

Targeting ketone drugs towards transport by the intestinal peptide transporter, PepT1†

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Received 21st October 2008, Accepted 5th January 2009

First published as an Advance Article on the web 21st January 2009

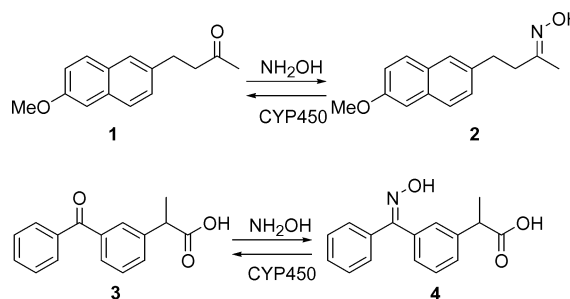
DOI: 10.1039/b818606e

Thiodipeptide prodrugs of the ketone nabumetone are shown to have affinity for, and be transported by, PepT1 *in vitro*.

The oral bioavailability of a compound is a crucial factor in its success or failure as a therapeutic agent, particularly given the convenience of this route of administration. There are two main mechanisms of absorption from the GI tract: passive diffusion and carrier mediated transport. The physicochemical properties of molecules required to facilitate good passive diffusion have been well studied and are summarised by Lipinski's "rule of five".¹ Intestinal carrier mediated transport, be it passive or active, is usually reserved for important nutrients like glucose (*e.g.* SGLT-1), amino acids (*e.g.* ATB₀) and short peptides (*e.g.* PepT1).² These transporters can also sometimes accommodate compounds that structurally mimic their natural substrates. The oral bioavailability of poorly absorbed drugs can be improved either by modifying their physicochemical properties to aid passive diffusion and/or by targeting of the compounds towards carrier mediated transport.

The prodrug approach is a common tool used to overcome poor bioavailability or other properties of the parent drug which adversely affect its clinical use.³ Prodrugs are inactive drug precursors that are metabolised to active drugs. The majority of prodrugs involve modifications to amine, hydroxyl or carboxylic acid functionality in the parent drug. Recently, the use of the hydroxyimine functionality as a prodrug for ketones has been described.⁴ *In vivo* studies suggested that the hydroxyimines **2** and **4** of the non-steroidal anti-inflammatory drugs nabumetone **1** and ketoprofen **3**, respectively, are metabolised by cytochrome P450 enzymes back to the active parent drug (Scheme 1). These authors also suggested that "the hydroxyimine is a useful intermediate prodrug structure for ketone drugs".

We initially planned to prepare prodrugs that could be hydrolysed directly to hydroxyimines, but the potential instability of (for example) hydroxyimine esters also led us to consider hydroxyimine ethers, for which oxidative release of the ketone might occur (see below). The transport mechanism we intended to exploit was *via* PepT1, which is a proton coupled oligopeptide transporter expressed principally in the small intestine, but also in the kidney



Scheme 1 The *in vivo* metabolism by cytochrome P450 of the hydroxyimine prodrugs of nabumetone **1** and ketoprofen **3**.

and liver.⁵ It has a broad substrate specificity including most di- and tripeptides, β -lactam antibiotics and ACE inhibitors.⁵

There are many examples of targeting PepT1 to improve the oral bioavailability of a compound.⁵ This has mostly been achieved by modifying compounds so that they resemble the natural di- or tripeptide substrates. We have recently lodged a patent⁶ for a set of thiodipeptide substrates that we hope can act as "carriers" for drug transport by PepT1 generally. The basic premise involves attaching the desired drug to the thiodipeptide through a hydrolysable linker. The prodrug formed is then recognised as a substrate of the transporter and absorbed from the intestine. Cellular metabolism may result in cleavage of the prodrug, followed by release of the drug moiety by passive diffusion or active transport into the bloodstream. Alternatively, basolateral oligopeptide transporters⁵ similar to PepT1 may transport the prodrug intact into the bloodstream, where metabolism will eventually release the active drug. This general approach overcomes the limitation that the prodrug must resemble a di- or tripeptide.

The use of ester or amide bonds to the carrier thiodipeptides restricts the range of suitable drugs to those containing alcohol, amine or carboxylic acid groups. We wished to investigate if this range could be expanded to ketones by way of the hydroxyimine previously described.⁴ If successful this method could then be applied in efforts to improve the oral bioavailability of a wide range of ketone drugs. We describe the synthesis and *in vitro* transport *via* PepT1 of two prodrugs **16** and **17** (Scheme 2). Nabumetone was chosen as a representative ketone drug as the *in vivo* studies on the hydroxyimine prodrug had already been performed.⁴ A glycol spacer was chosen to improve the water solubility of the prodrug to aid *in vitro* biological testing and to investigate the effect of chain length on transport.

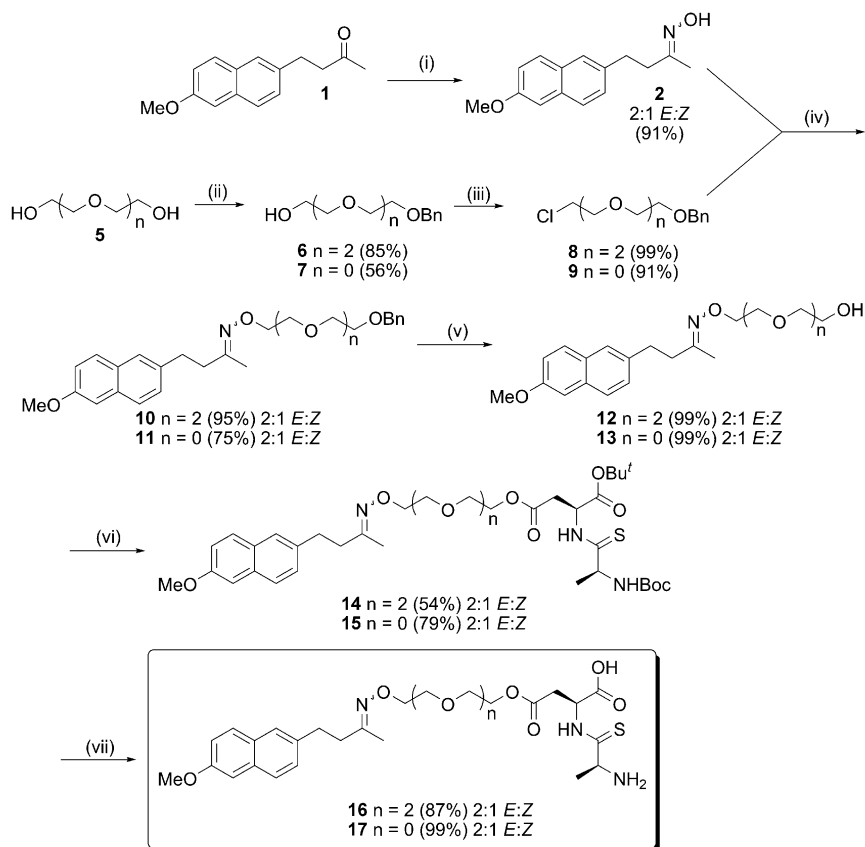
We initially investigated the possibility of attaching the hydroxyimine **2** directly to the aspartate thiodipeptide **23** in an effort to make the ester prodrugs. Despite evidence for the formation

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† Electronic supplementary information (ESI) available: Full experimental details for the synthesis, characterisation, testing and analysis of **16**, with characterisation data for **17**. See DOI: 10.1039/b818606e



Scheme 2 Synthesis of hydroxyimine prodrug linked to PepT1 carrier. (i) $\text{NH}_2\text{OH}\cdot\text{HCl}$, EtOH, rt, 72 h, then 4 M NaOH. (ii) $n = 0$ Ethylene glycol, NaH, KI, BnBr, 48 h. $n = 2$ Triethylene glycol, Ag_2O , BnBr, 72 h. (iii) SOCl_2 , cat. pyridine, 65°C , 4 h. (iv) KI, $\text{KO}t\text{Bu}$, DMF, rt, 24–30 h. (v) H_2 , 5% $\text{Pd}(\text{OH})_2\text{-C}$, EtOH, rt, 2 h. (vi) **23**, HBTU, DIPEA, rt, 2–5 days. (vii) TFA, DCM, rt, 5 h.

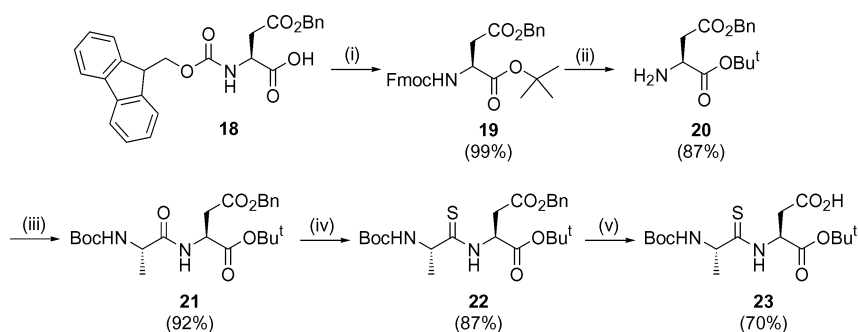
of the oxime ester from crude NMR and mass spectrometry, this compound proved too unstable to isolate or use. Whilst the ethers are considerably more stable, there is sufficient precedent for their chemical and metabolic degradation for us to be confident that release of free nabumetone will occur *in vivo* over a reasonable time-frame; this could take place through direct hydrolysis of the oxime,⁷ or through oxidation of the PEG spacer in the liver,⁸ which should liberate the oxime for further oxidative hydrolysis (as in Scheme 1). The oxime of nabumetone, **2**, was prepared from nabumetone and hydroxylamine hydrochloride, using a modified method to that previously reported.⁴ Stirring at room temperature in ethanol for three days followed by addition of aqueous sodium hydroxide gave **2** as a white solid in high yield (91%) by simple filtration (*c.f.* the literature method required the use of pyridine and column purification). The imine **2** was formed as a 2:1 mix of *E:Z* isomers and this ratio was unchanged throughout the rest of the synthesis.

The required monobenzyl glycol ethers **6** and **7** were synthesised from the corresponding glycols in moderate to good yields (56–85%) using either standard sodium hydride based desymmetrisation chemistry or the method of Bouzide.⁹ These were converted to the glycol chlorides **8** and **9** in excellent yields (91–99%) using the method of Mirviss.¹⁰ When forming the chloride, it was found that increasing reaction times led to decomposition and lower yields. A reaction time of four hours was found to give the optimum balance between conversion and decomposition.

Hydroxyimine ethers **10** and **11** were formed in excellent yield by the reaction of **2** with the glycol chlorides **8** and **9** in the presence of potassium *tert*-butoxide. Potassium iodide was added to accelerate the rate of reaction. No reaction at the imine carbon was detected under these conditions. The benzyl ether was then removed using Pearlman's¹¹ catalyst (palladium hydroxide on carbon) to give **12** and **13** in quantitative yields.

The synthesis of the thiodipeptide carrier **23** was carried out in five steps from Fmoc-Asp(OBzl)-OH, under conditions previously optimised in our group (Scheme 3).⁶ Lawesson's¹² reagent was used to effect the oxygen-sulfur substitution selectively at the amide carbonyl. This carrier was coupled to **12** and **13** to give **14** and **15** in moderate yields (54–79%). Finally acidolysis (50% trifluoroacetic acid in dichloromethane) was used to remove the Boc carbamate and the *tert*-butyl ester protecting groups. The resulting thiodipeptide conjugates **16** and **17** were lyophilised prior to *in vitro* testing.

The binding affinities of **16** and **17** 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 (Fig. 1). Inhibition constants were calculated from standard Michaelis-Menten kinetics.^{13,14} It is known that increasing the hydrophobicity of a PepT1 substrate usually increases its affinity.¹⁵ Our results are in agreement with this trend. PepT1 is a low affinity, high capacity transporter and compounds with an affinity < 1 mM are generally classed as high affinity substrates.⁵



Scheme 3 Outline of the synthesis of the thiodipeptide carrier **23**. (i) *tert*-butyl-2,2,2-trichloroacetimidate, DCM/Et₂O, 2 days. (ii) TBAF in THF, 6 h. (iii) Boc-Ala-OH, DPPA, TEA, DMF, 18 h. (iv) Lawesson's reagent, refluxing toluene, 4 h. (v) Na/NH₃(l) in THF, -78 °C, 3 h.

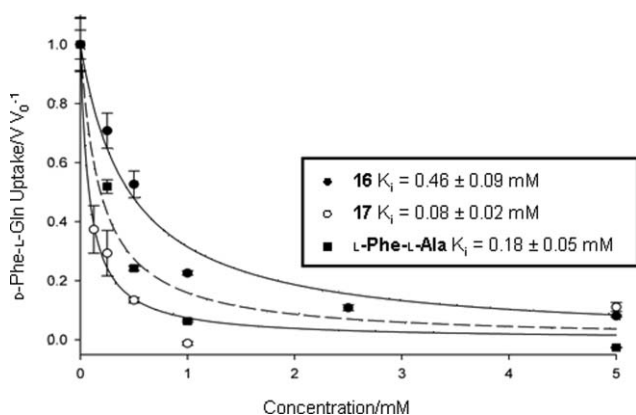


Fig. 1 Inhibition of the transport of [³H]-D-Phe-L-Gln into PepT1-expressing oocytes by **16** and **17**. Data obtained for the known PepT1 substrate, L-Phe-L-Ala is included for reference.

Both **16** and **17** were competitive inhibitors of PepT1 (data not shown), as indicated by an increase in the apparent K_M of D-Phe-L-Gln in their presence using methods previously reported.¹⁶

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 **16** or **17** were compared (Fig. 2).¹⁴ *Trans*-stimulation is a consequence of having PepT1 substrates on both sides of the membrane, removing 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.¹⁷ Since *trans*-stimulation of efflux can only occur if the test compound is a substrate, this simple assay strongly indicated that both **16** and **17** are transported by PepT1 with comparable efficiency to Gly-L-Gln. Free nabumetone did not cause *trans*-stimulation in this assay (data not shown).

To investigate further the extent and rate of *trans*-epithelial transport of these compounds, assays in Caco-2 cells were performed. Caco-2 cells were chosen as they are widely accepted as a good overall model for the small intestinal epithelium.¹⁸ This method also has 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.¹⁹

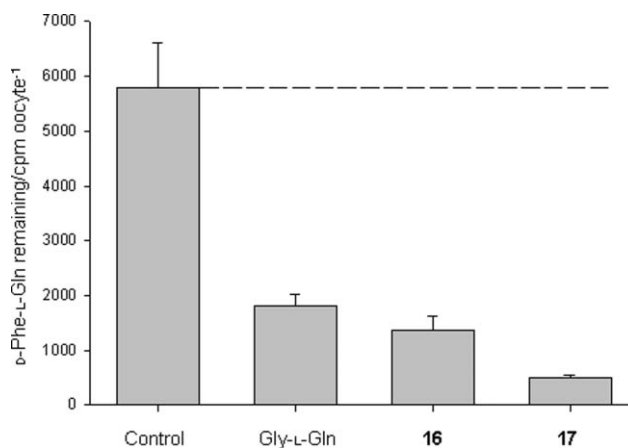


Fig. 2 Comparison of the efflux of 0.4 μM [³H]-D-Phe-L-Gln from PepT1-expressing oocytes in the absence (control) and presence of Gly-L-Gln, **16** or **17** (all 10 mM).

Apical to basolateral transport of 2 mM **16** or **17**, 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 **16** and **17** to a known hydrolysis resistant PepT1 substrate, Pheψ[CS-NH]-Ala (FSA),⁶ by normalisation (Fig. 3A). 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*.⁶ The rate of PepT1 mediated transport was determined by comparing the difference in apical to basolateral transport of 2 mM **16** or **17** with excess (20 mM) Gly-L-Gln. This level of excess Gly-L-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 (Fig. 3B).

The rate of PepT1 mediated transport of **17** was 1.3 times faster than FSA [P_{app} (**17**) = $4.4 \pm 0.1 \times 10^{-6}$ cm sec⁻¹ versus P_{app} (FSA) = $3.3 \pm 0.3 \times 10^{-6}$ cm sec⁻¹] with overall transport 1.7 times that of FSA. **16** gave a PepT1 mediated transport rate 6.3 times that of FSA [P_{app} (**16**) = $6.3 \pm 0.7 \times 10^{-6}$ cm sec⁻¹ versus P_{app} (FSA) = $1.0 \pm 0.1 \times 10^{-6}$ cm sec⁻¹] with overall transport 3.8 times that of FSA. These P_{app} values are similar in magnitude to other PepT1 (pro)drug substrates previously reported.²⁰ **16** was transported

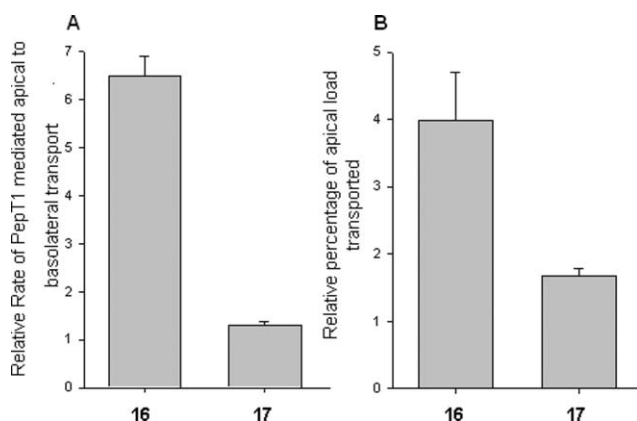


Fig. 3 A: Relative rate of mediated apical to basolateral transport of **16** and **17** in Caco-2 monolayers, normalised to the transport rate of FSA in the same culture of cells. B: Percentage of the 2 mM apical load of **16** and **17** that was transported across the Caco-2 monolayer in 1 hour by a mediated transport route (normalised to a mean value for 2 mM FSA of $1.22 \pm 0.30\%$, $n = 3$ cultures).

faster than **17**. This is consistent with it having lower affinity for PepT1 and implies that the release of the substrate to give an empty transporter for re-orientation is part of the rate-limiting step in the catalytic transport cycle of PepT1.²¹

The pH dependence of this transport was shown by examining the PepT1 mediated rate of transport after one hour (as per method above) at pH 5.5 and 7.4 (Fig. 4). If the compounds are substrates of the proton-coupled PepT1 transporter, as previous results suggested, the transport rates should be faster at pH 5.5 than at pH 7.4.⁵ In each case, a significantly slower transport rate was observed at pH 7.4, approximately 50% that of the pH 5.5 rate. This pH dependence data is fully consistent with the previous data in demonstrating that the compounds are substrates of PepT1.

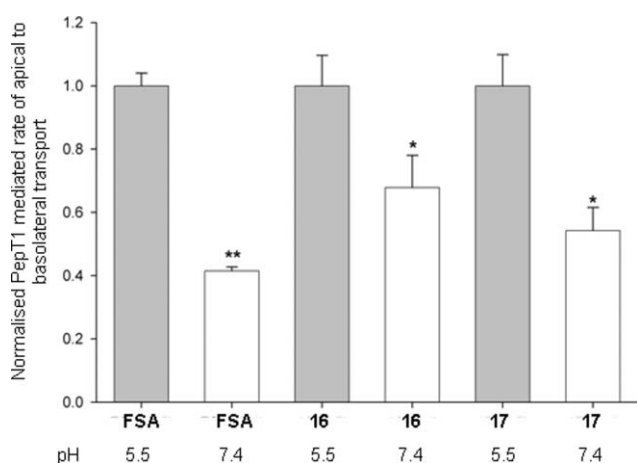


Fig. 4 pH dependence of apical to basolateral PepT1 transport rates across Caco-2 monolayers after one hour of FSA (standard), **16** and **17** (normalised to the mean rate of transport for each compound at pH 5.5, $n = 3$ cultures). ** $p < 0.01$, * $p < 0.05$.

In conclusion, the synthesis, *in vitro* binding and transport of hydroxyimine prodrugs of the anti-inflammatory ketone drug nabumetone *via* PepT1 has been shown. This is the first example of targeting a ketone prodrug towards PepT1 absorption. Whilst the model ketone drug studied is itself orally active, we feel this

approach could be used for other ketone containing drugs with low oral activity, such as the antibiotic pleuromutilin and certain cathepsin K inhibitors under development by GSK.²² Additionally, as prodrugs continue to play an ever increasing role in drug development and optimisation, expanding the range of functionality that can be converted into prodrugs will increase the number of tools available to medicinal chemists for drug development. Furthermore, active targeting of these prodrugs to membrane transporters such as PepT1 may overcome a variety of problems such as oral bioavailability, specific tissue distribution and excretion.

The authors wish to acknowledge the University of Oxford, where most of the biological testing described was carried out by DM and MP. The Wellcome Trust is thanked for generous financial support.

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