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Rapid Communications

In Vitro and Pharmacophore-Based Discovery of Novel hPEPT1 Inhibitors

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Purpose. The human proton-coupled small peptide carrier (hPEPT1) is a low-affinity, high-capacity transporter with broad substrate specificity. We have taken an iterative *in vitro* and *in silico* approach to the discovery of molecules with hPEPT1 affinity.
Methods. A pharmacophore-based approach was taken to identifying hPEPT1 inhibitors. The well-characterized and relatively high affinity ligands Gly-Sar, bestatin, and enalapril were used to generate a common features (HIPHOP) pharmacophore. This consisted of two hydrophobic features, a hydrogen bond donor, acceptor, and a negative ionizable feature.
Results. The pharmacophore was used to search the Comprehensive Medicinal Chemistry (CMC) database of more than 8000 drug-like molecules and retrieved 145 virtual hits mapping to the pharmacophore features. The highest scoring compounds within this set were selected and tested in a stably transfected CHO-hPepT1 cell model. The antidiabetic repaglinide and HMG CoA reductase inhibitor fluvastatin were found to inhibit hPEPT1 with sub-millimolar potency (IC_{50} 178 ± 1.0 and 337 ± 4 μM, respectively). The pharmacophore was also able to identify known hPEPT1 substrates and inhibitors in further database mining of more than 500 commonly prescribed drugs.
Conclusions. This study demonstrates the potential of combining computational and *in vitro* approaches to determine the affinity of compounds for hPEPT1 and, in turn, provides insights into key molecular interactions with this transporter.

KEY WORDS: data mining; hPepT1; peptide transporter; pharmacophore; QSAR.

INTRODUCTION

Optimizing new chemical entities for human oral bioavailability is generally confounded by molecules that are substrates for intestinal uptake or efflux transporters. At present, with few transport proteins crystallized, structural details and affinity requirements are generally unknown, and this has significantly hampered rational understanding of drug transport mechanisms. Attempts at the prediction of human oral bioavailability from molecular structure alone also has been extremely challenging for transporter substrates (1,2). However, advances in computational approaches have made it possible to use empirical data for predicting binding computationally, using quantitative structure-activity relationships (QSAR) (3,4).

The human intestinal small peptide carrier (hPEPT1) is a proton-coupled, low-affinity, high-capacity oligopeptide transport system with broad substrate specificity. In addition to transporting its natural substrates, di- and tri-peptides occurring in food products (5), it shows affinity toward a broad range of peptide-like pharmaceutically relevant compounds, such as β-lactam antibiotics (6) and angiotensin converting enzyme (ACE)-inhibitors (7). For this reason, hPEPT1 has been recognized as an important intermediate in the oral bioavailability of peptidomimetic compounds (8). However, the lack of knowledge regarding structural specificity toward its substrates has prevented the use of this transporter on a more rational basis. Recently, Zhang *et al.* (9) reported nine distinct single nucleotide polymorphisms for hPepT1; only one displayed a reduced transport capacity, inferring that hPEPT1 may be a pharmacologically relevant drug delivery target not confounded by genetic variability. Thus, there exists a keen interest in understanding the structural determinants for substrates and inhibitors of hPEPT1.

Despite the availability of several computational models for peptide transporters from various species as well as hPEPT1, little progress has been made in the design and elucidation of novel substrates for this key intestinal transport protein. In fact, discovery of most hPEPT1 substrates has remained remarkably and perhaps unacceptably serendipitous to date. In this current study, we present a novel approach using pharmacophore-based database searching for rapidly retrieving hPEPT1 inhibitors for this transporter. This method complements the many experimental approaches that are currently in use for identifying high-affinity hPEPT1 ligands as well as understanding the structural features for binding (10). This proof of principle study may be applicable

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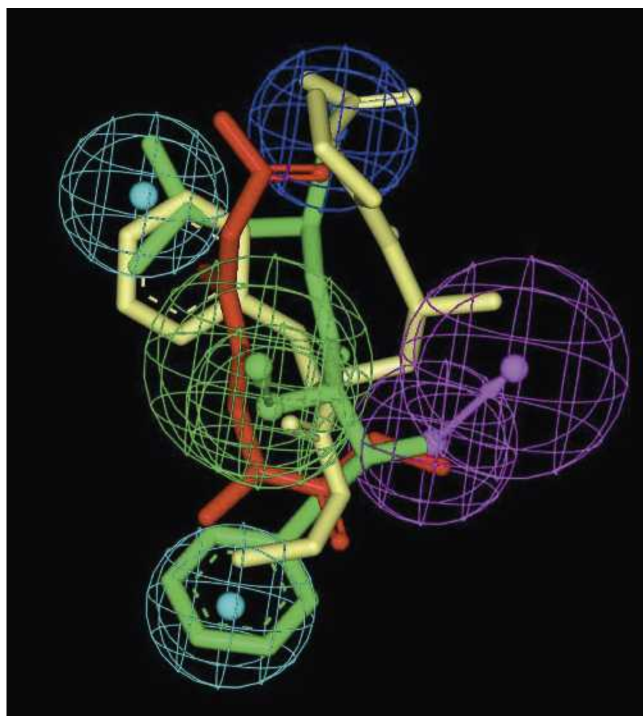

Fig. 1. HIPHOP pharmacophore for hPEPT1 substrates (red, Gly-Sar; green, bestatin; yellow, enalapril). Pharmacophore features: cyan, hydrophobe; green, HBA; purple, HBD; blue, negative ionizable.

to other human transporters for which there is significantly less data than for hPEPT1.

METHODS

hPEPT Pharmacophore Development

The computational molecular modeling studies were carried out using a Silicon Graphics (Palo Alto, CA, USA) Octane workstation. Briefly, models were constructed using Catalyst version 4.8 (Accelrys, San Diego, CA, USA) to generate a common features (HIPHOP) (11) pharmacophore for the well-known PEPT1 substrates Gly-Sar, bestatin, and enalapril (12). Structures for these three molecules were initially sketched in ChemDraw version. 7.0.1 (CambridgeSoft, Cambridge, MA, USA), exported into the MOL file format, and then imported into Catalyst. Up to 255 conformers were then generated with the fast conformer generation method, allowing a maximum energy of 20 kcal/mol. The three hPEPT1 substrate molecules were then aligned using hydrophobic, hydrogen bond acceptor, hydrogen bond donor, and negative ionizable features in the HIPHOP algorithm within Catalyst.

Pharmacophore-Based Database Searching

The resulting hPEPT1 HIPHOP pharmacophore was used for a fast-flexible search of the Comprehensive Medicinal Chemistry (CMC) "drug-like" database (MDL, San Leandro, CA, USA) of more than 8000 molecules. These molecules were implemented as a Catalyst searchable database by generating up to 100 conformers with the fast conformer generation method, allowing a maximum energy of 20 kcal/mol. The pharmacophore was used with the fast-flexible search approach and retrieved 145 virtual hits. This list was then sorted by molecular weight, and the top seven molecules were individually fast-fit to the hPEPT1 pharmacophore. As a means of comparison, the relatively high affinity substrates enalapril and bestatin were scored with the same method (3.74 and 2.59 arbitrary units, respectively). A search of the literature was carried out to ascertain the commercial availability of these molecules for purchase. A larger range of known hPEPT1 substrates or inhibitors not included in the pharmacophore but retrieved upon database searching were also scored using the same approach described above and included ampicillin (2.69 arbitrary units), Captopril (2.07), Cefaclor (3.24), Cefadroxil (3.14), Cefoperazone (2.60), Cefoxitin (2.89), Cephalexin (3.15), Cephradine (2.90), methyldopa (2.50), and valacyclovir (2.13).

The pharmacophore model was also used to search a database of 576 known drugs in clinical use in the United States derived from the *Clinician's Pocket Drug Reference* (13) in order to identify known peptide transporter substrates and inhibitors that fulfill the pharmacophore requirements. This database was created using structures in the SDF format prior to conversion to a 3D Catalyst database after generating up to 100 molecule conformations with the fast conformer generation method, allowing a maximum energy of 20 kcal/mol. The pharmacophore was then used with the fast-flexible or best search algorithms (Accelrys; Catalyst Tutorials 2003).

"Fast fit" means finding the optimum fit among the existing conformers of the molecule without performing an energy minimization. "Best fit" means that the conformers selected are manipulated to minimize the distances between tethered objects in the molecules, while keeping the resulting conformer energy within the energy limit.

Chemicals

All chemicals purchased were of the highest commercial purity. Repaglinide was purchased from TRC Biomedical Research (Toronto, Canada), and fluvastatin was obtained from Calbiochem (La Jolla, CA, USA). Aspartame was from Sigma-Aldrich (St. Louis, MO, USA). ^{3}H-GlySar (4 Ci/mmol) was purchased from Moravek Chemicals (Brea, CA, USA).

In Vitro hPEPT1 Bioassay

A stably transfected cell line (CHO-hPepT1; kind gift from Dr. Wolfgang Sadée, Ohio State University, Columbus,

Table I. Catalyst CMC Database Search Results for Selected Molecules After Fast-Flexible Searching and the *in Vitro* IC_{50} Data for Inhibition of Gly-Sar Uptake in CHO-PepT1 Cells (Mean ± SD of Three Experiments)

Molecule	Catalyst fast-fit score[a]	*In vitro* IC_{50} for CHO-PepT1 (mM)
Repaglinide	3.19	0.18 ± 0.01
Aspartame	2.39	7.69 ± 0.09
Fluvastatin	3.44	0.34 ± 0.04
Bumetanide	3.54	NT
Netobimin	1.26	NT
Pravastatin	3.39	NT
Cerivastatin	1.27	NT

NT, not tested.
[a] A higher score indicates a better fit to the pharmacophore.

Table II. Catalyst Search Results for 65 Molecules Returned from a Database of 576 Known Drugs in Clinical Use in the United States and Derived from the *Clinician's Pocket Drug Reference* (13)

Molecules	Best fit score	Fast fit score	Molecular weight
Fluvastatin	7.68	3.44	411.47
Argatroban	4.83	3.57	508.64
Bacitracin	4.70	2.82	1422.71
Cefdinir	4.61	3.40	395.41
Montelukast sodium	4.61	3.41	608.17
Ceftizoxime	4.57	2.95	383.40
Enalapril	4.57	3.74	376.45
Lisinopril	4.56	3.18	405.49
Cefixime	4.52	3.73	453.44
Pravastatin	4.52	3.39	424.53
Cefonicid	4.47	3.58	542.56
Mupirocin	4.41	3.31	500.63
Glucagon	4.38	2.92	3482.76
Mezlocillin	4.33	2.99	539.58
Epoprostenol	4.30	3.23	352.47
Repaglinide	4.30	3.19	452.59
Cefditoren	4.28	3.15	506.57
Piperacillin	4.27	3.11	517.56
Amoxicillin	4.21	3.13	365.40
Moexipril	4.20	3.57	498.58
Ceftriaxone	4.18	3.01	554.57
Quinapril	4.18	3.27	438.52
Ramipril	4.18	3.25	416.52
Octreotide	4.17	2.87	1033.23
Alprostadil	4.02	3.32	354.49
Bumetanide	4.01	3.54	364.42
Loracarbef	4.00	3.62	349.77
Aztreonam	3.99	3.43	435.43
Ceftazidime	3.96	3.29	546.57
Cefmetazole	3.95	3.41	471.52
Cefotaxime	3.95	3.37	455.46
Ceftibuten[a]	3.95	3.07	410.42
Dinoprostone	3.95	3.55	352.47
Cefpodoxime	3.94	3.25	427.45
Losartan	3.94	3.50	422.92
Ticarcillin	3.94	3.18	384.42
Cefuroxime	3.93	2.97	424.39
Fexofenadine	3.93	3.15	501.67
Benazepril	3.91	3.37	424.50
Nafcillin	3.89	3.43	414.48
Perindopril	3.89	3.36	368.47
Trandolapril	3.88	3.06	430.54
Cromolyn	3.86	3.27	468.37
Vancomycin	3.86	3.44	1449.27
Amphotericin B	3.85	3.32	924.09
Bivalirudin	3.84	3.19	2180.30
Furosemide	3.84	2.77	330.74
Tirofiban	3.79	3.24	440.60
Cefotetan	3.77	2.99	575.60
Cefazolin	3.73	3.52	454.50
Calcitonin, human	3.66	3.38	3417.85
Eptifibatide	3.66	3.28	814.93
Meropenem	3.52	3.20	383.46
Liothyronine	3.29	2.89	650.98
Cefaclor	3.24	3.24	367.81
Cephalexin	3.15	3.15	347.39
Cefadroxil	3.14	3.14	363.39
Levothyroxine	3.08	2.88	776.87
Etodolac	3.00	2.97	287.36
Nateglinide	3.00	2.93	317.43
Cefoxitin	2.89	2.89	427.45
Cephradine	2.89	2.90	349.40
Ampicillin	2.77	2.69	349.40
Captopril	2.67	2.07	217.28
Cefoperazone	2.57	2.60	645.66

Search results for selected molecules after fast-flexible and best searching. Molecules in bold are known peptide transporter substrates and or inhibitors either identified in the literature or in the current study.

[a] *cis* conformation, bioactive.

OH, USA) (14) was maintained in DMEM containing 100 μg/ml penicillin-streptomycin and 0.1% gentamycin to maintain selection of hPepT1. Because penicillin is a potential hPEPT1 substrate and its presence may interfere with transport experiments, before each experiment cells were seeded in 24-well plates at a concentration of 75,000 cells/well and allowed to grow in antibiotic-free DMEM supplemented with 9% FBS for approximately 48 h. Culture media was removed and replaced with uptake buffer [3 mM Hepes/Mes/Tris (pH 6.0) containing 100 mM NaCl, 2 mM KCl,, 1 mM $MgCl_2$, and 1 mM $CaCl_2$] for 10 min. After the equilibration period, buffer was removed and replaced with fresh buffer that contained ^{3}H-Gly-Sar (5 μM, 1 μCi/ml, S.A. 2 Ci/mM) and various concentrations of the substrate of interest (e.g., 0.033, 0.1, 0.33, 1.0, 3.3, 10, 33, and 100 mM, depending on compound solubility). After incubation with tracer and substrate for 30 min, the cells were rinsed several times with ice-cold buffer and lysed with Triton-X 100. Aliquots were then taken for liquid scintillation counting. The IC_{50} values (the concentration that resulted in 50% uptake as compared to control) were determined using a sigmoidal dose-response equation with variable slope in GraphPad Prism version 4.0a (GraphPad, Inc., San Diego, CA, USA). Gly-Sar was used as an internal control for hPEPT1 activity (mean IC_{50} 0.80 ± 0.07 mM).

RESULTS AND DISCUSSION

This work evaluates the capability of commercially available rapid computational approaches to identify ligands for transporters as well as the validation of this method *in vitro*. In the current study, we have used the molecular structures of well-known, relatively high affinity hPEPT1 substrates Gly-Sar, bestatin, and enalapril to generate a HIPHOP common feature 3D-pharmacophore (Fig. 1). These molecules represent three different classes of drugs: a dipeptide, a peptidomimetic, and an angiotensin converting enzyme (ACE) inhibitor, respectively. The pharmacophore alignment consisted of two hydrophobic features, a hydrogen bond donor, hydrogen bond acceptor, and a negative ionizable feature, which would indicate these are important features for interaction with this transporter *in vitro*.

Our (15,16) and other (5,17) groups have previously modeled peptide transport using conformational analyses to determine the molecular determinants and the distances between functional groups in substrates critical for affinity. Using relatively rigid β-lactam molecules, we previously suggested that a carboxylic carbon (likely to position in a posi-

tively charged pocket), 2 carbonyl oxygen atoms (hydrogen bond acceptors), a hydrophobic site, and finally an amine nitrogen atom (hydrogen bonding region) were important features of substrates (15,16). Other studies using expressed rabbit PEPT1 or hPEPT1 indicate a peptide bond is not essential for substrates of these ortholog transport carriers. Instead, two ionized amino or carboxyl groups with at least 4 carbon units between them, or amino acid esters of nucleosides, such as 5-amino-pentanoic acid and valacyclovir, permit transport (18–20). A more recent meta-analysis of K_i values for 42 substrates using PEPT1 data from many sources provided a template consisting of an N-terminal NH_3 site, a hydrogen bond to the carbonyl group of the first peptide bond, a hydrophobic pocket, and a carboxylate binding site (21). Other groups have confirmed these essential features for PEPT1 transport (22,23). A recent article (5) described comparative molecular field analysis (CoMFA) and a comparative molecular similarity indices analysis (CoMSIA) models for hPEPT1 using a series of 79 dipeptide-type substrates and test set of 19 dipeptides and dipeptide derivatives with acceptable model statistics. CoMSIA contour maps enabled the identification of the key elements for the binding of PEPT1 substrates. This model possibly provides a means to computationally predict the binding of other potential hPEPT1 inhibitors in the future; however, due to the structurally homologous training set and the expression of the transporter in Caco-2 cells (which also expresses other transporters), there may be some difficulty in extrapolating to more structurally diverse compounds.

To identify drugs that are also novel hPEPT1 inhibitors, we have used our pharmacophore to search the CMC database of more than 8000 "drug-like" molecules. Prior to screening, the molecules were first converted into a multiconformer three-dimensional database. This allowed us to take into consideration molecular flexibility, thereby ensuring that fast-fitting would not be limited to rigid molecules with conformations already aligned to the pharmacophore. We retrieved 145 (~1.8% of the total database) virtual hits mapping to the pharmacophore features. After fast fitting in Catalyst, the 7 (0.09% of the total database) best scoring molecules with drug-like molecular weight (<500) were selected (Table I) for purchase and *in vitro* testing. Three of these molecules (fluvastatin, aspartame, and repaglinide) were readily available and mapped well to the pharmacophore features (Fig. 2).

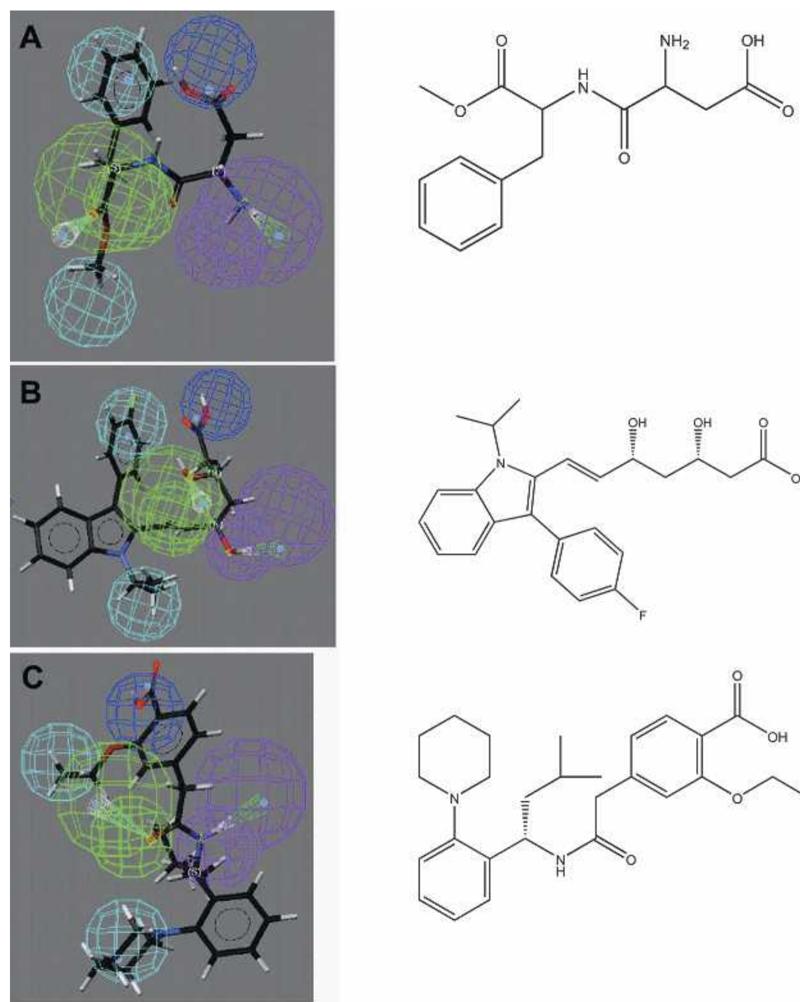

Fig. 2. Visualization of high-scoring molecules discovered with and fitted to the hPEPT1 HIPHOP pharmacophore: (A) aspartame, (B) fluvastatin, (C) repaglinide. Pharmacophore features: cyan, hydrophobe; green, HBA; purple, HBD; blue, negative ionizable.

These molecules were also experimentally identified as new inhibitors with affinity for hPEPT1 that is in a range similar to Gly-Sar [≤1 mM (8); Table I]. A set of 10 hPEPT1 substrates or inhibitors when fast fit to the hPEPT1 pharmacophore provide fit values between 2.07 and 3.24 arbitrary units. Repaglinide and aspartame possess fit values within this range (Table I), whereas fluvastatin is beyond the upper end of this range. As the known hPEPT1 substrates valacyclovir (2.13), enalapril (3.74), and bestatin (2.59) all have relatively similar fit values to the molecules we have tested, it is likely they may behave similarly as substrates. Interestingly, human oral bioavailability of fluvastatin is variable and low (29 ± 18%) (24), whereas repaglinide has a more pronounced systemic availability after oral administration (56 ± 7%) (24). Aspartame (*N*-L-alpha-aspartyl-L-phenylalanine, 1-methyl ester) bioavailability cannot readily be assessed, but the appearance of phenylalanine and aspartate in plasma suggests that the fraction absorbed from the gut is close to unity (25). The role of PEPT1 in the oral bioavailability of these three compounds had not been previously noted, and this may explain to some extent the relatively high bioavailability and/or the variable pharmacokinetic and pharmacodynamic profile for these compounds. Naturally, potential drug-drug interactions with other hPEPT1 substrates/inhibitors can be envisioned. It may be interesting to speculate that the widespread use of aspartame-based sugar substitute in food products and pharmaceuticals may potentially interfere with hPEPT1-mediated drug absorption. Although this possibility cannot be ruled out, the relatively high IC_{50} of aspartame combined with its milligram dosing and rapid intestinal metabolism into phenylalanine, aspartate, and methanol would minimize the potential impact on drug absorption via hPEPT1. After completion of this study, nateglinide, a molecule in the same therapeutic class and structurally similar to repaglinide, was shown to be an inhibitor but not a substrate of the rat PEPT1 (26). In the current study, we have identified 3 molecules as inhibitors of hPEPT1, and in order to test our hypothesis that these molecules are also substrates, further experiments are required. Based on a comparison of the predicted scores with the pharmacophore, these molecules are in the range of the known substrates used to build the model.

We also performed a computational validation of our hPEPT1 pharmacophore by searching a database of more than 500 commonly prescribed drugs that we created based on a clinician's reference (Table II). We were able to select 65 molecules using the pharmacophore; of these, 27 were known PEPT1 substrates or inhibitors based on literature or our own studies. Obviously, some of the other molecules that scored well using either best fit or fast fit algorithms may be considered for future testing.

The use of pharmacophore-based database searching with hPEPT1 has demonstrated that this approach is ideal for identifying the role of peptide transport in absorption of new chemical entities prior to *in vitro* testing of these compounds. The method used is amenable to searching large databases of molecules with 3D conformers and has been previously applied to discovering active molecules for therapeutic targets of commercial interest, lead selection, as well as understanding the key features on P-glycoprotein substrates (4). In turn, this method may also be useful for determining the role of other transporters by quickly searching for new ligands when little experimental data is available in the literature. By focusing on the key features for binding to the transporter, we have been able to find molecules that are either as active or more active for hPEPT1 than previously known compounds used in model building. Obviously, we are now in a position to iteratively improve the model using these newly discovered hPEPT1 inhibitors and ultimately to optimize the selectivity and specificity for future ligand identification.

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