Identification of high affinity, highly selective bicyclic peptides (*Bicycles*®) to transmembrane proteins using phage display screening on whole cells

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ABSTRACT#

bicycle therapeutics

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- Historically, phage display panning has been used in conjunction with purified proteins immobilised on a solid phase support, such as plates or beads [1].
- Bicycle Therapeutics have previously described a proprietary phage display platform which allows the initial selection and optimisation of high affinity bicyclic peptides (*Bicycles* ®)[2]
- Here we describe how this platform was adapted to screen a range of transmembrane targets by panning on whole cells and show the output from three separate screening campaigns:
  1) To a receptor tyrosine kinase (EphA3) where we show the ability to rapidly generate potent molecules.
  2) To a matrix metalloproteinase (MT1-MMP) where we show the ability to identify novel binding sites.
  3) To a chemokine class A GPCR (CCR4) which makes accessible target classes like ion channels and GPCRs, which are mostly intractable to antibodies.

INTRODUCTION

Phage display is a powerful technology that has been used extensively to screen isolated proteins to identify high affinity / high selectivity molecules[1]. However, many integral membrane proteins (e.g. GPCRs & ion channels) require membrane localisation to stabilise their secondary and tertiary structures and therefore cannot be screened as soluble proteins. We have adapted the Bicycle Therapeutics proprietary phage display platform to enable whole cell-based phage panning to enable screening against important integral membrane protein targets.

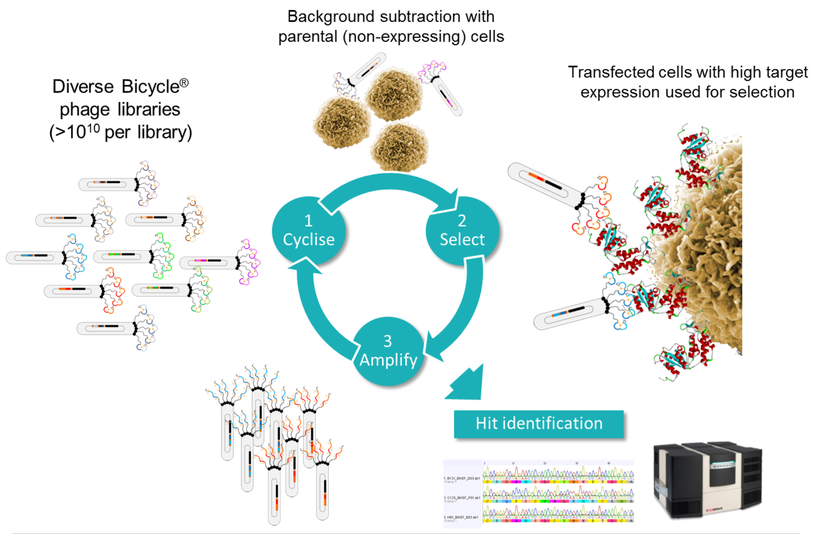

Figure 1: High throughput *Bicycle* phage display process on whole cells.

METHODS

- Phage libraries containing >10^{10} unique *Bicycle* were screened against cells ectopically expressing the full length transmembrane proteins: EphA3, MT1-MMP, CCR4
- Phage outputs were characterized using pyrosequencing, and their affinities determined using either an ALPHAscreen binding assay or a cell-based ELISA
- Peptides were synthesized by Fmoc solid phase synthesis, cleaved and cyclised with a tri-functional small molecule scaffold
- Peptides were characterized using a fluorescence polarization binding assay
- DiscoverX PathHunter® eXpress CCR4 CHO-K1 β-Arrestin GPCR Assay was used to characterised peptides against CCR4

RESULTS

Rapid identification of high affinity *Bicycles* using cell-based screening

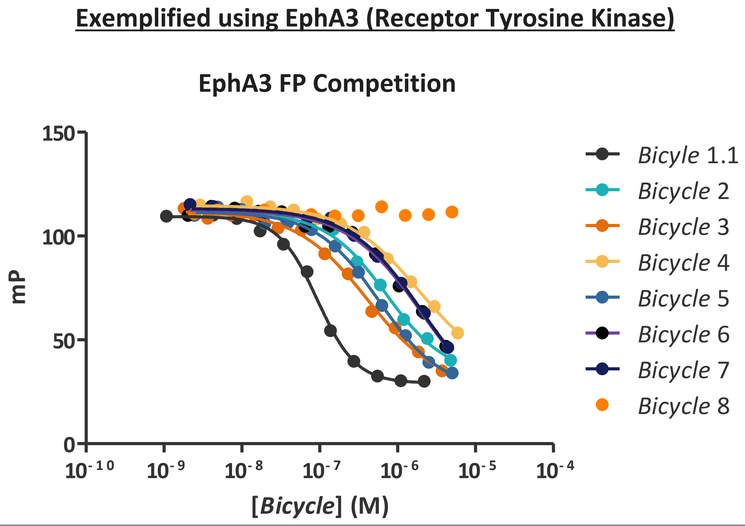

Figure 2: Competition fluorescence polarization (FP) assay against soluble ectodomain EphA3 protein using *Bicycle* 1.1 fluorescein labelled showing competition with unlabelled *Bicycle* peptides identified by cell-based selection.

Peptide	Loop size	Ki (nM)
Bicycle 1.1	6X6	5.39
Bicycle 2	2X7	104
Bicycle 3	2X7	47
Bicycle 4	2X7	238
Bicycle 5	6X6	77.9
Bicycle 6	6X6	231
Bicycle 7	6X6	263
Bicycle 8	6X6	No binding

Table 1: Loop sizes and affinities of *Bicycles* identified by cell-based selection against soluble ectodomain EphA3

EphA3 ectodomain soluble protein was used to identify *Bicycle* 1 which was further affinity matured leading to the identification of *Bicycle* 1.1 which was then used as a labelled tracer in FP assays.
After four round of naïve selections using the cell-based selection process, six peptides were identified in four weeks process.
Cell-based selections process enabled rapid discovery of high affinity *Bicycles*. Most of *Bicycles* identified via cell-based selections show competition with the *Bicycle* identified on soluble EphA3 protein.

Identification of a novel binding site for *Bicycles* on MT1-MMP using cell-based selection

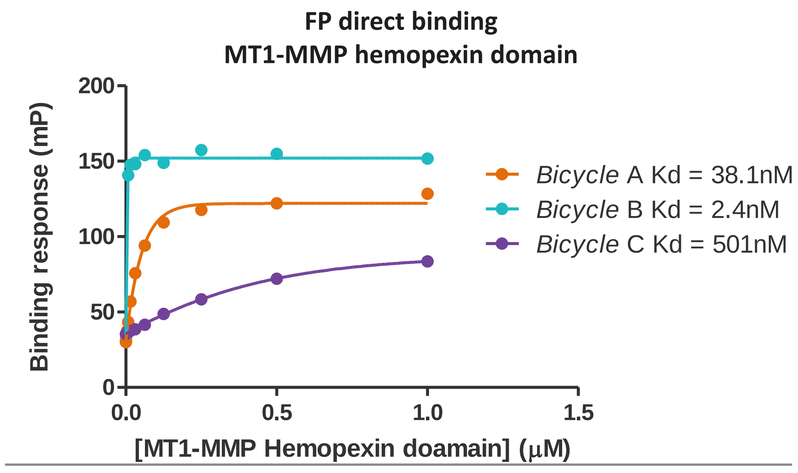

Figure 3: Fluorescence polarization assays were used to characterise binding using the MT1-MMP hemopexin domain and *Bicycles* labelled with fluorescein identified by soluble (*Bicycle* A and B) or cell-based selection (*Bicycle* C)

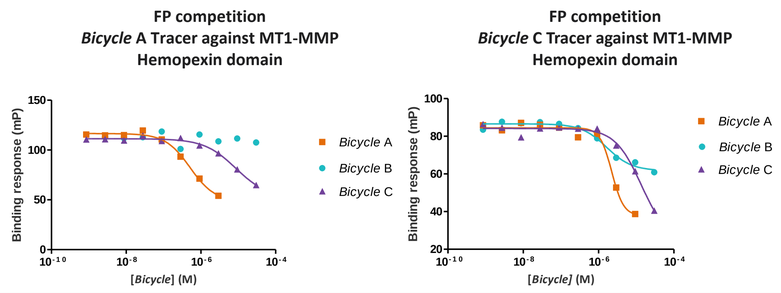

Figure 4: Competition Fluorescence polarization assay on MT1-MMP hemopexin domain showing competition between the different families

Two *Bicycles* A and B were initially identified using conventional phage display and shown to bind to the soluble hemopexin domain of MT1-MMP. These two peptides bound to distinct sites (the collagen binding site and a new previously undescribed site the "neutral site" Figure 5) on the protein sub-domain but did not compete one another's binding. The same peptide described above and a third family of *Bicycles, Bicycle* C were identified when cell-based selection was used against ectopically expressed full length MT1-MMP. This peptide competed the binding of both *Bicycle* A and *Bicycle* B which is indicative of binding to a second novel site which either overlaps the two previous binding sites or has an allosteric effect on binding.
This shows the power of screening against a native conformation of a given target, allowing the full complexity of the target to be observed and accessed by the phage libraries to identify binding pockets.

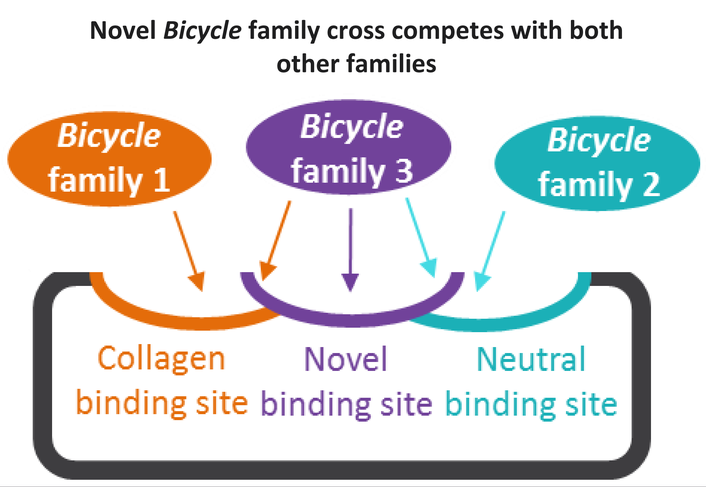

Figure 5: Three distinct binding sites have been identified on MT1-MMP using Bicyclic library screens: two distinct binding sites were identified using soluble protein (orange and blue) the same sites were identified using cell based screens along with an additional novel binding site which was only identified by cell-based selection. This site showed cross competition with both previous binding sites (purple).

Identification of functional Bicycle against a chemokine GPCR: CCR4

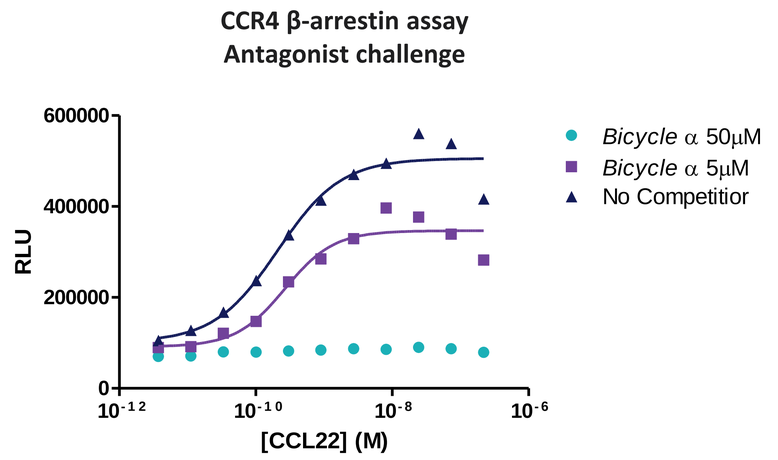

Figure 6: DiscoverX PathHunter® eXpress CCR4 CHO-K1 β-Arrestin Assay. Increasing concentration of *Bicycle* α have been applied on CCL22 dose response.

Cell-based selection and ELISA was performed on CCR4 expressing cells and several hits were identified including *Bicycle* α. *Bicycle* α reduced the maximum signal of the agonist in a dose dependent manner leading to complete inhibition of the signal at 50 μM.

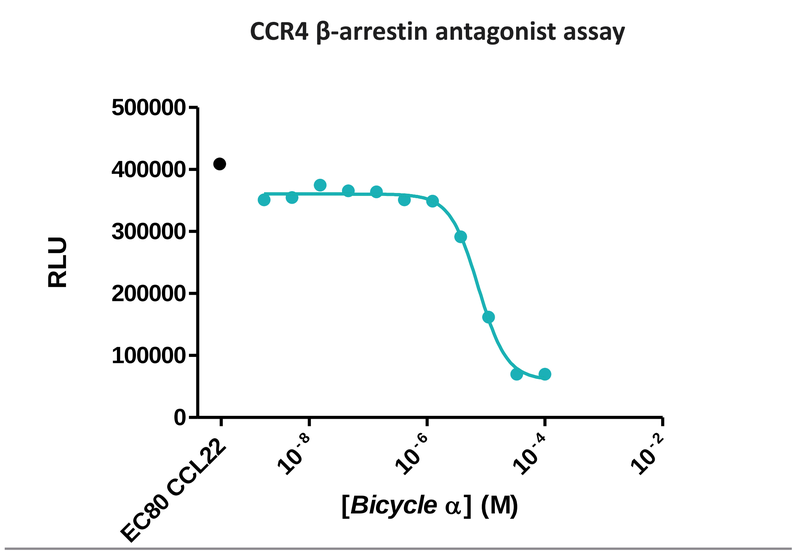

Figure 7: DiscoverX PathHunter® eXpress CCR4 CHO-K1 β-Arrestin Assay. Dose response of *Bicycle* α has been applied on CCL22 EC80 (10nM).

Bicycle α showed a functional effect in a DiscoverX PathHunter® eXpress (CCR4 CHO-K1 β-Arrestin) assay and antagonized CCL22 at an EC_{80} concentration exhibiting an IC_{50} of 7.4μM.
This micromolar activity is currently being optimised by further affinity maturation at Bicycle Therapeutics.

Cell-based selection using *Bicycle* phage display platform allowed the discovery of pharmacologically active bicyclic peptides for an integral membrane protein class A GPCR. This approach opened the doors for Bicycle Therapeutics to target previously intractable targets involved in many diseases.

CONCLUSION/SUMMARY

Bicycle Therapeutics has successfully adapted its *Bicycle* phage display platform to screen transmembrane proteins on whole cells.

Using this novel cell-based selection technique we have:

1) Identified high affinity *Bicycles* targeting EphA3 (receptor tyrosine kinase) in a short cycle of screening taking only 4 weeks

2) Identified novel previously undescribed binding sites on MT1-MMP (matrix metalloproteinase)

3) Shown the utility and versatility of the platform to target chemokine class A GPCRs such as CCR4

These three examples showed that cell-based phage display screening is a powerful and attractive technology with which to screen previously intractable target classes (such as GPCRs and ion channels) many of which are important therapeutic targets involved in diseases. Bicycle Therapeutics is using this platform to extend its pipeline in oncology and other important disease areas.

REFERENCES

[1] Xiaolei Wang et al. *Biochemistry and Biophysics Reports* 3:pp 169–174 (2015)
[2] Heinis *et al. Nature Chemical Biology*. 5:pp502-507 (2009)

–**Targets** like an antibody –**Performs** like a small molecule –**Excretes** like a peptide

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