DEVELOPMENT AND OPTIMISATION OF A BISPECIFIC *BICYCLE*®-LIGAND FOR CELLULAR INTERNALISATION

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ABSTRACT

Bicycles have shown unprecedented modularity in function over recent years, from *Bicycle*-Drug Conjugates (BDCs) to Tumour-targeted Immune Cell Agonists (TICAs™) (Mudd et al., 2020). Further modularity to the *Bicycle* platform could be introduced by developing methods to internalise *Bicycles* into the cell.

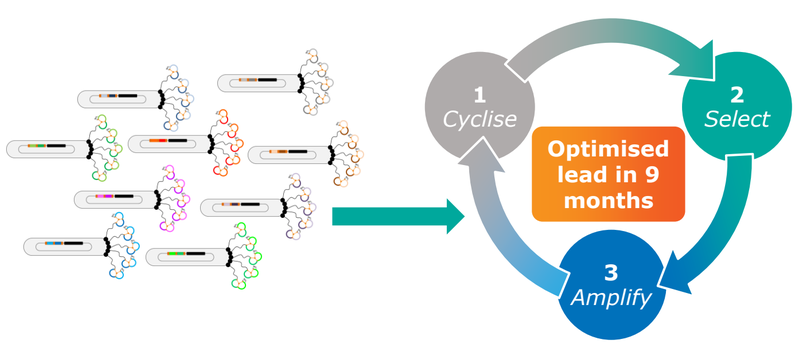
The study presented here centres first on optimising a *Bicycle* binder to Protein 1, followed by development and optimisation of a biotinylated Ligand 1 tool compound, internalised by Protein 2. This would enable the functional combination of the two as a bispecific *Bicycle*-Ligand for use as a proof of concept for *Bicycle* internalisation.

BICYCLE PLATFORM

Bicycle Therapeutics has further evaluated, developed and commercialised Sir Gregory Winter's pioneering work on phage display (Heinis et al., 2009). M13 Bacteriophage are used to display peptides cyclised in a bicyclic format, usually 8-20 amino acids in length. These bicyclic peptides – *Bicycles* – are selected against a target protein of interest. The peptide backbone of the *Bicycle* allows for tremendous, modular diversity within phage libraries, and the scaffolded nature of the cyclised *Bicycle* introduces various degrees of constraint which drives exquisite selectivity and affinity. To date, the company has achieved very highly, with success against more than 80% of its targets.

Diverse *Bicycle* phage libraries (>10^{15})

Evolution driven, informed selection



METHODOLOGY

- Protein 1 binding *Bicycles* were identified via iterations of phage selections and affinity maturation
- A lead *Bicycle* sequence underwent SAR analysis and was screened in fluorescence polarisation (FP) competition assays using Protein 1 (Figure 2)
- Multiple amino acid substitutions were subjected to affinity determination via FP direct binding (Figure 3)
- Biotinylated tool compound internalisation was demonstrated and quantified using IncuCyte™ live-cell imaging (Figure 4)
- Bispecific activity of *Bicycle*-Ligand was shown via a dual ligand binding AlphaScreen using Proteins 1 and 2 (Figure 5)

CONCLUSIONS

- Several high affinity SAR Bicycle sequences were identified
- Studies using biotinylated tool compounds drove the eventual synthesis of bispecific *Bicycle*-Ligand
- *Bicycle*-Ligand conjugate showed bispecific activity by co-binding Proteins 1 and 2
- The bispecific *Bicycle*-Ligand provides opportunity for novel extension of the already acclaimed *Bicycle* platform

ACKNOWLEDGEMENTS

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REFERENCES

Heinis, C., Rutherford, T., Freund, S. and Winter, G., 2009. Phage-encoded combinatorial chemical libraries based on bicyclic peptides. *Nature Chemical Biology* [Online], 5(7), pp.502–507. Available from: https://doi.org/10.1038/nchembio.184.

Mudd, G.E., Brown, A., Chen, L., van Rietschoten, K., Watcham, S., Teufel, D.P., Pavan, S., Lani, R., Huxley, P. and Bennett, G.S., 2020. Identification and Optimization of EphA2-Selective Bicycles for the Delivery of Cytotoxic Payloads. Journal of medicinal chemistry [Online], 63(8), pp.4107–4116. Available from: https://doi.org/10.1021/acs.jmedchem.9b02129.

APPROACH OVERVIEW

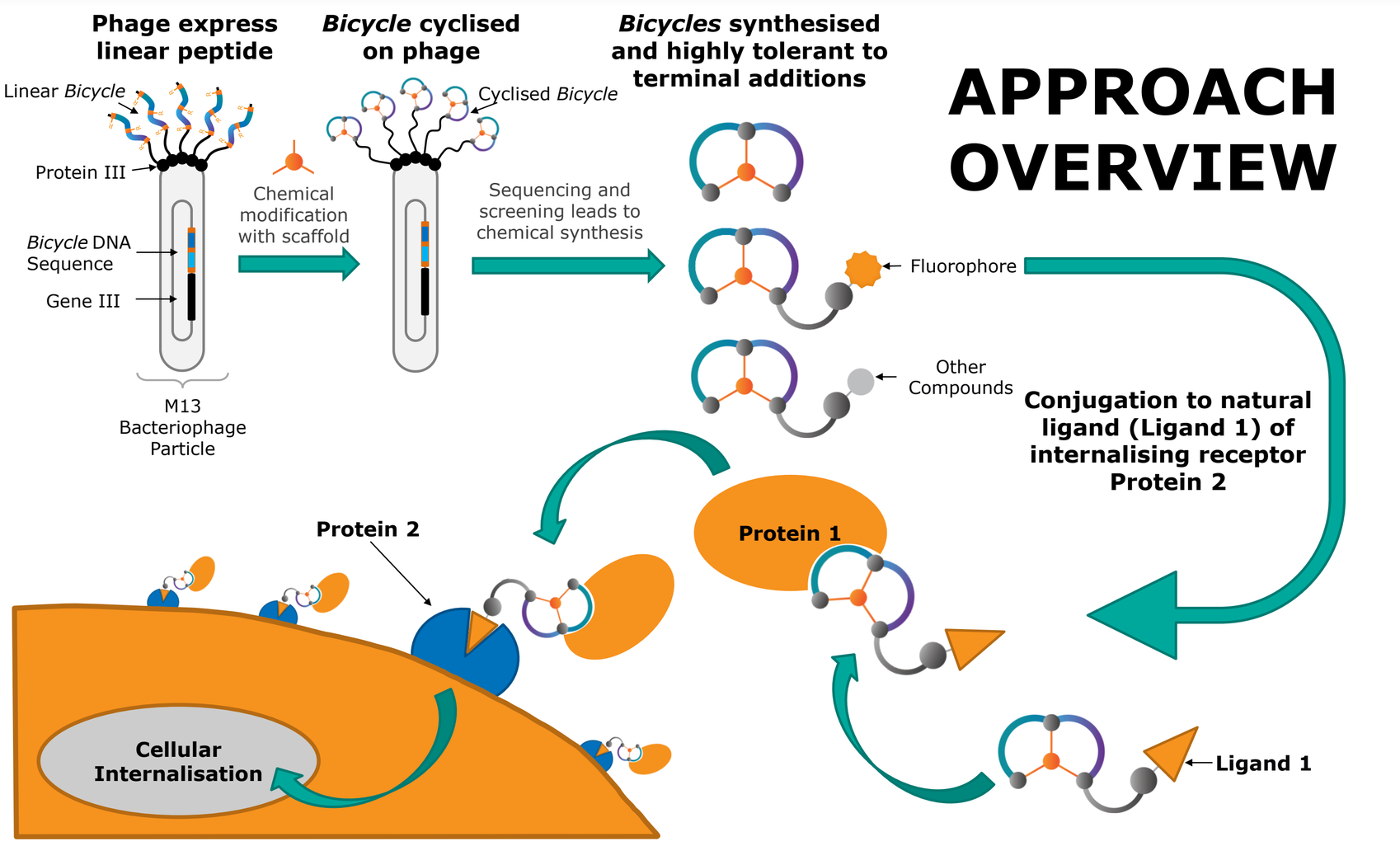

Figure 1: Overview of *Bicycle* generation and modification to form a bispecific *Bicycle*-Ligand. M13 bacteriophage virus particles have a *Bicycle* DNA sequence inserted into their pIII coat protein gene. Orange dashes represent selectively placed cysteine residues which separate the two loops of the *Bicycle* and act as attachment points for a trivalent chemical scaffold. This scaffold cyclises the *Bicycle.* As *Bicycles* are initially screened whilst attached to megadalton phage particles, they are very tolerant to terminal additions. The *Bicycle* in this case was selected to bind Protein 1 with high affinity. Ligand 1 binds Protein 2, targeted to internalise. The *Bicycle* in complex with Ligand 1 binding Protein 2, drives targeted cellular internalisation of the target Protein 1.

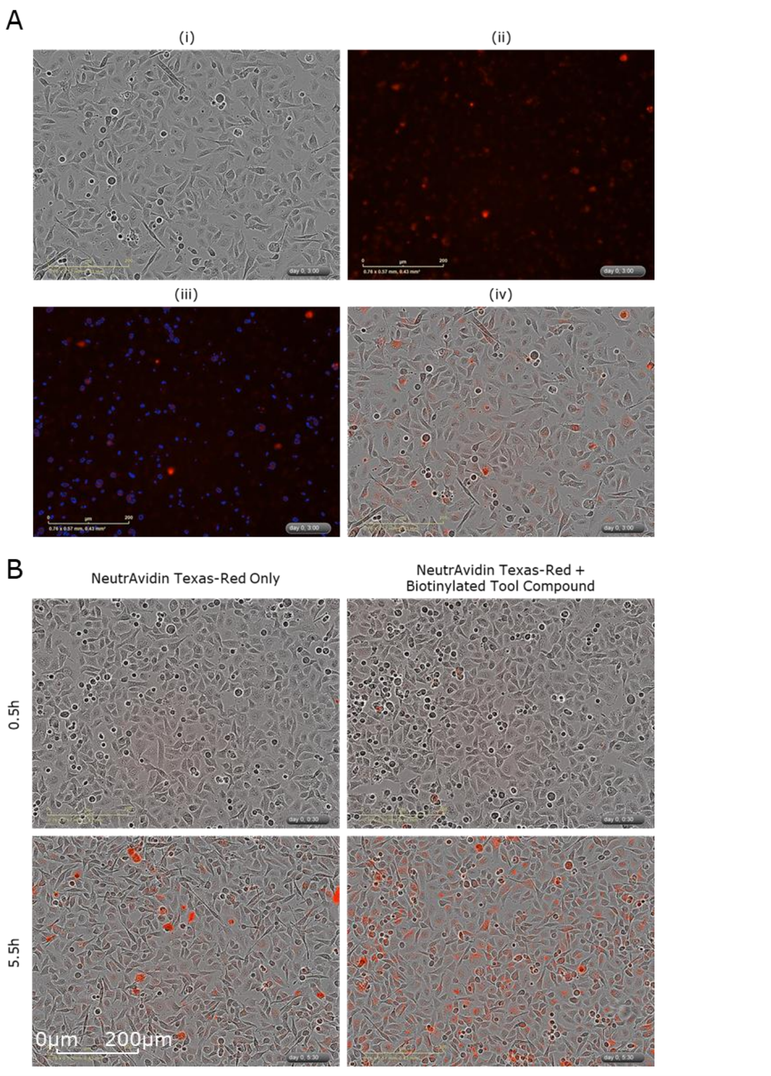

Figure 4: Internalisation of NeutrAvidin Texas-Red (NA-TR) using biotinylated tool compound on HT-1080 cells. Internalisation assay data collected and analysed on an IncuCyte™ Zoom. (A) A processing definition set with specific parameters to filter unwanted phase contrast and red phase objects was used (i) Phase contrast only (ii) Red phase only (iii) Red phase objects recognised by processing definition indicated with blue outline (iv) Phase contrast and red phase composite overlay. (B) A representative comparison of -/+ biotinylated tool compound at 0.5 h and 5.5 h incubation showed clearly observable greater red phase signal in the presence of Ligand 1 tool compound.

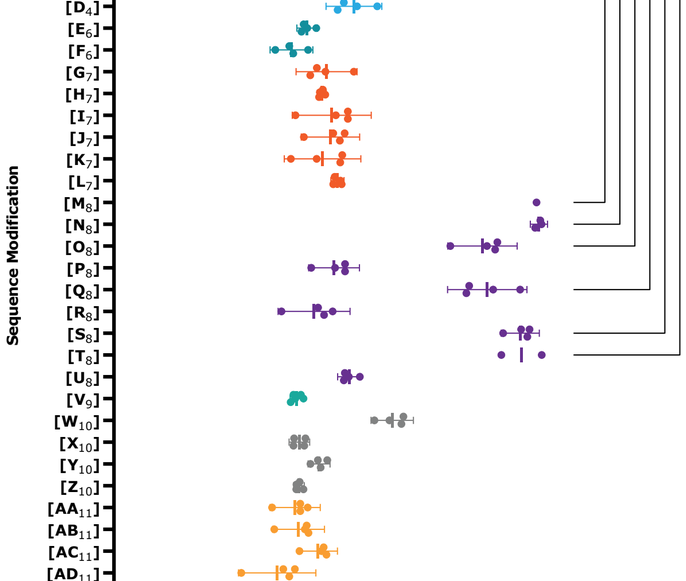

Figure 2: Affinity determination of SAR peptide sequences. Inhibitory constant (K_i) determination by FP competition assays. Data shown is geomean average with error bars denoting the 95% confidence intervals. Each affinity determined representative of at least n=4 biological repeats. Sequence data highlighted in format [X_α], where X= substituted amino acid, α = position of substitution. Statistical analysis was performed using a one-way ANOVA, Dunnett's multiple comparisons test using Sequence 2 as a reference. **** denotes p≤0.0001.

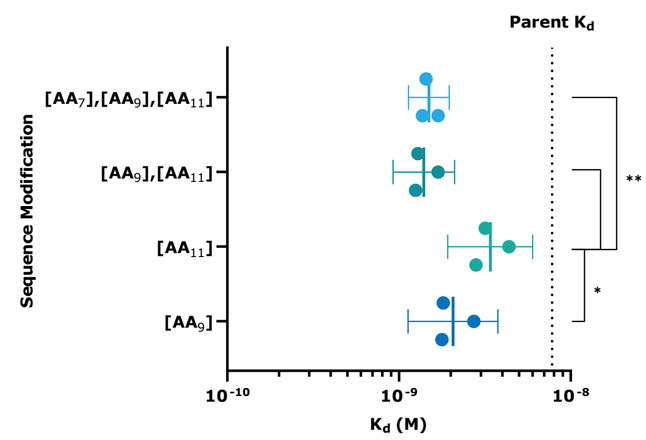

Figure 3: Affinity determination of multiple SAR replacements. Dissociation constant (K_d) determination used FP direct binding assays. Data shown represents geomean K_d with error bars denoted by the 95% confidence intervals (n=3). Sequence data highlighted in bold and format [AA_β] represented where AA = Amino Acid, β = position of substitution. Statistical analysis was performed using a one-way ANOVA, Tukey's multiple comparisons test. * denotes p≤0.05, ** denotes p≤0.01.

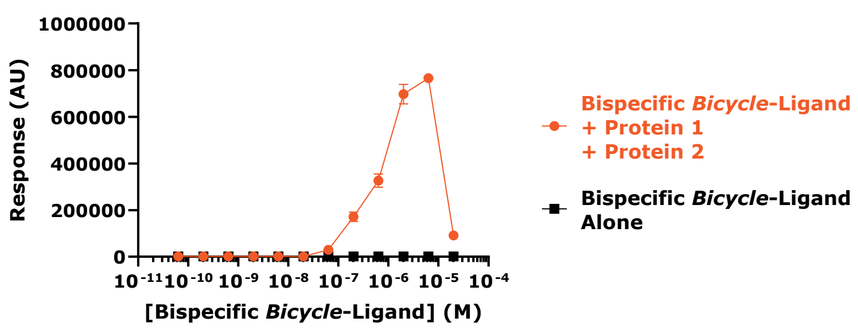

Figure 5: Bispecific activity of the *Bicycle*-Ligand conjugate was observed using an AlphaScreen assay. AlphaScreen Dual Ligand Binding assay. Data points represent n=2 ± SD. Signal generation in this assay was described in arbitrary AlphaScreen Units (AU).

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