Novel Multimers of Bicyclic Peptides Cluster and Activate CD137 (4-1BB): A Costimulatory T -Cell Checkpoint Receptor

ABSTRACT# A8

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ABSTRACT

- Agonism of CD137 to activate T cell mediate anti-tumour effects is a promising immunotherapeutic approach validated by numerous *in vivo* models
- CD137 agonism requires clustering of receptors into trimeric complexes; would be difficult achieve with small molecule drugs.
- CD137 agonistic antibodies currently in clinical testing but have shown hepatotoxicity and limited efficacy.
- Bicycles are bicyclic peptides constrained via a trifunctional chemical scaffold and bind targets with high affinity and selectivity.
- Bicycle binders to CD137 were identified by phage display before chemical optimisation to generate plasma stable monomeric antagonists of CD137.
- Systematic chemical elaboration generated of a matrix of dimeric, trimeric and tetrameric synthetic CD137 agonists with a broad range of activity in a cell-based functional assay.
- Bicycle multimers exhibit high avidity to CD137 on the cell surface resulting in receptor agonism even with short exposure.
- The Bicycle platform is efficient, flexible and adaptable and lends itself to multiple applications in oncology and beyond.

INTRODUCTION

CD137 (4-1BB/TNFRSF9) is a co-stimulatory T cell receptor expressed on activated CD4+ & CD8+ cells. Ligation and clustering of the receptor by CD137L (4-1BBL), expressed on antigen presenting cells (APCs) promotes T cell survival and proliferation *in vivo* (Fig.1). In an immune oncology setting, agonistic antibodies of CD137 have demonstrated strong anti-tumour activity in mouse models, including inducing a memory response (Melero *et al.* 1997). Human trials have been less successful due to hepatotoxicity (urelumab, BMS, Segal *et al.* 2017) or lack of single agent activity (utomilumab, Pfizer, Segal *et al.* 2018). Development of an agonist of CD137 that can overcome weak agonism and excessive activity in the liver could lead to a potent anti-tumour agent.

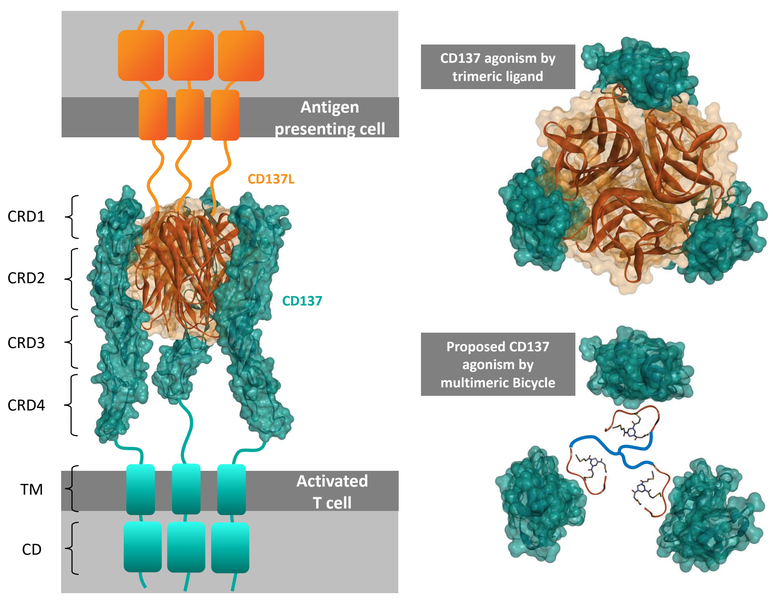


Fig. 1: Cross-linking of CD137 is required for receptor activation. CD137 (teal surface) comprises 4x cysteine-rich domains (CRD), a transmembrane domain (TM) and a cytoplasmic domain (CD). The CD137 signaling complex requires organization of the receptor into trimers by CD137L (orange surface). A multimeric Bicycle binder to CD137 could mimic the agonist action of CD137L by bringing together CD137 receptors on the surface of T cells. PDB accession 6CPR (Bitra *et al.* 2018)

Bicycles are a new class of drugs–constrained bicyclic peptides that show antibody-like affinity and exquisite target specificity, but with the advantages that come from being fully synthetic with tunable PK and built-in tolerance to conjugation. The Bicycle platform combines highly diverse phage display libraries with chemical optimisation to rapidly identify and optimise binders for affinity, biological function and physicochemical properties. Through novel chemical approaches, Bicycles can be joined together in a modular fashion to generate multimers that cross-link receptors.

METHODS

Phage selections: Bicycle phage libraries were panned against biotinylated soluble recombinant human CD137. After 4 rounds of selection and amplification, individual phage clones were sequenced and a phage-based ALPHAscreen used to identify binders.

Bicycle synthesis: Peptides made using standard solid phase Fmoc peptide chemistry followed by cyclisation on TATA scaffold. Lysines or D-lysines were substituted at various positions of the Bicycle for the attachment of discrete lengths of PEG linker and multimerization on central hinges.

Fluorescence polarisation: Bicycles were made with a short linker to a fluorescein probe. Binding to CD137 indicated by an increase in mP in assay

Surface plasmon resonance: Bicycles flowed over biotinylated CD137 immobilised on a streptavidin chip in a Biacore instrument

CD137 agonism reporter gene assay: CD137 NF-kB luciferase reporter assay cells used following manufacturer's instructions (Promega).

Synegeneic humanised mouse CDX model: Mice expressing humanised CD137 implanted with MC38 cell line xenografts. Tumours grown to ~100 mm^3 before dosing with CD137 agonists.

RESULTS

A diverse library of >10^{12} Bicycles were screened on phage against human CD137 protein. Hits from phage panning were synthesised. A peptide sequence was identified with K_D of 1.4 µM as determined by fluorescence polarisation (FP) and chosen for optimisation on phage by use of custom phage libraries. An improved Bicycle binder with K_D of 67 nM (FP) was found and a single amino acid substitution gave a plasma stable lead Bicycle with K_D of 32 nM (measured by surface plasmon resonance, SPR). Competition experiments showed that the Bicycle binding site had overlap with both the natural ligand and utomilumab but not urelumab (data not shown).

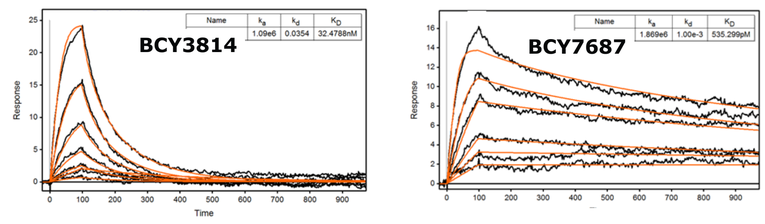
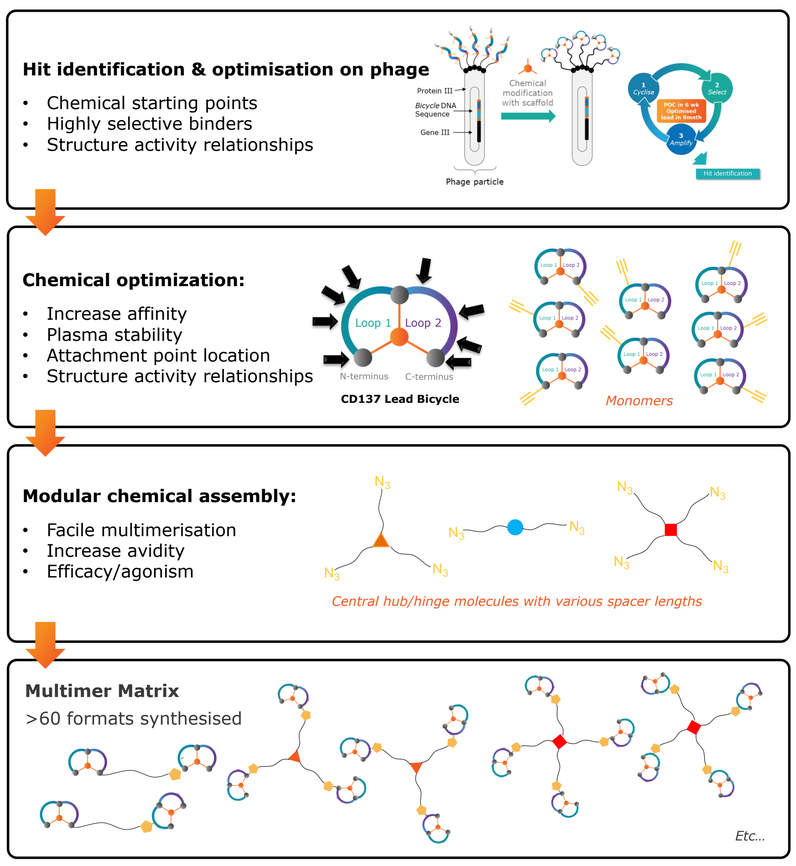


Fig. 2: Homotandem Bicycles show increased affinity to CD137 versus monomeric Bicycles. SPR measurements with mono- and di-meric Bicycles show that the dimer form exhibits similar association rates but much reduced rate of dissociation leading to an increase in affinity of over 20 fold. Testing with trimeric and tetrameric Bicycles showed even slower k_d values and higher affinities (data not shown, poor fit to binding models as result of multiple interactions).



Conjugation of a fluorescent probe linked by a PEG linker was found to be tolerated at 8 positions within the Bicycle (affinity to CD137 retained within 10x). These permissible regions of the peptide sequence were used as attachment points to assemble multimeric Bicycles using click chemistry. Combining CD137 Bicycles together gave an avidity advantage and a homotandem/dimer of the lead Bicycle improved affinity to ~0.5nM (Fig. 2). Monomeric and dimeric Bicycles were found to be functional antagonists of CD137 in a reporter gene assay (data not shown). Assembly of Bicycles into trimers and tetramers yielded agonists of CD137 with a range of efficacies (Fig. 3) between that of the two control antibodies used in the assay (urelumab and utomilumab) and similar to soluble CD137L (4-1BBL).

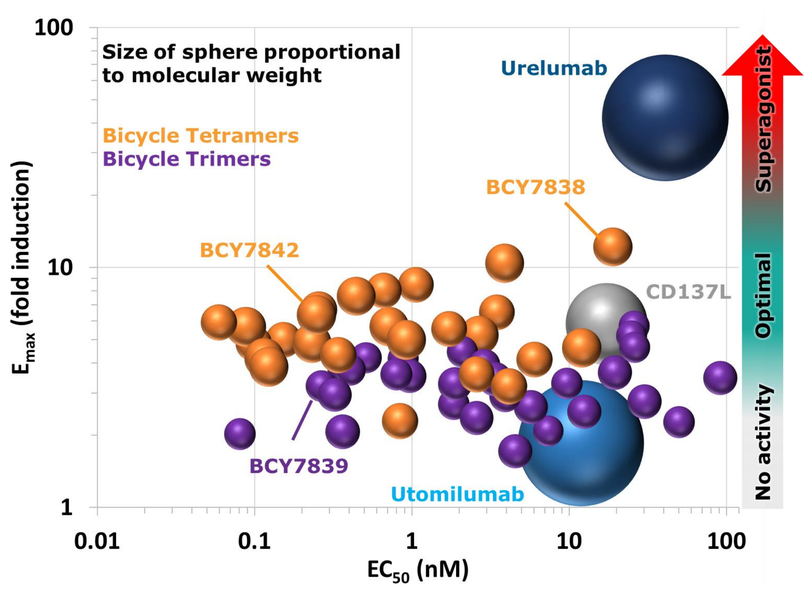


Fig. 3: Activity of trimer and tetramer Bicycles in a CD137 agonism reporter gene assay. Synthetic Bicycle trimers and tetramers are significantly smaller than biological activators of CD137 (soluble CD137L, urelumab and utomilumab) and demonstrate a range of activities. Monomeric and dimeric Bicycles show no agonism (not shown); tetrameric Bicycles generally give higher fold induction at lower concentrations versus trimeric Bicycles.

Compound	Multimer state	Attachment point	Linker	Mol. weight (kDa)
BCY7842	Tetramer	D-Lysine4	PEG23	14.1
BCY7838	Tetramer	Lysine3	PEG23	13.8
BCY7839	Trimer	D-Lysine4	PEG10	8.9
BCY7687	Dimer	C-terminus	PEG24	5.4
BCY3814	Monomer	N/A	N/A	2.1
Soluble CD137L	Trimer	N/A	N/A	60.1
Urelumab	Dimer*	N/A	N/A	150

*Urelumab has 2x CD137 binding sites; interaction with Fc receptors may produce higher order clusters *in vivo*

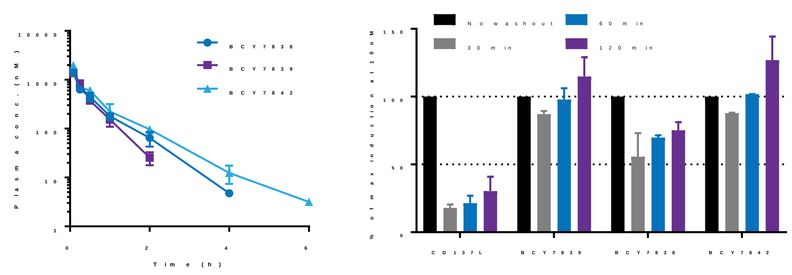


Fig. 4:
***In vivo* PK in mice** (left-hand graph): concentrations of Bicycle multimers in plasma after i.v. dosing at 5mg/kg. Bicycles show half-lives of 20-40 minutes.
CD137 Bicycle multimers maintain activity after washout (right-hand graph): Bicycle multimers were incubated with reporter cells (same as in Fig. 3) for 30, 60 or 120 min followed by washout of the compound or the compound was left on the cells for the full duration of the assay (no washout). Activity was measured 6 h after start of assay experiment. In all cases the Bicycle multimers were retained better on the cells and showed higher induction versus the CD137L control. For BCY7839 and BCY7842, 30 min exposure was sufficient to produce almost the same level of agonism as the 6 h no washout sample.

Multimeric Bicycles can overcome issues hindering development of an anti-tumour agent based on CD137 agonism. By tuning cellular activity to be around that of the natural ligand, they can avoid the lack of activity observed with very weak CD137 agonist antibodies. In addition, due to their tuneable half life and lack of an Fc domain that could interact with Fc receptors, it is anticipated that Bicycles would not illicit the on-target hepatotoxicity seen with superagonists like urelumab.

Limitations of multivalent biologics	Bicycles ideal as T cell activators
• Limited tumour penetration due to large size (150-275 kDa)	• Large volume of distribution (total extracellular water) due to small size (9-14 kDa as tri/tetra-mers)

• Liabilities associated with biologics
  • Immunogenicity
  • Interaction with Fc receptors
  • Difficulty of development and manufacture
	• Fully synthetic (NCE classification)

• Rigid biological scaffolds limit versatility	• Facile chemical synthesis using standard peptide chemistry

• Fixed PK profile, long half life; may be undesirable in activation pathways (T cell exhaustion)	• Highly flexible format, alter level of activation by changing:
	  • Affinity
	  • Attachment point
	  • Linkers
	  • Multimerization state

	• Tunable *in vivo* exposure for safety and efficacy

CONCLUSIONS

- Phage display of Bicycle peptides identified binders specific for human CD137 protein that shows tolerance to conjugation.
- Chemical optimisation of the affinity matured phage hit lead to generation of a plasma stable antagonistic monomer Bicycle with well defined permissible sites of chemical elaboration.
- Assembling multimers of Bicycles by click chemistry generated trimer and tetramer Bicycles that were potent agonists of CD137 in an *in vitro* cell-based assay, comparable to or better than the natural ligand and clinical antibodies.
- When dose i.v. multimer Bicycles exhibit *in vivo* half-lives typical of an unmodified Bicycles of 20-30 mins.
- Avidity advantage of multimeric Bicycles mean that even when exposed to CD137 on cells for a short period of time, they are able to be retained on the receptor and potentiate a long lasting effect.
- Bicycle multimers represent novel small molecule agonists of CD137 that could form the basis of safe and effective anti-tumour agents.

REFERENCES

1. Melero *et al.* Nat Med, 1997, 3(6): 682–685
2. Segal *et al.* Clin Cancer Res, 2017, 23(8): 1929–1936
3. Segal *et al.* Clin Cancer Res, 2018, 24(8): 1816–1823
4. Bitra *et al.* J Biol Chem, 2018, 293: 9958–9969
5. Rabu *et al.* J Biol Chem, 2005, 280(50): 41472–41481

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