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A Novel High-Throughput PepT1 Transporter Assay Differentiates between Substrates and Antagonists

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Abstract: PepT1 is a transporter of proven pharmaceutical utility for enhancing oral absorption. A high-throughput, robust functional assay, capable of distinguishing PepT1 binders from substrates, allowing identification and/or prediction of drug candidate activation was developed. An MDCK epithelial cell line was transfected with rPepT1. The high level of stable rPepT1 expression that was achieved enabled development of a miniaturized PepT1 assay in a 96-well format, which could be scaled to 384 wells. The assay is based on measurement of membrane depolarization resulting from the cotransport of protons and PepT1 substrates. Membrane potential changes are tracked with a voltage-sensitive fluorescent indicator. Control (mock-transfected) cells are used to determine nonspecific membrane potential changes. A variety of fluorescent dyes were tested during initial assay design, including intracellular pH and membrane potential indicators. A membrane potential indicator was chosen because of its superior performance. Upon PepT1 activation with glycylsarcosine, dose-dependent membrane depolarization was observed with an EC_{50} of 0.49 mM. Maximum depolarization was dependent on the level of PepT1 expression. Testing of 38 known PepT1 substrates, binders, and nonbinders demonstrated that this assay accurately distinguished substrates from binders and from nonbinders. Initial validation of this novel assay indicates that it is sensitive and robust, and can distinguish between transporter substrates and antagonists. This important distinction has been previously achieved only with lower-throughput assays. This assay might also be used to determine substrate potency and establish a high-quality data set for PepT1 SAR modeling.

Keywords: Prodrug; intestinal oligopeptide transporter; PepT1 substrate; functional transporter assay; oral bioavailability; carrier-mediated transport; structure−transport relationship

Introduction

In recent years, there has been considerable research on the role of intestinal oligopeptide transporters in the active absorption of peptide-like drugs.[1,2] The most extensively studied oligopeptide transporter to date, in both intestinal tissue and cell culture models, is the peptide transporter-1 (PepT1). PepT1 is a low-affinity, high-capacity nutrient

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transporter which mediates the active transport of di- and tripeptides across the intestinal wall via a transmembrane electrochemical proton gradient.[3−6] PepT1 expression is also found in kidney,[7] where it may play a role in the re-uptake of filtered peptides, pancreas,[8] and bile duct.[9]

Many orally active drugs share structural features with the physiologic substrates of the peptide transport system and owe their oral bioavailability to peptide transporter-mediated permeation of the intestinal mucosa. These include many β-lactam and cephalosporin antibiotics, ACE inhibitors, renin inhibitors, and 5′-nucleoside esters of amino acids.[1,10−12] To understand and fully explore the role of peptide transporters in the absorption of pharmaceutically relevant drugs, their substrate specificity should be characterized and transporter structure−activity relationships developed. This information could eventually allow the creation of predictive *in silico* models for guiding the design of compounds toward enhanced bioavailability. To achieve this goal, characterization of PepT1 structural requirements must be derived from functional assays that can distinguish between transporter inhibitors and substrates, between different peptide transporters, and between peptide transporters and other transport systems.

The purpose of this work was to generate an assay allowing rapid and accurate identification of putative PepT1 substrates. The existing methodologies either are labor intensive (standard patch−clamp recording), provide insufficient information (inhibition of [^{3}H]Gly-Sar uptake by tested compounds, a binding assay which does not predict if a drug is transported by PepT1), or require the use of radiolabeled compounds and extensive analytical procedures (drug uptake into or across cell monolayers). PepT1, a proton-coupled transporter, cotransports protons and substrates into cells. This proton movement causes intracellular acidification and membrane depolarization.[13] Several fluorescent indicators which can detect changes in either intracellular pH or membrane potential have been described.[14] Two of these, BCECF[15] and CMFDA,[16] have been used to detect intracellular acidification caused by PepT1 activation in Caco-2 cells and in CHO/PepT1 cells, respectively. BCECF was used in a microscopy-based assay, which does not lend itself easily to a high-throughput environment. The CMFDA assay was performed in 96-well plates but required extensive manipulation after dye loading to reduce background noise and may not be able to be easily adapted to a screening environment. We investigated whether other fluorescent dyes could be used to measure changes in membrane potential or intracellular pH induced by the electrogenic response of PepT1 to substrates. To maximize the signal-to-noise ratio, an epithelial cell line (MDCK), stably overexpressing rat PepT1, was used as a model for development of the membrane potential assay.

Experimental Section

MDCK (NBL-2) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell culture reagents and growth media were purchased from Mediatech, Inc. (Herndon, VA). Membrane potential dye (MP dye, included in the Membrane Potential Assay Kit) was purchased from Molecular Devices Corp. (Sunnyvale, CA). [^{14}C]-Glycylsarcosine (Gly-Sar) was purchased from Moravek Biochemicals (Brea, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Black, clear bottom 96-well Costar assay plates were purchased from Corning Inc. (Corning, NY). A microplate reader coupled to a fluid transfer system (FLEXstation) was purchased from Molecular Devices Corp. Liquid scintillation supplies, including CulturPlates (96-well plates), Microscint-40 (liquid scintillation cocktail), and Topcount (scintillation counter), were purchased from Perkin-Elmer Life Sciences

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(Boston, MA). Floxuridine prodrugs were synthesized as described previously.[17]

Cell Culture. A stably transfected MDCK/rPepT1-V5/His clonal cell line expressing the rPepT1 transporter was obtained as described previously.[18] Cells were grown in DMEM supplemented with 10% FBS, 0.2 mM L-glutamine, 1% nonessential amino acids, and 0.3 mg/mL Geneticin. MDCK cells transfected with vector cDNA alone (mock-transfected cells) served as a control and were grown under the same conditions as rPepT1-MDCK cells. Cell cultures were maintained in T-75 culture flasks under standard conditions (37 °C, 90% humidity, 5% CO_2), until they reached 80−90% confluence. The growth medium was changed every other day of culture. rPepT1-MDCK and mock-transfected cells were seeded into 96-well plates at densities of $0.4-4 \times 10^4$ cells/well in complete DMEM without Geneticin. Cells were cultured for 1−3 days. Prior to the assay, the culture medium was aspirated and cells were washed once with buffered Bicarbonated Ringer's Solution (BRS, pH 6.0) which consisted of 107.4 mM NaCl, 5.4 mM KCl, 0.782 mM $NaH_2PO_4 \cdot H_2O$, 25 mM $NaHCO_3$, 50 mM MES, 5.55 mM glucose, 1.8 mM $CaCl_2 \cdot 2H_2O$, and 0.81 mM $MgSO_4$.

HeLa cells overexpressing hPEPT1 transporter were prepared as described previously.[19] Briefly, HeLa cells were seeded onto six-well plates at a density of 3×10^4 cells/cm^2 and grown for 24 h. The cells were infected with 5000 viral particles (Ad.RSVhPept1) per cell, and uptake experiments were conducted 48 h postinfection.

[^{14}C]Gly-Sar Cell Uptake. Prior to being assayed, cells were washed twice and preincubated with BRS for 1 h at 37 °C. The buffer was then replaced with a solution containing 10 μM [^{14}C]Gly-Sar and, where indicated (for binding assays), unlabeled competitor. After incubation for 10 min at 37 °C, the cells were washed twice with ice-cold PBS and solubilized *in situ* with 200 μL of Microscint-40, and the radioactivity in each well was measured. The IC_{50} values were estimated by nonlinear regression analysis using Xlfit for Microsoft Excel (IDBS, Guilford, U.K.). Michaelis−Menten-like kinetic parameters (K_m and V_{max}) were determined by nonlinear curve fitting (using Xlfit) of specific uptake data to the equation $V_0 = V_{max}[S]/(K_m + [S])$, where V_0 is the initial uptake velocity, V_{max} is the maximal uptake velocity at saturating substrate concentrations, K_m is a constant analogous to the Michaelis−Menten constant, and [S] is the substrate concentration.

Inhibition of [^{3}H]Gly-Sar uptake in HeLa/hPEPT1 cells was performed as described previously.[20] Briefly, HeLa cells were seeded (80 000 cells/well) onto a 12-well plate. After 24 h, cells were transfected with hPEPT1 plasmid by directly incubating them with a Fugene−DNA complex (3:1) in DMEM with 10% FBS. After a 48 h transfection, cells were washed twice with uptake buffer and incubated with 10 μmol/L Gly-Sar (9.94 μmol/L Gly-Sar and 0.06 μmol/L [^{3}H]-Gly-Sar) and various concentrations (0.01−5 mM) of the prodrugs in uptake buffer for 30 min at 25 °C. After 30 min, the uptake was stopped by the addition of 1 mL of ice-cold uptake buffer. The cells were washed three times with ice-cold uptake buffer and lysed using 0.5 mL of 1% Triton X-100. A portion of the cell suspension was used for scintillation counting.

Uptake of Floxuridine and Its Prodrugs in HeLa/hPEPT1 Cells. Uptake of floxuridine and its prodrugs was screened in HeLa/hPEPT1 cells as described previously for Caco-2/hPEPT1 cells.[21] Briefly, 48 h postinfection, the growth medium was removed and cells were washed twice with pH 6.0 uptake buffer (145 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM MES). To each well was added 1 mL of a 1 mM drug solution in uptake buffer, and culture plates were agitated on a plate shaker at 25 °C. After 45 min, the drug solution was aspirated and cells were washed three times with ice-cold phosphate-buffered saline (PBS). To each well was added 0.5 mL of 0.1% SDS, and plates were agitated for 30 min. The cell lysates were treated with ice-cold trifluoroacetic acid (final concentration of 5%), vortexed, and centrifuged for 5 min at 10 000 rpm. The supernatant was filtered through a membrane filter (0.45 μM), and samples were analyzed for amounts of both the prodrug and parent drug by HPLC as described previously.[22] The amount of protein in each sample was determined with the protein assay DC kit (Bio-Rad, Richmond, CA). Control experiments in HeLa cells were carried out in the exact manner described above.

Measurement of Membrane Potentials. After the contents of the wells had been washed, 60 μL of MP dye reconstituted in BRS buffer was added to wells, and cells were incubated for 30−60 min at 37 °C to ensure dye distribution across cell membranes. Assays were carried out

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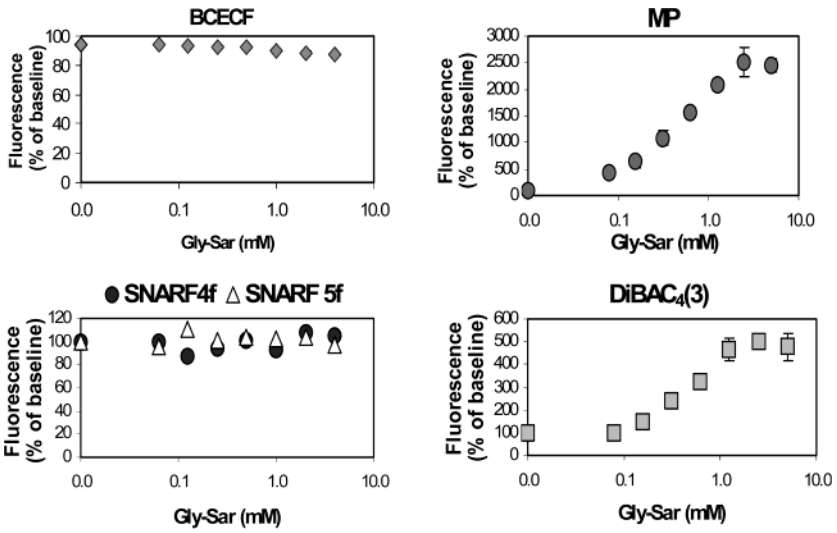

Figure 1. Gly-Sar modulation of fluorescence in PepT1-expressing cells loaded with intracellular pH indicators (BCECF, SNARF4, and SNARF5) or membrane potential indicators [$DiBAC_4(3)$ and MP]. The response is plotted as the percentage of control baseline fluorescence (12000 RFU). The effect of increasing concentrations of Gly-Sar on fluorescence intensity was measured as described in the Experimental Section.

at 37 °C and were initiated by the addition of 60 μL of test compounds. Compound stock solutions were prepared in aqueous buffer containing reconstituted MP dye or first reconstituted in DMSO before serial dilution. The final concentration of DMSO was less than 1%. The assay was not affected by DMSO concentrations of up to 2%. Fluorescence was measured every 2 s for 2−5 min, using wavelengths of 530 (excitation) and 565 nm (emission). Fluorescence responses were corrected for background changes in fluorescence caused by the addition of a dye solution containing no substrate to blank wells. Raw fluorescence data were analyzed with SOFTmax PRO 4.0.1 (Molecular Devices Corp.). The EC_{50} values (concentration of compound that results in a half-maximum fluorescence response) were obtained by fitting the data to a four-parameter logistic, where the baseline response (A), the maximum response (Fl_{max}), the slope (B), and EC_{50} can be described by the equation $y = (A - Fl_{max})/[1 + (X/EC_{50})^B] + Fl_{max}$. None of the curve fit parameters were constrained.

Results

Fluorescent Indicators of Proton-Coupled Transport. PepT1 is a proton-coupled transporter. This proton movement causes intracellular acidification and membrane depolarization.[13] Several fluorescent indicators are available, which detect changes in either intracellular pH or membrane potential.[14,22] We compared the increase in fluorescence elicited by increasing doses of the dipeptide Gly-Sar using either membrane potential indicators [$DiBAC_4(3)$ and MP] or pH indicators (BCECF, SNARF4, and SNARF5). In Figure 1, the response of the tested indicator is represented as the percent increase in fluorescence over control. No dose-dependent change in fluorescence was obtained with either SNARF dye, and only a 10% decrease in fluorescence was achieved with BCECF. In our model system, membrane potential indicators were more robust indicators, with a greater dynamic range and a better signal-to-noise ratio. Although $DiBAC_4(3)$ exhibited a 5-fold increase in fluorescence, a greater increase in fluorescence (25-fold) was obtained with the MP indicator. Further, the response kinetics of MP were also superior to those of $DIBAC_4(3)$. While MP required only 2 min to achieve peak fluorescence, $DIBAC_4$-(3) required a reading time of 5 min (data not shown). Therefore, the MP dye was chosen for further studies.

Assay Optimization. The manufacturer's protocol recommends addition of a small volume of test compound to a relatively large volume (200 μL) of MP dye in the well, resulting in substantial dilution of test compounds (5-fold). Since PepT1 is a low-affinity transporter ($K_m = 1-5$ mM), it would be necessary to prepare aqueous solutions of compounds at concentrations of 5−25 mM, which may be above the achievable solubility. Therefore, experimental conditions were modified to minimize the dilution of the test compound, and a final volume of 120 μL (60 μL of MP dye for loading and 60 μL of tested compound in dye solution) was chosen as a standard condition for the assay. Reducing the total volume per well had no measurable effect on assay performance (data not shown). Cell density and duration of cell growth were also optimized. rPepT1-transfected and mock-transfected MDCK cells were seeded onto six 96-well plates at three densities (0.4, 1.6, and 3.8 $\times$ 10^4 cells/well). Two plates were used for each assay on the first, second, and third day after seeding. The results were compared between plates and within plates between various cell densities. The lowest-density plates (0.4 $\times$ 10^4 cells/well) showed low fluorescence signals on all three days of the culture. This cell density also yielded high plate to plate variability. When cells were seeded at the medium density (1.6 $\times$ 10^4 cells/well), consistently high fluorescence readings were observed only on the second and third day of the

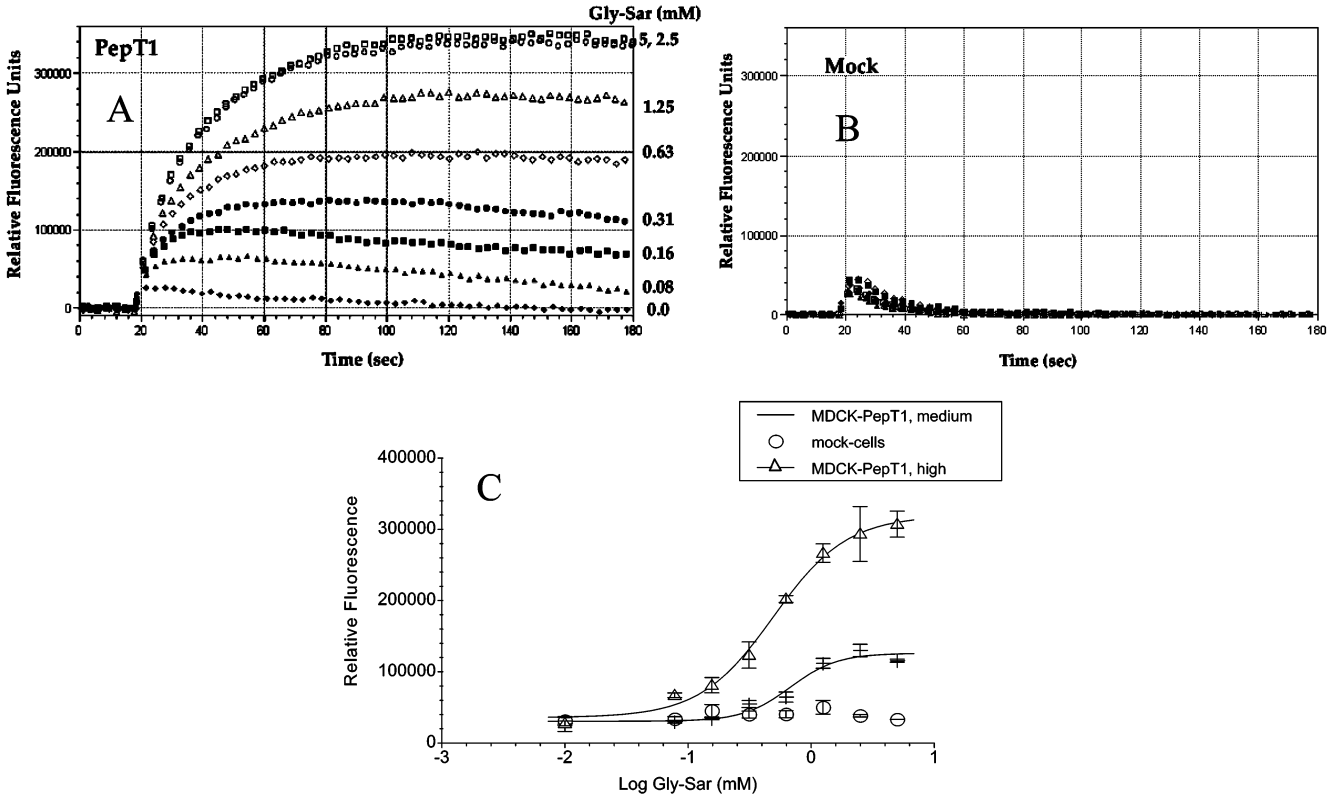

Figure 2. Gly-Sar modulation of membrane potential in PepT1-transfected cells and in mock-transfected cells. Gly-Sar (0−5 mM) was added to MP-loaded cells, and the fluorescence intensity was measured in real time as described in the Experimental Section. Fluorescence is expressed in relative fluorescence units. In panels A and B, fluorescence is plotted vs time. Gly-Sar caused a dose-dependent, saturable increase in fluorescence which reached a peak at 2 min. There was no corresponding change in mock cells. The data shown in panels A and B were used to construct the plot in panel C, where relative fluorescence units are plotted vs Gly-Sar concentration.

culture. Plates seeded at the high density (3.8×10^4 cells/well) were highly reproducible and responsive on all the days they were tested. However, considering the large amounts of cells necessary for preparation of assay plates, we chose the medium cell density of 1.6×10^4 cells/well and 2 days of culture as standard conditions. All conditions that were tested showed a tight fit to a four-parameter dose−response curve ($r^2 \geq 0.96$).

Gly-Sar Modulation of Membrane Potential. The response of the indicator dye (MP) to Gly-Sar adition was compared in MDCK-rPept1 and MDCK mock-transfected cells (Figure 2). In MDCK-rPepT1 cells, there was a dose-dependent fluorescence increase, reflecting membrane depolarization. This response reached a peak at ~2 min (Figure 2A) and was stable for up to 30 min. There was no corresponding change in fluorescence intensity in mock cells in response to Gly-Sar (Figure 2B). The peak fluorescence intensity (Figure 2A) was plotted against Gly-Sar concentration, and the resulting data were fitted to a four-parameter nonlinear dose−response curve (Figure 2C). The maximum fluorescence response (Fl_{max}) was dependent on the amount of PepT1 present, as determined by kinetic evaluation of Gly-Sar uptake, since another cell line with lower levels of PepT1 expression had a lower Fl_{max} (Figure 2C and Table 1). The cells expressing high PepT1 levels had a 2.6-fold higher Fl_{max} value than the medium expressers. This is very similar to the V_{max} ratio (2.3, Table 1) between the two lines, indicating that Fl_{max} is proportional to the concentration of the transporter. The EC_{50} values for Gly-Sar activation of MDCK-rPepT1 in the high- and medium-density expresser were similar, 0.49 and 0.67 mM, respectively, which are comparable to the K_m values for Gly-Sar uptake determined in these cells (1 mM, Table 1).

Validation plates treated with MP dye only ("low signal") or 4 mM Gly-Sar ("high signal") were used to evaluate the variability and signal window for the membrane potential assay. The signal window measures the separation between different levels of activity that can be detected with an assay (the distance between two distributions in terms of the standard deviations).[23] For a screen to be able to detect midrange hits, there must be enough room to fit the distribution of midrange hits between the minimum and maximum signal distributions. The midrange distribution will occupy six standard deviations. Thus, to detect midrange hits, the signal window between the totals and the blanks should

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Table 1. Kinetics of PepT1 Activation in Cells Transfected with Different Levels of PepT1 Expression[a]

cell line	EC_{50} (mM)	K_m (mM)	Fl_{max} (fold over baseline)	V_{max} (nmol mg^{-1} p^{-1})
high PepT1 expresser	0.49 ± 0.05	1.08 ± 0.23	10.6 ± 0.4	90.61 ± 6.23
medium PepT1 expresser	0.67 ± 0.13	1.04 ± 0.04	4.1 ± 0.2	38.73 ± 1.70
high/medium ratio			2.59	2.33

[a] EC_{50} and Fl_{max} values were obtained by fitting the data to a four-parameter sigmoidal dose−response curve, where the baseline response (A), the maximum response (Fl_{max}), the slope (B), and EC_{50} can be described by the equation $Y = (A - Fl_{max})/[1 + (X/EC_{50})^B] + Fl_{max}$. Fl_{max} is expressed as the fold increase over the baseline fluorescence. The affinity of PepT1 for the Gly-Sar dipeptide and the levels of PepT1 in the medium- and high-expressing cell lines were determined by the Michaelis−Menten-like kinetic parameters (K_m and V_{max}). These values were determined by nonlinear curve fitting of specific uptake data to the equation $V_0 = V_{max}[S]/(K_m + [S])$, where V_0 is the initial uptake velocity, V_{max} is the maximal uptake velocity at saturating substrate concentrations, K_m is a constant analogous to the Michaelis−Menten constant, and [S] is the substrate concentration.

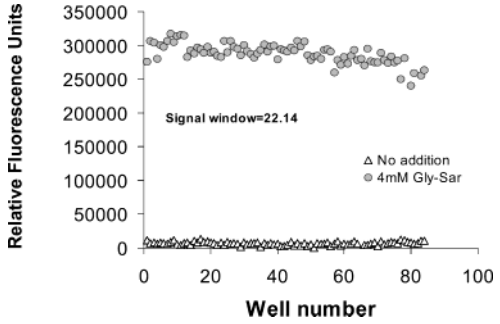

Figure 3. Assay variability and signal window. Two 96-well plates were treated with MP dye alone or 4 mM Gly-Sar with dye, and the peak fluorescence was measured. The average minimum signal was 6713 ± 2684. The average maximum signal was 287 909 ± 14 436. The distance between the two distributions was 22.1 in standard deviation units, indicating that this assay should be sufficiently sensitive to identify substrates producing medium- to low-amplitude signals.

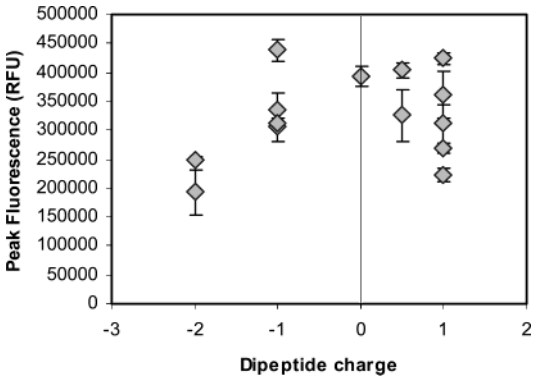

Figure 4. Effect of substrate charge on fluorescence intensity in rPepT1 cells. The peak fluorescence obtained for each of 14 dipeptides (Gly-Asp, Asp-Gly, Asp-Asp, Arg-Gly, Gly-Arg, Gly-Gly, Gly-Lys, Gly-Glu, Gly-His, Glu-Glu, Lys-Gly, His-Gly, His-His, and Glu-Gly, used at a concentration of 10 mM) is plotted vs their charge at pH 6.

be at least seven standard deviations.[23] According to these criteria, the membrane potential assay showed a robust signal window (22.14, Figure 3), indicating that it should be sufficiently sensitive to identify substrates producing medium- to low-amplitude signals.

Effect of Substrate Charge on the Fluorescent Signal. In addition to the concentration of PepT1 transporters, another possible factor affecting the magnitude of the fluorescent signal is the charge of the compound. Since the assay measures membrane potential changes, it is conceivable that the magnitude of these changes could be affected by the charge in the compound being tested. Fourteen dipeptides (Gly-Asp, Asp-Gly, Asp-Asp, Arg-Gly, Gly-Arg, Gly-Gly, Gly-Lys, Gly-Glu, Gly-His, Glu-Glu, Lys-Gly, His-Gly, His-His, and Glu-Gly) with charges ranging from −2 to +1 at pH 6 were evaluated, and the peak fluorescence at 10 mM was determined. All of these dipeptides were capable of activating the transporter with EC_{50} values ranging from 0.3 to 3.5 mM. There was no correlation between charge and fluorescent signal (Figure 4), indicating that the amount of charge in these compounds is not a significant factor in assay sensitivity.

The Assay Differentiates PepT1 Substrates from Nonbinders. The effect of valacyclovir (a known PepT1 substrate, capable of binding and activating transport activity) and acyclovir (does not bind PepT1) on membrane potential was evaluated (Figure 5). Acyclovir failed to increase fluorescence in either mock- or PepT1-transfected cells. In contrast, valacyclovir elicited a dose-dependent membrane potential increase exclusively in PepT1-transfected cells but not in mock-transfected cells. Testing of 13 other known PepT1 binders/substrates and nonbinders demonstrated that this assay was capable of distinguishing between these two classes of compounds. In Table 2, the response of these compounds in the functional assay is compared with their IC_{50} values determined in an inhibition of the [^{14}C]Gly-Sar uptake assay. Since PepT1 is a low-affinity transporter, it was not possible to determine EC_{50} values for a substantial fraction of the compounds that were tested because no maximum plateau activation was obtained at the highest achievable compound concentrations. We are currently working on an alternative way to rank compounds with low affinity. Until that goal is achieved, compounds are scored as (+) or (−) depending on whether they activate the transporter. Determination of transporter activation is based on fulfilling both of these criteria: (1) a dose-proportional fluorescence increase after addition of the compound to PepT1-overexpressing cells, relative to (2) a lack of equivalent response in mock-transfected cells. Eight of the 15 compounds that were tested had moderate to high affinity

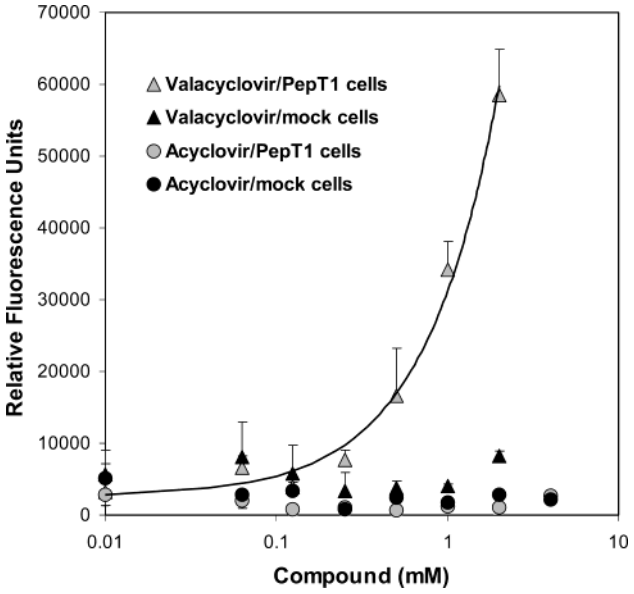

Figure 5. Effect of valacyclovir and acyclovir on membrane potential. The effects of increasing compound concentrations on fluorescence of MP-loaded cells were measured as indicated in the Experimental Section. Fluorescence is plotted in relative fluorescence units. There was no increase in fluorescence in response to acyclovir. Valacyclovir elicited a dose-dependent fluorescence increase in PepT1-expressing cells only.

Table 2. Activation of Transporter Correlates with PepT1 Affinity[a]

compound	transporter activation	IC_{50} (mM)
SQ-29852	+	0.7 ± 0.09
Gly-Sar	+	0.9 ± 0.07
valacyclovir	+	1.2 ± 0.12
cefadroxil	+	3 ± 0.49
bestatin	+	4.1 ± 1.10
cephalexin	+	4.9 ± 0.33
cephradine	+	7.3 ± 2.43
enalapril	+ (weak)	7.3 ± 1.15
cephaclor	+	8.2 ± 2.55
cefuroxime	−	>30
cephalothin	−	>40
lisinopril	−	−
acyclovir	−	−
ampicillin	−	−
amoxicillin	−	−

[a] Determination of the level of transporter activation is based on fulfilling both of these criteria: (1) a dose-proportional fluorescence increase after addition of the compound to PepT1-overexpressing cells, relative to (2) a lack of equivalent response in mock-transfected cells. For affinity measurements, cells were incubated with 10 μM [^{14}C]Gly-Sar and serial dilutions of the above compounds (15−0.1 mM). The radioactivity in each well was measured and used to estimate IC_{50} values by nonlinear regression analysis.

for PepT1 (IC_{50} < 10 mM; Table 2). All of these compounds, with the exception of enalapril, showed clear activation of PepT1 in the membrane potential assay. Compounds with very low PepT1 affinity (IC_{50} > 30 mM) scored as negatives

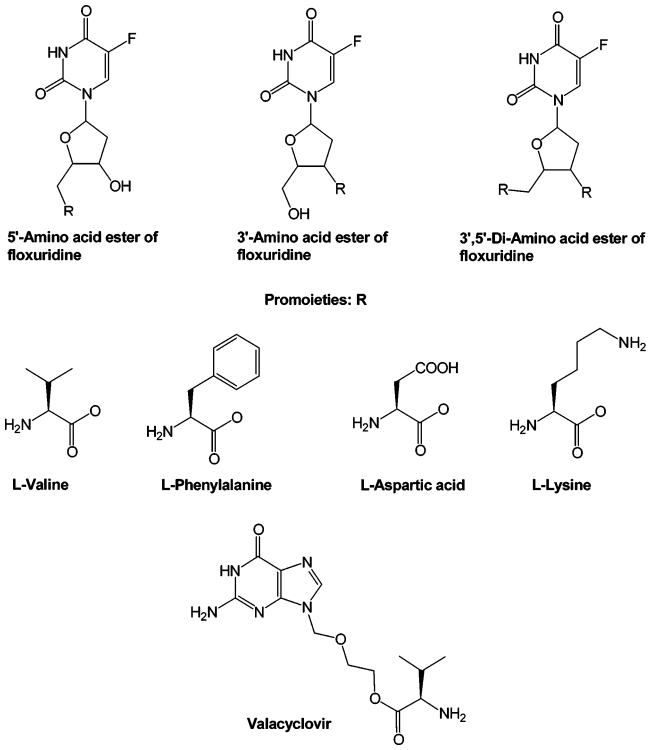

Figure 6. Structures of amino acid ester prodrugs of floxuridine and promoieties.

in the assay. This could be a sensitivity issue since the highest compound concentrations tested in the membrane potential assay were 10 mM.

The Assay Differentiates PepT1 Substrates from PepT1 Antagonists. As the enalapril data suggest, PepT1 may not necessarily actively carry compounds with measurable binding affinity for this transporter. Although ligand displacement assays have been used extensively to identify compounds that bind to PepT1, they cannot predict whether a compound is actively transported. The membrane potential assay was designed to distinguish between compounds that activate PepT1 (substrates) and compounds which bind to this transporter but do not activate it (antagonists). To test the usefulness of this assay in supporting future directed drug design efforts, we evaluated whether this assay could correctly differentiate a previously characterized set of substrates and antagonists. A series of amino acid ester prodrugs of the nucleoside anticancer agent floxuridine (Figure 6)[17] were used to perform this test. These compounds were designed with the goal of enhancing both oral absorption and targeting by taking advantage of active transport by PepT1 in the gut and in metastatic cancer. Nine of these floxuridine prodrugs were tested, all of which were previously found to bind to PepT1 in a ligand displacement assay.[24]

Table 3 lists binding IC_{50} values, cell uptake ratios in

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Table 3. Floxuridine Prodrugs[a]

amino acid ester prodrugs	short name	IC_{50} (mM)	hPepT1/control	membrane potential assay
3′,5′-di-*O*-phenylalanyl	FF1	0.89 ± 0.09	9.53	substrate
3′-*O*-phenylalanyl	FF2	0.84 ± 0.01	2.23	nonsubstrate
5′-*O*-phenylalanyl	FF3	0.62 ± 0.01	12.34	substrate
3′-*O*-valyl	VF2	0.70 ± 0.08	8.24	substrate
5′-*O*-valyl	VF3	0.99 ± 0.08	19.24	substrate
3′,5′-di-*O*-aspartyl	DF1	1.13 ± 0.05	no uptake	nonsubstrate
3′-*O*-aspartyl	DF2	1.27 ± 0.01	no uptake	nonsubstrate
5′-*O*-aspartyl	DF3	1.09 ± 0.01	no uptake	nonsubstrate
3′,5′-di-*O*-lysyl	KF1	1.16	1.91	nonsubstrate
floxuridine		no inhibition	no uptake	ND[b]
valacyclovir		0.46 ± 0.13	4.73	substrate

[a] The binding affinity (IC_{50} values) for hPepT1 in transiently transfected HeLa cells was determined as described in the Experimental Section. hPepT1/control is the ratio of compound uptake by hPepT1-transfected HeLa cells relative to control untransfected cells. Classification as a substrate in the membrane potential assay was made according to the following criteria: (1) a dose-proportional fluorescence increase after addition of the compound to rPepT1-overexpressing cells, relative to (2) a lack of equivalent response in mock-transfected cells. Compounds not fulfiling both of these conditions were classified as nonsubstrates. [b] Not determined.

PepT1-expressing cells (HeLa/hPepT1) versus control HeLa cells, and the responses evoked in the membrane potential assay for each of the nine prodrugs. Cellular uptake of drugs was assessed by HPLC quantitation. The parent drug floxuridine and the PepT1 substrate valacyclovir were also included in the series as controls. Compounds (FF1, FF3, VF2, and VF3) exhibiting substantial cellular uptake enhancement (>8-fold) in PepT1-expressing cells were also classified as substrates by the membrane potential assay. For comparison, valacyclovir, which was clearly positive in the membrane potential assay, exhibited a 4.7-fold increase in the rate of uptake in Hela/hPept1 cells over control cells. The aspartyl prodrugs (DF1−DF3) which cells did not measurably take up were categorized as nonsubstrates in the membrane potential assay. Two compounds (FF2 and KF1) eliciting an ∼2-fold increase in the rate of cellular uptake were not positive in the membrane potential assay.

Discussion

Realization of the critical role that active absorption mechanisms play in the oral absorption of several marketed drugs has spurred great interest in further understanding and exploring these mechanisms.[25] One of these transporters, PepT1, has been the subject of detailed characterization which has led to the *a posteriori* identification of its contribution to the oral bioavailability of several marketed drugs, including β-lactams and ACE inhibitors.[26] PepT1's large capacity and potential for recognition of a large diversity of structures and chemistries have made this transporter a promising target for enhancement of drug absorption by directed drug design.[27] One of the limiting factors in this approach is the lack of high-throughput functional assays, capable of testing the large number of compounds produced by combinatorial chemistry approaches while using a minimum of resources, so that early stage testing can lead to more effective drug optimization. Since the existing methodologies are laborious and resource intensive, we have chosen an alternative strategy that exploits the electrogenic properties of PepT1 and successfully adapted a method originally designed to detect ion channel-mediated modulation of membrane potential.[22,28−30] This novel assay measures membrane potential changes related to H^+/substrate cotransport by PepT1. A similar principle, detecting intracellular acidification, had been reported previously.[15,16] In the first example, pH_i was detected by measuring fluorescence changes in BCECF-loaded perfused Caco-2 cells by optical microscopy.[15] Because of the extremely low throughput of this assay, compounds had to be assayed serially, at a single dose, on the same cell preparation. In the second example, CHO cells overexpressing PepT1 were used.[15,16] In this paper, the assay was performed in a 96-well plate format, enabling the rapid assessment of multiple compounds and conditions. However, to decrease background, the cells required extensive manipulation during the 24 h period prior to the assay. The change in fluorescence in response to transporter activation required 15 min to peak, and the magnitude elicited by Gly-Sar, which in our hands was a

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very potent substrate (25-fold enhancement of the signal with MP dye), was very small, making identification of less potent PepT1 substrates problematic. In our hands, pH indicators were not sensitive to PepT1 activation (Figure 1). Given both the published data and our data, it was concluded that pH indicator assays are not amenable to a high-throughput environment and/or had an insufficient signal-to-noise ratio to be easily adapted to a screening environment where only a limited number of compound concentrations may be tested. The membrane potential assay described in this paper relies on a cell line with high levels of PepT1 expression and real-time, kinetic reading of membrane potential modulation. These features maximize the signal-to-noise ratio and minimize the time required to obtain the data, which facilitates the assay miniaturization required for an HTS environment.

Compounds evoking dose-proportional fluorescence intensity changes measured in cells overexpressing the PepT1 transporter while failing to induce equivalent changes in mock-transfected cells were classified as PepT1 substrates. For Gly-Sar, a compound for which Fl_{max} could be determined, a detailed examination of the response of the assay was carried out. As expected, the Fl_{max} was proportional to the total amount of PepT1 present, highlighting the importance of using a cell line expressing high levels of PepT1. For Gly-Sar, the assay has a signal window of 22 which is well above minimum recommendations for HTS assays. Testing of dipeptides with different charges did not uncover a correlation between assay performance (Fl_{max}) and charge. If such a correlation exists, it might only be seen with compounds carrying greater net charge at pH 6.

The performance of the assay was initially evaluated by testing 15 known PepT1 substrates/nonbinders. The membrane potential assay was capable of distinguishing known PepT1 substrates from nonbinders, as the assay results correlated well with compound PepT1 affinity. Ampicillin and amoxicillin, which are reported to be PepT1 substrates, were negative in the membrane potential assay. However, when tested in the binding assay at concentrations of up to 15 mM, these compounds could not displace [^{14}C]Gly-Sar from PepT1 either, and therefore, in our model system, we could not show that these compounds bound PepT1. Other researchers have reported these two compounds have low affinities for PepT1 in a ligand displacement assay.[31] Acyclovir, cefuroxime, cephalothin, and lisinopril were negative in our assay, confirming their reported status as nonsubstrates.[11,15,32] Enalapril showed only a modest activation in the membrane potential assay despite being able to bind PepT1. It is possible that enalapril is not efficiently transported despite having a measurable affinity for PepT1. It has been argued that, in the gut, enalapril may not be actively absorbed via PepT1 but instead through other intestinal transporters.[2,33]

Nine floxuridine prodrugs, all capable of binding Pept1 were correctly classified by the membrane potential assay as substrates/antagonists. All the prodrugs exhibiting greater than 8-fold enhancement in cellular uptake were found to be substrates. Valacyclovir, which was clearly positive in the membrane potential assay (see Figure 5), had a cellular uptake ratio of 4.7, indicating that this assay requires an at least 4.7-fold difference in the rate of compound uptake to detect PepT1 activation. The assay classified as antagonists compounds shown to have no cell uptake or only a small improvement in cell uptake (2-fold enhancement). The aspartyl prodrugs (DF1−DF3) exhibited high affinities for the hPepT1 transporter in binding assays; however, these prodrugs were categorized as antagonists in the membrane potential assay. Interestingly, these prodrugs showed no increase in the rate of uptake in cells overexpressing the hPepT1 transporter. Two compounds (FF2 and KF1) eliciting an ∼2-fold increase in the rate of cellular uptake were not positive in the membrane potential assay. It is not clear whether this result is due to a lack of sensitivity in the membrane potential assay for poor PepT1 substrates or whether the 2-fold enhancement is not significant given the experimental variability associated with the cellular uptake experiments. It could be argued, however, that a 2-fold enhancement in uptake may not be sufficient improvement in compound absorption/targeting and that it may be desirable to select for drugs showing better transport characteristics. Therefore, it can be said that within the small data set tested, the membrane potential assay predicted potent transporter substrates accurately, confirming its usefulness for identifying compounds that are actively transported by PepT1. These data also confirm that this assay is capable of distinguishing between transporter substrates and antagonists, and highlight its potential usefulness in predicting compounds with potentially good oral absorption.

Although this method could be used for identifying potential PepT1 substrates, at this stage of assay development it can only be used for ranking compounds for which an EC_{50} can be determined. Ongoing statistical analysis of the data may enable the generation of criteria for rank ordering of lower-affinity PepT1 substrates. Although this assay was initially developed in a cell line expressing rat PepT1, initial cross validation with cells expressing human PepT1[18] shows no essential difference in transporter function between the two species.

In conclusion, our initial assessment of this novel assay indicates that it is sensitive and robust, and can distinguish between transporter substrates and antagonists. This important distinction has been previously achieved only with lower-

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throughput assays. For higher-affinity substrates, this assay can be used for rank ordering and establishing a high-quality data set for PepT1 modeling. Similar methodologies could potentially be expanded to the characterization of other relevant drug transporters.

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