Transferrin Receptor 1-targeting *Bicycles*: A New Platform for Transcytosis

Abstract Number

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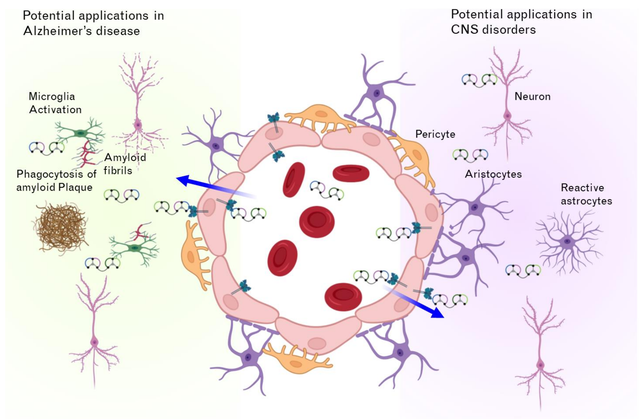
ABSTRACT

Bicycles are a class of novel synthetic compounds that have the potential to be precision-targeted therapeutics. There is an unmet need for therapeutics targeting brain diseases that is hindered by difficulties in transporting drugs to the required areas. By applying the new technology of *Bicycles* to existing ideas on using Transferrin Receptor 1 to penetrate the blood brain barrier, we hope to produce a new generation of multivalent brain targeting therapeutics.

INTRODUCTION



▲ Figure 1: Cartoon showing intended mode of action, with *Bicycles* transporting compounds across the cerebral vasculature. example of how *Bicycles* can be used is by activating microglia into phagocytosing amyloid plaques

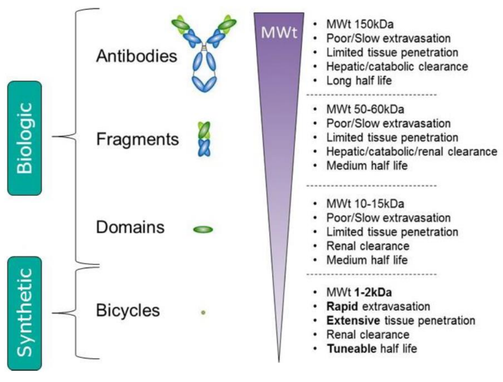
The blood brain barrier (BBB) plays a critical role in controlling movement of select substances into the brain. This is achieved by limiting paracellular passage with tight endothelial junctions, pericyte and astrocyte endfoot placement (Fig 1). In addition, there is reduced transcellular passage through lack of pinocytosis and expression of efflux pumps. These properties impede delivery of therapeutic drugs to treat brain disorders such as brain tumours, Parkinson's and Alzheimer's disease. Specific transcytosis mechanisms do exist, with Transferrin Receptor 1 (TfR1) being one example. TfR1 is highly expressed on cerebrovascular endothelial cells, and the strategy of using TfR1 as a 'Trojan horse' carrying therapeutic molecules across the BBB has been pursued for over 30 years ([1]Pardridge et al). This is supported by substantial data on TfR1 antibodies as CNS delivery agents in animal models with efficacy/surrogate readouts. The current aim is to adopt this strategy onto the Bicycle® platform with hopes of improving therapeutic penetration.

ABOUT *BICYCLES*

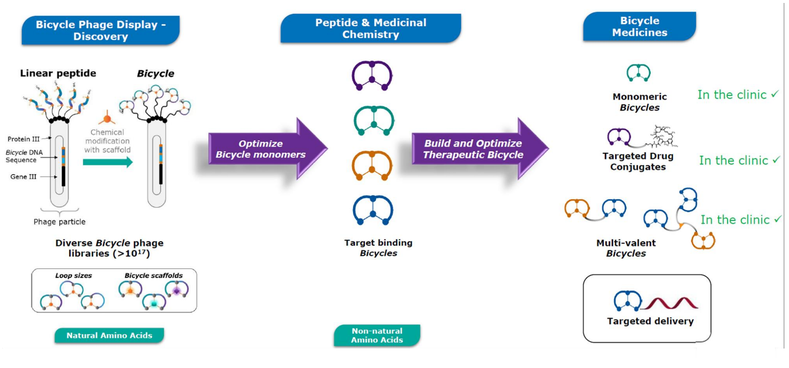
Bicycles are a class of small (~2kDa) peptide-based therapeutic modality (Fig 2 A.). Peptides are selected through a phage display screening process (Fig 2 B. [2] Winter et al). The unique attributes of *Bicycles* include:

- High selectivity to target and tuneable affinity
- Large binding footprint to biologically relevant 3D structures
- Readily conjugated to toxin payload, fluorochrome, other *Bicycles*, radionuclides, biotin/affinity tags etc.
- No complications associated with the Fc as commonly seen with antibodies
- Compatible with multiple routes of administration, including IV, SQ, and inhalation
- Fully synthetic and scalable manufacturing

A. *Bicycles* relative to other therapeutic compounds

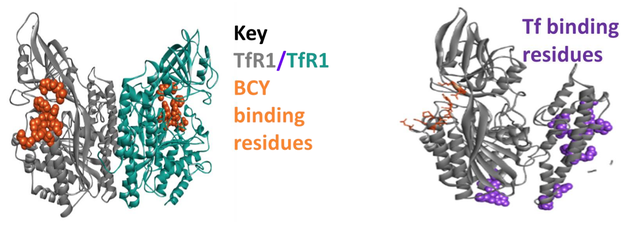


B. Phage display screening process and multi-valent *Bicycles*



▲ Figure 2 A. *Bicycles* relative to other therapeutic compounds, B. Phage display screening process and multi-valent *Bicycles*.

CRYSTAL STRUCTURE AND *BICYCLES* BINDING SITE



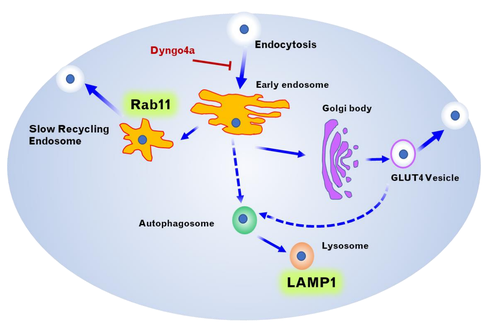
▲ Figure 3: Crystallography structure of the transferrin receptor and binding sites of *Bicycles* and the natural ligand, transferrin

Bicycles undergo a selection strategy designed to prevent blocking the binding sites of natural transferrin ligands. Peptides identified are tuned to have optimal affinity to hTfR1.

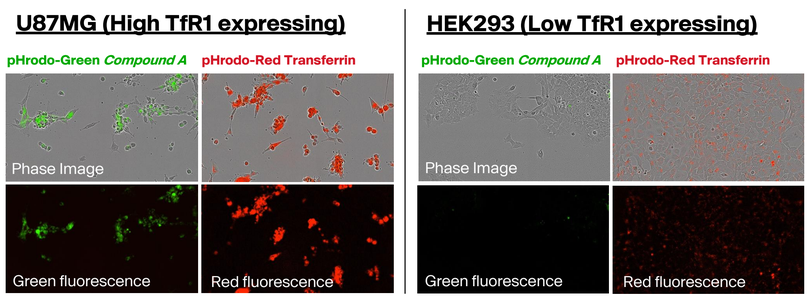
IMAGING INTERNALISATION AND LOCALISATION OF *BICYCLES*

A key objective was to demonstrate *Bicycles* colocalise with transferrin and transferrin receptor when internalised, and to determine the path taken within the cell.

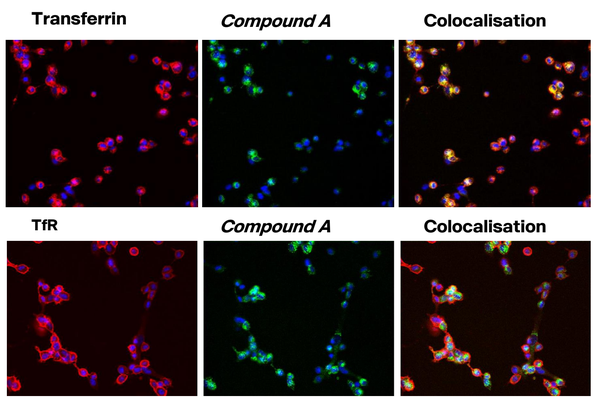
A. Graphical representation of internalisation routes



B. Internalisation of *Bicycles* is higher in cells with high TfR1 expression

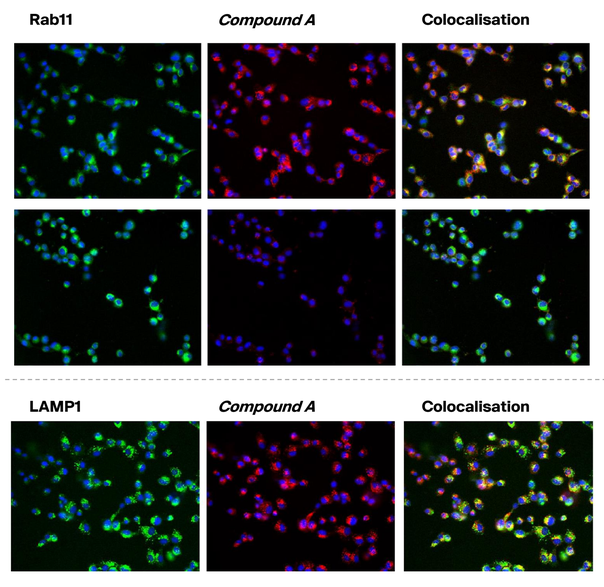


C. HT1080 cells incubated with fluorophore-labelled *Bicycles*



Compound A (KD 29 nM) colocalises with both transferrin (without competing for uptake) and with Transferrin receptor

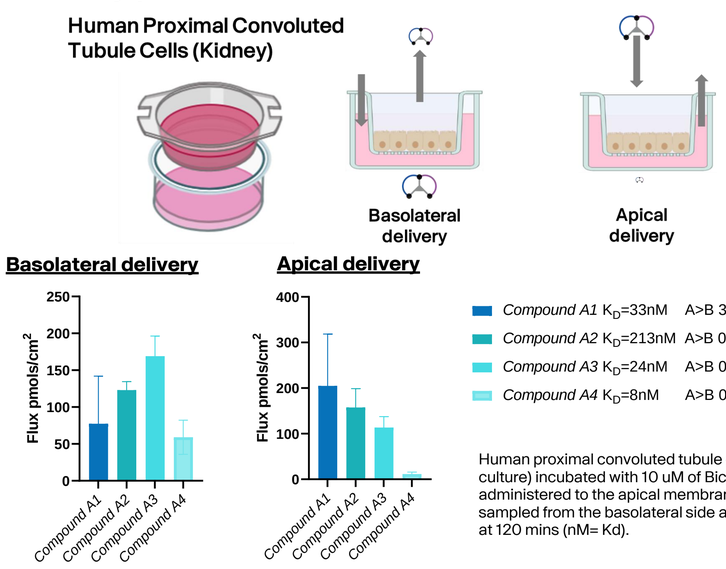
D. Co-localisation of *Bicycles* with Recycling (Rab11) and Lysosomal (LAMP1) markers



- Higher levels of internalisation of *Bicycles* (*Compound A*) labelled with pH sensitive pHrodo dye was observed in U87MG cells with high levels of TfR1
- *Bicycles* are shown to co-localise with transferrin receptor and binding does not inhibit transferrin binding
- Association of binding with Rab11 indicates recycling, which is likely to facilitate transcytosis
- These internalisation pathways appear to be mediated by dynamin, as treating with Dyngo4a inhibits signal
- A proportion is also transported to the lysosome (LAMP1)

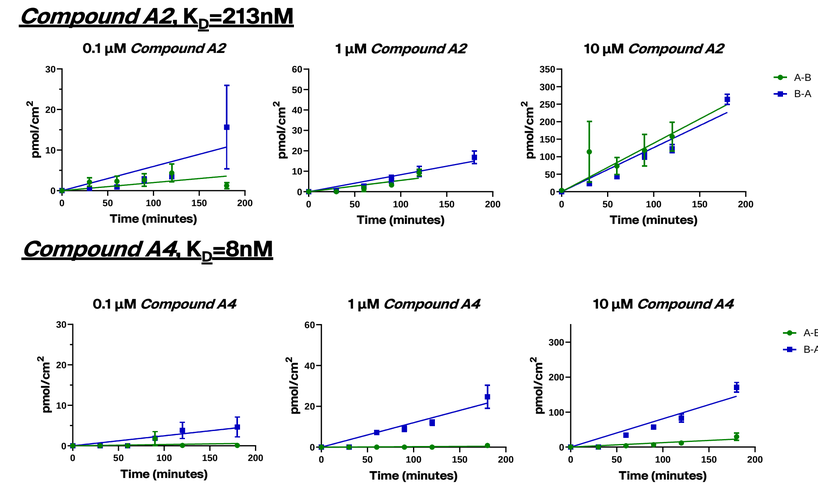
TRANSCYTOSIS ASSAY SHOWS INDICATIONS OF *BICYCLES* MOVING ACROSS A CELL MEMBRANE BARRIER

A. TfR1 binding *Bicycles* show transcytosis in human kidney proximal convoluted tubule cells



Human proximal convoluted tubule cells (primary culture) incubated with 10 uM of Bicycles administered to the apical membrane side and sampled from the basolateral side and vice versa at 120 mins (nM= Kd).

B. Representative *Bicycles* showing dose dependent transport



A= Apical B= Basolateral

▲ Figure 5: A. TfR1 binding *Bicycles* (*Compounds A1-4*) show transcytosis in human kidney proximal convoluted tubule cells, B. Representative *Bicycles* showing dose dependent movement of *Bicycles* from the apical to basolateral chamber and vice versa

◀ Figure 4: A. Diagram showing various internalisation routes (adapted from [3] Zhang et al), B. Internalisation of *Bicycles*-pHrodo is improved in cells with high TfR1 expression, 5µM, 20X objective, 18 hours timepoint
C. Co-localisation of *Bicycles* with TfR1, D. Co-localisation of *Bicycles* with recycling endosomal markers Rab11 and lysosomal marker LAMP1. Dyngo 4a shows that internalisation of *Bicycles* occurs via a dynamin dependent mechanism

- *Bicycles* showed transcytosis across proximal convoluted tubule cells with different affinities potentially influencing preferential direction of movement
- Literature has shown that mid-lower range antibody affinity results in better transcytosis (possibly because high affinity leads to lysosomal degradation) ([4] Yu et al and [5] Bien-Ly et al), but the affinity of *Bicycles* does not appear to have a correlation with amount transported
- With higher doses of *Bicycles*, the concentration measured from basolateral to apical and vice versa is increased.

CONCLUSIONS

- *Bicycles* bind to sites on TfR1 that do not interfere with transferrin ligand
- Internalised TfR1 *Bicycles* co-localise with recycling endosomal markers
- The mechanism for internalisation is likely to require dynamin mobilisation
- *Bicycles* are currently the only known small molecules able to employ the TfR1 system for shuttling across endothelial cell barriers
- Data shown represents a significant step towards generating cargo-bearing peptides that can be potentially utilised to cross the BBB to treat brain related diseases

METHODS

(◀ Figure 4) B. *Bicycles* and transferrin conjugated with pHrodo dye were incubated with cells for 18 hours and analysed on Incucyte. C. and D. HT1080 cells were seeded overnight. Cells were incubated in serum free media for 60 minutes at 37 °C. For D. Cells were pretreated with either vehicle (0.1% DMSO) or Dyngo 4a (30 µM) for 30 minutes at 37 °C. Cells were then incubated with conjugated *Bicycles* (1.0 µM; red) for 1 hour at 4 °C. Cells were then transferred to 37 °C for 1.5 minutes to allow endocytosis. After washing, cells were fixed and permeabilised using 80% acetone for 10 minutes at -20 °C. Cells were then blocked for 1 hour in 10% goat serum and labelled with primary antibody (indicated). Cells were then washed and labelled with secondary antibody (green) plus Hoechst (blue). Representative images from triplicate culture wells. x40 magnification. (▲ Figure 5) A. and B. Freshly isolated human proximal convoluted tubule cells seeded in a transwell insert with *Bicycles* tested at 10µM. Transepithelial absorptive and secretory flux across the polarized cells were measured by mass spectrometry. Results were normalised to baseline FITC labelled transferrin uptake (AB and BA). Monolayer integrity (80-120 Ω.cm^2 transepithelial electrical resistance) was measured as a quality control.

REFERENCES

1. Pardridge et al, Pharmaceuticals (2020)
2. Winter et al, Nature Chem Bio (2009)
3. Zhang et al, Nanoscale (2017)
4. Yu et al, Sci Transl Med (2011)
5. Bien-Ly et al, J Exp Med (2014)

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