PBP inhibitors discovered using a modified phage display platform (*Bicycles*)

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Introduction

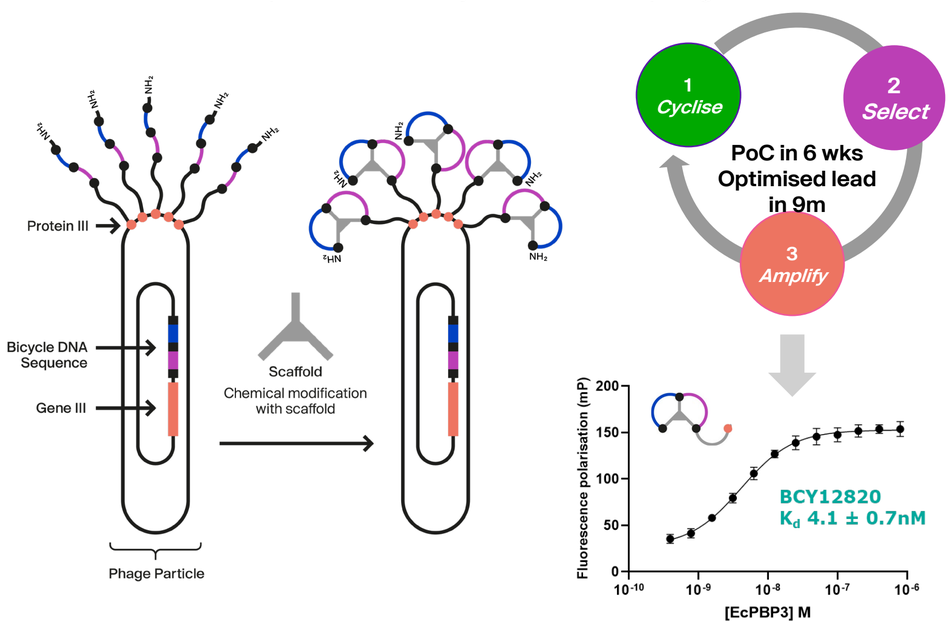
Bicycle Therapeutics is developing a unique class of chemically synthesised medicines based on its proprietary bicyclic peptide (*Bicycle*®) phage display platform. Given the strong precedence for cyclic peptide moieties in successful antimicrobials, *Bicycles*® are a promising new modality for potential novel antimicrobials.

Penicillin binding proteins (PBPs) are one of the key classes of enzymes involved in synthesis of peptidoglycan in bacterial cell walls. Escherichia coli PBP3 is an essential transpeptidase in the division process of peptidoglycan biosynthesis. Beta-lactam antibiotics inhibit PBPs and have been a highly successful class of antibiotics. However, resistance to beta-lactams has arisen in Gram negative bacteria by mechanisms including expression of beta-lactamase enzymes, which cleave the pharmacophore of these antibiotics.

EcPBP3-binding *Bicycles* offer a potential new class of antimicrobials against a well-validated target.

Phage Selections Identify Hit *Bicycles*

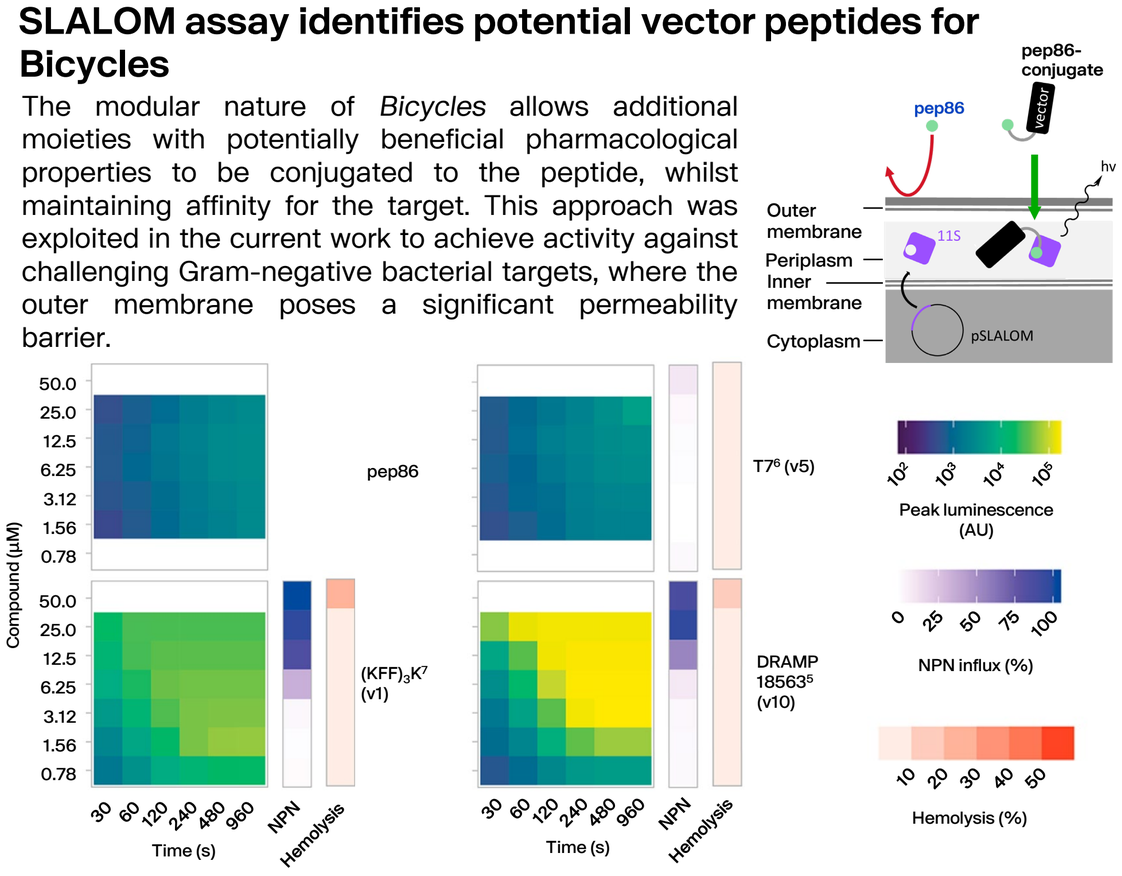
Initial phage selections against the Escherichia coli (Ec) PBP3 target identified two conserved sequence motifs and a variety of enriched sequences. 15 binding hits with diverse sequences were synthesised off-phage for further studies.



Screening in a fluorescence polarisation (FP) competition assay using BOCILLIN FL (Bodipy-penicillin) identified peptide 101-08-00 as a binder at the transpeptidase active site of EcPBP3. The corresponding Fluorescein-labelled peptide BCY12820, with high affinity to EcPBP3 in direct binding assays, was used as a tracer for characterization in FP of subsequent peptides in the series.

SLALOM assay identifies potential vector peptides for Bicycles

The modular nature of *Bicycles* allows additional moieties with potentially beneficial pharmacological properties to be conjugated to the peptide, whilst maintaining affinity for the target. This approach was exploited in the current work to achieve activity against challenging Gram-negative bacterial targets, where the outer membrane poses a significant permeability barrier.



Antimicrobial peptides from the literature and the DRAMP database were screened as potential vectors using a periplasm entry assay (SLALOM – Split Luciferase Assay for Live monitoring of Outer Membrane transit), MIC and hemolysis assays.

$(KFF)_3K$	K F F K F F K F F K
T7	N A G S L L S G W G
DRAMP18563	K S L R R V W R S W W

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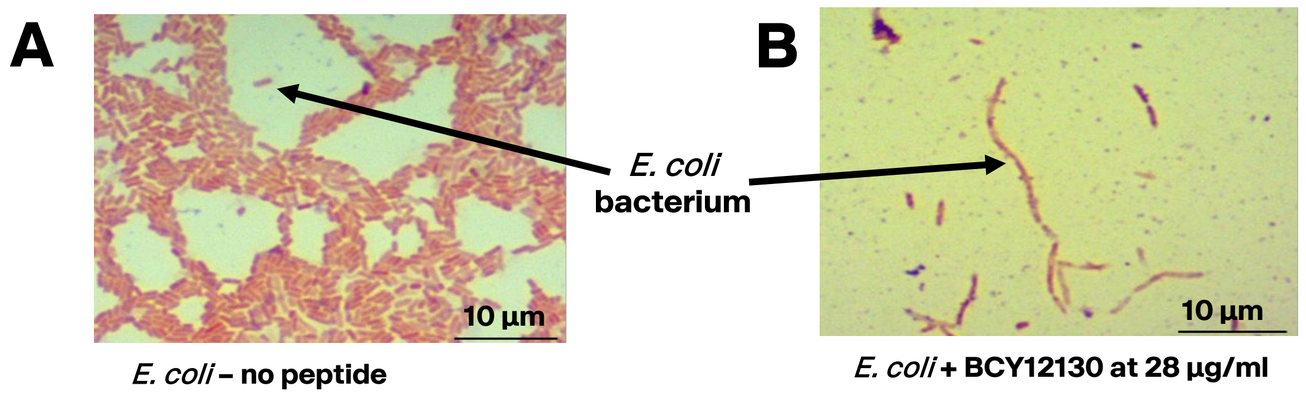
References

(1) Heinis, C. *et al.* Nature Chemical Biology. 5:502–507 (2009); (2) Natale *et al.* Environmental Microbiology. 15: 3169-3182 (2013); (3) Krishnamoorthy, G. *et al.* Antimicrobial Agents and Chemotherapy. 60:7372-7381 (2016). We would like to thank the laboratory of Helen Zgurskaya for *E. coli* strains.; (4) Shi *et al.* Nucleic Acids Research. 50: D488-496 (2022); (5) Wagstaff *et al.* ACS Infectious Diseases. 6: 2355-2361 (2020); (6) Li *et al.* Appl Environ Microbiol. 84:e00052-18 (2018); (7) Good *et al.* Nature Biotechnology. 19:360-364 (2001)

Bicycles Inhibit Bacterial Growth

Compound	MIC (μg/ml)	
	E. coli GKCW101 (WT)	E. coli GKCW102 (Ec-Pore)
BCY12130	> 128	0.5 - 2
Carbenicillin	16	1

'Hyperporinated' tool strains with expression of a constitutively open FhuA pore[3] were used to evaluate cell death via inhibition of EcPBP3 in whole cells, without the OM as a barrier to access the periplasm. BCY12130 showed potent growth inhibition of the hyperporinated strain GKCW102.

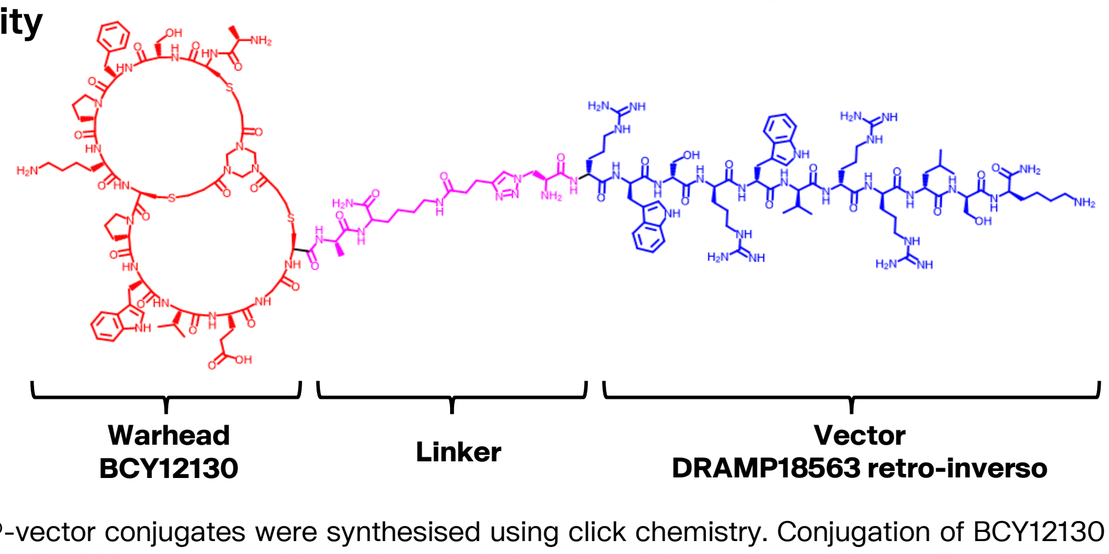


E. coli – no peptide

E. coli + BCY12130 at 28 μg/ml

Morphology studies using E. coli bacteria incubated with BCY12130 resulted in filamentous growth (B). This morphology is indicative of PBP3 inhibition, wherein cell division is incomplete due to inhibition of septal peptidoglycan biosynthesis.

Bicycle-Conjugate Has Potent Whole-Cell Activity Without In Vitro Toxicity



Bicycle®-vector conjugates were synthesised using click chemistry. Conjugation of BCY12130 to the DRAMP18563 vector peptide conferred whole-cell activity against wild-type *E. coli* strains, in addition to activity in related *Enterobacteriaceae*.

Organism	% sequence homology of ftsI* with *E. coli*	BCY13246 MIC (μg/mL)
Escherichia coli	100	4-32 (n=4 including one NDM-1 producer)
Citrobacter freundii	96	4-8 (n=2 including one meropenem resistant)
Klebsiella pneumoniae	94	4-8 (n=4 including one KPC producer)
Enterobacter cloacae	94	2-4 (n=4 including 2 meropenem resistant)
Proteus mirabilis	76	>128 (n=2)
Pseudomonas aeruginosa	45	>128 (n=2)

**PBP3 is the gene product of* ftsI

Cytotoxicity against HT1080 cells was evaluated using an ATP-dependent luminescence assay, with normalization to vehicle and staurosporine. No significant cytotoxicity was observed with the conjugate at concentrations 10-fold higher than MIC by comparison to vehicle control (n=3, unpaired t-test, two tailed; p=0.326).

PK + Early *in vivo* Activity

Conjugates were stabilised in mouse blood by switching the stereochemistry of the vector (retroinverso format), N-terminal capping, and exchange of residues outside of the binding motif for non-natural amino acids. Conjugates were thus stabilised with half-life in mouse blood >5h. An early conjugate in the lead series was progressed through pharmacokinetics, tolerability and activity, showing good exposure and being well tolerated up to 60mg/kg. Stasis was achieved with the top dose of 60mg/kg (SC) q2h in a 9h mouse neutropenic thigh model of efficacy with E. coli ATCC 25922. Subsequent conjugates in the series with improved properties are currently under evaluation in model tolerability and activity studies.

Summary

We believe this early data highlights the potential of the *Bicycle* platform to generate a new modality of antibacterial agents.

Potent *Bicycle*® binders have been obtained with functional activity against EcPBP3. Conjugation of BCY12130 to an uptake vector confers bacterial cell penetration and enhanced whole-cell activity against *Escherichia coli* and related *Enterobacteriaceae*. An optimised example from the series has demonstrated activity in an in vivo model. Further work is ongoing to develop a lead *Bicycle* candidate.

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