Small Synthetic, Multivalent Bicyclic Peptides That Activate T Cell Costimulatory Protein CD137

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- CD137 agonism is a promising immunotherapeutic approach and there are currently two agonistic antibodies in clinical trials.
- CD137 receptors on immune cells form trimeric complexes in the activated state. Peptides binding to human CD137 ligand-binding site were identified by phage screening using proprietary Bicycle technology.
- Further chemical optimisation allowed systematic generation of a matrix of dimeric, trimeric and tetrameric CD137 synthetic agonists with a broad range of cell-activity properties.
- CD137 synthetic multimers were shown to be stable in plasma and to have in vivo half-life of approximately 30 minutes.
- A trimer and tetramer using the Lysine 3 attachment point were efficacious in preventing syngeneic tumour growth in the hCD137 mouse model.

INTRODUCTION

CD137 (4-1BB/TNFRSF9) belongs to the TNF receptor superfamily and provides costimulatory signalling for T cells and NK cells. Agonistic anti-CD137 antibodies have shown potent, often curative anti-tumour activity in preclinical mouse models (1). These effects are mainly mediated by cytotoxic T cells and generate long lasting, memory responses. Two human anti-CD137 antibodies urelumab (BMS) and utomilumab (Pfizer) are currently undergoing clinical testing. Urelumab has shown several single-agent partial responses, but its use has been hampered by on-target hepatotoxicity (2). The agonist antibody utomilumab lacks hepatotoxicity, but has shown little or no single agent activity in solid tumours (3).

Bicycles® are a new class of drugs - fully synthetic, constrained bicyclic peptides that show antibody-like high affinity binding and exquisite target specificity (4). The Bicycle platform uses phage display and chemical optimization to rapidly identify and improve CD137 binders for affinity and physicochemical properties. Through novel chemical approaches, peptides can be attached to generate agonistic multimers that cross-link and thus activate the trimeric CD137 receptor complex on immune cells. We hypothesized that fully synthetic Bicycle CD137 agonist multimers may induce CD137-mediated anti-tumour immune responses. By generating an extensive multimer matrix, we aimed to modulate properties that are desirable for CD137 agonism as a novel immunotherapeutic approach.

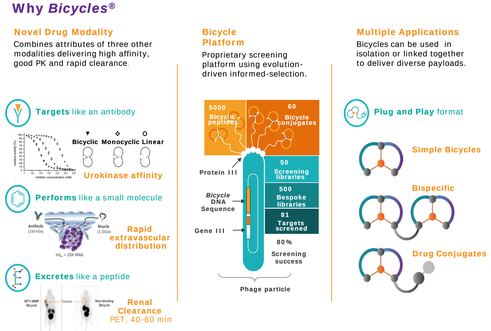

Figure 1: Bicycle peptides are flexible therapeutic modules. The generation of synthetic multimers as agonists enable Bicycle peptides to target trimeric TNFR-superfamily immune receptors such as CD137.

- Enormous (>10^{15}) & diverse libraries generate multiple chemical start points.
- Fully synthetic, faster, more versatile production than antibodies.
- Bicyclic multimers have different in vivo profile from antibodies. The clinical development of urelumab has been hampered by on-target hepatotoxicity. We expect the risk of liver inflammation to be minimal with CD137 synthetic multimers.

METHODS

Phage selection: Bicycle phage libraries were used to identify binding peptides to the target human CD137 protein. The hits were characterized by alphascreen and pyrosequencing. Further rounds of affinity maturation identified CD137 binders in the 50-100 nM range.

Chemistry: The CD137 binding peptides were further optimized to improve peptide stability by substitutions with non-natural amino acids. Monomeric peptides with different attachment points were generated based on peptide SAR information. The peptides were linked using discrete PEG spacers of various lengths to generate fully synthetic dimers, trimers and tetramers.

Protein binding: Binding affinities were determined by fluorescent polarization (FP) of FITC-labelled peptides. Alternatively, surface plasma resonance (SPR) Biacore was used for determination of KD-values.

In vitro activity: Cell-activity was analysed using CD137 NF-kB luciferase reporter assay cells (Promega). The activity was normalized to untreated cells as fold induction.

In vivo profiling: Multimer stability in mouse plasma and the half-life upon i.v. dosing 5 mg/kg was determined by LC-MS/MS. For efficacy studies, hCD137 (Biocytogen) mice were inoculated s.c. with MC38 cells and when the tumours reached ~100 mm^2 the mice were dosed with CD137 agonists. Synthetic multimers were dosed i.v. at 20-30 mg/kg biweekly and compared to urelumab dosed i.v. at 3 mg/kg biweekly.

RESULTS

10^{10-11} Bicycles per round were screened on phage against human recombinant CD137 protein. Initial hits in the µM range underwent affinity maturation which identified peptides binding to CD137 with affinities below 100 nM. After chemical optimization, a high affinity lead BCY3814 (K_D ~30 nM) was selected. BCY3814 competed for binding with the CD137 ligand and utomilumab (known to bind to CD137 ligand binding site) but did not compete with urelumab which binds an alternative epitope. CD137 activation requires receptor crosslinking and we aimed to generate synthetic multimers that would emulate the natural trimeric ligand. The versatility of the Bicycle format allowed us to rapidly generate more than 60 different dimer, trimer and tetramer assemblies of BCY3814. The multimers exhibited strong avidity driven binding characterised by extremely slow off-rates typical of higher order multimers. The linker lengths and attachment points were explored while maintaining a compact molecular size (4-15 kDa).

The resulting molecules exhibited a wide range of potencies and efficacies in a cell-based CD137 activation assay. Several of these synthetic Bicycle CD137 agonists were more potent than the clinical antibodies or the natural ligand in the cell reporter assay. Initially, two multimers containing attachment points at the third amino acid position in the peptide that showed potent Emax-values and in vitro cell activity profile similar to CD137L, were selected for mouse proof-of-concept experiments. The in vivo PK profile of the two Bicycle multimers indicated rapid kinetics typical of peptide metabolism. Crucially, the Lysine 3 trimer and tetramer demonstrated anti-tumour efficacy against the MC38 syngeneic tumour in the humanized CD137 mouse model.

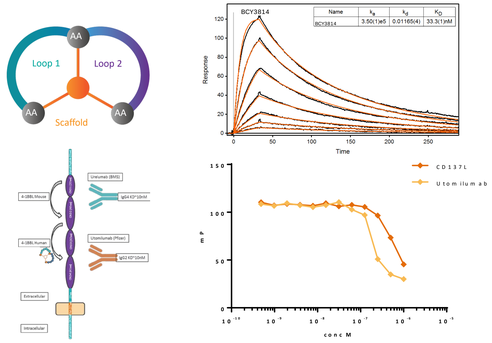

Figure 2: Top: Phage screening identified initial hits from the 6x6 phage library in the uM range that that underwent affinity maturation. The lead peptide BCY3814 showed KD=33.3 nM (SPR) after chemical optimization. Bottom: The general structure of the human CD137 protein indicating the binding sites of agonist antibodies. The parental lead peptide bound to the CD137L-binding site in the CD137 receptor as shown by FP competition against CD137L and utomilumab.

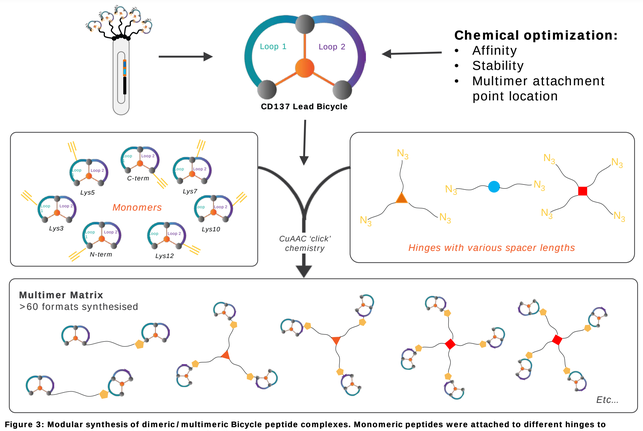

Figure 3: Modular synthesis of dimeric / multimeric Bicycle peptide complexes. Monomeric peptides were attached to different hinges to generate dimers, trimers and tetramers with flexible spacer lengths.

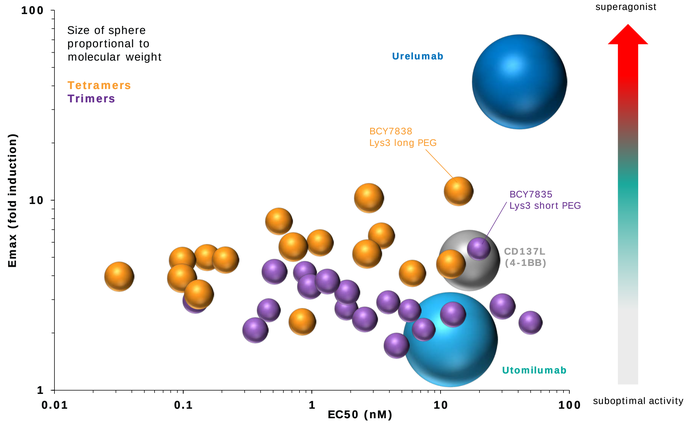

Figure 4: The cellular activity of trimers and tetramer compared to known agonists CD137L, urelumab and utomilumab. The most potent multimers have 100-fold lower EC50-values compared to the natural ligand (below 0.1 nM compared to 10 nM). Size of sphere proportional to the molecular weight. Synthetic dimers showed no / little biological activity in vitro (data not shown). There is trend towards tetramers being more potent than trimers. The multimer matrix enabled rapid generation of molecules with a broad range of cell activity properties.

BCY ID	Multimer	Attachment point	Molecular weight (Da)	EC50 nM (in vitro)	Emax (in vitro)
BCY7835	trimer	Lysine3	8691	19.8	5.55
BCY7838	tetramer	Lysine3	13816	13.7	11.2
CD137L	trimer		60800	16.7	6.8

Table 1: The two Lysine 3 attachment point multimers selected for in vivo profiling compared to the natural ligand CD137L.

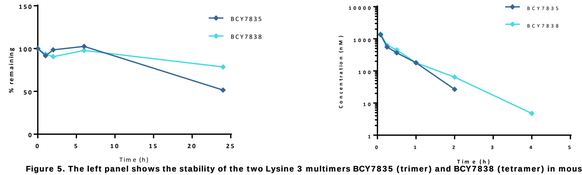

Figure 5. The left panel shows the stability of the two Lysine 3 multimers BCY7835 (trimer) and BCY7838 (tetramer) in mouse plasma over time measured by LC-MS/MS. The data was normalized relative to Time 0. The right panel shows the plasma concentration of the BCY7835 and BCY7838 after dosing at 5 mg/kg i.v. The multimer half-life was calculated to be 20-30 minutes, typical of the rapid clearance kinetics of peptides.

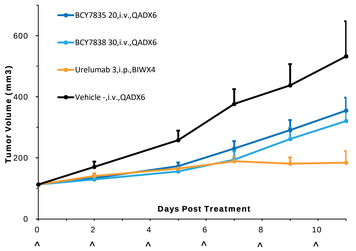

Figure 6. CD137 Bicycle multimers inhibited tumour growth in humanized hCD137 syngeneic mouse tumour model.

Human CD137 (hCD137) knock-in mice were generated by Biocytogen with expression of hCD137 under the control of endogenous mouse regulatory elements. The resulting construct expresses human CD137 that bind Bicycle multimers (data not shown).

The hCD137 mice were inoculated s.c. with syngeneic MC38 cells. Mice were treated when the tumours reached a size of ~100 mm^3. The Bicycle multimers were dosed i.v. at 20-30 mg/kg every other day. Urelumab (3 mg/kg, twice a week) was used as a positive control and vehicle as a negative control.

BCY7835: Lysine 3 attached trimer
BCY7838: Lysine 3 attached tetramer

CONCLUSION / SUMMARY

Bicycle® peptides specific for human CD137 protein were identified by phage screening. Amino acid substitutions of the parental lead peptide revealed potential attachment sites for generation of synthetic multimers. Different attachment points and linkers were selected as a matrix to comprehensively explore the biological properties of synthetic multimers. Trimers and tetramers showed potent cell-activity agonism that was comparable or better than the natural ligand and clinical antibodies.

Lysine 3 side-chain attached trimer BCY7835 and tetramer BCY7838 were stable in plasma and showed rapid clearance in mice. These multimers also showed potent anti-tumour activity in MC38 syngeneic tumour model in a humanized CD137 knock-in mouse model. These results demonstrated the *in vivo* proof-of-concept (POC) of short-acting CD137 peptide agonists as a promising, novel cancer immunotherapeutic candidate. Moreover, this POC paves the way for development of small synthetic, short-acting agonists for other TNF and immunomodulatory receptors.

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