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COMMUNICATION

A cyclosporine derivative is a substrate of the oligopeptide transporter PepT1†

 Received 00th January 20xx,
Accepted 00th January 20xx
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DOI: 10.1039/x0xx00000x

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The mammalian proton-coupled oligopeptide transporter PepT1 is recognised as an important route of oral drug delivery. Peptide-based compounds offer great potential as drugs but their application is limited by poor membrane permeability, amongst other challenges. Using cyclosporin A as a proof-of-concept, we demonstrate for the first time that peptidic molecules over 1000 Da in size can be targeted towards and transported by PepT1.

The vast majority of drugs on the market can be classed as small molecule (< 500 Da) or biological (> 5000 Da). Between these two extremes is the relatively unexploited class of peptidic compounds. These compounds combine many of the advantages of small molecules (ease of synthesis) and biologics (exquisite potency and selectivity).¹ However, a major challenge to the application of these compounds as therapeutics is their poor oral bioavailability, caused by a combination of GI and metabolic instability and poor membrane permeability. Research to develop systems that can allow for oral administration of these molecules is extensive and ongoing and has been comprehensively reviewed by several authors previously.^{2–4} These reviews are consistent in highlighting transporter targeting as a potential practical method of achieving high oral bioavailability of these types of molecules. Included in the list of possible targets is the intestinal peptide transporter, PepT1.

PepT1 is a proton coupled oligopeptide transporter expressed principally in the small intestine.⁵ It has a broad substrate specificity including most di- and tripeptides, β -lactam antibiotics and ACE inhibitors.⁵ We have previously demonstrated that drugs can be modified with a thiodipeptide

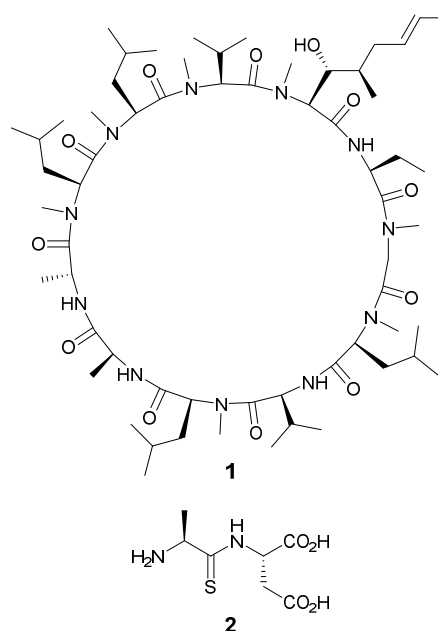


Figure 1 A PepT1 targeting prodrug of Cyclosporine A (**1**) was envisioned through attachment to our thiodipeptide carrier (**2**).

“carrier” so as to become substrates of the PepT1 transporter,^{6,7} and have shown that PepT1 can accommodate substrates of up to 700 Da. We were intrigued by the possibility of applying this approach to peptidic drug space and to investigate if PepT1 would be capable of transporting molecules in the range of 1–5000 Da.

We elected to synthesise a derivative of cyclosporine A (**1**), modified with our aspartate thiodipeptide carrier (**2**) (**Figure 1**). Cyclosporine A was chosen as a proof-of-principle compound as it gave an ideal balance of size (1202 Da), stability, commercial availability, poor and variable oral bioavailability (20–50%)^{8,9} and synthetic challenge.

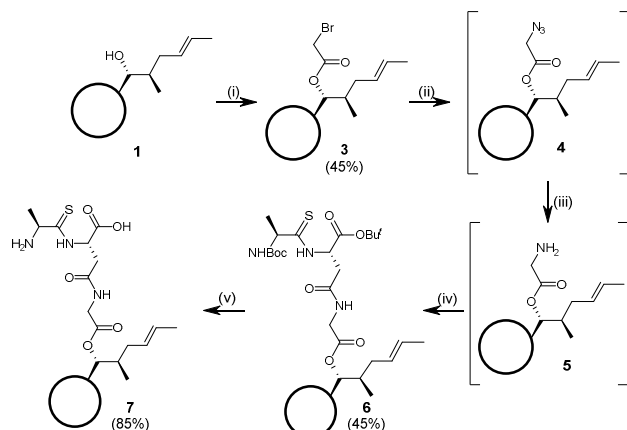
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Synthetic Chemistry



Scheme 1 Synthesis of cyclosporine A prodrug. (i) Bromoacetyl bromide, DMAP, 0 °C-rt, 1 h (ii) NaN_3 , DMF, rt, 24 h (iii) Ph_3P , H_2O , THF, rt, 24 h (iv) Boc-AlaΨ[CS-NH]-Asp-OBu, DPPA, TEA, DMF, 0 °C-rt, 24 h (v) TFA, DCM, rt, 5 h.

The synthesis of the protected aspartate thiodipeptide carrier has been previously described.^{6,7} Attempts were made to directly attach cyclosporine A to the aspartate carrier, however the cyclosporine alcohol is unreactive to various coupling conditions. A search of the literature revealed some peptide prodrugs of cyclosporine A.¹⁰ The protocol identified was followed, with some minor modifications, in our synthesis of a cyclosporine thiodipeptide conjugate (**Scheme 1**). Cyclosporine was first reacted with neat bromoacetyl bromide to form the bromo ester (**3**). This was displaced by the azide anion to form (**4**), which was reduced¹¹ to form the amine (**5**). The amino group was then coupled using diphenylphosphylazide to the acid side chain of the aspartate thiodipeptide. At this point the conjugate was purified by HPLC and then pure (**6**) was cleanly deprotected using a 33% solution of TFA in DCM to give the cyclosporine A thiodipeptide derivative (**7**) as a TFA salt. All of the intermediates were fully characterised by NMR in CDCl_3 . However, (**7**) was insoluble in this solvent and gave very broad NMR spectra in polar solvents such as CD_3OD and $\text{DMSO}-d_6$. This can be explained by the multiple conformations that cyclosporine is known to adopt in polar solvents.¹² Electrospray mass spectrometry, however, gave strong parent ions for (**7**) at 753.8 $[\text{M} + 2\text{Na}]^{2+}$ and 1484.3 $[\text{M} + \text{Na}]^+$ (Exact Mass $\text{C}_{71}\text{H}_{124}\text{N}_{14}\text{O}_{16}\text{S} = 1460.9040$), with no evidence of the free carrier (Exact Mass $\text{C}_7\text{H}_{12}\text{N}_2\text{O}_4\text{S} = 220.0518$) or the expected thiodipeptide product of ester hydrolysis (Exact Mass $\text{C}_9\text{H}_{15}\text{N}_3\text{O}_5\text{S} = 277.0732$).

We were able to confirm the absence of any free cyclosporine A by HPLC using literature conditions¹³ and no cyclosporine A was detected from samples of (**7**) stored in pH 5.5 buffered solutions for several months at -18 °C. However, in our hands and in collaboration with AstraZeneca, (**7**) itself consistently eluted with the solvent front under various HPLC conditions.

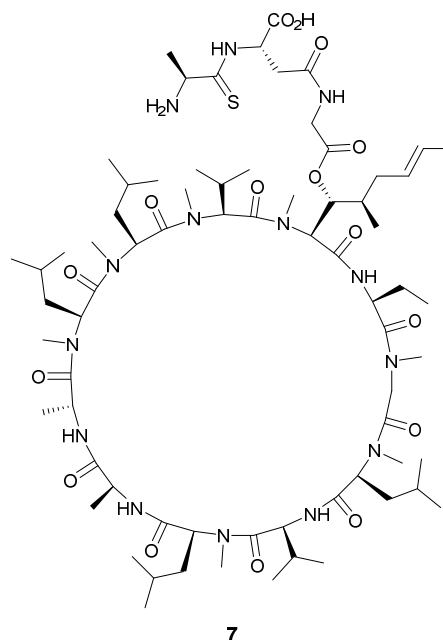


Figure 2 Structure of the Pept1 targeting cyclosporine A prodrug (**7**).

Biological Testing

The K_i of the compound was determined by measuring the uptake of $[^3\text{H}]\text{-D-Phe-L-Gln}$ into *Xenopus* oocytes in the presence of increasing concentrations (0 – 5 mM, 5 oocytes per data point) of compound (**7**) in pH 5.5 uptake medium (in mM: NaCl 95, KCl 2, CaCl_2 1, MgCl_2 0.1, Tris-Mes 20, pH 5.5). After incubation of 1 hour, the oocytes were washed five times in 1 mL of ice-cold 0.12 M NaCl solution, before being individually lysed in 25 μL 2% (w/v) SDS in 96 well scintillation counter plates (GE Healthcare, Amersham, UK). After lysis, 200 μL of scintillation fluid was added to each well (Optiphase, GE Healthcare) and scintillation counted. Non-injected oocytes were run in parallel as a control. The K_i was obtained from a plot of fractional uptake versus substrate concentration, using standard Michaelis-Menten kinetics as previously described.^{14,15}

Transport of the compounds was determined using *trans*-stimulation of efflux studies.¹⁶ Five oocytes expressing rabbit PepT1 were injected with 4.6 nM of $[^3\text{H}]\text{-D-Phe-L-Gln}$ (37.0 MBq mL^{-1}). The oocytes were then placed in 100 μL of a pH 5.5 solution containing 5 mM compound (**7**), 5 mM Gly-L-Gln (as a positive control) or with nothing added (negative control) and incubated for 90 minutes. The oocytes were then washed, lysed and the amount of $[^3\text{H}]\text{-D-Phe-L-Gln}$ remaining assessed by scintillation counting as above. A significant drop in the amount of radioactivity remaining in the oocyte after the 90 minutes indicates *trans*-stimulation, which is indicative of transport of the extracellular unlabelled substrate.

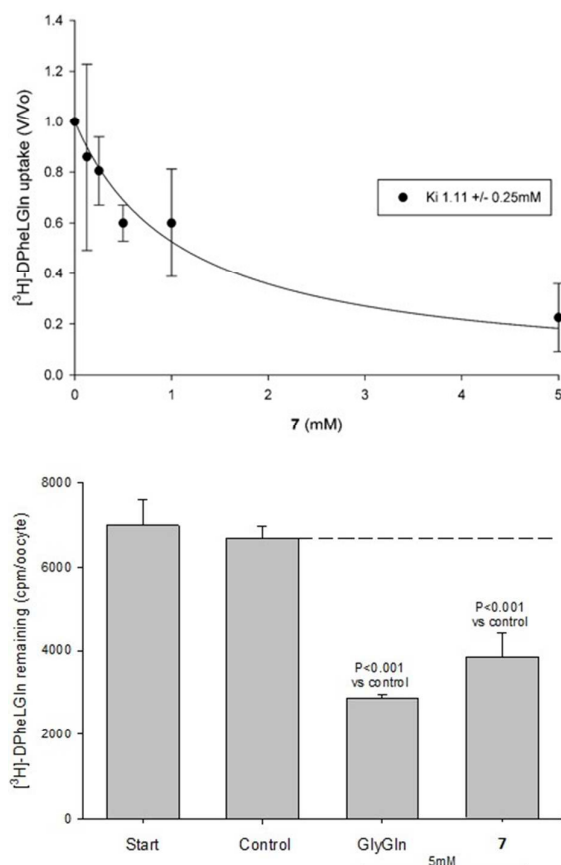


Figure 3 Affinity and *trans*-stimulation efflux data for (**7**). Data represents the average of two experiments each with 5 oocytes per data point.

Confirmation of transport was provided by electrophysiology experiment. Oocytes were placed in a 0.1 ml recording chamber and perfused with pH 5.5 uptake solution at a rate of 15 ml min⁻¹. Oocytes were impaled by two agarose-cushioned microelectrodes filled with 3 M KCl (0.3–2.0 MOhms) and voltage-clamped at –30 mV using a Geneclamp 500B amplifier (Axon Instruments, CA, U.S.A.). Compound (**7**) (2 mM) and Gly-L-Gln (0.5 mM) as a positive control (both in pH 5.5 uptake medium) were applied by gravity perfusion using a manually activated valve. Traces were recorded in real time on a chart recorder. All experiments were carried out at room temperature on PepT1-expressing and non-injected control oocytes.

Results and Discussion

The binding affinities of (**7**) for PepT1 were determined by measuring the concentration at which it inhibited uptake of radiolabelled D-Phe-L-Gln in *Xenopus laevis* oocytes expressing rabbit PepT1. Inhibition constants were calculated from standard Michaelis-Menten kinetics.^{14,15} PepT1 is a low affinity, high capacity transporter and compounds with an affinity < 1 mM are generally classed as high affinity substrates.⁵ (**7**) gave

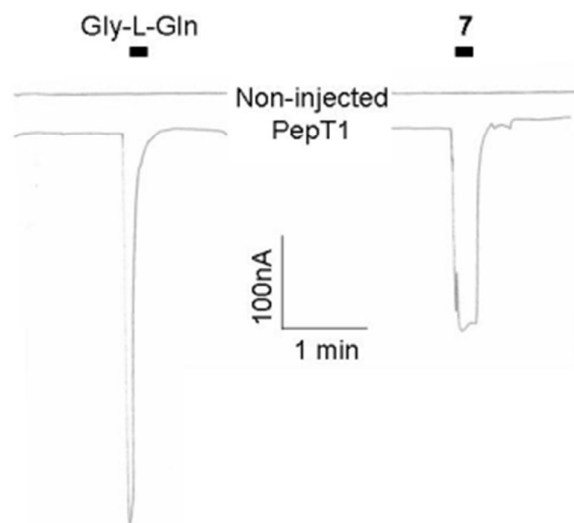


Figure 4 Representative trace for the electrophysiological response to application of 0.5 mM Gly-L-Gln and 2 mM (**7**) in non-injected (control) and PepT1-expressing *Xenopus* oocytes with their membrane potential clamped to –30 mV.

a K_i value of 1.1 ± 0.3 mM as shown in **Figure 3**. Based on this, it can be concluded that (**7**) has medium to high affinity for PepT1. Whilst a relatively high concentration of DMSO (5% v/v) was required to solubilise (**7**), control experiments showed no effect on the oocytes (data not shown).

As binding studies only show affinity for PepT1 and do not provide information as to whether the compound is a substrate or an inhibitor, we measured the *trans*-stimulation of radiolabelled D-Phe-L-Gln efflux from rabbit PepT1 expressing oocytes in the presence of 5 mM Gly-L-Gln (a standard PepT1 substrate) and 5 mM (**7**). A pH of 5.5 was chosen as this pH range has been used to study PepT1 expressed exogenously in oocytes¹⁷ and endogenously in cell lines such as Caco-2.¹⁸ (**7**) reproducibly triggered significant *trans*-stimulation efflux, comparable to a standard PepT1 substrate, Gly-L-Gln. This indicates that (**7**) is a substrate of PepT1 (**Figure 3**). Again, the control containing 5% (v/v) DMSO showed no effect on the signal. This was the first evidence to support our hypothesis that peptidic molecules of this size could be targeted towards transport by PepT1.

To confirm this observation we conducted electrophysiology experiments, which have been widely used in the study of PepT1 mediated transport. Oocytes were perfused with normal uptake buffer (pH 5.5) + 1% methanol and held at –30 mV membrane potential by two-electrode voltage clamping. Addition of 0.5 mM Gly-L-Gln + 1% methanol gave a current of 347 ± 32 pA, whereas 2 mM (**7**) gave 322 ± 55 pA (**Figure 4**). The four-fold difference in the concentration of Gly-L-Gln (K_i (Gly-L-Gln) = 0.2 mM) and (**7**) applied allows for the difference in affinity of these compounds for the transporter. 2 mM cyclosporine A + 1% methanol alone gave no depolarisation (data not shown). The considerable and significant change in membrane potential upon application of (**7**) is consistent with PepT1 transport.

Conclusions

Taking the poorly orally bioavailable peptidic drug cyclosporine A as a model, we have demonstrated that a thiodipeptide derivative of this drug is a substrate of rabbit PepT1, and hence that drugs with molecular weight >1000 might be delivered using our technology. This result is of particular interest because the therapeutic exploitation of peptidic molecules is generally limited by their poor membrane permeability. Targeting such molecules towards transport by PepT1 may be a solution to this challenge, and allow more of these promising molecules to progress in drug discovery. Whilst our study was conducted using rabbit PepT1, the sequence similarity between rabbit and human PepT1 of 92%¹⁹ means our result provides encouraging preliminary data for continued research into the exploitation of PepT1 transport as a means of improving the pharmacokinetic profile of peptidic drugs in humans. Future work will revolve around reproducing this result with cloned human PepT1 and extending the scope to other pharmacologically important peptide drugs of this molecular weight range.

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Cyclosporine was attached to a thiodipeptide carrier, yielding conjugate **7**; this is a substrate for PepT1 with oral bioavailability potential.

