Chapter 5: Development of CuAAC cyclized one bead one compound peptide libraries and their application in the epitope targeting strategy

5.1 Introduction

Cyclic peptides have been frequently used for drug discovery[1,2], as molecular probes[3], and as a protein detection agent. Whereas small molecules bind to proteins that have well developed hydrophobic pockets, a significant amount of protein interaction surfaces are relatively flat and distributed over a large contact surface area. So for surface binding, peptides perform better than small molecules. However, for peptide protein interactions to be high affinity, it is essential to have structural constraints in the peptide[4]. Cyclizing a peptide provides this constraint, at the same time increasing the protease resistance of the peptide[5].

For cyclic ligand development against proteins, phage display libraries have been frequently used. But a limitation of the phage display method is in incorporating unnatural amino acids such as D amino acids in the library. While innovative approaches, such as mirror image phage display [6], have been explored to overcome this disadvantage, it is by no means a trivial to introduce unnatural amino acids into phage display libraries. One Bead One Compound (OBOC) peptide libraries[7,8] overcome this disadvantage, allowing easy incorporation of unnatural amino acids and other chemical modifications. However on bead screening and sequencing processes are not compatible with several categories of cyclization processes[9-11].

Here we describe the development of CuAAC cyclized peptide libraries that are compatible with OBOC screening. The developed cyclic library is used in epitope targeting strategy. In the epitope targeting strategy, we are trying to detect peptide-peptide interaction, which is typically weaker and tougher to detect than peptide-protein interaction. The property of cyclic peptide to have higher affinity than its linear one is particularly attractive for optimizing of the epitope targeted PCC agent development process. To demonstrate of this process cyclic ligands were developed against a peptide epitope that has already been demonstrated to be good for developing peptide based ligands. This epitope is the C terminal fragment from the protein Akt2, amino acids 450-481, that contains pSer474.

Another motivation of this study is to study the cell penetrating properties of the isolated cyclic peptide inhibitor. Along with the robustness and compatibility of the cyclization procedures with on bead peptide chemistry, an important characteristic of the developed cyclic libraries is the nature of the cyclization linker. It has been found that an all hydrocarbon cross-linker stabilizes the peptide and increases the cell penetrating property of the peptide[12,13]. For the triazole cyclized library, the hydrocarbon part, like the stapled peptide, should have a positive effect on the intracellular uptake of the cyclized peptide. The triazole unit acts as an amide bond[14], and may or may not hinder the uptake of the cyclic peptide. The triazole, is, moreover, not susceptible to protease cleavage as amide bonds are. We have preliminary data that suggest the branched multiligand developed against Akt, as described in chapter 3 and 4, can be effective inside cells when tagged with the HIV derived TAT peptide sequence. However the ideal situation would be to have a peptide based drug that has significant cell permeating property by itself. CuAAC cyclized peptides are promising in this respect. Among the four cyclic ligands isolated, one peptide Cy(yytytTz4) exhibits selective binding to both the peptide epitope and the Akt2 protein. We have preliminary data that the ligand acts as an inhibitor of kinase activity.

5.2 Materials and Methods

5.2.1 Materials

Materials as described in sections 3.2.1 and 4.2.1 are used.

5.2.2 Methods

5.2.2.1 Synthesis of randomized peptide library and on bead cyclization

Three comprehensive linear peptide libraries A, B and C were synthesized on Tentagel-S-NH_2 resin using standard SPPS library synthesis method. Each library was synthesized at a 10-fold excess to ensure adequate representation of each library element. After the synthesis of each

library, all the beads in the linear library were subjected to an on bead CuAAC reaction, for 6 hours at room temperature with 1.5 equivalents of CuI, 2.5 equivalents of ascorbic acid in 20% piperidine in DMF (Figure 5.1). After washes to remove the adsorbed Cu, the library was washed with DMF, methanol and DCM and dried. Random beads were picked from the library to be sequenced. The rest of the library was stored in NMP. The structure and details of the three libraries are shown in Table 5.1.

Figure 5.1: Peptide cyclization on bead by the Cu catalyzed Azide Alkyne Cycloaddition (CuAAC) reaction.

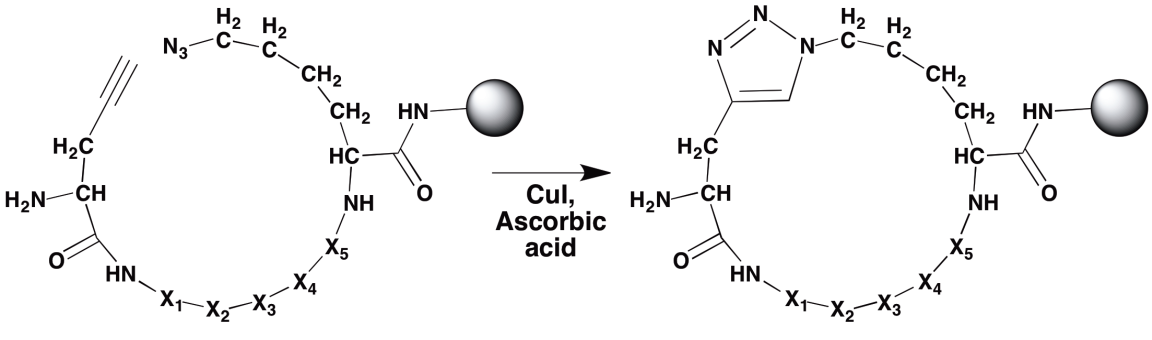
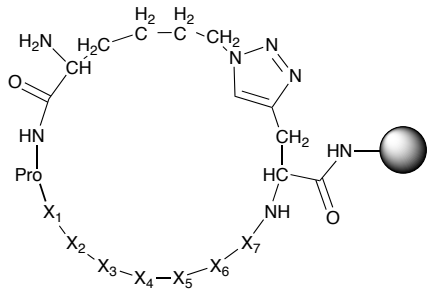
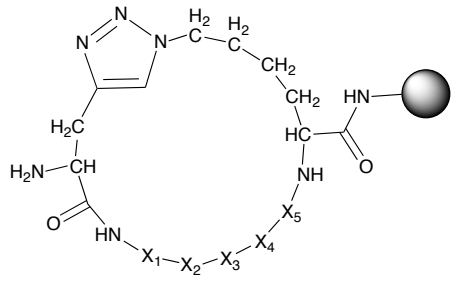
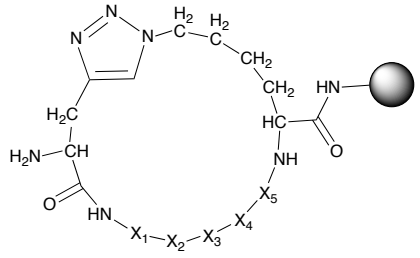


Table 5.1: One Bead One Compound (OBOC) cyclic peptide libraries used in screens

Library (notation)	**Sequence**	**Amino acids used**	***Number of unique sequences***
A Cy(Pro-$X_1X_2X_3X_4$ $X_5X_6X_7$–Tz4)		X_i =D-Ala, Gly, D-Leu, D-Ile, D-Val, D-Phe, D-Trp, D-Arg, D-His, D-Lys, D-Asp, D-Glu, D-Asn, D-Gln, D-Ser, D-Thr, D-Tyr, D-Pro.	612,220,032
B Cy($X_1X_2X_3X_4$ X_5–Tz4)		X_i =D-Ala, Gly, D-Leu, D-Ile, D-Val, D-Phe, D-Trp, D-Arg, D-His, D-Lys, D-Asp, D-Glu, D-Asn, D-Gln, D-Ser, D-Thr, D-Tyr, D-Pro.	1889568
C Cy($X_1X_2X_3X_4$ X_5–Tz4)		X_i =L-Ala, Gly, L-Leu, L-Ile, L-Val, L-Phe, L-Trp, L-Arg, L-His, L-Lys, L-Asp, L-Glu, L-Asn, L-Gln, L-Ser, L-Thr, L-Tyr, L-Pro.	1889568

The linker containing the amino acid azidolysine clicked to L-Pra in a 1,4 disubstituted orientation is referred to as Tz4.

5.2.2.2 Synthesis of scrambled phospho-epitope and target phospho-epitope

Akt2 450-481 (ITPPDRYDSLGLLELDQRTHFPQF(pS)YSASIRE) is used as the target phospho-epitope. The scrambled non-phosphorylated epitope has sequence ITPPDRYDSLGLLEL-QRTHYFFASQPSSIRE. For screening both peptides are synthesized on Rink Amide MBHA resin with a polyethylene glycol spacer (PEG_2) and biotin on the N terminal.

5.2.2.3 IR spectra

HPLC purified lyophilized peptides were ground with KBr powder to make pellets. Measurements were made on a Vertex 70 FT-IR spectrometer (Bruker Optics). Scans of air were served as the background and were subtracted from the spectra.

5.2.2.4 Screening cyclic OBOC library with peptide epitope

For isolating cyclic ligands against the peptide epitope, three consecutive libraries A, B and C were screened against the phospho-peptide. Each of the screens followed the following protocol: 500 mg of library resin were dried with DCM and afterwards, swelled and shaken overnight in binding buffer (25 mM trisCl, pH = 7.5, 150 mM NaCl, 0.1% BSA, 0.05% Tween20) at room temperature. Beads were incubated overnight with a solution of 500 nM of the scrambled phosphopeptide in binding buffer. The beads were washed three times, for fifteen minutes each, with the binding buffer. They were then treated for 1 hour at room temperature with a 1:1000 dilution of mouse monoclonal Anti-biotin antibody conjugated with Alkaline Phosphatase (mAb-AP) in screening buffer. Following the screen, the beads were successively washed three times with each of the following buffers: 25 mM trisCl, pH = 7.5, 150 mM NaCl, 0.1% BSA, 0.05% Tween20; 25 mM trisCl, pH = 7.5, 150 mM NaCl, 0.05% Tween20; 25 mM trisCl, pH = 7.5, 150 mM NaCl; 100 mM Tris-HCl, pH 9.0, 150 mM NaCl, 1 mM $MgCl_2$) (AP buffer). The library was treated with a 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (33 uL BCIP in 10 mL AP buffer) for fifteen minutes. The AP reaction was stopped using 0.1 M HCl, which denatures AP protein. Blue background hits were removed from clear ones using a

pipette. Clear beads were combined and transferred to a peptide synthesis vessel. The beads were shaken in 7.5 M of guanidine-HCl pH = 2, which serves as a common protein denaturant, for 2 hours at room temperature. Afterwards, the library was washed with deionized water and treated with NMP to decolorize the blue beads. Following a wash with DCM, the clear beads were dried, then shaken overnight in binding buffer.

The beads were screened overnight with a solution of 500 nM of the target phospho-peptide in binding buffer. Following washes with the binding buffer, the beads were treated with a solution of mAb-AP. The same procedure as in the prescreening was conducted to obtain blue click hits. The beads were manually picked, washed with 7.5 M guanidium hydrochloride (pH 2) and water, and sequenced using the Edman Sequencer.

Table 5.2: List of sequences obtained from screen against target epitope using cyclic library C.

X_1	X_2	X_3	X_4	X_5	X_6	X_7
-	Y	V	Y	K	S	Tz4
–	V	F	A	K	V	Tz4
–	I	R	Y	Y	S	Tz4
–	Y	Y	T	Y	T	Tz4

5.2.2.5 ELISA for cyclic peptide Cy(yytytTz4) against full length Akt2 protein

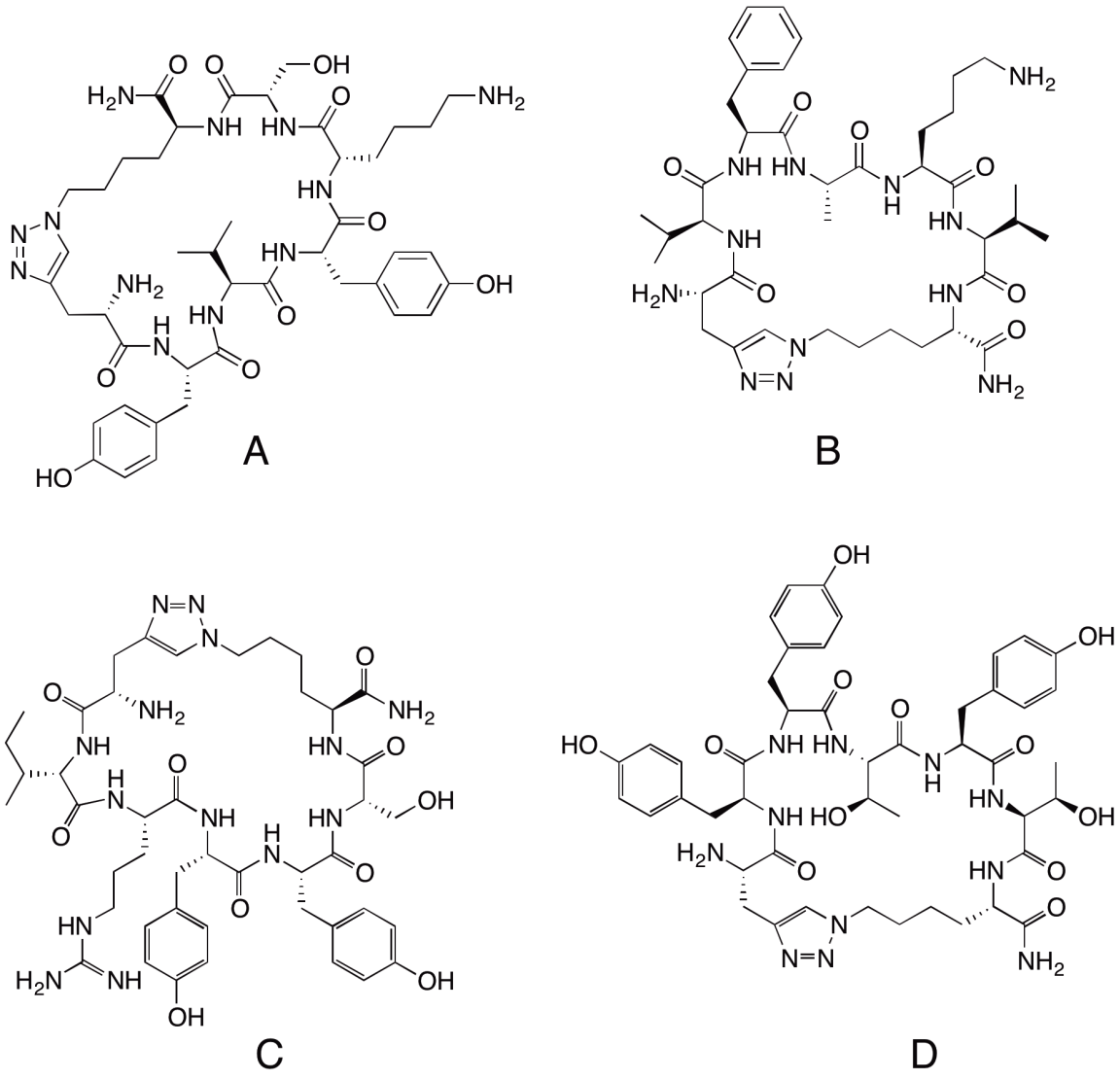
2 μM solution of the biotinylated cyclic peptide was prepared in binding buffer (TBS pH 7.5, 0.05% Tween 20/0.1% BSA. 100 μL of the solution was added to each well of a High Capacity Streptavidin 96 well plate (Thermo Scientific) and the plate shaken for two hours at room temperature. The wells were blocked with 1% BSA / TBS pH 7.5 / 0.05% Tween 20. The

wells were washed with binding buffer five times. Serial dilutions, from 1.15 nM to 450 nM, of active His_6 tagged Akt2 (Abcam) were made in the binding buffer. 100 µl of each solution was added per well 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 His_6 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 H_2SO_4, and the absorbance at 450 nm wavelength measured. From the A450 value of each protein concentration, the corresponding blank A450 (with no ligand, same amount of protein) was subtracted. All the A450 values were normalized w.r.t the maximum A450 value. They were then fitted by non-linear regression in Graphpad Prism 6.

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

To evaluate if the cyclic ligand had any effect on the kinase activity of Akt2, we performed a preliminary non-radioactive kinase assay with a single concentration of the cyclic ligand. The protocol as described in A solution of 400 ng Akt2, 400 ng GST-GSK-3α/β fusion protein, 500 mM ATP and 0.5 µL of 90 mM peptide solution or DMSO (control) was made in kinase buffer (25 mM trisHCl (pH 7.5), 10 mM $MgCl_2$, 0.01% Triton-X, 1X Roche Phosstop phosphatase inhibitor, 1X COMplete protease inhibitor), so that the final concentration of the peptide in the reaction mixture was 1.8 mM. The kinase assay protocol as described in Chapter 4, subsection 4.2.2.5 was followed.

Figure 5.2: Cyclic peptide ligands isolated by screening against target peptide epitope.
A) Cy(YVYKS-Tz4), B) Cy(VFAKV-Tz4), C) Cy (IRYYS-Tz4), D) Cy(YYTYT-Tz4)



5.3 Results and discussion

5.3.1 Verification of on bead cyclization by Edman Peptide Sequencing

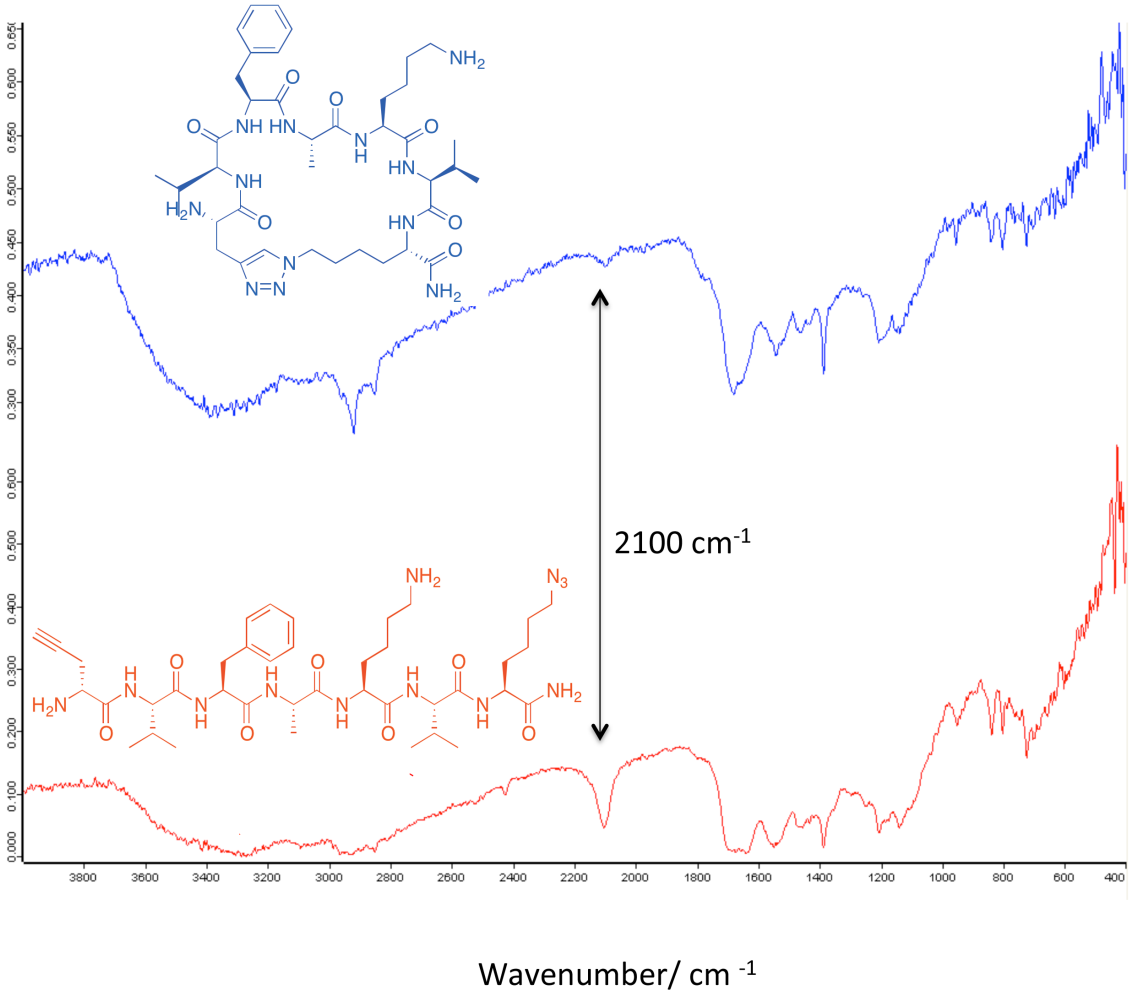
A single bead of tentagel resin from the library was loaded on a cartridge of the Edman Sequencer for sequencing. A peptide from library A or C, on Edman sequencing, is expected to

generate a particular pattern of signals. During the first sequencing cycle, the amino acid with triazole in the side chain should be converted to its PTH derivative. Since the amino acid is still attached to the peptide on bead, there should be no peak in cycle 1. From the 2nd cycle onwards, the amino acids of the peptide should get sequenced like in a linear peptide. The 7th cycle will give the peak corresponding to the triazole side chain derivative. It will cleave as a diphenylthiohydantoin derivative. The diphenylthiohydantoin derivative of D-propargylglycine amino acid elutes at 25 minutes using the standard amino acid calibration curve. Several random beads were picked from library A and sequenced on the Edman. All the random beads from the library yielded this pattern, with no peak in cycle 1, then after sequencing 5 amino acids, there is a characteristic peak on the 7th cycle. This indicates that the cyclization of peptides on bead by CuAAC is a viable and robust and that an on bead CuAAC cyclized library can be readily synthesized.

5.3.2 IR verification of on bead cyclized peptide formation by CuAAC reaction

The MBHA resin, with higher molar capacity than the Tentagel-S-NH_2 resin, was used in this synthesis, so that any issues of dimerization, which could happen from the on bead cyclization, would be more apparent in this case. The cyclized and linear peptides on HPLC purification showed one major peak each, which on mass spectral analysis, showed the correct mass for the monomeric compound. These pure compounds were analyzed by solid phase IR and the spectra compared. While the linear peptide had a distinct peak at ~ 2100cm^{-1} corresponding to azide triple bond rotation, the cyclized peptides did not have this characteristic peak. Thus it was demonstrated that the azide on the bead is efficiently cyclized during the click reaction.

Figure 5.3: IR spectra of linear peptide L-Pra-VFAKV-L-Az4 and the cyclic peptide Cy(VFAKVTz4).



5.3.3 Using on bead cyclic peptide library for ligand discovery

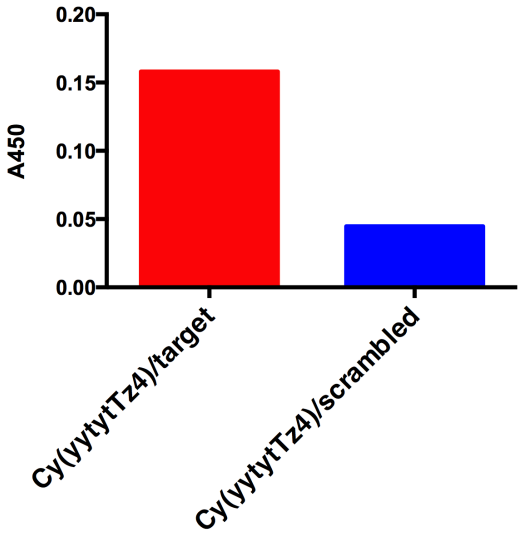
To eliminate binding to a different region than the phosphorylation site and to prevent background binding to the detection antibody, each library was screened against the scrambled phospho-epitope, a peptide that does not contain the phosphate and is scrambled in the AGC kinase specific motif. In the prescreen the beads binding to the detection antibody and the peptide regions other than the hydrophobic motif are detected by the coloration on BCIP treatment. These blue beads are separated from the rest of the library that remains colorless. The colorless beads

are washed with protein denaturants and then screened with the target phospho-peptide epitope. Libraries A and B on screening in this protocol did not yield any hit beads against the target phospho-epitope. Only library C yielded four hits. The hits are listed in Table 5.2.

5.3.4 Determination of best cyclic binder to the target Akt2 C terminal fragment

The four peptide candidates were tested for their capacity to capture the target peptide in a sandwich ELISA platform. Biotinylated capture agents were immobilized on Streptavidin plates and titrated with 1 μM of either the His_6 tagged target peptide or the scrambled peptide. Binding was detected by mouse anti His_6 tag antibody. Only peptide Cy(YYTYT-Tz4) showed binding to the target peptide and no binding to the scrambled peptide.

Figure 5.4: Selectivity of the cyclic peptide Cy(YYTYT-Tz4) for the target peptide.

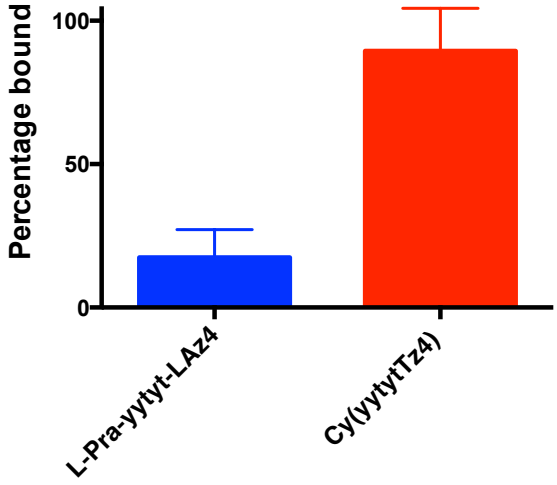


5.3.5 Effect of cyclization on binding specificity

To determine whether the cyclization of the peptide binder was vital to its binding to the

target, the binding of both the linear peptide L-Pra-YYTYT-L-Az4 and the cyclic peptide Cy(YYTYT-Tz4) of the peptide ligand with the target were determined. The linear peptide exhibited low binding to the target peptide, thus demonstrating that the cyclization of the peptide ligand was significant for getting specificity in binding.

Figure 5.5: The cyclized ligand Cy(YYTYT-Tz4) shows significant binding to the epitope compared to its linear version L-Pra-YYTYT-L-Az4.



5.3.6 Effect of ring size on binding

To determine whether the ring size of the cyclic peptide binder is an important factor in its specificity for the target peptide, the linear peptides L-Pra – YYTYT – L-Az1 and L-Pra – YYTYT– L-Az2 were synthesized on resin and subjected to the CuAAC reaction overnight. The peptide L-Pra – YYTYT – L-Az1 failed to cyclize on bead. The peptide L-Pra –YYTYT– L-Az2 could be cyclized. This cyclic peptide of smaller ring size did not exhibit significant binding to the target peptide, and thus it could be demonstrated that the ring size of the cyclic peptide is an important criteria for binding the target peptide.

Figure 5.6: Ring size affects cyclization efficiency.

L-Pra – YYTYT– L-Az2 cyclizes on bead as the major product while L-Pra – YYTYT– L-Az1 does not cyclize efficiently on bead

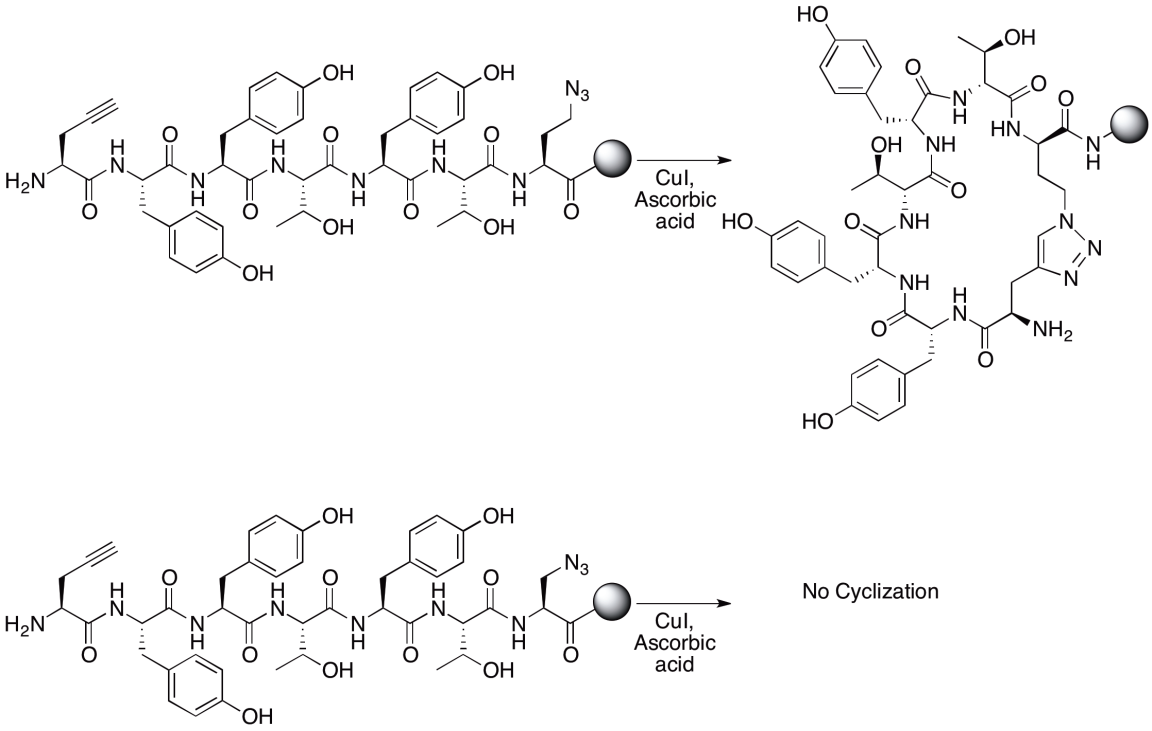
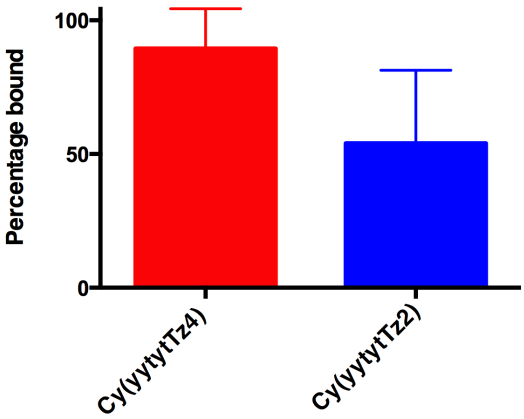


Figure 5.7: Effect of ring size on epitope selectivity.

Cy(YYTYT-Tz4) is a better binder than Cy(YYTYT-Tz2)



5.3.7 Characterization of the cyclic ligand Cy(YYTYT-Tz4)

To determine the affinity of the cyclic monoligand for full length Akt2 protein, the ligand was titrated against immobilized protein. The ligand acts as a detection agent and has a low nanomolar (124 nM) binding affinity compared to the low micromolar affinity (3.6 μM) of the linear monoligand previously developed against Akt. Thus there is a ~ 30 fold improvement in the binding affinity using a cyclic instead of linear peptide monoligand for Akt2. It will be interesting to see if the same improvement can be attained in the biligand stage using a cyclic peptide as the 2 °arm.

We demonstrate the selectivity of the cyclic monoligand for Akt2 protein by comparing the binding of the ligand to the proteins Akt1, Akt2 and Akt3. As the proteins are highly homologous, even in the targeted C terminal region, it is not surprising that binding to all the three proteins is observed to some extent. However there is a distinct preference for Akt2.

Figure 5.8: Determination of binding affinity of cyclic ligand Cy(YYTYT-Tz4) for Akt2 protein

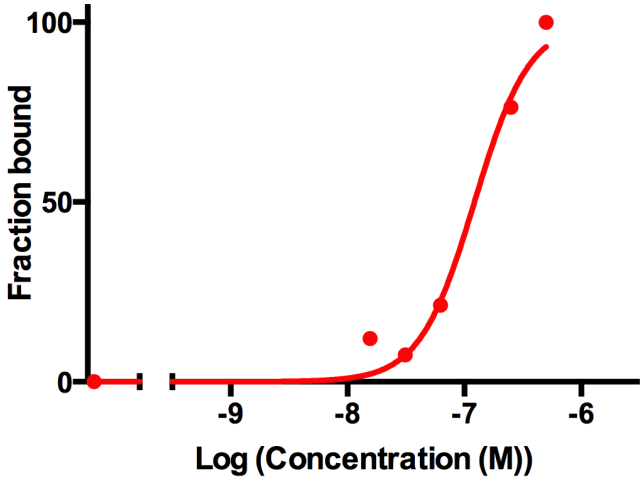
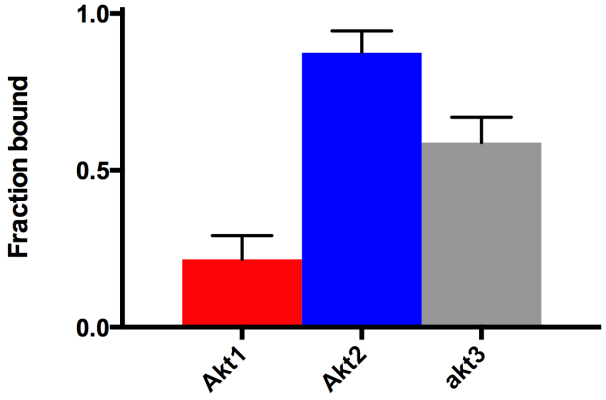


Figure 5.9: Cy(YYTYT-Tz4) is selective for the Akt2 protein over the Akt1 and Akt3 proteins.



5.3.8 Effect of cyclic monoligand on Akt2 kinase activity

To evaluate if the cyclic ligand has any effect on the kinase activity of the Akt2 protein, we performed the non-radioactive kinase assay described in Chapter 4. Briefly, the protein is treated with the ligand, ATP and an enzyme substrate, in this case, GST-GSK-3α/β fusion protein. The reaction mixture is analyzed using western blot to detect the amount of substrate phosphorylated. Under normal conditions (as in the DMSO control) the GST-GSK-3α/β fusion protein will be phosphorylated by Akt2. If, however, the ligand acts as an inhibitor, it will decrease the formation of phospho-Ser21/9- GST-GSK-3α/β fusion protein. The cyclic monoligand, Cy (YYTYT-Tz4) inhibits the phosphorylation of the substrate. As this was a preliminary experiment, we used a high concentration (2mM) of the cyclic ligand. Further verification with lower concentrations of the cyclic peptide is required.

Figure 5.10: Cyclic ligand Cy(YYTYT-Tz4) inhibits Akt kinase activity.

5.3.9 Optimization of monoligand anchor

To introduce an azide handle on the monoligand to screen against an OBOC library containing Alkyne handle, 6- azido hexanoic acid is coupled to N terminal of cyclic peptide to develop the monoligand anchor. The protein is incubated with the monoligand anchor and screened against library D. There was very little background binding to the antibody in the preclear screen, and in the actual screen no hits were obtained. During the purification of the monoligand anchor peptide on the HPLC using a standard gradient, we observed a significant difference in the elution time. While the monoligand eluted at 50 minutes, the monoligand anchor eluted at 65 minutes using the same gradient. The significant change in elution times on addition of a single amino acid to a peptide probably implies that this addition is altering the nature of the peptide. It is possible that the click handle in the monoligand anchor is not available for reaction with the on bead alkyne because the azide containing side chain is binding to the cyclic part of the peptide. It is observed that use of an alternative version of the monoligand anchor with a glycine and azidoalanine (instead of the 6-azidohexanoic acid) decreases the gap in HPLC elution, eluting close to the monoligand. This is chosen as the monoligand anchor candidate for the biligand screen.

Figure 5.11: Monoligand anchor candidate peptides developed for biligand screen.
The length of the azide side chain is same for both

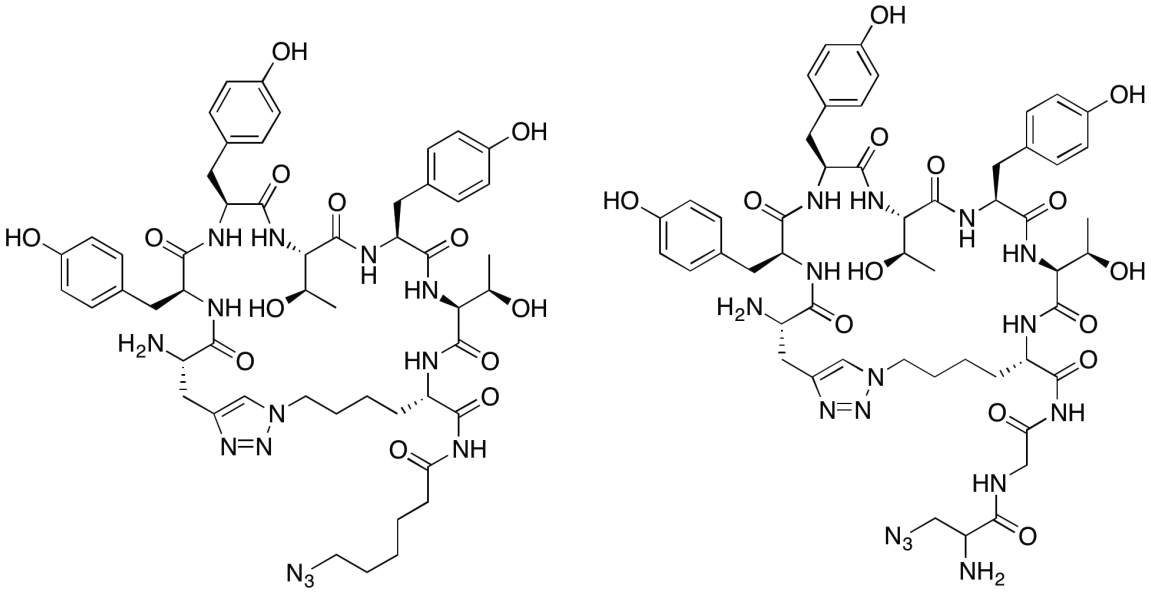
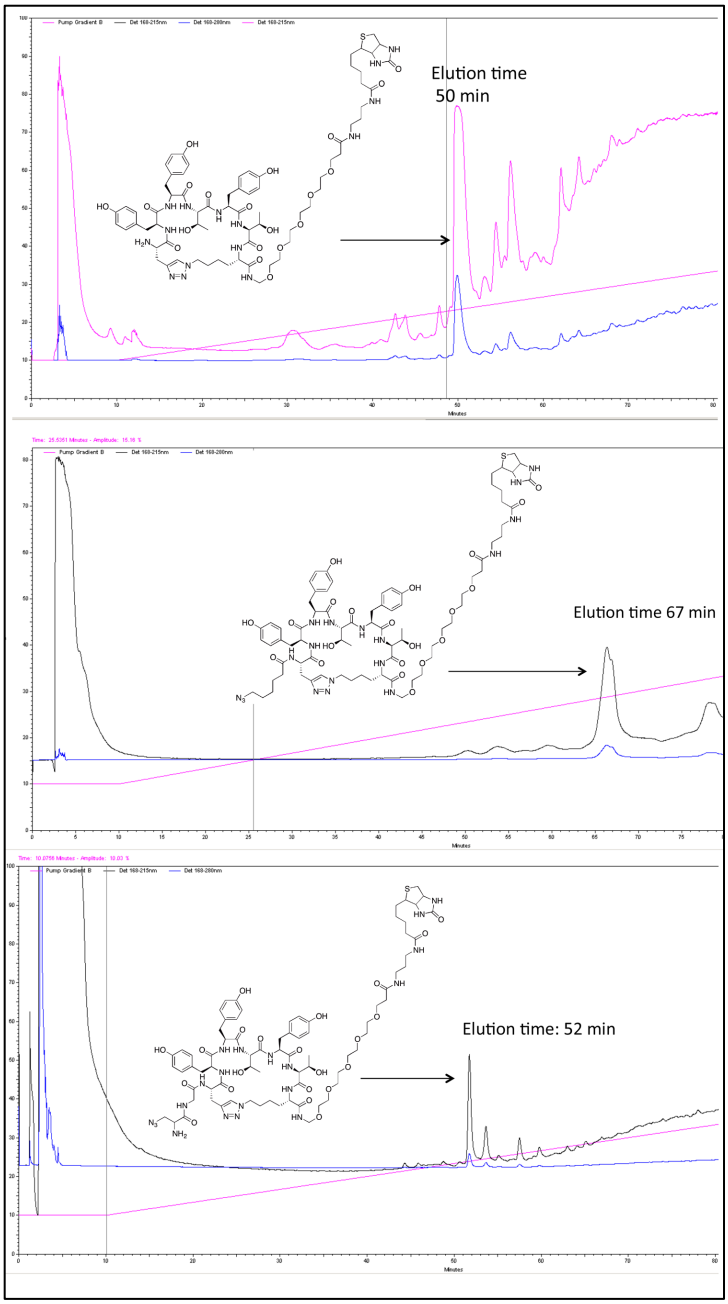


Figure 5.12: Comparison of HPLC traces to determine best monoligand anchor candidate. Monoligand anchor A is significantly different in character from the monoligand Cy(YYTYT-Tz4) while monoligand anchor B is similar in character.



5.4 Conclusion

In this chapter we have described the development of a Cu catalyzed cyclic library and demonstrated its use in OBOC screening. Although on bead cyclization of some specific peptide sequences through CuAAC have been demonstrated, we are not aware of use of CuAAC cyclized comprehensive libraries in OBOC screening. The libraries were developed with a specific aim in mind, isolating monoligands with high affinity to render the epitope targeting strategy more effective. This aim has been fulfilled. We were able to demonstrate the binding of the PCC agent to the epitope in the monoligand stage itself. The cyclic monoligand has a significantly higher binding affinity for the protein compared to the linear monoligand developed using the peptide epitope. It is also selective enough to distinguish between various Akt isoforms. Finally, we have preliminary data that the cyclic ligand is an inhibitor of the kinase activity of the Akt2 enzyme. We have optimized the monoligand anchor to be used in developing the biligand. The biligand will be bicyclic, and hopefully, will have affinity and specificity similar to a monoclonal antibody.

REFERENCES:

(1) Gause, G. F.; Brazhnikova, M. G. *Nature* **1944**, *154*, 703.

(2) Sewald, N.; Jakubke, H.-D. *Peptides : chemistry and biology*; Wiley-VCH: Weinheim, 2009.

(3) Dijkgraaf, I.; Rijnders, A. Y.; Soede, A.; Dechesne, A. C.; van Esse, G. W.; Brouwer, A. J.; Corstens, F. H. M.; Boerman, O. C.; Rijkers, D. T. S.; Liskamp, R. M. J. *Organic & Biomolecular Chemistry* **2007**, *5*, 935.

(4) Verdine, G. L.; Walensky, L. D. *Clin Cancer Res* **2007**, *13*, 7264.

(5) Rozek, A.; Powers, J. P. S.; Friedrich, C. L.; Hancock, R. E. W. *Biochemistry* **2003**, *42*, 14130.

(6) Schumacher, T. N. M.; Mayr, L. M.; Minor, D. L.; Milhollen, M. A.; Burgess, M. W.; Kim, P. S. *Science* **1996**, *271*, 1854.

(7) Aina, O. H.; Liu, R.; Sutcliffe, J. L.; Marik, J.; Pan, C. X.; Lam, K. S. *Mol Pharm* **2007**, *4*, 631.

(8) Lam, K. S.; Lebl, M.; Krchnak, V.; Wade, S.; Abdul-Latif, F.; Ferguson, R.; Cuzzocrea, C.; Wertman, K. *Gene* **1993**, *137*, 13.

(9) Joo, S. H.; Xiao, Q.; Ling, Y.; Gopishetty, B.; Pei, D. *Journal of the American Chemical Society* **2006**, *128*, 13000.

(10) Lambert, J. N.; Mitchell, J. P.; Roberts, K. D. *Journal of the Chemical Society-Perkin Transactions 1* **2001**, 471.

(11) Roberts, K. D.; Lambert, J. N.; Ede, N. J.; Bray, A. M. *Tetrahedron Letters* **1998**, *39*, 8357.

(12) Walensky, L. D. *Science* **2004**, *305*, 1466.

(13) Schafmeister, C. E.; Po, J.; Verdine, G. L. *Journal of the American Chemical Society* **2000**, *122*, 5891.

(14) Brik, A.; Alexandratos, J.; Lin, Y. C.; Elder, J. H.; Olson, A. J.; Wlodawer, A.; Goodsell, D. S.; Wong, C. H. *Chembiochem* **2005**, *6*, 1167.