The *in vitro* transport of model thiodipeptide prodrugs designed to target the intestinal oligopeptide transporter, PepT1†

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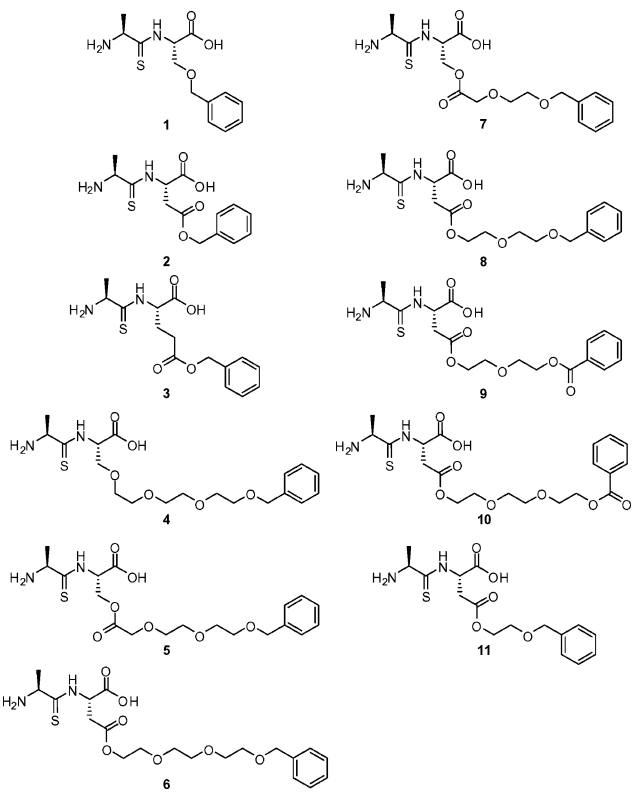
A thiodipeptide carrier system is shown to be effective at enabling a range of covalently bound molecules, including benzyl, benzoyl and ibuprofen conjugates, to be transported *via* the intestinal peptide transporter PepT1, demonstrating its potential as a rational drug delivery target.

PepT1 is a membrane transporter located principally on the luminal cell membrane of the intestine. The broad substrate capacity of the transporter, including most di- and tripeptides, β-lactam antibiotics and antivirals, has made it a promising target for improving oral drug delivery.[1]

Several examples of drugs and prodrugs that are transported by PepT1 *in vitro* and *in vivo* have been reported.[2] In most of these cases, the increase in oral bioavailability of the various prodrugs was first observed, with PepT1 mediated transport proposed as an explanation at a later date. While the idea of rationally targeting drugs towards transport by PepT1 has been investigated by various groups and methods,[3-7] it is only recently that we have demonstrated the designed transport by PepT1 of a prodrug of the non-steroidal anti-inflammatory drug (NSAID) nabumetone,[8] using our thiodipeptide "carrier" prodrug approach.[9] Amidon's recent work on floxuridine prodrugs represents the only other example we could find of rational drug delivery *via* PepT1.[10]

Additionally, despite a wealth of empirical and modelling data on substrate affinity for PepT1,[7,11] very little is known about the substrate structural features important to transport.[12] This field has been particularly hampered by the lack of a three dimensional structure of the transporter, although recent homology models offer the promise of improvements in this area in the near future.[13]

Following on from our work with nabumetone prodrugs,[8] we wished to investigate the limits of capacity of the transporter, in relation to its ability to bind and transport thiodipeptide prodrugs in particular. Our initial study involved the synthesis and *in vitro* testing of a range of thiodipeptides (**1–11**) for PepT1 affinity and transport. Benzyl alcohol or benzoic acid were chosen as simple probes for potential drugs in these studies, as this type of system has been used in the investigations of other potential PepT1 "carrier" compounds, although on a more limited scale.[3,6] We focussed on glycol spacers, since our previous work on nabumetone prodrugs had indicated that the PepT1 transporter could accommodate hydrophobic groups placed a considerable distance (*e.g.* triethylene glycol spacer) from the peptide backbone.[8] This work also indicated that the length of the glycol spacer affected the rate of PepT1 mediated transport.[8] Therefore we elected to synthesise compounds **4–11** to probe both of these effects in more detail. We also anticipated that polyethylene glycol spacers may prove more useful generally in modifying other physicochemical properties important to oral delivery.[14]



The synthesis of the fully protected thiodipeptides **24–26** has been reported previously[9] and is included in the ESI (Scheme 1).† Target compounds **1–3** were isolated as their TFA salts following standard acidolysis of the Boc carbamate and OBu^t ester using TFA in DCM. A dissolving metal reduction of intermediates **24** and **25** yielded the protected serine and aspartate thiodipeptide "carriers" **27** and **28** respectively, to which we envisioned a variety of drugs could be attached. The five step route to **27** and **28** has now

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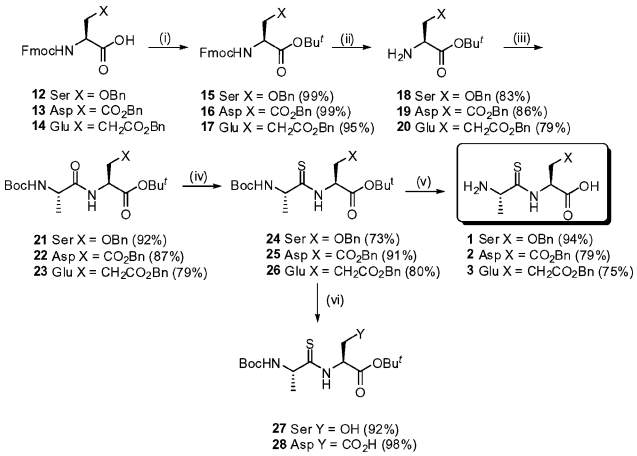
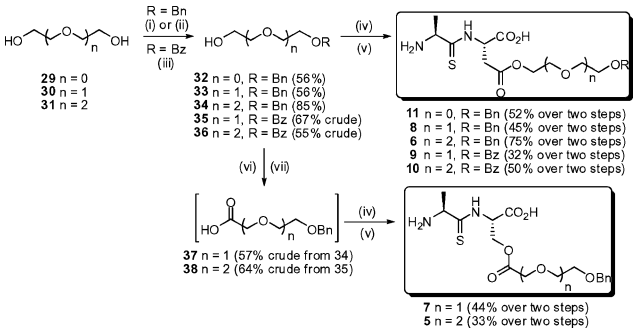
† Electronic supplementary information (ESI) available: Full experimental details for the synthesis of all compounds, with characterisation data. Full details of *in vitro* experiments, with affinity and *trans*-stimulation data. See DOI: 10.1039/b909221h



Scheme 1 Synthesis of target compounds **1–3** and thiodipeptide "carriers" **27** and **28**. (i) *tert*-Butyl-2,2,2-trichloroacetimidate, DCM/Et_2O. (ii) TBAF in THF. (iii) Boc-Ala-OH, DPPA, TEA, DMF. (iv) Lawesson's reagent, refluxing toluene. (v) 33% TFA in DCM. (vi) $Na/NH_3(l)$ in THF, –78 °C.

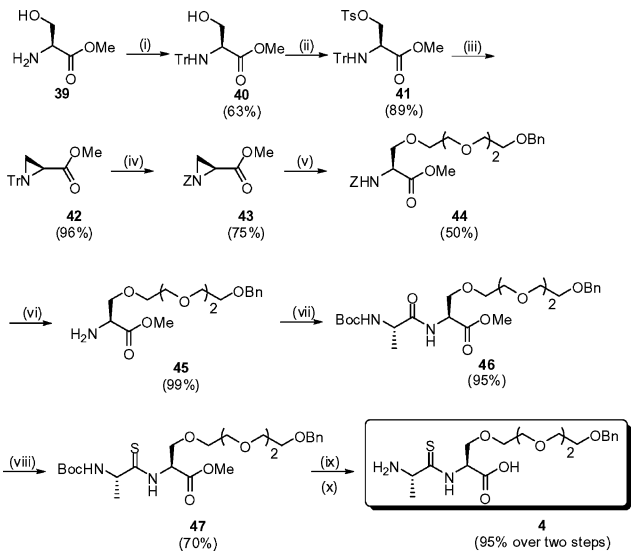
been optimised in our laboratory so that multi-gram quantities can be brought through in less than two weeks.

The synthesis of **5–11** was carried out using similar methods to those we employed for the synthesis of thiodipeptide prodrugs of nabumetone (Scheme 2).[8] In brief, mono-, di- or triethylene glycols were mono-functionalised with benzyl bromide using standard sodium hydride methods or the method of Bouzide.[15] Di- and triethylene glycols were mono-functionalised with benzoic acid using concentrated Mitsunobu conditions and sonication.[16] These mono-etherified or esterified glycols were then coupled to the aspartate carrier **28** using standard coupling conditions (typically HBTU, DIPEA in DMF). These same glycols were subjected to Swern[17] oxidation followed by silver oxide oxidation of the crude aldehyde[18] and the resultant glycolic acids coupled to the serine carrier **27**. TFA deprotection of these conjugates yielded the desired compounds **5–11** as TFA salts.



Scheme 2 Synthesis of target compounds **5–11**. (i) NaH, KI, BnBr. (ii) Ag_2O, BnBr. (iii) Benzoic acid, DIAD, Ph_3P, THF, sonication. (iv) **27** or **28**, HBTU, DIPEA, DMF. (v) 33% TFA in DCM. (vi) $(COCl)_2$, DMSO, TEA, –60 °C. (vii) Ag_2O, NaOH, H_2O, reflux.

We wished to synthesise an extremely hydrolysis resistant compound, such as **4**, to reduce any effect that metabolism of the prodrugs may have on the transport results. The synthesis of the serine ether **4** was complicated by the fact that standard methods of etherification resulted in either elimination from **27**, to form an α,β-unsaturated ester, and/or significant racemisation. The procedure reported below, employing a key aziridine ring-opening step, gives reliable access to this important type of compound, and may be of value to those wishing to access other seryl ethers (Scheme 3). A known literature route was used to form aziridine **43**.[19] For the crucial ring-opening step, we initially employed the conditions reported by Ho *et al.*[20] which in their hands gave yields of 64% with glycol nucleophiles. In our hands, this procedure resulted in the formation of compound **44**, but in only 18% yield. A study of the effects of temperature, concentration and catalyst loading resulted in an optimised method with an improved yield of 50%. Following removal of the Cbz group by catalytic hydrogenation, coupling of the serine ether to Boc-Ala-OH was readily achieved in high yield using diphenylphosphoryl azide as a coupling reagent.[21] Lawesson's reagent[22] was used to selectively effect an oxygen–sulfur exchange at the amide carbonyl. Hydrolysis of the methyl ester with lithium hydroxide in aqueous THF, followed by TFA mediated acidolysis of the Boc group yielded ether **4** as a TFA salt.



Scheme 3 Synthesis of serine ether **4**. (i) TrCl, TEA, $CHCl_3$, 0 °C, 24 h. (ii) TsCl, pyr, 0 °C, 48 h. (iii) TEA, THF, 75 °C, 48 h. (iv) TFA, Et_3SiH, DCM, –5 °C, 1 h then DIPEA, –5 °C, 20 min, concentrate under vacuum and add benzylchloroformate, DIPEA, 0 °C, 3 h. (v) **34**, $BF_3{\cdot}OEt_2$, DCM, 20 h. (vi) H_2, 10% Pd-C, EtOH, 3 h. (vii) Boc-Ala-OH, DPPA, TEA, DMF, 0 °C–rt, 18 h. (viii) Lawesson's reagent, refluxing toluene, 4 h. (ix) $LiOH{\cdot}H_2O$, THF/H_2O, 18 h. (x) 33% TFA in DCM, 5 h.

The PepT1 binding affinity of compounds **1–11** was determined by measuring the concentration at which they inhibited uptake of radiolabelled D-Phe-L-Gln in *Xenopus laevis* oocytes expressing rabbit PepT1 (Table 1). Inhibition constants were calculated from standard Michaelis–Menten kinetics.[23,24]

As binding studies only show affinity for PepT1 and do not provide information as to whether the compound is a substrate or an inhibitor, further transport assays were undertaken. *Trans*-stimulation of radiolabelled D-Phe-L-Gln efflux from rabbit PepT1 expressing oocytes in the presence of 10 mM Gly-L-Gln (a standard PepT1 substrate) and 10 mM of test compound **1–11** were compared (Table 1).[24] *Trans*-stimulation is a consequence of having PepT1 substrates on both sides of the membrane, removing

Table 1 Results of affinity, *trans*-stimulated efflux and Caco-2 monolayer studies

Compound	K_i (mM)	*trans*-stimulation	Overall P_{app} ($\times 10^{-6}$ cm s^{-1})	Overall P_{app} (normalised)	PepT1 P_{app} ($\times 10^{-6}$ cm s^{-1})	PepT1 P_{app} (normalised)
1	0.25 ± 0.04	Yes	3.57 ± 0.74	2.81 ± 0.58	[a]	[a]
2	0.22 ± 0.04	No	11.91 ± 4.45	0.52 ± 0.19	[a]	[a]
3	0.03 ± 0.005	No	4.15 ± 0.51	1.29 ± 0.16	2.61 ± 0.56	1.02 ± 0.20
4	0.20 ± 0.03	Yes	4.01 ± 1.28	1.25 ± 0.40	2.70 ± 1.34	1.06 ± 0.53
5	0.79 ± 0.44	No	0.95 ± 0.11	0.34 ± 0.04	0.36 ± 0.11	0.47 ± 0.14
6	0.59 ± 0.10	No	0.42 ± 0.12	0.42 ± 0.11	[a]	[a]
7	0.10 ± 0.01	Yes	4.09 ± 0.87	1.44 ± 0.31	1.99 ± 0.78	1.01 ± 0.40
8	0.09 ± 0.02	Yes	0.92 ± 0.18	0.33 ± 0.07	0.34 ± 0.06	0.44 ± 0.08
9	0.30 ± 0.14	No	3.17 ± 0.01	2.05 ± 0.01	1.06 ± 0.01	3.26 ± 0.04
10	0.84 ± 0.43	No	1.08 ± 0.16	0.70 ± 0.10	0.75 ± 0.20	2.29 ± 0.62
11	0.04 ± 0.01	Yes	1.02 ± 0.16	0.37 ± 0.06	0.81 ± 0.16	1.04 ± 0.21

[a] The PepT1 mediated component of transport was statistically insignificant ($p > 0.05$, $n = 3$).

the need for the re-orientation of an empty PepT1 transporter (the rate-limiting step in the transport cycle) and therefore in this case increasing the efflux rate of the radiolabelled dipeptide injected into the cell compared to when no substrate is present extracellularly.[25] Since *trans*-stimulation of efflux can only occur if the test compound is a substrate, this simple assay can be used to demonstrate PepT1 transport. However, it can give false negatives and is only a qualitative assay.

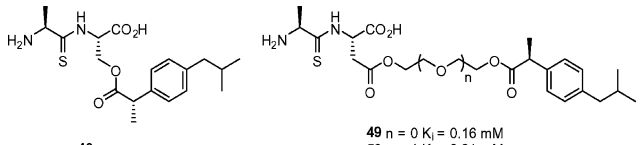
To overcome both of these limitations, assays in Caco-2 monolayers were performed. Caco-2 cells were chosen as they are widely regarded as a good model of absorption from the human intestine.[26] They also have the advantage of being high-throughput, although it has recently been suggested that Caco-2 cells may underestimate the *in vivo* trans-epithelial rate of transport.[27] Apical to basolateral transport of 2 mM **1–11**, applied to the apical side, was monitored by high performance liquid chromatography (HPLC) after one hour. This allowed us to rapidly determine if the intact compounds were crossing the membranes. We compared the extent of transport of compounds **1–11** to that of a known hydrolysis resistant PepT1 substrate, PheΨ[CS-NH]-Ala (FSA),[9,28] by normalisation (Table 1). This normalisation allowed direct comparison between different batches of Caco-2 monolayers and gives an indication as to how well the compounds were crossing the monolayer, relative to a compound known to be transported *in vivo*.[9,28] The rate of PepT1 mediated transport was determined by comparing the difference in apical to basolateral transport of 2 mM **1–11** with excess (20 mM) Gly-Gln. This level of excess Gly-Gln completely saturates the PepT1 transporter, so the reduction in overall transport (after one hour) of the test compounds corresponds to the PepT1 mediated component of transport. This was again compared to the PepT1 mediated rate of FSA transport by normalisation.

PepT1 is regarded as a low affinity, high capacity transporter and compounds with an affinity < 1 mM are generally classed as high affinity substrates.[1] Therefore all of our compounds have high affinity for PepT1, validating the use of thiodipeptides to target drugs generally towards absorption by PepT1. Whilst it is known that peptides ranging in size from Gly-Gly to Trp-Trp-Trp interact with the transporter, it has recently been shown that not all di- and tripeptides are substrates, indicating that there are limits to the capacity of the PepT1 transporter.[12] Our results show the unprecedented capacity of the PepT1 as a transporter of thiodipeptides and clearly indicate that these limits should not adversely affect the rational delivery of small molecule drugs *via* a thiodipeptide approach. With the exceptions of compounds **1**, **2**, and **6** all of our model prodrugs were well transported by PepT1, with PepT1 mediated transport from 0.3–2.7 times FSA (entries **4** and **8**, Table 1), an orally absorbed thiodipeptide.[9,28]

Compound **1** induced significant *trans*-stimulated efflux in our oocyte assay. Surprisingly, this did not translate into significant PepT1 mediated transport across Caco-2 monolayers. We believe that the high overall transport of **1** (2.8 times that of FSA) means that other routes of absorption, not accounted for in the relatively simple oocyte system, are masking the PepT1 mediated component of transport. We have demonstrated that **1** is absorbed intact into rat bloodstreams upon oral administration (data not shown). Our *in vitro* data shows that whilst **1** is a substrate of PepT1, PepT1 mediated transport is not the major pathway of absorption of this compound. Compounds **2** and **6**, however, appear not to be PepT1 substrates, and it appears that they are binding to the transporter in a subtly different way than similar high affinity substrates (**3**, **4** and **10**), preventing transport whilst retaining affinity. The lack of a three-dimensional structure for the transporter prevents a more detailed explanation of this effect.

Prior to this study, we had concerns that the aspartate thiodipeptide may prove less useful generally as a PepT1 carrier, based on poor *trans*-stimulation efflux results, compared to analogous serine derivatives (Rachel Pettecrew and Richard Price, unpublished results). With the exception of **2** and **6** it is clear from our results that this concern was unfounded and that PepT1 can accommodate a variety of aspartate thiodipeptides of various sizes. Indeed our results clearly show that PepT1 can accommodate an aromatic "drug" separated from both serine and aspartate thiodipeptides by a variety of ethylene glycol linkers. This offers a significant range where the choice of linker between thiodipeptide and drug could be modified to optimise diverse properties such as affinity, rate of transport, lipophilicity, solubility and stability.

For the set of monoesters (compounds **3**, **5–8** and **11**) the rate of PepT1 mediated transport drops as the spacer length is increased, and becomes insignificant once a triethylene glycol spacer (**6**) is employed. However the precise distance of the aromatic from the backbone and its conformation appears crucial to transport, as demonstrated by the fact that PepT1 mediated transport can be restored for triethylene glycol spaced diether (**4**) and diester (**10**).



48
K_i = 0.04 mM

49 n = 0 K_i = 0.16 mM
50 n = 1 K_i = 0.21 mM
51 n = 2 K_i = 0.25 mM
52 n = 3 K_i = 0.28 mM
53 n = 4 K_i = 0.48 mM

To probe this effect further and demonstrate the capacity of the PepT1 transporter to accommodate real drugs, we synthesised and tested for affinity and transport in oocytes a set of ibuprofen thiodipeptide prodrugs, **48–53**. The synthetic route employed was similar to that discussed for **5–11** and is given in the ESI.† Ibuprofen is an orally active NSAID, however there is considerable interest in the development of drugs of this class with reduced gastrointestinal toxicity.[29] A PepT1 targeted prodrug may allow retention of the high oral bioavailability of these drugs, whilst reducing interaction of the drug with cyclooxygenase enzymes in the GI tract, especially the stomach, thus reducing toxicity.

With the exception of **48**, the compounds are all diester prodrugs similar to model compounds **9** and **10**. These compounds all induced *trans*-stimulated efflux in our oocyte assay, and to a greater extent than the positive control Gly-Gln (see ESI), suggesting that **48–53** are transported by PepT1 as well as, if not better than, Gly-Gln. Whilst this assay can overestimate the importance of PepT1 transport due to the simplicity of the oocyte system (as discussed for **1**), this assay confirms that these compounds are accepted by PepT1 as substrates, even when a pentaethylene glycol spacer (**53**) is employed, giving even more scope for optimisation of parameters important to PepT1 targeted delivery specifically and oral drug delivery in general. We have also carried out some additional experiments with the ibuprofen prodrug **48**, demonstrating significant PepT1 mediated transport in Caco-2 monolayers (PepT1 $P_{app} = 2.10 \pm 0.27 \times 10^{-6}$ cm s^{-1}; 1.07 ± 0.29 times FSA), and preliminary *in vivo* testing in rats indicating rapid transport of the conjugate **48** into the blood plasma (unpublished data).

We report here a systematic investigation of the potential of targeting the intestinal PepT1 transporter to improve oral drug delivery. Whilst the majority of our data were collected using compounds with benzyl alcohol or benzoic acid in place of actual drugs, we have also demonstrated the PepT1 mediated transport of actual prodrugs, giving the data an immediate application to real medicinal chemistry problems. As expected, the broad substrate capacity of the transporter resulted in it being able to transport the majority of compounds tested. Despite the wealth of information in the literature allowing prediction of a compound's affinity for PepT1, little is known about its transport capacity. Although we could not determine any specific rules governing PepT1 transport, as has been described for affinity,[7] we believe the general trends and observations reported here will be of benefit in the design of rational drugs or prodrugs targeting this transporter. To this end we have specifically demonstrated *in vitro* the ability of PepT1 to transport thiodipeptide prodrugs of ibuprofen, consistent with our previous work on nabumetone.[8]

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