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Design of high-affinity peptide conjugates with optimized fluorescence quantum yield as markers for small peptide transporter PEPT1 (SLC15A1)

Praveen M. Bahadduri, Abhijit Ray, Akash Khandelwal and Peter W. Swaan*

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, MD 21201, USA

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Abstract—We employed a computational approach to design and synthesize a series of fluorescently labeled hPEPT1 substrates. Five Alexa Fluor-350™-labeled peptides were assessed for their in vitro inhibitory activity in hPEPT1-transfected CHO cells. At least four labeled peptides show potent inhibitory activity toward hPEPT1-mediated uptake of [^{3}H]-GlySar and three compounds displayed a significant cellular uptake specifically mediated by hPEPT1.

The human small peptide transporter, hPEPT1, is a clinically important intestinal solute carrier protein critically involved in the absorption of its natural substrates, food protein-derived di- and tripeptides. Additionally, it mediates the intestinal uptake of numerous drug classes, such as β-lactam antibiotics, ACE-inhibitors, several antivirals,[1] as well as various prodrugs. For example, valyl prodrugs of the nucleoside antiviral drugs acyclovir and ganciclovir display 3- to 5-fold and 10-fold higher bioavailability upon oral administration in humans compared to their parent compounds, respectively.[2,3] The increased intestinal permeability can be attributed solely to their affinity for intestinal hPEPT1. This transporter's relaxed substrate specificity, relatively high capacity and low affinity ($K_m \sim 1.1$ mM for the prototypical PEPT1 substrate glycylsarcosine (GlySar)) makes it an ideal target for the design of peptidomimetic drugs with increased intestinal permeability and bioavailability.

In the absence of a crystal structure for hPEPT1, the rate at which new substrates and inhibitors can be identified or rationally designed is expectedly slow. Alternative experimental strategies to rapidly screen large compound database for hPEPT1 affinity can be carried out using stably expressed cell lines in a high-throughput screening (HTS) setup. The success of this approach will be determined by assay sensitivity for prototypical substrates or new chemical entities (NCEs) when assessing inhibition or transport via hPEPT1. The most commonly applied assay techniques involve laborious liquid chromatography–mass spectrometry or costly radiolabeled tracer methodologies. However, fluorescence offers a convenient alternative for HTS applications due to its sensitivity and applicability to a wide variety of cell-based systems.

Several other groups have reported fluorescently labeled peptides directed to develop hPEPT1 HTS assays, albeit with limited success. Abe and colleagues[4] synthesized FITC and coumarin-3-carboxylic acid conjugated peptide analogs that showed affinity for hPEPT1 expressed in Caco2 cells, but were not transported. Other studies reported AMCA (7-amino-4-methylcoumarin-3-acetic acid) peptide conjugates[5] and peptide analog-FITC esters[6] with hPEPT1 affinity. However, the fluorescent dyes used in these studies have limited quantum yields and may not be particularly suitable for HTS implementation.[7] In fact, fluorescein dyes are known to display self-quenching properties at high concentrations, thereby limiting potential assay sensitivity. The present study was designed to develop a metabolically stable fluorogenic substrate for hPEPT1 with superior fluorescence quantum yield at a range of intracellular pH values. Based on our previous knowledge of the hPEPT1 pharmacophore and structure-activity relationship,[8] we

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* Corresponding author. Tel.: +1 410 706 0103; fax: +1 410 706 5017; e-mail: pswaan@rx.umaryland.edu

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designed five novel Alexa Fluor 350™ (AF)-labeled peptide analogs using computational design, synthesis, and in vitro analysis of AF-labeled substrates designed specifically to target PEPT1.

We built a three-dimensional quantitative structure–activity relationship (3D-QSAR) using Comparative Molecular Field Analysis (CoMFA) from the extensive dataset by Gebauer and colleagues.[9] Molecules were built and subsequently energy minimized in SYBYL (v.7.1) using the Tripos force field.[10] The most active compound in the dataset, *o*-(methylphenyl)-D-tyrosyl-L-alanine[9] was used as a template and all the other molecules were superimposed to it using atom-based alignment. The resulting CoMFA steric and electrostatic contour plots are shown in Figure 1. The model developed has good descriptive ability as evidenced by a r^2 value of 0.989 with six components.

The overall steric contribution (53%) to binding is higher than the electrostatic contribution (47%). The AF analogs (Table 1) using pK_i ($-\log K_i$) (M) as dependent variable and CoMFA descriptors as independent variables in the partial least square (PLS) analysis (data not shown). Out of a set of 50 permutations, the five highest scoring compounds were selected for synthesis and in vitro testing.

The synthesis of dipeptide-conjugated AF (Molecular Probes, Eugene, OR) was carried out by dissolving BocLys(Z)OSu in a mixture of *N*,*N*-dimethyl formamide (DMF), 1,4-dioxane, diisopropylamine (DIEA), and an appropriate amino acid or dipeptide (Scheme 1). The progress of the reaction was monitored by mass spectroscopy for the disappearance of a peak at *m*/*z* 478.5. The dipeptide is then deprotected by catalytic hydrogenation using 10% Pd/C to remove the carbonyloxybenzyl (Z) group. The deprotected compound was dissolved in DMF, dioxane, and diisopropylethylamine followed by the addition of AF carboxylic acid succinimidyl ester to give the fluorescently labeled dipeptide. This was finally deprotected using TFA in dichloromethane to give the

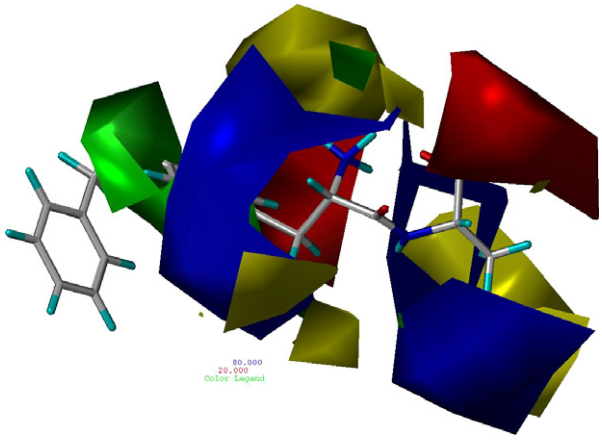


Figure 1. CoMFA contour map. The green and yellow regions indicate that an increase in steric bulk favors or disfavors binding to hPEPT1, respectively. The increase of negative charge near red region or positive charge near blue region favors binding. The template compound is shown in capped stick model.

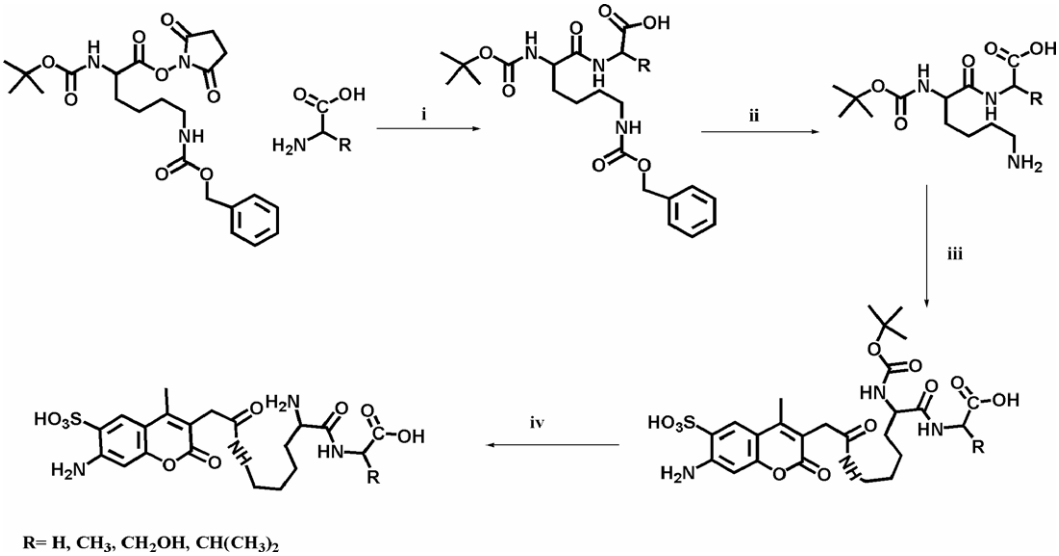
Table 1. Predicted activities of Alexa Fluor-350™ derivatives from CoMFA analysis

Compounds	$-\log K_i$	K_i (mM)
AF–LysGly	4.550	0.028
AF–LysVal	4.459	0.035
AF–LysSer	4.590	0.026
AF–LysAla	4.611	0.024
AF–LysGlyGly	4.700	0.019

desired product. All in vitro studies were conducted with stably transfected CHO cell lines expressing hPEPT1 as described previously.[8] Competitive inhibition studies were performed at pH 6.0 for 30 min using [^{3}H]-glycylsarcosine (GlySar) (1 μM, 1 Ci/mmol) (Moravek, Brea, CA) as a tracer. Initially, parent peptides were assessed for their inhibitory potencies of [^{3}H]-GlySar uptake in hPEPT1-CHO cells. Subsequently, the inhibition of 100 μM and 1000 μM AF-labeled peptides (in DMSO, not exceeding 1% final DMSO concentration) on [^{3}H]-GlySar (1 μM) accumulation in hPEPT1-CHO cells was determined. To determine the cellular uptake of fluorescently labeled peptides and to assess their mutual competitive inhibition of GlySar, the effect of 10 mM GlySar (Sigma, St. Louis, MO) on the uptake of 100 μM AF-labeled dipeptide was assayed in hPEPT1-CHO cells. Cells were washed three times and lysed with 1 N NaOH for 1 h and neutralized with 10% HCl solution. Total cellular fluorescence was determined using a Spectramax Gemini XS (Molecular Devices, Sunnyvale, CA). Data were calibrated using fluorescence standard curves and normalized to protein content.

AF-labeled peptides have strong affinity for hPEPT1 as demonstrated by their potent inhibition of [^{3}H]-GlySar uptake in hPEPT1-transfected CHO cells (Fig. 2). Reduction in hPEPT1 affinity imparted by AF-conjugation is minimal as compared to the activity of the parent peptide (Fig. 2). Of the compounds tested, the LysVal, LysSer, and LysAla analogs showed the strongest relative inhibition of [^{3}H]-GlySar uptake. Interestingly, the cellular uptake of AF-labeled LysVal was significantly greater (2- to 3-fold) compared to the other peptides (Fig. 3). This would indicate that inhibition data do not correlate with hPEPT1-mediated substrate translocation. Inhibition of AF-labeled peptide uptake by a 100-fold GlySar excess indicates mutual inhibition between GlySar and AF-labeled analogs, indicative of a shared translocation pathway, that is, hPEPT1 (Fig. 3). Interestingly, the LysVal conjugate of AF displays relatively high cellular uptake while only modestly inhibiting [^{3}H]-GlySar uptake (~40% at 1 mM), whereas LysAla and LysSer conjugates exerted stronger [^{3}H]-GlySar uptake inhibition (<~22%) with only modest cellular permeation. This would suggest that AF–LysVal is a good substrate for PEPT1 compared to the other dipeptide conjugates, but further studies are warranted to fully characterize this phenomenon.

Overall, AF offers significant advantages over other reported fluorescently labeled peptides such as a large Stokes shift (100 nm for AF vs 30 nm for FITC) and a relatively high fluorescence quantum yield (0.89 AF–



Scheme 1. Reagents and conditions: (i) DMF, 1,4-Dioxane, DIEA; (ii) 10% Pd/C, EtoAc, MeOH; (iii) DMF, 1,4-Dioxane, Alexa Fluor 350 carboxylic acid succinimidyl ester; (iv) CH_2Cl_2, TFA.

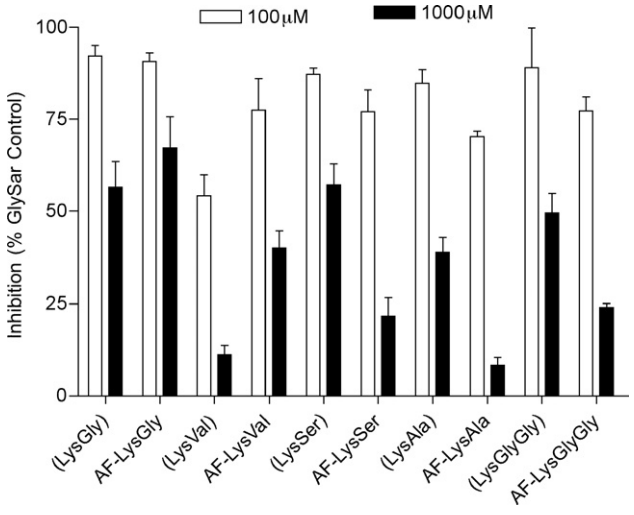


Figure 2. Relative inhibition of 1 μM [^{3}H]-GlySar uptake (% control) in hPEPT1-transfected CHO cells in the presence of fluorescently labeled peptides ($n = 3 \pm$ SD).

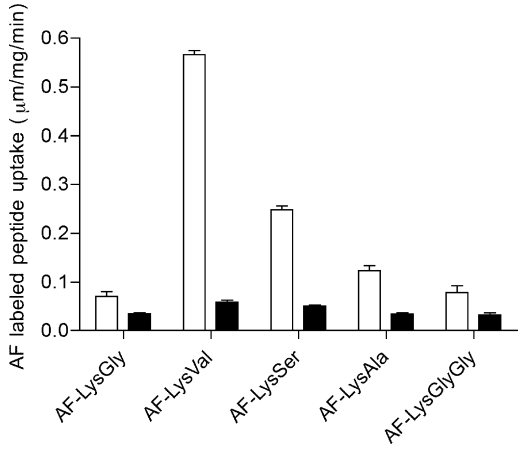


Figure 3. AF-labeled peptide uptake in hPEPT1-CHO cells. Bars indicate cellular uptake of 100 μM labeled peptide in the absence (open bars) and presence (closed bars) of 10 mM GlySar. ($n = 3 \pm$ SD).

LysVal vs AMCA reference in water), which may enable the use of these novel conjugates in a HTS setting. In summary, this report describes the design and synthesis of five novel AF-conjugated peptides with sub-millimolar affinity levels for hPEPT1. Future studies should determine whether cellular uptake can be monitored without cellular disruption and in real-time to facilitate HTS screening.

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