Development of a CD39 Inhibition Assay to Evaluate Novel Bicyclic Peptides (Bicycles®)

ABSTRACT#

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Bicycle Therapeutics' novel platform for the discovery and optimisation of bicyclic peptide drugs (Bicycles®) has been applied to >100 targets, with over 80% success rate.

We present here, method development for an enzyme inhibition assay for the ectonucleotidase, CD39. Ectonucleotidases are plasma membrane-bound enzymes with extracellular active sites that metabolise nucleotides to nucleosides and are crucial for maintaining immune homeostasis. Blockage of this pathway may promote anti-tumour immunity.

Two assay methods were identified as potential routes to measure CD39 enzymatic activity; a colourimetic phosphate detection assay, where free phosphate combines with reagents to produce a green dye, and a nucleotide displacement method, using fluorescence polarisation. Both methods were evaluated for robustness and reproducibility.

BICYCLE PLATFORM

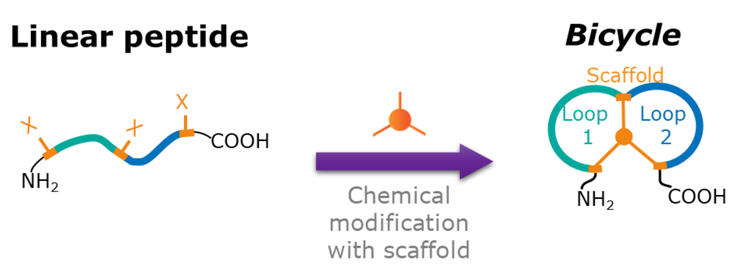


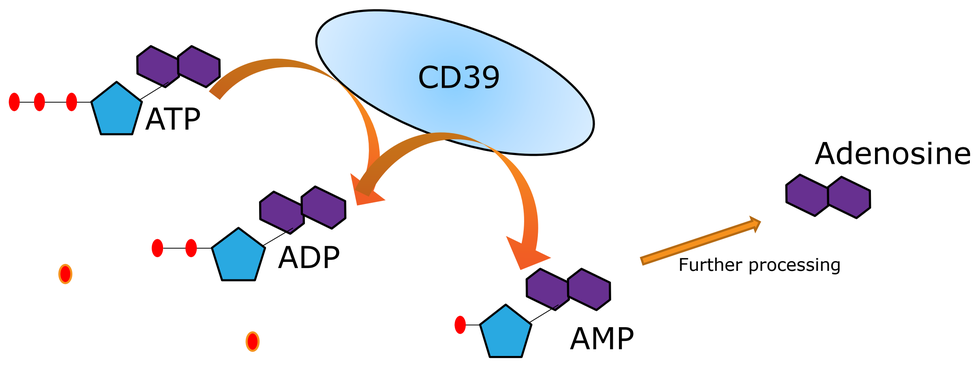
Figure 1.

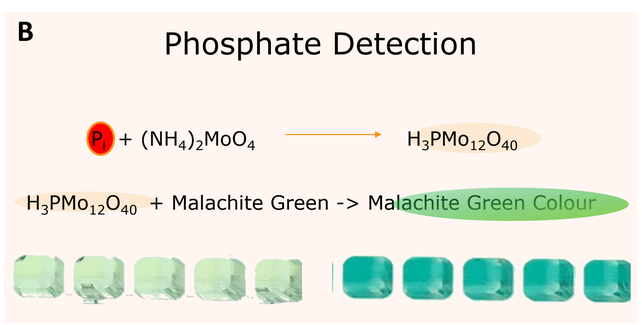
Figure 1. The Bicycle technology is based on an original concept conceived and developed in the laboratory of Sir Gregory Winter, at the MRC Laboratory of Molecular Biology, Cambridge, UK[1].

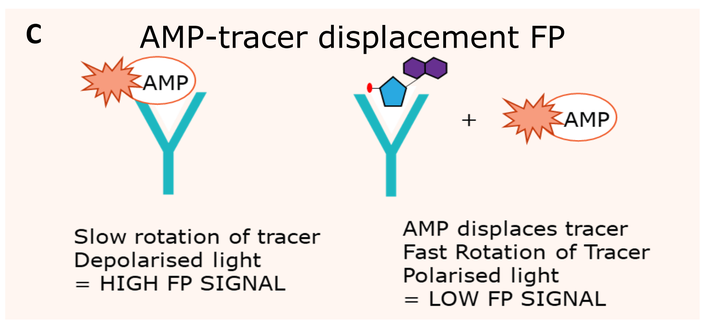
Bicycles® are peptides of typically 8 – 20 amino acids with three strategically-placed cysteine moieties. These residues are reacted with a variety of trivalent scaffolds to form bicyclic structures, conferring advantages in affinity, selectivity and stability.

IMMUNE - ONCOLOGY FOCUS

Figure 2. A







Recent studies have shown a key role for adenosine in immunosuppression in the tumour microenvironment, and ectonucleotidases are emerging as promising immuno-oncology targets. Blockage of the adenosine pathway may promote anti-tumour immunity.

To validate CD39 target material and ensure effective workflow, a screening assay needed to be created. Several technologies were considered and two were selected to evaluate CD39 inhibition.

ASSAY TECHNOLOGIES

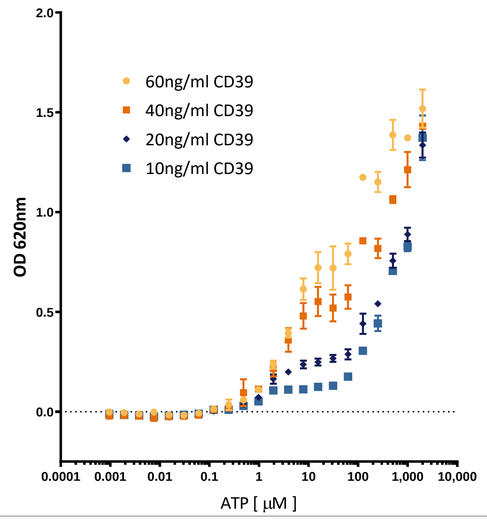
Figure 2. CD39 enzymatic pathway (**A**). Transcreener AMP fluorescence polarisation (FP) Kit (Bellbrook Labs) and Malachite Green Phosphate Detection Kit (R&D systems) were evaluated for robustness and suitability for identifying inhibitors within the CD39 programme. The assays vary in readout and in the reaction product used for detection. Both detection methods were terminated after 20mins reaction time and read using BMG pherastars.

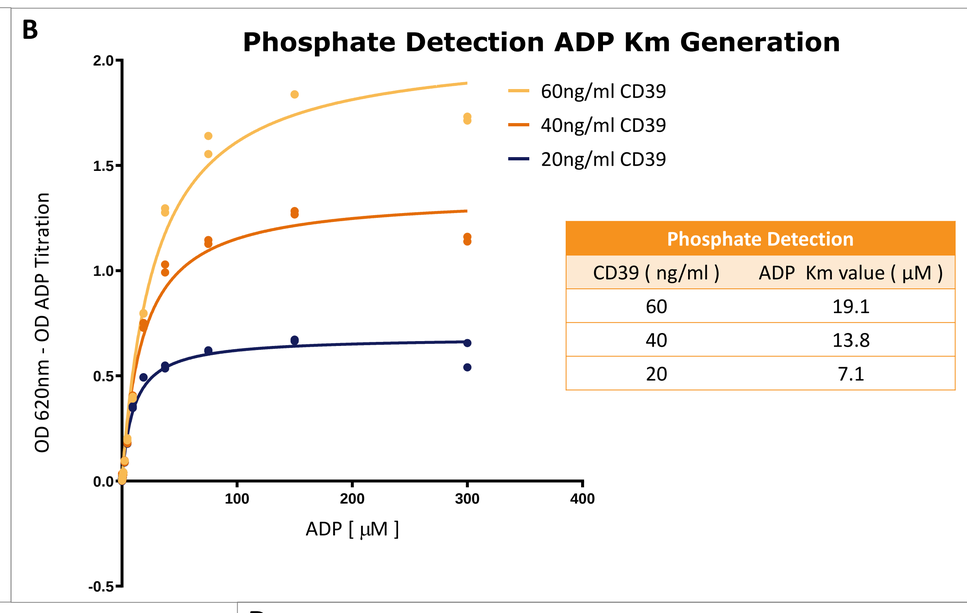
Malachite Green (**B**): Free phosphate and malachite green molybdate react to form green molybdophosphoric acid. The colourimetric change is read via absorbance at 620nm.

Transcreener AMP-tracer FP (**C**): An AMP-FarRed (675nm) tracer is displaced from a highly selective antibody in the present of AMP, resulting in changes of the fluorescence polarisation signal.

ASSAY DEVELOPMENT

Figure 3. A





Phosphate Detection	
CD39 (ng/ml)	ADP Km value (µM)
60	19.1
40	13.8
20	7.1

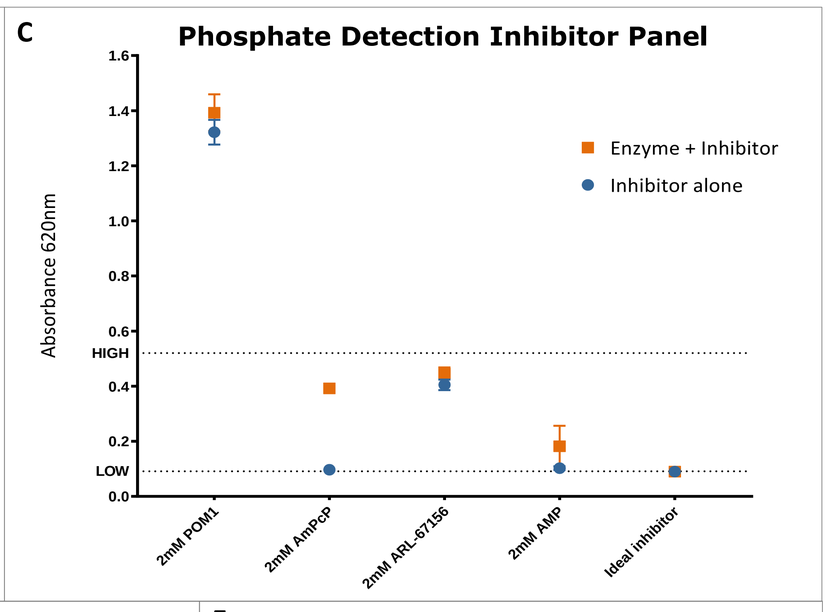
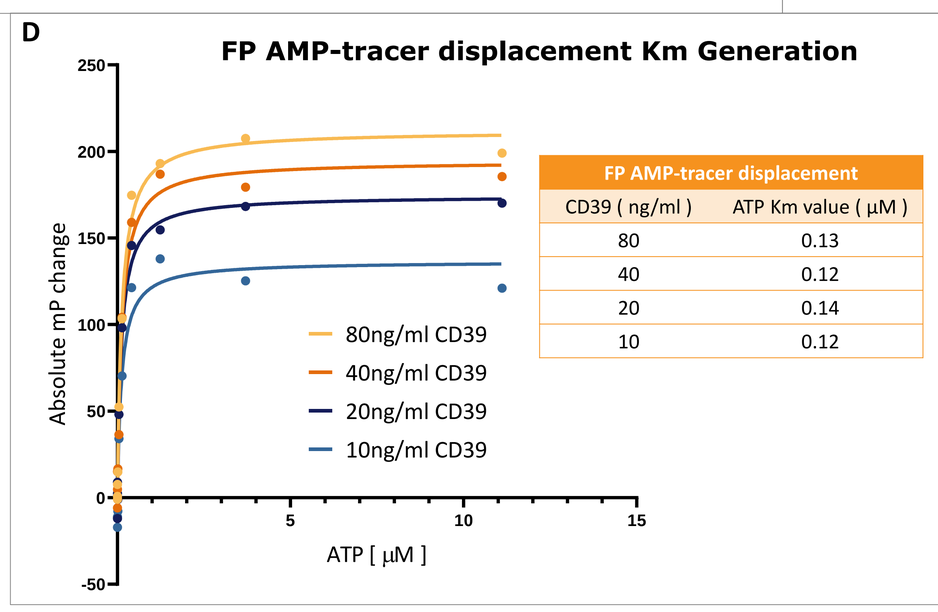


Figure 3. ATP substrate kinetics measured by the phosphate detection system proved difficult to interpret, with a biphasic curve (**A**). Results from experiments using ADP as the substrate allowed 1:1 measurements of phosphate release, generating Km values with good reproducibility across multiple enzyme concentrations and between repeats (**B**).

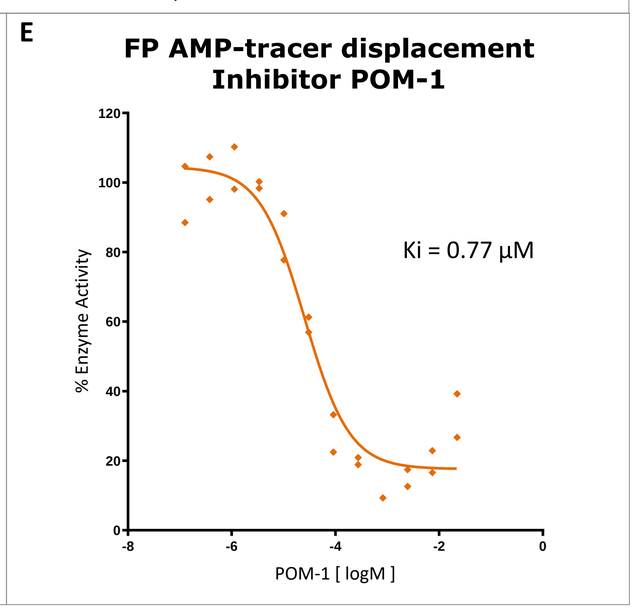
A panel of commercial CD39 inhibitors with known Ki values were identified and were evaluated for assay interference, robustness and suitability for the Bicycle project (**C**). Due to lack of inhibition or high background, attributed to presence of inherent phosphate, no suitable inhibitor was identified. This method design was abandoned.

Robust Km values were generated using ATP in the FP AMP-tracer displacement assay (**D**). POM-1 (sodium polyoxotungstate)(Tocris UK), an inhibitor of CD39, produced Ki values which were in line with literature values (**E**). This method was chosen for screening of Bicycle CD39 inhibitors.

Neither method evaluated here uses a kinetic readout, the gold standard for an enzymatic screen. Both assays required the termination of the enzymatic reaction before separate reagents are added to elicit the measurable read out. Our preferred method of detection would be a kinetic read, taken over the duration of the enzyme reaction.



FP AMP-tracer displacement	
CD39 (ng/ml)	ATP Km value (µM)
80	0.13
40	0.12
20	0.14
10	0.12



SUMMARY

Ectonucleoside triphosphate diphosphohydrolase-1 (CD39) is an attractive target in oncology. We have evaluated two methods to assess peptides for enzymatic inhibition.

Phosphate detection via malachite green was determined to be unsuitable for validating new peptides for the CD39 project. However, the assay modality is robust and has potential to be used for alternative targets, such as other ectonucleotidases.

The Transcreener AMP FP kit proved to a robust assay format, suitable for screening enzymatic inhibition of CD39. This will be the preferred assay to screen Bicycle peptides.

REFERENCES

Reference (1) Heinis et al, *Nat Chem Biol* 5(7): 502-7 (2009)

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