptide: Calculated mass: 4858.2 Observed mass: 4858.1 Scrambled phospho-peptide: Calculated mass: 5071.5 Observed mass: 5071.5

4.2.2.2 ELISA for *mono-L* and *bi-L*

On a nunc 96 well Immunosorp plate, 1 µg of active Akt2 per well was immobilized for an hour in carbonate buffer (pH 9.6). The wells were blocked with ethalonoamine for two hours and then with SuperBlock blocking buffer in PBS (Thermo Scientific) overnight at 4°C. The wells were washed with PBST. A half dilution series of the ligand (concentrations from 50 nM to 10 µM) were made in the buffer (1% Superblock/ 0.05% Tween 20/PBS). The immobilized protein was shaken with the ligand solution series for 6 hours at 4°C, then washed five times with binding buffer. The wells were treated with Antibiotin-HRP antibody (1:10,000 dilution of 1 mg/ml stock) in binding buffer for an hour at 4°C. After four washes with PBS and one wash with

PBST (0.05% Tween-20/PBS), the wells were treated with TMB substrate. The reaction was quenched with 0.5 M H₂SO₄. Absorbance was measured at $\lambda = 450$ on a 96-well plate reader. The Net A₄₅₀ was obtained by subtracting the respective absorbance values for no immobilized protein from the values obtained for the ligand protein interaction. These values were normalized and the data fitted by non-linear regression in GraphPad Prism software.

4.2.2.3 ELISA for *N-term-tri-L* and *C-term-tri-L*

200 nM solutions of the biotinylated triligand in binding buffer (1% Superblock, 0.05% Tween 20/ TBS pH 7.5) were added to the wells of a High Capacity Streptavidin 96 well plate (Thermo Scientific) and shaken for two hours at room temperature. The plate was blocked with 1% BSA/ 0.05% Tween 20/ TBS pH 7.5. The wells were washed with binding buffer five times. Active His₆ tagged Akt2, from 1.15 nM to 450 nM concentrations, was added and the plate shaken overnight at 4°C. The wells were washed three times with the binding buffer, then treated for an hour with a 1:1000 dilution of anti His₆ mouse monoclonal antibody. A 1:10,000 dilution of goat anti mouse antibody-Horse Radish Peroxide conjugate (Abcam) in binding buffer was added to the wells for an hour. The plate was washed, treated with TMB and quenched with H₂SO₄, and the absorbance measured as described in the previous paragraph. The Net A₄₅₀ (subtracting the absorbance value for no immobilized ligand) was fitted by non-linear regression in GraphPad Prism software.

4.2.2.4 OVCAR3 lysate pulldown assay

OVCAR3 cells were grown in RPMI-1640 media containing 10% fetal bovine serum and 0.01 mg/ml bovine insulin. Passage five cells were grown to ~ 80% confluence. Cells were lysed with lysis buffer (10 mM Tris-Cl (pH = 7.5), 100 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS (w/v), 0.5% deoxycholate, 1 mM DTT, 1 mM EDTA, 1X PhosStop phosphatase inhibitors

(Roche), 1X Complete protease inhibitors (Roche)) and the amount of protein measured using BCA Protein Assay Kit (Pierce Biotechnologies, Inc).

Streptavidin-agarose resin solution (EMD) was swelled in TBST (25 mM tris chloride, pH= 7.5, 150 mM NaCl, 0.05% Tween-20). Each of the biotinylated ligands was immobilized on the Streptavidin-agarose resin by adding 7.2 μ L of 1 mM ligand stock (DMSO) to 10 μ L of the swelled Streptavidin-agarose resin. After shaking overnight at 4°C, 50 μ M D-biotin was added to the resin to block any remaining sites. The resin was washed with TBST five times, for fifteen minutes each. The resin was then swelled in binding buffer for two hours. To each of the immobilized ligands 150 μ L OVCAR3 cell lysate (0.8 mg/ml) and binding buffer was added to a final volume of 250 μ L. The tubes were shaken at 4°C for 18 hours. The beads were then extensively washed, three times for fifteen minutes in binding buffer, three times (fifteen minutes) in TBST, and three times (fifteen minutes) in TBS to remove unbound proteins. The resin bound proteins were eluted by adding 40 μ L of 2X SDS-PAGE sample loading buffer (BioRad) and heating at 95°C for 10 minutes. 10 μ L of each sample was loaded on a 12% SDS-PAGE gels (BioRad) and run for 50 minutes at 120 volts. The gel was transferred to a nitrocellulose membrane, blocked for 2 hours at 4°C with 5% non-fat milk, and treated overnight at 4°C with a 1:1000 dilution of pan-Akt rabbit monoclonal antibody (11E7, Cell Signaling Technology) in 0.5% non-fat milk. The membrane was washed and treated for an hour with a 1:10,000 dilution of monoclonal mouse anti-rabbit- HRP secondary antibody (Cell Signaling) in 0.5% milk. After five washes of five minutes each with TBST and one wash of five minutes with TBS, the blot was developed with West Dura ECL substrate (Thermo Scientific) and imaged on film.

4.2.2.5 Non-radioactive kinase assay to evaluate effect of ligands on Akt2 kinase activity

The non-radioactive kinase assay kit for Akt2 was purchased from Cell Signalling. Serial dilutions of 2 mM ligand solution were made in filtered DMSO. Kinase reactions were set up in

1X kinase buffer (25 mM trisHCl (pH 7.5), 10 mM MgCl₂, 0.01% Triton-X, 1X Complete protease inhibitor (Roche), 1X Phosstop phosphatase inhibitor (Roche)), each 25 µL reaction mixture containing 400 ng Akt2, 400 ng GST-GSK-3 α /β fusion protein, 500 mM ATP and 0.5 µL of peptide solutions in DMSO or DMSO only. Reactions were allowed to proceed for 30 minutes at 30°C. The reactions were quenched by addition of 2.5 µL of a 500 mM DTT/ 20% SDS solution.

For dot blot, 2 µL of each of the reaction mixtures were spotted in triplicate on a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk/TBST for an hour at 4°C, and treated overnight at 4°C with a 1:200 dilution of Phospho GSK-3 α /β Ser (21/9) rabbit antibody (Cell Signaling) in 0.5% milk/TBST. Following washes, the membrane was treated with a 1:2000 dilution of mouse anti-rabbit –HRP antibody for an hour at 4°C. After four five minute washes with TBST, and one five minute wash with TBS, the membrane was treated with West Dura ECL substrate (Thermo Scientific) and imaged on film.

For the Western blot, an additional reaction mixture was also prepared using a 1:2.5 dilution of anti pS473 antibody (Cell Signaling) in the kinase buffer instead of the peptide ligand or DMSO. This reaction mixture was treated similar to the other kinase reaction mixtures. After 30 minutes the reactions were quenched with addition of 3X SDS sample buffer. The solution was heated at 95°C for five minutes. 10 µL from each sample was loaded on a any KD SDS gel (Biorad) and run for an hour at 110 volts. Following semidry transfer of the gel to a nitrocellulose membrane, the membrane was blocked, treated with Phospho GSK-3 α /β Ser (21/9) rabbit antibody and anti-rabbit –HRP antibody as described in the previous paragraph. After four five minute washes with TBST, and one five minute wash with TBS, the membrane was treated with West Dura ECL substrate (Thermo Scientific) and imaged on film.

4.2.2.6 Epitope Targeting Selectivity Assay

1.25 μ M biotinylated ligand was prepared by diluting the 1 mM stock in binding buffer (25 mM tris chloride, pH= 7.4, 150 mM NaCl, 0.1% BSA, 0.05% Tween 20). The prepared ligand solution or 0.125 % DMSO in buffer (buffer control) was immobilized on a High Capacity Streptavidin 96 well plate (Thermo Scientific). After washing away the excess ligand, the plate was blocked overnight with 1% BSA /25 mM tris chloride, pH= 7.4/ 150 mM NaCl, 0.05% Tween 20. 2.5 μ M solutions of the His₆ tagged target phosphopeptide epitope Akt2 amino acids 450 – 481 or the His₆ tagged off target peptide Akt2 amino acids 346-378 (His₆ – PEG – off-target) were added to each of the wells. Following three washes with the binding buffer, the plate was treated for an hour with a 1:1000 dilution of anti His₆ mouse monoclonal antibody. A 1:10,000 dilution of goat anti mouse antibody-Horse Radish Peroxide conjugate in binding buffer was added to the wells. The plates were washed four times, five minutes each, with 0.05% Tween 20/ TBS (25 mM tris chloride, pH= 7.5, 150 mM NaCl) and once with TBS (25 mM tris chloride, pH= 7.5, 150 mM NaCl). Color was developed by adding TMB substrate (KPL) to each well. The reaction was quenched with 0.5 M H₂SO₄. The A450 measured on a 96-well plate reader. The Net A450 was obtained by subtracting the absorbance value for the blank control (no immobilized ligand) from each of the triplicate values obtained for the ligand-epitope interaction.

4.2.2.7 Protein Isoform Selectivity Assay

The selectivity assay with full length Akt1, Akt2 and Akt3 is performed following the protocol in section 4.2.2.6, using 25 nM protein instead of 2.5 μ M His₆ tagged peptide epitope.

4.2.2.8 Measurement of binding affinity of N-term-tri-L and N-term-tri-L-dimer by Surface Plasmon Resonance

A Biacore T100 machine was used for SPR experiments. A Streptavidin Chip (Series S, G.E. Healthcare) was conditioned as per manufacturer's recommendation. A stock of 1 mM biotinylated ligand was diluted into HBSP+ Buffer (G.E. Healthcare) to a final concentration of

100 nM and ~150 RU was immobilized on the chip. Serial dilutions (450 nM to 1 nM) of the Akt2 protein were made in HBSP+ buffer for the experiment with the immobilized *N-term-tri-L*. For the *N-term-tri-L-dimer*, serial dilutions of Akt2 from 45 nM to 0.0086 nM were made. The solution was flowed over the chip at 50 μ L/min. Binding and dissociation were carried out at 10°C with a contact time of 350 sec, a dissociation time of 390 sec, and a stabilization time of 200 sec, with buffer blanks between each concentration. The response was corrected using an unmodified reference flow cell. Biacore Evaluation software was used to calculate the dissociation constants.

4.3 Results and discussion

4.3.1 Characteristics of PCC ligands developed against Akt2

Biligand candidates were prepared from the 1° ligand and each of the 2° ligands using the Cu(I)-catalyzed click reaction,^{28,29} and the biligands were then compared in an immunoprecipitation assay for their ability to pulldown Akt2 from OVCAR3 cell lysate. The highest performing peptide biligand (*bi-L*) exhibited superior performance to *mono-L* (Figure 4.2), and was selected for further characterization. The affinity of *bi-L* was estimated, from a direct ELISA involving the full Akt2 protein, to be 1.3 μ M. The *N-term-tri-L* exhibited the best performance in the selectivity immunoprecipitation assays from OVCAR3 cell lysate (Figure 4.1). The affinity of the *N-term-tri-L* and *C-term-tri-L* were estimated from single component ELISA assays to be 19 nM and 124 nM, respectively (Figure 4.2). We note that the 3° ligand on the *C-term-tri-L* is remarkably similar in sequence to the 2° ligand arm, indicating that both arms may be competing for the same spot on the protein for this triligand.

We carried out selectivity assays on *bi-L* and both triligands to determine the level of epitope selectivity. To this end, we performed an ELISA assay, comparing the binding of the ligands to either the C-terminal 32-mer polypeptide fragment of Akt2, or an off target 32-mer

polypeptide Akt2 fragment (corresponding to residues 346 through 378) that is similarly surface exposed and unstructured in the full length protein. All the three capture agents exhibited a high selectivity for the C-terminal 32-mer relative to the other polypeptide fragment (Figure 4.3)

The selectivity of the *N-term-tri-L* and *C-term-tri-L* for Akt2 over its isoform Akt1 and Akt3 was determined at the epitope level and against the full-length protein (Figure 4.4). One of the triligands, *C-term-tri-L* is highly selective for the peptide epitope of Akt2, and bind significantly less to the highly homologous C terminal epitopes of isomeric proteins Akt1 and Akt3. The same trend is observed in the binding of the ligands to the full-length Akt isoform proteins.

The absolute affinities of the high affinity ligands, *N-term-tri-L* and *N-term-tri-L-dimer* were measured by Surface Plasmon Resonance (SPR), by immobilizing the biotinylated ligand on the SPR chip and measuring the responses for various concentrations of the analyte, the full length active Akt2 protein. The absolute affinity of *N-term-tri-L* was obtained as 10 nM from steady state fit on Biacore T100 evaluation software, which is very close to the affinity measured in the ELISA (19 nM). The *N-term-tri-L-dimer* for Akt2, having two binding sites on the ligand, could not be fitted to the simplest 1:1 Langmuir binding model. The sensograms, when fit to the more appropriate model of 1:1 binding with mass transfer¹, yielded a K_D of 6.25 nM (Figure 4.5).

Figure 4.1: Immunoprecipitation and Western Blot assays showing the performance of the various PCC Agents.

Akt2 is pulled down from the lysate of an ovarian cancer cell line Acetyl-glycine-biotin is used as the control.

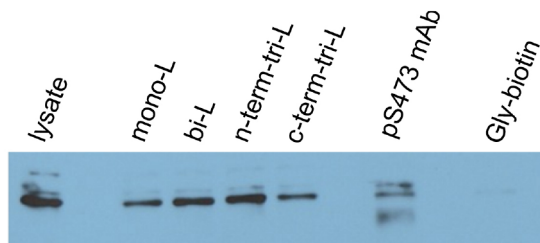


Figure 4.2: ELISA assays demonstrating use of PCC Agents.

PCC agents used as surface-localized capture agents (for *N-term-tri-L*, *C-term-tri-L*) or as detection agents (for *mono-L*, *bi-L*). This type of assays provides relative affinity measurements, which are given as EC_{50} values in the key.

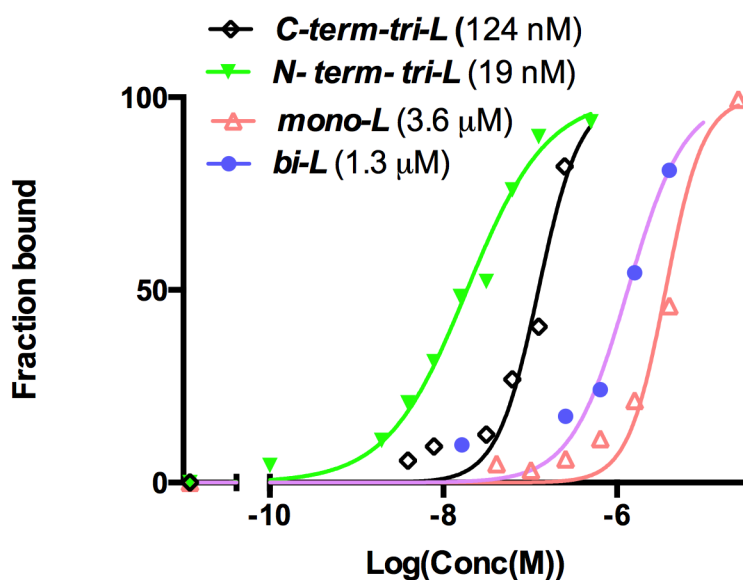


Figure 4.3: Epitope specificity assay.

The various triligand PCC agents are used as capture agents for the peptide epitope. The biotinylated capture agents are immobilized on a Streptavidin plate, and then probed with the His₆ tagged target or control peptides. The target peptide (Akt2 450-481) with pS474 shows a distinct binding affinity for the capture agents compared to the control peptide (Akt2 346-378). The *C-term-tri-L* also significant selectivity for the Akt2 epitope compared to the corresponding regions in Akt1 (449-480) and Akt3 (448-479).

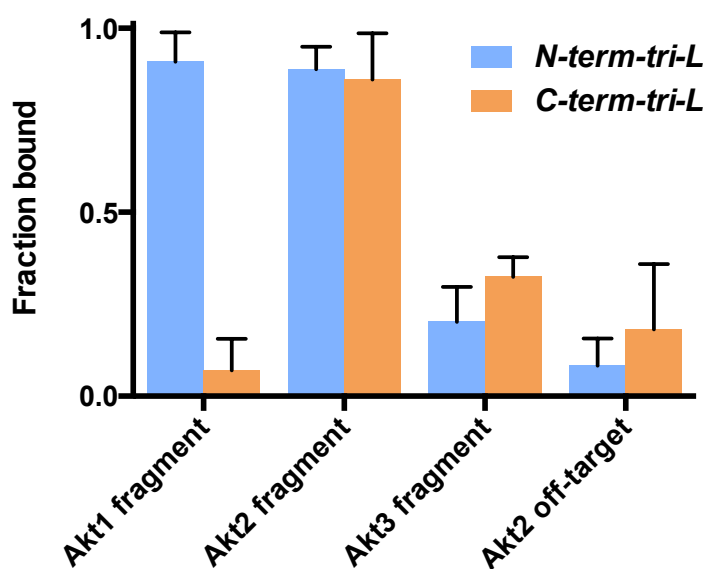


Figure 4.4: Selectivity of triligands for the full length Akt isoform proteins.

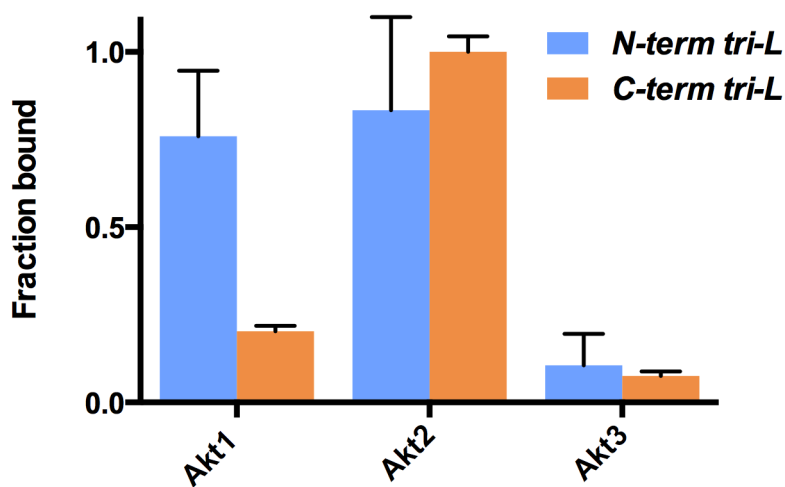
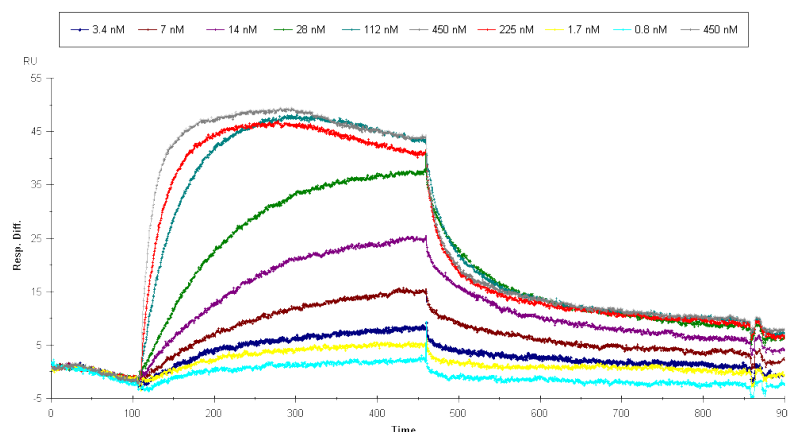


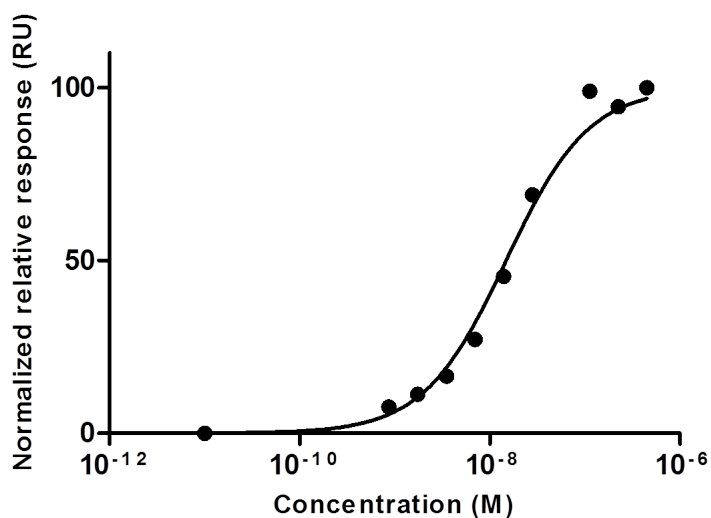
Figure 4.5: SPR measurements of triligands.

A. Sensograms from Surface Plasmon Resonance (SPR) experiment, immobilizing *N-term-tri-L* on SA chip and using Akt2 protein as the analyte. B. Steady state dissociation constant is measured by taking an average of the RUs in steady state over 3 time points, 150s, 200s and 250s, for each concentration of the protein. C. Sensograms from Surface Plasmon Resonance (SPR) experiment, immobilizing *N-term-tri-L-dimer* on SA chip and using Akt2 protein as the analyte. Because of the dimeric nature of the ligand, the sensograms fit best with the heterogenous ligand parallel model.

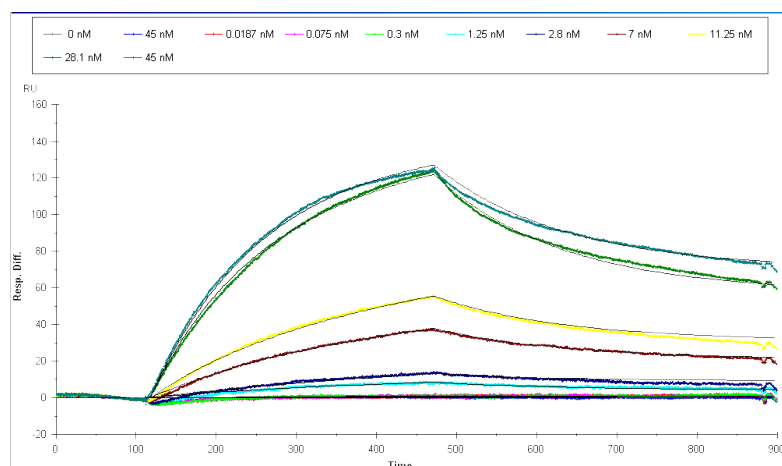
A.



B.



C.



4.3.2 Inhibitory Characteristics of the Akt2 Capture Agents

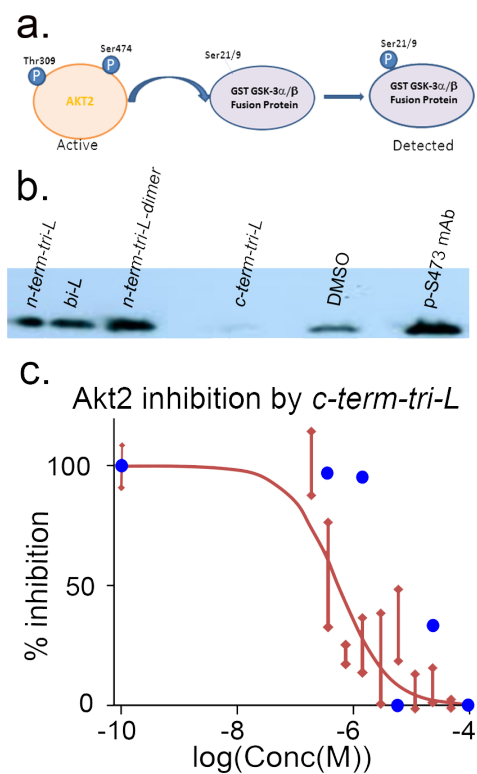
Phosphorylation of Akt2 at Thr309 activates Akt2, but further phosphorylation at Ser474 increases its enzymatic activity four to ten fold^{2,3}. In cancer cell lines^{4,5} and tissue samples⁶ the constitutively active PKB is phosphorylated at S473. Overexpression and/or activation of Akt have been found to increase resistance of tumors to chemo⁷ or radio⁴ therapies. The importance of Akt as a therapeutic target has prompted several approaches towards selective Akt inhibition⁸. Akt small molecule kinase inhibitors are classified into two general classes: type I ATP competitive inhibitors, and type II allosteric inhibitors that bind to the interface between the membrane docking pleckstrin homology domain (PHD) and the kinase domain⁹. These allosteric inhibitors exhibit no influence on the enzymatic activity of Akt when the PHD is removed⁸. We recently reported on a peptide based allosteric inhibitor ($K_{\text{inhibition}} \sim 3 \mu\text{M}$) of the kinase domain of Akt¹⁰ that did not compete with either the ATP or peptide substrate binding sites, but appeared

to overlap at least partially with a commercial mAb that was directed at the C-terminus of the protein. As the phosphorylated hydrophobic motif around S474 (near the C-terminus) of Akt2 has been identified to act as an allosteric activator, we hypothesized that a ligand that was targeted near S474 would influence the enzymatic activity of Akt2. In this section, we explore the inhibitory characteristics of the various ligands developed here.

The Akt kinase in vitro non-radioactive assay kit (Cell Signaling Technology) was used to estimate the influence of the ligands on the kinase activity of Akt2. In this assay, the active Akt2 is combined with its downstream effector GSK-3 α/β substrate (GST-GSK3 α/β fusion peptide substrate), ATP, and one of the peptide ligands (or dimethylsulfoxide (DMSO) for a control). The level of phosphorylation of GSK-3 α/β , as measured by spotting the reaction mixture on a nitrocellulose membrane or by Western Blot, provides a readout for the Akt2 kinase activity (Figure 4.6). The *bi-L* and *N-term-tri-L* capture agents were found to activate Akt2, as did the commercial anti-p-Ser473 mAb (Figure 4.6). However, the *C-term-tri-L* inhibits Akt2, with an IC₅₀ (~500 nM) (Figure 4.6). Thus, the enzymatic activity of Akt2 is clearly hypersensitive to perturbations near the C-terminus, whether it is phosphorylation of S474, or structural perturbation of that (phosphorylated) terminus via ligand binding. This hypersensitivity apparently works in both directions; perturbations of the ATP binding site can influence phosphorylation at the C-terminus. Mechanistic studies of traditional ATP-competitive small molecule Akt inhibitors have highlighted this intricate structure/activity balance; ATP-competitive inhibitors like A-443654 cause paradoxical hyperphosphorylation of Akt at both Thr308 and Ser473, and thus lead to reactivation of the protein after inhibitor dissociation¹¹.

Figure 4.6: Influence of the PCC Agents on the enzymatic activity of the Akt2 kinase.

A. The activity of Akt2 directly influences the phosphorylation state of GSK-3 α/β , and detecting p-GSK-3 α/β provides an assay readout, as a function of varying the nature or the concentration of a potential p-Akt2 inhibitor. B. Western blot analysis of p-GSK-3 α/β levels as the various PCC Agents are added to influence the activity of p-Akt2. DMSO is used as the control. The p-GSK-3 α/β levels for the *N-term-tri-L*, the *bi-L*, the *N-term-tri-L-dimer*, and the commercial p-S473 mAb all imply that these agents activate p-Akt above baseline, with the *N-term-tri-L-dimer* and the mAb being the strongest activators. The *C-term-tri-L*, however, serves as an inhibitor. C. Quantitation of the inhibitory characteristics of the *C-term-tri-L* PCC Agent on p-Akt2. Red points with error bars are from a dot blot analysis of p-GSK-3 α/β , while the blue points are from a Western Blotting analysis (which only yielded a limited dynamic range in terms of % inhibition detectable). The red line is a fit to the dot-blot data points. The assays yield similar IC₅₀ values of 500 nM (dot blot) and 2 μ M (Western).



4.4 Conclusions

We report here on an epitope targeting strategy that, when combined with sequential in situ click chemistry, permitted the development of a series of peptide multi-ligands (called PCC Agents) that are targeted near the key activating phosphorylation site of Akt2. The strategy relies on the use of dinuclear Zn(II)-dipyridylamine complex that binds to the phosphorylated residue¹² of interest, and presents both a biotin label and an azide functionality near that site. An advantage of the strategy is that it can be initiated using only a polypeptide fragment that represents the phosphorylated epitope of the target. This permits stringent chemical characterization of all of the relevant chemical species used in the process.

The strategy is utilized to identify an initial PCC Agent monoligand, a PCC Agent biligand and two PCC Agent triligands. One of those triligands is further expanded into a dimer structure. All of affinity agents are effective at detecting total Akt from ovarian cancer cell lysate, with improved performance being increasingly observed through the biligand and triligand stages. The best triligand (and triligand dimer) exhibits an affinity in the 10 nM range, and epitope specificity is shown for the biligand and triligand PCC Agents.

The p-Ser474 region near the C-terminus of Akt2 was explored here because phosphorylation of this hydrophobic motif leads to allosteric activation of Akt2, and so provides an interesting therapeutic epitope on the protein. However, the epitope itself is not obviously amenable towards the development of traditional small molecule inhibitors. Indeed, we find that PCC Agents developed against this epitope exhibit both inhibitory and activating characteristics, depending upon the detailed structure of the PCC Agent. An obvious next step is to develop approaches for delivering these PCC Agents into live cells, and to further explore their potential as therapeutic inhibitors of Akt2. Such studies are currently underway.

REFERENCES:

- (1) Khalifa, M. B.; Choulier, L.; Lortat-Jacob, H.; Altschuh, D.; Vernet, T. *Anal Biochem* **2001**, *293*, 194.
- (2) Sarbassov, D. D.; Guertin, D. A.; Ali, S. M.; Sabatini, D. M. *Science* **2005**, *307*, 1098.
- (3) Yang, J.; Cron, P.; Good, V. M.; Thompson, V.; Hemmings, B. A.; Barford, D. *Nat Struct Biol* **2002**, *9*, 940.
- (4) Brognard, J.; Clark, A. S.; Ni, Y. C.; Dennis, P. A. *Cancer Research* **2001**, *61*, 3986.
- (5) Sun, M.; Wang, G.; Paciga, J. E.; Feldman, R. I.; Yuan, Z. Q.; Ma, X. L.; Shelley, S. A.; Jove, R.; Tsichlis, P. N.; Nicosia, S. V.; Cheng, J. Q. *American Journal of Pathology* **2001**, *159*, 431.
- (6) Yuan, Z. Q.; Sun, M.; Feldman, R. I.; Wang, G.; Ma, X. L.; Jiang, C.; Coppola, D.; Nicosia, S. V.; Cheng, J. Q. *Oncogene* **2000**, *19*, 2324.
- (7) Clark, A. S.; West, K.; Streicher, S.; Dennis, P. A. *Molecular Cancer Therapeutics* **2002**, *1*, 707.
- (8) Liu, Y.; Gray, N. S. *Nat Chem Biol* **2006**, *2*, 358.
- (9) Lubben, M.; Feringa, B. L. *Journal of Organic Chemistry* **1994**, *59*, 2227.
- (10) Millward, S. W.; Henning, R. K.; Kwong, G. A.; Pitram, S.; Agnew, H. D.; Deyle, K. M.; Nag, A.; Hein, J.; Lee, S. S.; Lim, J.; Pfeilsticker, J. A.; Sharpless, K. B.; Heath, J. R. *Journal of the American Chemical Society* **2011**, *133*, 18280.
- (11) Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat Chem Biol* **2009**, *5*, 484.
- (12) Sakamoto, T.; Ojida, A.; Hamachi, I. *Chemical Communications* **2009**, 141.