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Bioavailability through PepT1: the role of computer modelling in intelligent drug design

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Abstract

In addition to being responsible for the majority of absorption of dietary nitrogen, the mammalian proton-coupled di- and tri-peptide transporter PepT1 is also recognised as a major route of drug delivery for several important classes of compound, including β -lactam antibiotics and angiotensin-converting enzyme inhibitors. Thus there is considerable interest in the PepT1 protein and especially its substrate binding site. In the absence of a crystal structure, computer modelling has been used to try to understand the relationship between PepT1 3D structure and function. Two basic approaches have been taken: modelling the transporter protein, and modelling the substrate. For the former, computer modelling has evolved from early interpretations of the twelve transmembrane domain structure to more recent homology modelling based on recently crystallised bacterial members of the major facilitator superfamily (MFS). Substrate modelling has involved the proposal of a substrate binding template, to which all substrates must conform and from which the affinity of a substrate can be estimated relatively accurately, and identification of points of potential interaction of the substrate with the protein by developing a pharmacophore model of the substrates. Most recently, these two approaches have moved closer together, with the attempted docking of a substrate library onto a homology model of the human PepT1 protein. This article will review these two approaches in which computers have been applied to peptide transport by attempting to integrate them and suggest how such computer modelling could affect drug design and delivery through PepT1.

1. INTRODUCTION

Any compound that is not hydrophobic, and therefore cannot diffuse directly through the lipid bilayer, must interact with an integral membrane protein (a transporter, channel or pump) if it is to cross the plasma membrane to enter (or exit) a cell. In particular, this is vital if a hydrophilic pharmaceutically active compound is to be taken up from the intestinal lumen into the body, i.e. to have oral bioavailability, a point not lost on the pharmaceutical industry (for example, see Ayrton & Morgan [1] for a recent review). Furthermore, although not considered further in this review, drug-drug interactions concerning membrane transporters are taken as seriously by regulatory agencies worldwide as any other kind of potential drug-drug interaction.

When trying to model drug-transporter interactions, there are several approaches that can be taken. One is to conduct studies which investigate the relation between the structure of a number of substrates and their interactions with its transporter to generate a quantitative structure-activity relationship (QSAR). The aim is to identify the regions of the molecules

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that play a key role in the substrate binding and thus allow predictions of interactions of novel compounds. The logical extension of the QSAR is to generate a pharmacophore model, whereby the structures of the multiple substrates studied are reduced to a single model that has areas with particular characteristics in the correct 3D orientation, for example hydrophobic or hydrophilic pockets, positive or negative ionisable sites and so on. An advance on the straight pharmacophore are the techniques of comparative molecular field analysis (CoMFA) and comparative molecular similarity index analysis (CoMSIA), which correlate the major predicted interactions of the substrate with its binding site with the bioactivity. The QSAR and pharmacophore/CoMFA/CoMSIA approaches to modelling transporters with respect to drug transport were recently reviewed by Bhatia *et al.* and Chang & Swaan, and are discussed relative to PepT1 below [2, 3].

A second approach is the computer modelling of the transporter protein itself, necessary due to the lack of crystal structures currently available for membrane transporters, and especially those of mammalian membrane proteins from the major facilitator superfamily (MFS) of which PepT1 is a member [4]. While it would be attractive to be able to model *ab initio*, i.e. from the order of amino acids (primary sequence) of the protein, the size and complexity of membrane transporters makes this an over-ambitious approach. More recently, homology modelling has been attempted for a number of members of the MFS, spurred by the publication of two crystal structures of bacterial transporters, the proton-coupled lactose permease LacY and the glycerol-3-phosphate/inorganic phosphate antiporter GltP [5, 6]. The strikingly similar gross structure of these two transporters, despite very different substrates (lactose and glycerol-3-phosphate, respectively), lead to the suggestion that all MFS members may share the same basic structure, and thus homology modelling might be valid despite a low sequence identity. While more recent MFS crystal structures, such as the bacterial sodium-linked amino acid transporter LeuT [7], sodium-galactose cotransporter vSGLT [8] and sodium benzyl-hydantoin cotransporter Mhp1 [9], have disproved the premise that all MFS transporters will share the same protein structure, it does seem apparent that there are going to be common protein folds amongst subtypes of MFS proteins, as all of the sodium-linked transporters share similar structure despite very different substrates. However, it is still important to remember that these homology models are just that, and it is necessary for the model to be consistent with biochemical data and further validated or refined by experiments designed to test it. Despite its obvious limitations, the technique of homology modelling may prove useful if there is an already crystallised protein in the database with sufficient structural similarity, and this approach was also recently reviewed by Chang & Swaan [3] and its application to PepT1 will be discussed subsequently.

2. THE POTENTIAL OF PEPT1 AS A DRUG DELIVERY TARGET

Since the expression cloning of PepT1 from rabbit small intestine by Fei *et al.* in 1994 [10], a major area of interest has been the binding site for substrates. Originally it was thought that all physiological di- and tri-peptides (400 and 8000 respectively, based on 20 amino acids) were transported from the intestinal lumen, and despite more recent evidence to suggest that perhaps this is not the case [11, 12], the fact remains that PepT1 carries a remarkable range of dietary protein-derived substrates. Thus the substrate binding site must be able to accommodate a range of molecules, with particularly different chemical characteristics of the side chains of the amino acid residues.

However, the interest in the PepT1 substrate binding site is not limited to endogenous substrates, as PepT1 is a major route of oral absorption of pharmaceutically active compounds (see Brandsch *et al.* [13] and Rubio-Aliaga & Daniel [14] for recent reviews). The major clinically administered drugs or drug types that are carried include:

- i. β -lactam antibiotics e.g. cefadroxil [15].
- ii. Antivirals, most notably valacyclovir, the valine ester prodrug of the active compound acyclovir [16].
- iii. Angiotensin-converting enzyme (ACE) inhibitors: ACE inhibitors have long been assumed to be absorbed from the intestine / reabsorbed from the kidney by PepT1 and PepT1, largely based on their binding affinities. However, a recent study by Knütter *et al.* [17] has demonstrated that this may not in fact be the case, with enalapril being one of the few of the ACE inhibitors tested that caused a significant current in PepT1-expressing *Xenopus laevis* oocytes, while binding with a low affinity. It should be noted that these findings are contrary to earlier work (eg Zhu *et al.* [18]) using similar techniques, and so further studies are needed to resolve the route of oral absorption of these therapeutically important compounds.
- iv. Hypotensive agents such as midodrine [19].
- v. The dopamine receptor antagonist sulpiride [20].

The rational design of prodrugs to target the PepT1 transporter and improve oral delivery is also a major area of research [21-28]. Given the capacity of the PepT1 transporter, this approach has the potential to make any drug a PepT1 substrate and consequently orally active. With the daily uptake of drugs through PepT1 estimated to have a commercial value of over a billion US dollars worldwide, it is obvious why there is interest in this potential route of uptake. Thus PepT1 has been extensively studied and is relatively well understood compared to other intestinal nutrient membrane transport systems.

3. SUBSTRATE AFFINITY MODELS

3.1 Critical Substrate Features for Binding

Prior to the cloning of PepT1 from rabbit DNA in 1994 [10], very little was known about the type of compounds that interacted with the transporter. Following this breakthrough, several key structural features for high affinity binding have been established. Several excellent reviews detail these features [13, 29, 30] and what follows is a brief discussion of the key discoveries.

A peptide bond is not a fundamental requirement for binding, as demonstrated first by Temple *et al.* [31] and Meredith *et al.* [32] on 4-aminophenylacetic acid **1** and 4-aminomethylbenzoic acid **2** respectively. Additional support and refinement was provided by Döring *et al.* [33] on ω -amino fatty acids such as **3** which again showed that a peptide bond was not necessary but also suggested that the distance between amino and carboxy terminus should be 5-6 Å, although this is a very structurally unconstrained molecule. Final

confirmation was provided by Våbenø *et al.* [34] on ketomethylene dipeptide analogues such as **4**.

The importance of a free amino terminus to binding affinity was conclusively demonstrated by Meredith *et al.* [35]. N-terminal acetylation **6** of the dipeptide Phe-Tyr **5** resulted in a dramatic loss of affinity. Alterations at the carboxy terminus are better tolerated as exemplified by compounds **7-9** [35, 36]. These results indicate that the amino terminus is the primary binding feature, which orientates the rest of the molecule in the binding site.

The introduction of a D-amino acid residue at the N-terminus of a dipeptide, such as **10-12**, has been shown by Taub *et al.* [28] in 1998 to have a detrimental effect on binding affinity. A similar effect has been reported for C-terminal D-amino acid residues, with D-X_{AA}-D-X_{AA} dipeptides having almost no affinity for the transporter. Early evidence of this effect had been reported by Lister *et al.* [37] in 1995, by monitoring the rates of absorption of the various dipeptide diastereomers, but no affinity data was reported. Bailey *et al.* [38] went on to probe this effect further in 2005 using cyclic (hence conformationally restricted) dipeptide analogues **13-16**, to elucidate the precise conformation, in which the amino group is directed back into the plane, that the N-terminal residue must adopt for high affinity binding.

That PepT1 binds the *trans* isomer of dipeptides preferentially has been demonstrated by a variety of groups and methods. Brandsch *et al.* [39, 40] and Bailey *et al.* [41] used thiodipeptides, such as **17**, in which the percentage *trans* conformation can be controlled by the storage temperature, the incorporation of a proline residue or pH. Niida *et al.* [42]

reported a study on the synthesis and binding of fluoroalkene **18** and alkene **19** containing dipeptide mimics to unequivocally probe this interaction. In all cases compounds that existed mainly or completely in the peptide *trans* conformation had higher affinities for the PepT1 transporter.

A general trend exists between lipophilicity and PepT1 affinity for a variety of substrates as determined by several groups and methods [12, 42-44]. Additionally, the highest affinity substrates **20** and inhibitors **21** of the PepT1 transporter reported to date all share large, hydrophobic areas and a C-terminal proline residue [43, 45-48]. In general, the incorporation of proline at the C-terminus leads to increase affinity, but its effect at the N-terminus is more diverse [40, 45, 49-51].

3.2 A Qualitative Template Binding Model

A detailed analysis of the published substrates of PepT1 as previously discussed combined with *in vitro* investigations of over 100 further substrates led to a seminal paper by Bailey *et al.* [52] in 2000 which proposed a substrate model to predict the affinity of binding. It consists of several features, the key four being: 1) a strong binding site for the N-terminal NH^+_3 group; 2) a hydrogen bond to the carbonyl group of the first peptide bond; 3) a carboxylate binding site which controls the preferred stereochemistry of the adjacent chiral centre and 4) a hydrophobic pocket with a strong directional vector, Figure 1Fig. (1).

This template was successful at predicting high, medium or low affinity for a variety of PepT1 substrates including dipeptides, cephalosporins and other β -lactam antibiotics and also the 4-substituted alanyl anilides (**8** and **9**). By assigning simple numerical values corresponding to the presence or absence of the various features needed for binding, then aggregating, a simple linear relationship between the score and the free energy of binding emerged [53]. This template thus became the first qualitative and semi-quantitative method for predicting affinities for PepT1 binding, allowing compounds targeting this transporter to be more rationally designed.

Quantitative Structure Activity Relationships

For a training set of 79 dipeptides, a CoMFA and CoMSIA model was developed by Gebauer *et al.* [54] in 2003. The pharmacophore model was derived using DISCO with L-Ala-L-Ala as the reference compound. Whilst this compound had six unique low energy conformers, comparison with Ala- Ψ [CS-NH]-Pro (which has only two conformers with a *trans* thioamide bond) indicated that only a single spatial arrangement, Fig. (2), Figure 2was

shared. The values obtained were later supported by independent studies by Våbenø *et al.* [55] in 2005. DISCO analysis of the 79 training compounds in general gave conformers similar to the reference, but where exceptions were found, manual alignment by fitting of the peptide backbone to the template was carried out. Both CoMFA and CoMSIA models were successful at predicting affinities for the training set and an additional test set of 19 dipeptide and dipeptide mimics.

The model generated went some way towards explaining several of the previously observed results. For example, the drop in affinity caused by the introduction of D-amino acid residues at either the N- or C-terminal position [37] was attributed to their side chains occupying a disfavoured steric region. Steric bulk, as expected, was favoured at the side-chains of L-amino acid residues. The vital importance of an unmodified N-terminal amine was confirmed. The effect of lipophilicity on affinity was ascribed to a favourable pocket at the C-terminal end. Perhaps most interestingly, the reason why alterations to the carboxy terminus had such a small effect on affinity was rationalised by the fact that it is electron density, not specifically negative charge, which is important at this site. Most modifications to the carboxy terminus tested *in vitro* involved replacing the acid with a primary amide **7** or aromatic ring **8** & **9**, which are still electron rich and so should only have a minor effect on affinity.

This model was limited to dipeptides, whereas PepT1 can bind a range of substrates ranging in size from Gly-Gly to Trp-Trp-Trp. To overcome the limitations of this first QSAR model, Biegel *et al.* [56] extended their work further in 2005. Crucially the training set for this CoMSIA model contained 98 substrates of affinity ranging from 0.01 to 100 mM, with examples of dipeptides, dipeptide derivatives, tripeptides and β -lactam antibiotics. The L-Ala-L-Ala dipeptide reference conformer was employed as an initial starting point. When the relatively rigid tripeptide Val-Pro-Pro (three low energy conformers in *trans* arrangement) was aligned onto this structure, the conformer ($\Psi_1 = 147^\circ$; $\omega_1 = 180^\circ$; $\phi_2 = 286^\circ$; $\Psi_2 = 172^\circ$; $\omega_2 = 180^\circ$; $\phi_3 = 309^\circ$; $d_{N-C} = 7.8 \text{ \AA}$) with the lowest root-mean-square deviation was selected and used as a reference for all tripeptides, Figure 3Fig. (3). Independent work by Andersen *et al.* [57] in 2006 employing Volsurf descriptors supported this conformation.

Since β -lactams sterically resemble tripeptides, they were initially aligned onto the reference conformer for Val-Pro-Pro. It quickly became apparent that the β -lactams could not all be accommodated into one preferred binding conformer. A distinction was made between those β -lactams containing an aminothiazole ring, such as ceftibuten **22**, and those that did not, for example cephadrine **23**. The need for this separation was identified by the fact that the affinity of **22** was attributed almost entirely to its butenyl carboxylic acid, a structural feature unique to this compound and not the β -lactams generally. Employing separate reference conformations for these two sets of β -lactams did solve the problem, allowing a CoMSIA model to be generated that was successful at predicting the affinities of a test set of 8 β -lactams, 10 tripeptides and 6 dipeptides. However, no study was carried out as to the energy difference between the two possible conformations of aminothiazole β -lactams, so it is difficult to ascertain the prevalence of the modelled conformation *in vivo*. However the model did predict that 2-aminothiazole-4-acetic acid **24** itself would have affinity for PepT1, indicating that this structural feature may direct the binding orientation of β -lactams with aminothiazole rings.

The model showed the expected three sterically favourable binding areas into which the side-chains of high affinity di- and tripeptides of L-stereochemistry as well as the bulk around position **X** of the β -lactams are accommodated. The electrostatic fields generated were similar to the dipeptide model and again the importance of a hydrogen bond donor (the amino terminus of peptides or in position **Y** of β -lactams) was shown.

The QSAR models discussed provide highly detailed information of the subtle substrate features affecting affinity between groups of closely related molecules. In order to generate the model, a considerable amount of computational time and software is required and indeed, most of these models involved a significant element of manual optimisation. In order to successfully design compounds that target the PepT1 transport, it may always be necessary to study them in this level of detail, due to the broad substrate capacity of the transporter. In this case, simpler qualitative and semi-quantitative models exist which are useful in getting a new project “off the ground” and do not require a large investment in computational resources. The general trends identified by the QSAR models, such as lipophilicity and the importance of a free amino group, are all accommodated by these simpler models.

Models to predict PepT1 affinity at various levels of quantification have been successfully developed over the past decade. Affinity does not always mean that a compound will be transported and a high affinity inhibitor of PepT1 has been reported [45]. The substrate structural features required for transport remains an under developed area of PepT1 research, with only one paper to date on the problem. This paper, by Vig *et al.* [12] in 2006, is remarkable as the first to suggest that not all di- and tripeptides are substrates of the transporter under physiological conditions, although most have affinity for it. In particular, dipeptides are better substrates of the transporter generally than tripeptides and PepT1 appears incapable of transporting dibasic dipeptides such as Arg-Lys and Arg-Arg. Additionally dipeptides with Trp at the N-terminus were poor substrates. Earlier studies by Amasheh *et al.* [58] have shown large currents induced by Lys-Lys in PepT1-expressing *Xenopus* oocytes, but only when the extracellular pH is non-physiologically high (above pH 7.4).

Whilst the properties of the individual residues at both the N- and C-termini are important and each site appears to have its own preferred characteristics, the transport activity of dipeptides is not merely equal to the sum of independent contributions from both residues. For example, Ala-Trp, Trp-Ala and Trp-Trp all have similar affinities but Trp-Trp is not transported. This implies that PepT1 has one contiguous binding pocket.

Whilst in general higher affinity compounds had higher levels of transport, confirming that most of the features important to affinity are also important to transport, these exceptions allow some separation between features required for affinity and features required for transport. In particular, the inability of PepT1 to transport dibasic dipeptides was rationalised by modelling of the lowest energy conformations. As previously discussed and confirmed in this paper, the optimum configuration for PepT1 binding places the side-chain residues on the same side of the peptide chain [12, 54, 56]. As such, interactions between these side chains themselves and the binding pocket will have an impact on affinity and

transport. Hydrophobic interactions, π - π stacking and salt-bridge formation between the side-chains will stabilise this conformation and improve affinity and transport. Dibasic dipeptides adopting this conformation will pay an energy penalty from the repulsion of their electrostatic charges. However the fact that Glu-Glu is a substrate indicates that this theory does not fully explain all cases. This maybe due to the fact that our understanding of the structure and residues comprising the PepT1 binding pocket is extremely limited, meaning the interactions between peptide backbone, side-chains and the binding pocket are not fully understood. Our current understanding of the structure of PepT1 will be discussed in the next section.

4. PEPT1 STRUCTURAL MODELS

4.1 Initial Hydropathy Model and Experimental Validation

Once PepT1 was cloned from rabbit small intestine in 1994, investigations into the structure of the protein could begin [30]. Hydropathy analysis of rabbit [10] and human [59] isoforms of PepT1, using a Kyte & Doolittle [60] hydropathy plot, indicated that the protein consisted of twelve transmembrane domains (TMDs), Fig. (4).

This idea was extended by work from Lee's group with a study of pairwise interactions between residues of all possible adjacent helices in the membrane to develop a computational model of the arrangement of the TMDs in the membrane, Fig. (5) [61-63]. The model indicated the possibility of half a binding site from TMDs 7, 8, 9 and 10.

As the earliest model for PepT1 structure, Lee's model has been the subject of intense experimental validation. The intracellular position of the C- and N-terminals was confirmed by epitope labelling work using permeable and non-permeable cells in 1998 [64].

Chimera proteins comprised of varying amounts of PepT1 and its renal counterpart PepT2, have been used by several groups to demonstrate the importance of transmembrane domains (TMDs) 7-9 [65-67]. Cysteine scanning mutagenesis has been used to confirm the importance of TMDs 3, 5 and 7 [68-70]. Crucially these experiments indicated that some of the TMDs were tilted away from perpendicular to the axis of the membrane, a result not accommodated by Lee's modelling technique.

Site-directed mutagenesis studies over the last decade have confirmed the importance of Tyr-12, Tyr-91 and Glu-295 on affinity and transport. Cysteine scanning mutagenesis has indicated the importance of Asn-171, Ser-174, Phe-293, Leu-296 and Phe-297 to PepT1 function [68-71]. The critical importance of His-57 (located in TMD-2) has been repeatedly demonstrated in several experiments by various groups [71-74]. Any mutation or chemical modification of this residue results in non-functional protein being produced. Since it was known that tyrosine residues can stabilise nearby cationic residues, mutations of tyrosine residues neighbouring His-57 were carried out by Chen *et al.* [72] in 2000. Mutations of Tyr-56 and Tyr-64 to phenylalanine had a minor effect on transport, but mutations to alanine had a major effect. It is widely supposed that His-57 is the site of proton binding [71-75].

Independent mutations on Arg-282 and Asp-341 were shown to alter the pH dependence, stoichiometry and extent of transport of D-Phe-L-Gln in rabbit PepT1 [51, 76] and transport of Gly-Sar in human PepT1 [77]. The double mutant R282D-D341R, in which the positions of the charges are reversed, had no effect on transport or pH profile, implying these two residues are involved in a salt bridge [76, 77]. When the positive charge held normally at Arg-282 is removed the transport, proton:substrate stoichiometry and pH profile are altered which implies a role in proton binding for this residue [76]. When Asp-341 is changed to a positively charged residue, transport is reduced in human PepT1 [77], which implies this

residue is involved in substrate binding and/or translocation, although the same result was not seen in rabbit PepT1 [76]. These results led to the publication of two independently conceived models for proton and substrate binding and transport which both propose that it is the breaking and reforming of the salt bridge between Arg-282 and Asp-341 that drives the conformational changes needed for substrate transport and transporter resetting [76, 77]. The actual sequence of substrate binding, proton binding and conformational change remains in some doubt.

The original model from Lee's group [61-63] suffers from limitations inherent in the assumptions used to generate it. It assumes that only TMDs adjacent in sequence are adjacent in space and able to interact. It also assumes that the TMDs are perpendicular to the axis on the membrane, which as shown by SCAM experiments is most definitely not the case. Finally, this model cannot explain why His-57 is so fundamentally important to the function of the transporter as neither the residue nor its TMD is involved in the proposed substrate binding pocket. Regardless, there has been considerable experimental validation of the bulk of Lee's model, laying a foundation that must be met by any future efforts.

4.2 Homology Models and Experimental Validation

Membrane transporters such as PepT1 are extremely difficult to analyse by either X-ray or multi-dimensional NMR techniques. Complete three dimensional structures of such proteins are therefore exceedingly rare in the Protein Database. The recent publication of the three dimensional structures of two twelve transmembrane domain transporters, namely the proton-coupled lactose permease (LacY) [5] and the phosphate coupled glycerol-3-phosphate antiporter (GlpT) [6] gave a glimpse into the structure of these types of transporter. It is noteworthy that LacY and GlpT are members of the same major facilitator superfamily as PepT1 [78] and it has been suggested that members of this family may have structural similarity [79].

On this basis, Meredith and Price [80] have published a recent paper in which they superimpose a modified form of rabbit PepT1 (rPepT1-trunc) onto the structures of LacY and GlpT. The modification consisted of the removal of residues mainly comprising the large extracellular loop of PepT1 between TMDs 9 and 10, as this was not present in either of the other two transporters. This was done by sequence line-up with PepT1 homologues from lower order organisms such as bacteria which lack this large extracellular loop, and PepT1 with the majority of the large extracellular loop removed was shown to be effective transporter, although with a much higher binding affinity than the wild-type (S. Williams & D. Meredith, unpublished data). A bacterial proton oligopeptide transporter similar to PepT1 has recently been purified [81], making it a good candidate for further structural studies. Even though there is biophysical data [82] and molecular evidence [83] to suggest PepT1 is a multimer, it seems probable that each subunit would have a similar three dimensional structure and active site. Indeed LacY was actually crystallised as a dimer [5].

The structural model produced by this homology modelling, Fig. (6), Figure 6 is quite different from that proposed by Lee's group [61-63]. When the sequence was run through more advanced prediction programs (MEMSAT3) than that used during the original hydropathy analysis [10], it indicated that TMD 1 may actually occur later in the sequence (from residues 24-42 rather than 7-25). This later position for TMD 1 explains why previous epitope labelling experiments at position 39 gave non-functional protein [64], but when an epitope tag (FLAG) was placed at position 49, functional protein was expressed (D. Meredith, unpublished data). The model predicts that TMDs 1, 4, 5, 7 and 8 in particular are tilted from perpendicular to the membrane consistent with substituted cysteine accessibility method (SCAM) experiments [69, 70]. It shifts the critical residue Tyr-91 away from the substrate binding site but involves TMD 2 and the crucial His-57 residue in the binding site.

The remaining critical residues (such as Arg-282, Asp-341, Tyr-167 and Glu-594 for example) are all still predicted to be in the binding site. The model also predicts that TMD 7 will play a significant role in the binding site and be quite solvent accessible, as demonstrated by SCAM experiments [70].

One major limitation of the above model was the use of a truncated version of PepT1, despite experiments demonstrating that the truncated form had transport activity (the full PepT1 sequence has now been modelled using SYBYL which also mapped onto LacY as the best available structure and thus gave an identical transmembrane helix arrangement; J. Rajamanickam & D. Meredith, unpublished data). Recently, a similar homology model has been published using the full PepT1 sequence which also used the LacY crystal coordinates as a template [84] and therefore produced an identical model regarding the layout of the transmembrane helices. The model was generated by fragmenting the amino acid sequence of human PepT1 into 25 segments (twelve transmembrane domains, six extracellular loops, five cytoplasmic loops and the two termini). A rather unusual approach was taken, using the computer program Fugue to homology model each of these segments separately against all of the proteins in the database, before assembling the fragments using *E. coli* lactose permease as a template. For example, this led to the large extracellular loop between TMDs 9 and 10 being modelled on a sucrose phosphatase, even though there is no experimental evidence to suggest that this domain has such enzymatic activity and thus might assume a similar conformation. Thus the 3D structures assigned by this approach must be considered highly speculative, especially for the intra- and extra-cellular loops where there is no equivalent structure in LacY, and the rationale behind this approach is far from clear.

The Pedretti *et al.* model [84] was validated by predicting the affinity of 50 PepT1 substrates. The substrates were docked in a side box of 8 Å around three residues claimed by the authors to be of known importance to ligand recognition, Glu-26, Trp-294 and Tyr-588. Of these however, only Trp-294 has had any experimental validation of its importance to PepT1 function, whereas Glu-26 was not expressed when mutated [85] (abstract) and Tyr-588 (Tyr-587 in rabbit PepT1) mutations transported as wild-type [86, 87, 88]. When the predicted and actual pK_i values were plotted, only a weak correlation ($r^2 = 0.75$) was observed. Additionally, if the reciprocals of the predicted and actual K_i values are plotted, allowing direct comparison with Brandsch's QSAR models [54, 56], no correlation is observed.

This substrate docking proposed a binding site that was quite different to anything previously published, consisting of the key residues Asn-22, Glu-23, Glu-26, Glu-291, Phe-293, Trp-294, Ala-295, Phe-296, Leu-297, Ile-331 and Tyr-588. However, only a few of these residues have been experimentally studied [70, 83, 88] so further site-directed mutagenesis experiments are required to confirm the role of these other residues identified by the Pedretti *et al.* model. Additionally, whilst most of the dipeptides appear to be docked in the same conformation, most of the β -lactams used in the study adopted a different binding conformation. This does not agree with the Brandsch group's QSAR work [56] or the concept of a template binding model [52], both discussed above, both of which suggest that all substrates have to bind in the same conformation. Furthermore, two dipeptides, Val-Phe and Tyr-Phe, are proposed to have the amino terminus interacting with Glu-23 and the carboxy terminus interacting with Asn-22, requiring a conformation in contrast to the accepted fully extended, *trans*-conformation known to be necessary for PepT1 binding [39, 41, 42]. This may explain the lack of good correlation between the actual and predicted affinities of the model, and suggests that free energy minimisation of substrates in an aqueous environment, rather than using substrate structures in a suitably constrained conformation, may not be a valid approach for docking studies.

Both of these models as they currently stand are highly speculative and in very early stages of development. The Meredith & Price model appears to explain most of the experimental data accumulated over the years and is generally consistent with the Lee group's model. The major difference, the sequence of TMD 1, has some experimental validation. As yet no binding site has been identified by *in silico* docking and hence it has yet to be validated by predicting affinity and transport of PepT1 substrates. The Pedretti *et al.* model is based on the complete sequence of PepT1 and has identified a binding site, however this site has not been experimentally validated, the binding conformation is variable and the predictive power of the model is not good. However, these models offer the prospect of potentially understanding how this incredibly important transporter works, and further research is clearly needed.

5. CONCLUSIONS

The types of computer modelling discussed here essentially fall into two areas: the modelling of the substrate, be it the endogenous di- and tri-peptides or the drug molecules currently known to be carried by PepT1, and the modelling of the transporter in the hope of in particular identifying the binding site. Currently, the substrate modelling has been very instructive and able to allow *in silico* prediction of the affinity of a compound to a reasonable degree based on its chemical structure. The main advances have been in the establishment of the QSAR/pharmacophore models that effectively determine the 3D shape that the substrate must adopt to bind to the PepT1 transporter. In contrast, modelling of the transporter itself is much more speculative, and while biochemical data supports homology models, in the absence of a crystal structure many regions of the model are unsatisfactorily characterised, including critically the substrate binding site. Until the binding site is clearly identified, it will not be possible to design compounds to interact in specific ways with the protein. Thus although PepT1 is widely accepted as an important route of drug absorption, this is for compounds that have been discovered to be PepT1 substrates after their development as effective and bioavailable therapeutic agents. The possibility of designing bioavailable drugs *de novo* remains a tantalising prospect, but one that may have to wait until the crystal structure of a peptide transporter is solved.

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Figure 1. Substrate template for binding to PepT1. Free dipeptide terminate with $\text{X} = \text{O}^-$ whilst tripeptides are extended by $\text{X} = \text{NH}$ [52].

Figure 2.
Reference conformation for dipeptide QSAR study. $\Psi = 165^\circ$, $\omega = 178^\circ$ and $\phi = -65^\circ$.

Figure 3.

Left: The reference conformation of Val-Pro-Pro as defined in the text. Right: Val-Pro-Pro overlaid with reference dipeptide Ala-Ala (orange); as can be seen, the peptide backbone of the molecules align well.

Figure 4.

Overall structure of PepT1 showing critical structural features. TMDs 1-5 and 7-9 have been implicated as domains important in determining the phenotype of PepT1 by PepT1-PepT2 chimera studies. TMD6 contains the PTR family signature motif characteristic of peptide transporters. The residues marked in TMD 2 (Tyr-56, His-57), TMD3 (Tyr-91), TMD5 (Tyr-167), TMD7 (Arg-282, Trp-294), TMD8 (Asp-341) and TMD10 (Glu-594) have all been shown to be important in PepT1 function by site-directed mutagenesis and are discussed in the text.

Figure 5.

Lee's model of the substrate binding site of PepT1. From left to right: (1) Schematic view looking into the cell of a computational model of TMDs buried in phospholipid membrane (●). (2) Schematic view showing arrangement of all 12 TMDs. (3) Proposed transport channel. Redrawn from [61-63]

Figure 6.

A: Side view of rPepT1-trunc overlaid onto LacY with His-57 and Glu-336 (Glu-594 in rabbit PepT1) highlighted. **B:** Plan view of rPepT1-trunc overlaid onto LacY. **C:** Cartoon of the positions of the TMDs of rPepT1-trunc overlain on LacY and reoriented to match Lee's model in Figure 5. **D:** Helical wheel plan of rPepT1-trunc drawn from B.