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Synthesis and Evaluation of a Dipeptide–Drug Conjugate Library As Substrates for PEPT1

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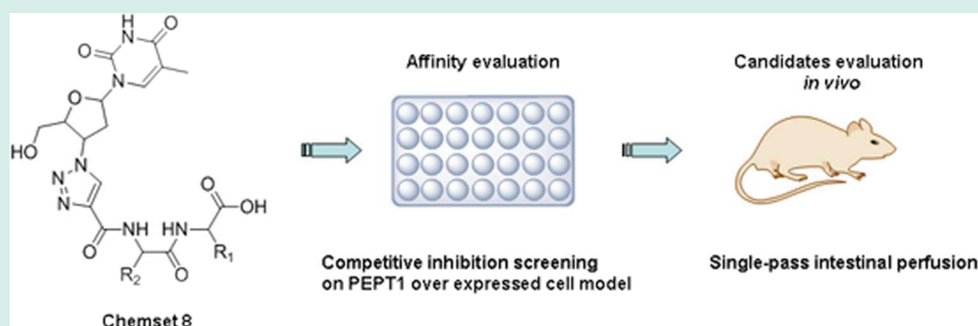
Supporting Information



ABSTRACT: The oligopeptide transporter PEPT1 is considered as a valuable target for prodrug design, but its 3D structure and substrate specificity of PEPT1 are not fully understood. In this study, we designed a focused dipeptide conjugated azidothymidine (AZT) library and described a convenient and efficient solid phase synthesis scheme based on click chemistry. Over 60 candidate structures containing various dipeptide sequences were obtained with high purity, and screened in a PEPT1 overexpressing cell model for their abilities to compete with the known ligand cephalexin. Some of the compounds selected to have medium or high affinity were tested for their in vivo transport in a single-pass intestinal perfusion experiment. Results showed that the designed library contained some new structure features that have high affinities toward PEPT1 and could be further explored for their application in prodrug design and development.

KEYWORDS: *azidothymidine–dipeptide conjugates, click chemistry, PEPT1, competitive inhibition, single-pass intestinal perfusion*

INTRODUCTION

A number of transporter proteins expressed in the intestinal epithelial cell have been identified, such as the nucleotide transporters, amino acid transporters, and peptide transporters (PEPT1 and PEPT2).[1−3] PEPT1 is found dominantly in the brush borders of the small intestinal epithelium in mammals and is believed to play an important role in the active transport of di- and tripeptides actively[4,5] and to serve as a main pathway for the absorption of dietary nitrogen. In addition, some recent studies revealed that PEPT1 is also overexpressed on malignant cancer cells, such as Capan-2, AsPC-1, and HT1080.[6,7] Therefore PEPT1 is considered as a very important target for prodrug design for improvement of oral bioavailability and also the development of targeted cancer therapeutics.[8−10]

Although PEPT1 was cloned in 1994, its 3D structure and substrate specificity are not yet clear. Most studies on candidate substrates have been done empirically with testing of various amino acid monoester and diester prodrugs of compounds such as acyclovir, gemcitabine, Ara-C, azidothymidine, and floxuridine.[11−16] Indeed, improved bioavailability of some conjugates was observed after oral administration. G. L. Amidon et al. have further suggested that dipeptide monoester prodrugs may have better stability and higher affinity for PEPT1 than amino acid monoester prodrugs.[17,18] Santos et al. selected the sequence Val-Ala as the best binder to the hPEPT1 transporter among eight different dipeptide prodrugs of azidothymidine.[19]

It was initially believed that the peptide bond, the amino terminal, the carboxyl group and a hydrophobic group were all important for PEPT1 recognition and transport.[20] However, later studies have separately cast doubt on the neccesity of the peptide bond,[21] the carboxyl group,[21] and the amino terminus[22] for PEPT1 recognition and transport. Therefore, more work is certainly needed to establish the structural requirements for specific hPEPT1 transport. We describe here an attempt to promote the uptake of nucleoside derivatives by the hPEPT1 pathway.

In many prodrug studies that used nucleoside analoges as model drugs, conjugation to dipeptides and other biocompatible units has been done via nucleoside 5′-hydroxyl group. We chose instead to design a focused dipeptide conjugate library based on the model drug azidothymidine(AZT), eliminating both the 5′-hydroxyl connection and the amine N-terminus. A

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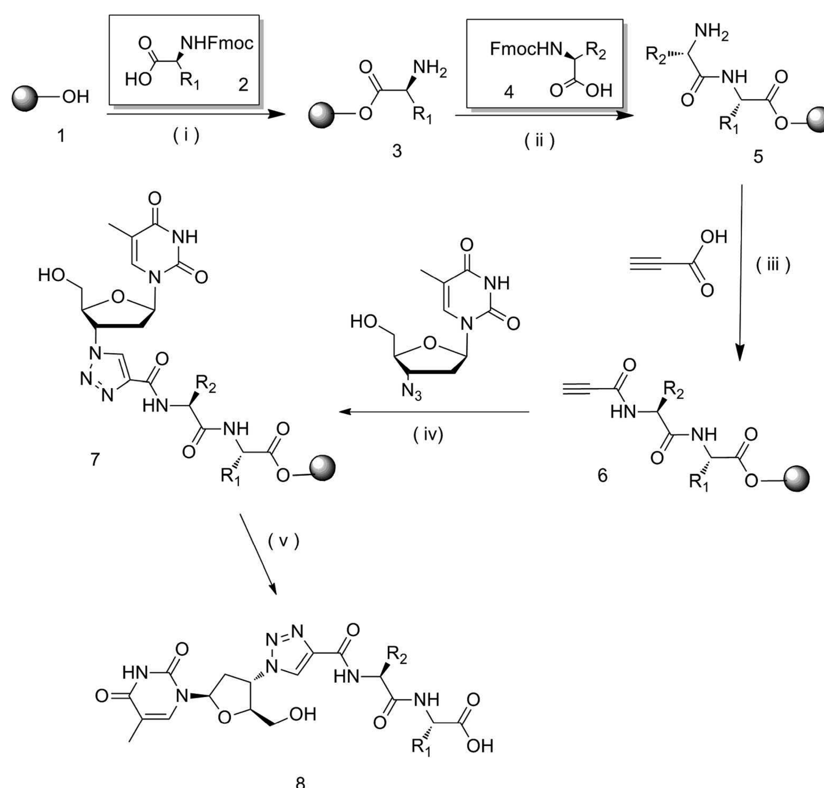

Figure 1. Strategy for synthesis of AZT−dipeptide conjugates. Reagents and conditions: (i) (1) DIC/HOBt/DMAP, DMF, rt, 3 h; (2) 20% piperidine/DMF, 30 min; (ii)(1)HBTU,NMM, DMF, rt, 2−4 h; (2) 20% piperidine/DMF, 30 min; (iii) DIC/DIPEA,DMF, photophobic, rt, overnight; (iv) CuI/DIPEA 2/50, THF, photophobic, rt, 3 h; (v) TFA/H2O/TIPS 95/2.5/2.5, rt, 1−2 h.

convenient solid phase synthesis scheme was devised based on click chemistry,[23−25] employing the copper(I)-catalyzed Huisgen 1,3-dipolar azide−alkyne cycloaddition reaction. Click chemistry with copper(I)-catalyzed Huisgen 1,3-dipolar azide−alkyne cycloaddition is quite efficient and can be incorporated in peptide synthesis schemes.[26,27] The library was screened for their ability to compete with the binding and uptake of the known PEPT1 substrate cephalexin, and selected compounds were also evaluated by single-pass intestinal perfusion studies to further characterize their PEPT1 transport specificity.

■ RESULTS AND DISCUSSION

AZT was selected as the model drug for the design of all the dipeptide-drug conjugates, since it is widely used and has been previously employed for PEPT1-based prodrug therapy.[19]A total of 61 AZT-dipeptide conjugates consisting different combination of amino acids were synthesized, using the general strategy outlined in Figure 1. Not all of the 20 natural amino acids were included. The amino acids Asn, Gln, Asp, Met, Cys, His, Trp, and Tyr were excluded because they are not very stable in the physiological environment.[28−30] But effort was made to include most of the amino acids that had been reported to be involved in PEPT1 activity,[11,15,19,31] that is, Phe, Val, Leu, Ile, Gly, and Ala.

The AZT-dipeptide conjugates library synthesis was started with commercially available wang resin **1**, which was converted to chemset **3** by condensation reaction with Fmoc amino acids comprising chemset **2**. Standard Fmoc deprotection and coupling to chemset **4** provided the dipeptide chemset **5**. Afterward, propiolic acid coupling and the [3 + 2] Huisgen 1,3-dipolar cycloaddition were carried out on resins based on typical click chemistry method[26,27] with minor modifications to afford chemset **7**. Cleavage from resin was conducted using TFA/H_2O/TIPS to afford chemset **8**, and the products were purified by RP-HPLC.

The purity of each member of chemset **8** was above 80% and over 90% after RP-HPLC purification (see Table 1). The isolated yields were on average 30−45%, except for the compounds containing Gly or Lys as the terminal residue. The yields in those cases were much lower (∼10%) because the RP-HPLC conditions used to collect the compounds were not suitable for highly soluble compounds. All compounds of chemset **8** were characterized by ESI-MS and ^{1}H NMR spectroscopy. Among them, 10 compounds were confirmed with further characterization, including ^{13}C NMR, HR-MS, and melting point.

To evaluate the bioactivities of the entire library as PEPT1 substrates, we adopted the method developed by Dr. Amidon's group using adenovirus Ad.RSVhPepT1 transfected Hela cells that transiently overexpresses PEPT1.[32] The cells were incubated with 2.5 mM of the known PEPT1 substrate cephalexin[33] for 45 min and then washed and harvested for HPLC assay of intracellular cephalexin content. The PEPT1 transfected cells usually take up at least three times the amount of cephalexin as the untransfected cells (9.7 ± 1.95 μg compared to 2.4 ± 0.24 μg per mg protein in 45 min).Then the abilities of various AZT-dipeptide conjugates to compete with cephalexin for PEPT1 transport were tested as the indirect measurements of their potential as PEPT1 substrates.

In our cell uptake study, the cephalexin concentration was maintained at 2.5 mM, while all the AZT-dipeptide conjugates were added at 1 mM to identify compounds with PEPT1 affinity higher than cephalexin. To rule out potential

Table 1. Characteristics of Resulted AZT−dipeptide Conjugates Library

chemset	dipeptide sequence	purity (%)[a]	yield (%)[b]	chemset	dipeptide sequence	purity (%)[a]	yield (%)[b]
8{1,1}	Arg-Ala	98.30	42.0	8{8,7}	Pro-Lys	93.97	40.8
8{1,5}	Arg-Ile	93.35	39.2	8{8,8}	Pro-Phe	91.49	41.2
8{2,1}	Glu-Ala	98.83	46.0	8{9,1}	Ser-Ala	95.98	39.6
8{2,2}	Glu-Arg	93.48	42.7	8{9,2}	Ser-Arg	92.69	39.5
8{3,1}	Gly-Ala	93.79	14.7	8{9,3}	Ser-Glu	94.08	41.2
8{3,2}	Gly-Arg	90.41	14.5	8{9,4}	Ser-Gly	96.40	40.8
8{3,3}	Gly-Glu	90.48	10.3	8{9,5}	Ser-Ile	92.27	42.4
8{3,12}	Gly-Val	92.17	14.7	8{9,6}	Ser-Leu	90.21	41.0
8{4,1}	Ile-Ala	94.21	38.0	8{9,7}	Ser-Lys	92.13	39.7
8{4,3}	Ile-Glu	99.40	37.2	8{9,8}	Ser-Phe	90.03	39.2
8{4,4}	Ile-Gly	98.81	33.2	8{9,9}	Ser-Pro	94.31	42.7
8{5,1}	Leu-Ala	98.32	42.0	8{10,1}	Thr-Ala	97.04	43.0
8{5,2}	Leu-Arg	98.81	41.6	8{10,2}	Thr-Arg	96.77	42.7
8{5,3}	Leu-Glu	97.19	34.5	8{10,3}	Thr-Glu	95.85	37.5
8{5,5}	Leu-Ile	99.48	35.8	8{10,4}	Thr-Gly	99.24	35.2
8{6,1}	Lys-Ala	91.75	9.7	8{10,5}	Thr-Ile	99.24	35.2
8{6,2}	Lys-Arg	94.45	8.0	8{10,6}	Thr-Leu	90.06	39.6
8{6,3}	Lys-Glu	86.30	9.0	8{10,7}	Thr-Lys	92.80	41.2
8{6,5}	Lys-Ile	97.82	10.3	8{10,8}	Thr-Phe	93.82	38.5
8{6,6}	Lys-Leu	91.70	10.7	8{10,9}	Thr-Pro	97.22	40.5
8{7,1}	Phe-Ala	94.23	41.8	8{10,10}	Thr-Ser	97.34	38.6
8{7,3}	Phe-Glu	92.88	38.0	8{11,1}	Val-Ala	97.40	37.8
8{7,4}	Phe-Gly	98.13	32.6	8{11,2}	Val-Arg	92.60	34.6
8{7,5}	Phe-Ile	99.50	33.2	8{11,3}	Val-Glu	99.07	34.5
8{7,6}	Phe-Leu	100.00	37.2	8{11,5}	Val-Ile	90.15	37.5
8{7,7}	Phe-Lys	93.27	32.0	8{11,6}	Val-Leu	96.04	38.7
8{8,2}	Pro-Arg	90.34	42.8	8{11,7}	Val-Lys	97.98	40.2
8{8,3}	Pro-Glu	96.39	34.5	8{11,9}	Val-Pro	98.40	38.6
8{8,4}	Pro-Gly	96.39	34.5	8{11,10}	Val-Ser	95.46	36.7
8{8,5}	Pro-Ile	95.19	37.8	8{11,11}	Val-Thr	92.89	39.2
8{8,6}	Pro-Leu	93.41	39.2				

[a]Purity was determined by analytical HPLC. [b]Isolated yield.

cytotoxicity effects, the compound library were tested using a Cell Counting Kit-8 (CCK-8, Dojindo) and only those giving cell viability greater than 85% were included in the cephalexin competition study. (Six compounds were ruled out in this screen.) The results of this study are shown in Figure 2.

The data in Figure 2 show that quite a few dipeptide compounds can interfere with PEPT1 mediated uptake of cephalexin. Phe-Gly and Val-Ser were highly active in cephalexin uptake inhibition, which agreed very well with previous studies of 5′-hydroxyl dipeptide prodrugs of floxuridine or cidofovir.[17,18] In addition, almost all of the compounds containing the first amino acid of Ile or Ala displayed significant activities. This is also consistent with previous reports that 5′-L-Ile- prodrugs and 5′-L-Ala- prodrugs of gemcitabine and floxuridine had higher activities.[11,15] However, a number of other dipeptide sequences(Arg-Ile, Ile-Ala, Leu-Ile, Phe-Ala, Phe-Lys, Pro-Ile, Ser-Pro, Ser-Glu, Thr-Ala, and Val-Arg) showed even greater activities and could inhibit up to 80% of cephalexin uptake by PEPT1 Hela cells. None of these sequences have been previously reported as PEPT1 substrates and may therefore have good potentials in prodrug and drug design and development.

Inhibition of cephalexin transport could be accomplished by PEPT1 binding rather than transport. To test for this possibility, we wished to directly measure PEPT1 mediated transport activity. Two effective compounds, the Ser-Glu (8{9,3}) and Pro-Ile (8{8,5}) adducts, along with the weak Glu-Ala (8{2,1}) inhibitor, were evaluated for in vivo trans-epithelia permeability in single-pass intestinal perfusion. The calculated effective permeability coefficients P_{eff} were ploted in Figure 3. Considering the most relevant metabolite from these conjugated structures is probably not AZT, but AZT coupled with propargyl acid (labeled as AZT-T), it was synthesized and tested too for comparison. In the tests of compound 8{9,3}, the known PEPT1 substrate Gly-Sar was also added to examine specific PEPT1 competition effect.

Compared to AZT-T, the Ser-Glu (8{9,3}), Pro-Ile (8{8,5}), and Glu-Ala (8{2,1}) conjugates all showed higher rat intestinal permeability. In addition, compound 8{9,3} indeed had higher in vivo permeability than compound 8{2,1} ($p < 0.01$) as suggested by the in vitro cephalexin competition assay (Figure 2). The difference between compounds 8{8,5} and 8{9,3}, however, was not exactly the same as seen in the in vitro assay, probably because many other factors including passive diffusion and other transporter involvements could contribute in the in vivo perfusion experiment. In addition, the study by Miyamoto et al. showed that there were some small sequence differences between rat PEPT1 and hPET1 (the homology score is 83%)[34] which may account for some discrepancies between these two data sets. But most importantly, transport of 8{9,3} was decreased significantly in the presence of Gly-Sar (20 μg/mL). The calculated numbers were $1.48 \pm 0.44 \times 10^{-3}$ and $4.30 \pm 1.08 \times 10^{-3}$ cm/s respectively in the presence and absence of Gly-sar ($p < 0.005$). The data clearly indicated that the

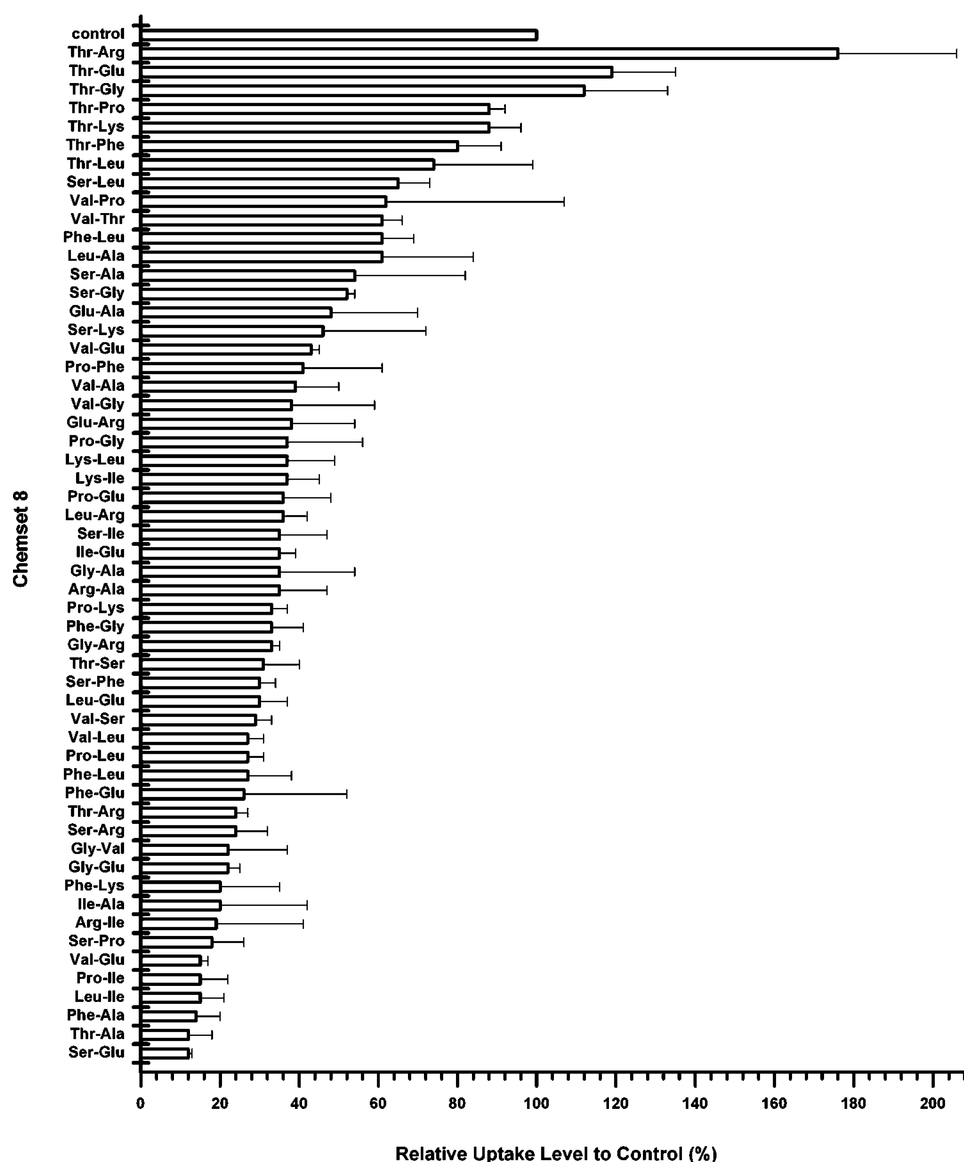

Figure 2. Competitive inhibition assay to evaluate uptake of cephalexin inhibited by AZT−dipeptide conjugates in adenovirus (Ad.RSVhPepT1) transfected Hela cells. "Relative uptake" is defined by the amount of drug found in the cells in the presence of the AZT-dipeptide conjugate divided by the amount of drug taken up in the absence of the AZT-dipeptide conjugate (x 100%). The indicated peptides represent the components used from Chemsets **2** and **4**, respectively (Figure 1).

transport of the dipeptide AZT conjugate **8**{9,3} was indeed mediated at least partly by PEPT1, which confirmed our finding that the dipeptide Ser-Glu could be a good substrate of PEPT1 and a good candidate for designing dipeptide prodrugs.

■ CONCLUSION

Over 60 compounds were synthesized, 55 compounds were examined for their ability to compete with the PEPT1 binding and tranporting of a known substrate cephalexin in vitro, and 3 compounds were tested for their intestinal permeability in vivo. Several new dipeptide sequences including Ser-Glu and Pro-Ile were found to have high affinity to PEPT1 and mediated significant active transport activity across intestinal epithelia.

■ EXPERIMENTAL SECTION

Solid-Phase Synthesis of Different Sequences of Dipeptide Conjugated with Propargyl Acid. Wang resin (1.2 mmol/g, Tianjin Nankai Hecheng S&T Co., Ltd., China) was used as solid phase, and two amino acids were sequentially conjugated based on the standard SPPS procedure and monitored using the ninhydrin Kaiser test. Deprotection of the Fmoc group was carried out using 20% piperidine/DMF for 30 min. The dipeptide containing resins were then filtered and dried. Meanwhile, based on mole number of wang resin, propargyl acid (10 equiv) was added into a 50 mL beaker containing DMF (ice-cold), and 5 min later, DIC (5.5 equiv) was added and kept reacting for 10 min. The mixture was then transferred into a reaction column filled with the dipeptide containing resins. Finally, DIPEA (5 equiv) was added into the reaction column in 5 min later and the reaction was kept overnight at room temperature protected from lights.

1,3-Cycloaddition Reaction of Dipeptides to AZT. The dipeptide and propargyl acid conjugated resins were added in THF, together with CuI (2 equiv) and DIPEA (50 equiv). Five minutes later, AZT (2 equiv) dissolved in THF was added into the reaction system. The reaction was maintained at room

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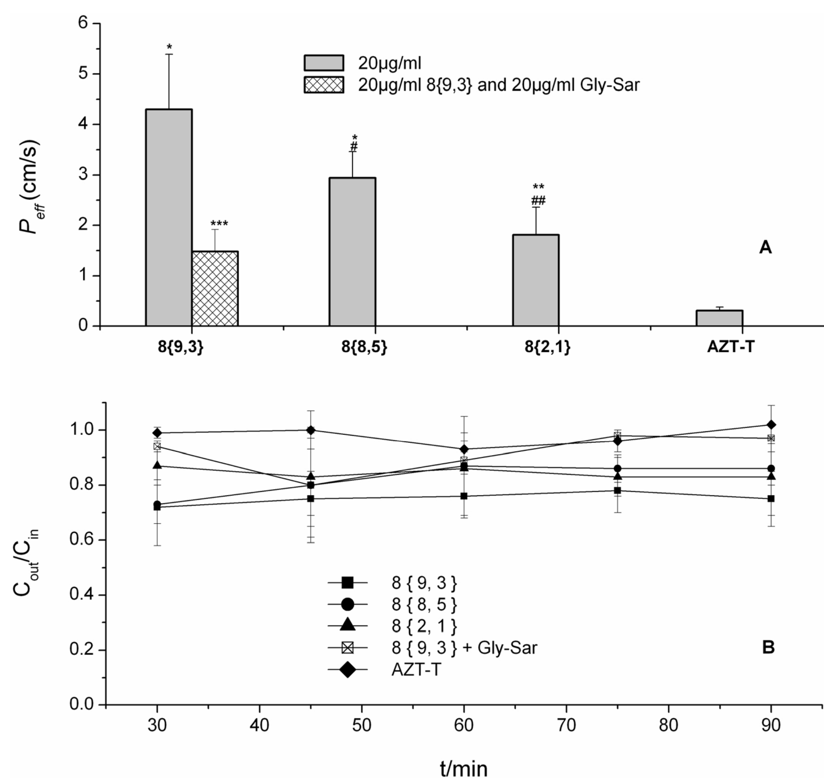

Figure 3. Single-pass intestinal perfusion. A: Effective permeability, P_{eff} ($P_{eff} \times 10^3$ cm/s) using single-pass intestinal perfusion. P_{eff} of compounds were determined at 20 μg/mL, and **8**{9,3} were tested in the presence or absence of 20 μg/mL Gly-Sar. The data were presented as mean ± SD (n = 5 in group **8**{9,3} and n = 3 in other groups): *, $p < 0.005$ versus AZT-T group; **, $p < 0.05$ versus AZT-T group, ***, $p < 0.005$ versus **8**{9,3} group, #, $p > 0.05$ versus **8**{9,3} group, ##, $p < 0.01$ versus **8**{9,3} group. B: Approach to steady-state for compounds existing in the intestinal perfusate (n = 5 in group **8**{9,3} and n = 3 in other groups).

temperature protected from lights for about 3−6 h. The exact reaction time may vary and were optimized for different dipeptide sequences.

The AZT-dipeptide conjugates were cleaved from the resin using cleavage reagent TFA/H_2O/TIPS(95/2.5/2.5) for 2 h and filtered. The filtrates were dried under vacuum. The crude conjugates were first cleaned up by precipitation in dry ice cold ether to afford crude products with the purity over 80%. Then, crude products were purified using a preparative RP-HPLC with a Agilent C18 column (150 × 9.4 mm, 5 μm) and gradient solvent wash (solvent A is water with 0.1% trifluoroacetic acid and solvent B is acetonitrile with 0.1% trifluoroacetic acid, Gradient: %B gradient start = 25.0, end = 45.0, duration = 25 min) at a flow rate of 1 mL/min with UV detection at 210 nm. Most of the AZT−dipeptide conjugates were obtained as white or off-white solids with the purity over 90%.

Competitions of Cephalexin Uptake by PEPT1 Over-expressing Hela Cells. The Hela cell line was purchased from IBCB (Institute of Biochemistry and Cell Biology, Shanghai, China) and maintained in RPMI 1640 (Gibco-BRL) containing 10% fetal bovine serum (FBS; Gibco-BRL). The Ad.RSVh-PepT1 adenovirus for transducing cells to express PEPT1 was a kind gift from Prof. Amidon at university of Michigan.[32] 5 × 10^4 Hela cells per well were planted in 48 well plates and transfected with the Ad.RSVhPepT1 24 h later. Two days (sometimes longer) after transfection, cells were washed once with fresh growth medium, twice with the uptake buffer[11] (pH 6.0, 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) and then incubated with 2.5 mM cephalexin at presence/absence of 1 mM AZT-dipeptide conjugate. After 45 min, the cells were again washed three times with ice-cold uptake buffer and lysed with 120 μL of deionized water containing 1% SDS in each well. After cell lysis, another 60 μL of ice-cold trifluoroacetic acid was added into each well (to reach final concentration of trifluoroacetic acid at 5%), and lysates were collected and centrifuged for 10 min at 6000 rpm. The supernatants were then filtered (0.45 μm) and analyzed for cephalaxin concentration by HPLC.

Total protein concentration was measured to control the amount of cells. Briefly, cells were collected and lysed by boiling in a sample buffer (0.303 g Tris and 0.8 g SDS in 10 mL of ultrapure water) at 100 °C for 20 min, and lysate was centrifuged for 10 min at 15000 rpm. The supernatants were then analyzed for total protein concentration by using Coomassie G-250 (TCI Development Co., Ltd. Shanghai). The cephalexin uptake activities were normalized per milligram of protein.

HPLC Quantification of Cephalaxin Concentration in Cells. The Agilent HPLC system 1100 Series workstation equipped with a Kromasil C_{18} column (150 mm ×4.6 mm, 5 μm, Sweden) and a guard column was employed for cephalaxin concentration determination. The mobile phase used for all samples was 8% acetonitrile and 2% methanol in 10 mM KH_2PO_4 buffer (adjusted to pH 4.0 with H_3PO_4). The flow rate was 1.0 mL/min and the detection wavelength was set at 254 nm. Under the chromatographic conditions described, retention time of cephalexin was about 5 min.

Single-Pass Intestinal Perfusion in Rats. Single-pass intestinal perfusion studies were done based on established

protocols as described in the literature.[35,36] Briefly, male Waster rats weighing 180−230 g were fasted overnight before the perfusion experiment. A 10−15 cm intestinal segment was isolated and cannulated at both ends with plastic tubing with careful surgery. The segment was rinsed with 37 °C saline and Kreb's buffer respectively. The experiment was initiated by rapidly filling (1.0 mL/min) the segment with the perfusate solution, start the perfusion at a rate of 0.2 mL/min. After approximately 30 min, when steady-state was reached, the outlet perfused samples were collected at 15 min intervals up to 90 min. Samples were centrifuged for 10 min at 6000 rpm. The supernatants were filtered (0.45 μm) and analyzed by HPLC.

Calculation of the Effective Permeability Coefficient. The steady-state intestinal effective permeability (P_{eff}, cm/s) was calculated according to the following equation based on the parallel tube model[37,38]

$$P_{\text{eff}} = \frac{-\upsilon \times \ln\left(\frac{C_{\text{out}}}{C_{\text{in}}} \times \frac{Q_{\text{out}}}{Q_{\text{in}}}\right)}{A}$$

where ν is the perfusion flow rate (0.2 mL/min), A is the mass transfer surface area within the intestinal segment, C_{in} and C_{out} are the inlet and outlet solution concentrations, respectively, Q_{in} is the volume measured that entering intestinal perfusate, and Q_{out} is the volume measured that exiting intestinal perfusate at the specified time interval.

Statistical Analysis. All experiments were conducted at least in triplicate and results are expressed as mean ± SD. Statistical comparison was performed by t test and $p < 0.05$ was considered to be statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

Characterization data, including a list of MS and ^{1}H NMR data of chemset **8**, ^{1}H NMR, ^{13}C NMR, and HRMS spectra and melting point data of ten compounds, MS and ^{1}H NMR spectra of AZT-T and data for transfected Hela cell model. This information is available free of charge via the Internet at http://pubs.acs.org/.

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