Intestinal Absorption of Low Permeability Drugs: A Transporter- and Enzyme-Targeted Approach

by

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hPEPT1, human peptide transporter 1;

OCT, organic cation transporter;

GOCarb, guanidine oseltamivir carboxylate;

CAT, cationic amino acid transporter;

HAT, heteromeric amino acid transporter;

hPEPT2, human peptide transporter 2;

ACE, angiotensin converting enzyme;

hVACVase, human valacyclovirase;

BDCRB, 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole;

p-NA, *p*-nitroanilide;

DFP, diisopropyl fluorophosphate;

AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride;

PCMB, *p*-chloromercuribenzonate;

3-HPG, [3-(hydroxymethyl)phenyl]guanidine;

Boc, *tert*-butyloxycarbonyl;

MES, 2-(*N*-morpholino)ethanesulfonic acid;

HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid;

TEA, tetraethylammonium;

FUdR, floxuridine;

3-APG, [3-(aminomethyl)phenyl]guanidine;

PNP, *p*-nitrophenyl.

Chapter 1

Introduction

Prodrug strategy to increase oral absorption

Prodrugs are bioreversible derivatives of drug molecules designed to overcome pharmaceutical, pharmacokinetic or pharmacodynamic barriers such as low oral absorption, lack of site specificity, insufficient chemical stability, poor solubility, toxicity, etc.[1] To improve oral absorption, a classic prodrug approach can be adopted to enhance drug lipophilicity and passive diffusion. For example, influenza neuraminidase inhibitor oseltamivir carboxylate is poorly absorbed from gastrointestinal tract due to high polarity and negative charge of the carboxyl group (Scheme 1.1). However, its ethyl ester prodrug, oseltamivir (trade name Tamiflu), can be effectively absorbed by passive diffusion and extensively metabolized to the parent drug.[2] With an absolute oral bioavailability of 80%, Tamiflu is the most effective orally administered drug for the treatment of influenza, including the currently widespread H1N1 flu. In recent years, the understanding of membrane transporters has promoted a novel targeted prodrug approach, utilizing carrier-mediated transport to increase intestinal permeability.[3] As examples, valacyclovir and valganciclovir, the valine ester prodrugs of poorly absorbed antiviral drugs acyclovir and ganciclovir, respectively, exhibit more than 50% oral bioavailability by hPEPT1-mediated membrane transport (Scheme 1.1).[4-7] After being

absorbed, prodrugs must be converted to their corresponding parent drugs to exert therapeutic effects. This activation process does not necessarily need to be specific; however, a thorough understanding of the possible activating enzymes will aid the rational design of successful prodrugs.

Guanidino-containing drugs and prodrug strategy

Due to the polar nature and positive charge of the guanidino functionality, it is hard for guanidino-containing drugs to penetrate biological membranes by passive diffusion. However, other transport mechanisms are employed to bring the oral bioavailability of some guanidino-containing drugs to a reasonable level. For example, the biguanide metformin, one of the widely used oral drugs for the treatment of type 2 diabetes, is mainly absorbed from small intestine with an oral bioavailability of 50-60%.[8] The absorption can be associated with a combination of passive diffusion and carrier-mediated transport.[9-11] Another example of orally available guanidino-containing drug is the H_2-receptor antagonist famotidine, which is an organic cation transporter 1 (OCT1) substrate.[12] On the other hand, the low-permeability guanidino-containing drugs cannot be given orally: the thrombin inhibitor argatroban and the influenza neuraminidase inhibitor zanamivir (Scheme 1.2) are dosed by intravenous administration[13] and inhalation[14] respectively. When oral route of administration is desired, the guanidino group has to be replaced in many cases, although activity may also be compromised. As an example, the more potent guanidino analog of oseltamivir carboxylate, GS 4116 (GOCarb), is not orally absorbed (Scheme 1.2).[15] Neither is its ethyl ester GS 4109 orally available, which suggests that the poor oral bioavailability is

partially attributed to the guanidino functionality. Finally, GOCarb had to be abandoned despite its potential as a very potent influenza neuraminidase inhibitor.

Considering the difficulty of finding novel orally available drug candidates, especially anti-influenza agents, it would be beneficial to solve the permeability problem with a prodrug strategy. Many research groups have applied the classic prodrug approach to mask the charge of the guanidino group and facilitate passive diffusion, following activation to the parent drug.[16-19] However, this strategy has met with only limited success, partly due to the insufficient membrane transport and complicated activation. Moreover, if the drug is zwitterionic such as argatroban, zanamivir and GOCarb, all the charges need to be shielded, further complicating both the synthesis and the *in vivo* activation. On the other hand, prodrugs can be designed to target mucosal transporters, which may tolerate the guanidino functionality and other charges. Such transporters include organic cation transporters, amino acid transporters and oligopeptide transporters.

Organic cation transporters (OCTs)

The organic cation transporters (OCTs) facilitate the diffusion of structurally diverse organic cations including endogenous substrates such as monoamine neurotransmitters and many pharmaceutical compounds.[20, 21] The substrate and inhibitor specificities of OCT1, OCT2 and OCT3 overlap extensively even though the affinity and maximal transport rates may be different.[20, 21] OCT substrates are mostly organic cations including guanidino-containing compounds such as metformin and guanidine.[20, 22] Although OCTs are expressed in the small intestine,[20, 21, 23] their involvement in oral drug absorption is still not well established. Furthermore, if a

guanidino-containing compound is not an OCT substrate itself, it is very unlikely that a prodrug designed to target OCTs will have a higher oral absorption.

Amino acid transporters

The guanidino-containing amino acid L-arginine is a semiessential or conditionally essential amino acid in humans. It is involved in many important metabolic pathways, such as nitric oxide, urea, ornithine, agmatine, creatine, proline, glutamate and polyamine synthesis.[24]

There are at least four different transport systems that accept arginine as a substrate (Table 1.1).[25] System y^+, a Na^+ independent transport system, is the only selective one for cationic amino acids. System y^+ comprises at least 3 cationic amino acid transporters (CATs): CAT-1, CAT-2B and CAT-3, all of which are widely expressed membrane glycoproteins. Both system y^+L and $b^{0,+}$ are heteromeric amino acid transporters (HATs), consisting of a glycoprotein (4F2 heavy chain in system y^+L and rBAT in system $b^{0,+}$) and a real transporter protein (y^+LAT1 or y^+LAT2 in system y^+L and $b^{0,+}AT$ in system $b^{0,+}$). System $B^{0,+}$ is encoded by $ATB^{0,+}$, which is the only known Na^+ dependent transporter for arginine. rBAT/$b^{0,+}AT$ is the major transporter for arginine absorption in the apical membrane of the small intestine.[25-27] 4F2hc/y^+LAT1, on the other hand, mediates the efflux of arginine on the basolateral membrane.[25-27]

Many studies suggested that amino acid transporters have potentials to serve as prodrug targets.[28-32] However, they are not as widely used as peptide transporters for oral drug delivery purposes, probably due to their stringent substrate requirements and low capacity.

Oligopeptide transporters

The absorption and reabsorption of di- and tripeptides are mediated by human peptide transporter 1 (hPEPT1) and human peptide transporter 2 (hPEPT2) in intestinal and renal epithelial cells, respectively.[33-35] hPEPT1 and hPEPT2 are members of the Proton-coupled Oligopeptide Transporter (POT) superfamily, which contains 12 predicted transmembrane domains with the N- and C-termini facing the cytosol.[34, 36] hPEPT1 is a low-affinity, high-capacity transporter; whereas hPEPT2 is a high-affinity, low-capacity transporter, and they share similar broad substrate specificity. In addition to numerous oligopeptides with different sizes and net charges, hPEPT1 and hPEPT2 can also accommodate peptidomimetics such as β-lactam antibiotics[37, 38] and angiotensin converting enzyme (ACE) inhibitors.[37, 39]

Because of its high level of expression in the intestinal epithelium, high capacity and broad substrate specificity, hPEPT1 is an ideal prodrug target for oral drug delivery. In addition to valacyclovir and valganciclovir, researchers have attempted to improve oral absorption of different poorly absorbed drugs via attachment to amino acids or dipeptides to resemble hPEPT1 substrates, mostly through ester bonds[40-50] and amide bonds.[51-54] Among the successful examples, most of the parent drugs are not structurally similar to amino acids or natural substrates of hPEPT1, and some of them are charged such as alendronate.[52] Therefore, applying the same strategy to the positively-charged guanidino-containing compounds may also be promising. We chose to conjugate the parent compounds with the amino acid promoiety by an ester bond for more predictable activation by hVACVase. Compared to the classic prodrug strategy, this targeted prodrug approach leaves the guanidino group intact, which avoids the complicated activation

process and guarantees good solubility of the prodrug. Also, the activation does not involve toxicity issues because the only activation byproduct is an amino acid.

Human valacyclovirase (hVACVase) as prodrug-activating enzyme

For a prodrug to be successful, it must be metabolized to the active parent drug *in vivo*. In 1995, Burnette[55] *et al* purified and characterized a 29-kDa enzyme from rat liver which activates L-alanine, L-methionine, L-leucine and L-valine ester prodrugs of acyclovir. Although this conversion can be catalyzed either chemically or by other esterases or proteases, the novel enzyme which was named valacyclovirase (VACVase) was believed to play a major role in the activation of valacyclovir, the L-valine ester prodrug of acyclovir. In contrast to α-amino acid esters of acyclovir, esters without a free α-amino group, such as valeryl, 2, 3-dimethylbutyryl, and *N*-formyl-L-valyl esters of acyclovir, as well as *p*-nitrophenyl acetate and *p*-nitrophenyl *N*-*tert*-butoxycarbonyl-L-alaninate are not substrates for VACVase. In addition, neither typical peptidase nor arylamidase activity was detected with di- or tripeptides and aminoacyl-β-naphthylamides. VACVase is expressed in rat liver, where it is most abundant, as well as kidney, lung, stomach, heart, brain and small intestine.

In 2003, our lab identified one of the major enzymes activating valacyclovir in human from Caco-2 cells,[56] and both the precursor protein with a leader sequence of 20 amino acid residues[56] and the mature form (residues 21–274)[57] were overexpressed in *Escherichia coli* and purified. This human valacyclovirase (hVACVase) enzyme was suggested to be the human homologue of rat VACVase, and was previously cloned from breast cancer tissue.[58] hVACVase is widely expressed in a variety of

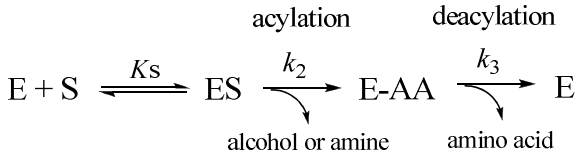
human tissues with highest levels in liver and kidney.[58] It can efficiently metabolize many amino acid ester prodrugs of nucleoside analogues such as floxuridine, gemcitabine, acyclovir, ganciclovir, zidovudine and 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (BDCRB) and seems to prefer amino acids with neutral side chains (valine, phenylalanine and proline) over charged ones (aspartic acid and lysine).[59] In a study of 12 different α-amino acid esters (mostly benzyl esters), hVACVase can accommodate all of them except L-Asn *p*-nitrobenzyl ester and L-Asp benzyl ester.[57] β-Amino acid esters, however, exhibited about 3 orders of magnitude lower activity than corresponding α-amino acid esters. Moreover, when the α-amino group was blocked or substituted by other functional groups (α-hydroxyl or hydrogen), the activity was lost. hVACVase did not exhibit significant hydrolytic activity towards common esterase substrates *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate or amino acid and dipeptide amides of *p*-nitroanilide such as Lys-*p*-NA, Leu-*p*-NA, Pro-*p*-NA, Phe-*p*-NA, Val-*p*-NA and Gly-Pro-*p*-NA. hVACVase activity can be inhibited by serine hydrolase inhibitors DFP and AEBSF as well as a free thiol group modifier, PCMB. Taken together, the substrate specificity of hVACVase was in accordance with rat VACVase and all the studies suggested that hVACVase was a specific α-amino acid ester hydrolyzing enzyme.

Via site-directed mutagenesis, it was confirmed that hVACVase was a serine hydrolase with a catalytic triad S122-H255-D227. The crystal structure of hVACVase binding to a product analogue revealed that the negatively charged side chain of the D123 residue formed an electrostatic interaction with the α-amino group of the product analogue, explaining the importance of the free amino group in the substrate structure.

Because D123 is adjacent to the catalytic serine, the free amino group in the substrate must be close to the carbonyl carbon, which may be the reason why hVACVase is not very active hydrolyzing β-amino acid esters. hVACVase contains a large open leaving group-accommodating groove, which is consistent with the broad specificity of the leaving groups we tested so far, and indicates that hVACVase may be an ideal target for prodrug activation. Also the negative electrostatic potential in the groove suggests that hVACVase may prefer positively charged leaving groups such as guanidino-containing drugs.

hVACVase-catalyzed reaction mechanism and the effect of leaving groups

The proposed mechanism of hVACVase was shown in Scheme 1.3. With the help of H255, the catalytic serine attacks the carbonyl carbon of substrate and forms the first tetrahedral intermediate. Then the leaving group is released from the enzyme and the amino acid will be covalently connected to the catalytic serine. Afterwards, a water molecule serves as a nucleophile, and through another tetrahedral intermediate, the enzyme is regenerated and the final product amino acid is formed. This reaction can also be shown as the following scheme:



The leaving group of the reaction will be corresponding to the drug part of the prodrug. In order to further explore the potential of hVACVase as a prodrug-activating enzyme, the effect of the leaving group on the binding and the reaction rate need to be understood. The rate-limiting step of hVACVase can be either acylation or deacylation,

with the leaving group only affecting the former. When studying the *p*-nitrophenyl acetate hydrolysis by chymotrypsin, Hartley and Kilby[60] observed an initial burst of *p*-nitrophenol, which reflected the much faster acylation step. However, after the reaction reached steady state, the reaction rate was only determined by the slower deacylation step. Instead of trying to observe the burst kinetics, which requires very poor substrates of the enzyme, we studied the kinetics of a series of amino acid analogues to find out (1) the rate-limiting step of hVACVase-catalyzed reaction and the effect of leaving groups on the reaction rate and (2) whether the leaving groups affect the binding to hVACVase. Based on previous studies, amino acid amides are not good substrates of hVACVase, which cannot be well-explained by the reaction mechanism. Since making an amino acid amide bond is a very promising prodrug strategy to increase oral absorption, an explanation on why hVACVase cannot efficiently activate amide prodrugs is crucial.

Specific aims

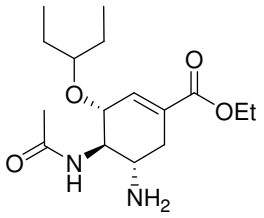
Amino acid ester and amide prodrugs have attracted a great deal of interest for improved oral bioavailability of low-permeability drugs. The purpose of this research was to increase the oral absorption of guanidino-containing compounds, and further understand the mechanism of a prodrug-activating enzyme, eventually expanding the application of the amino acid ester and amide prodrug approach.

There were two specific aims of this study:

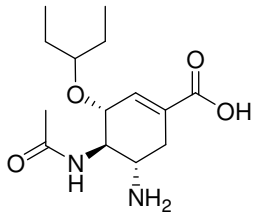
The first specific aim was to apply a transporter- and enzyme-targeted (hPEPT1 and hVACVase) prodrug strategy for poorly absorbed guanidino-containing molecules. A model parent compound and its amino acid esters were used to evaluate the feasibility of

this approach. The second specific aim was to investigate the effect of leaving group on the hVACVase-catalysed prodrug activation by comparing the kinetic data of a series of amino acid analogues.

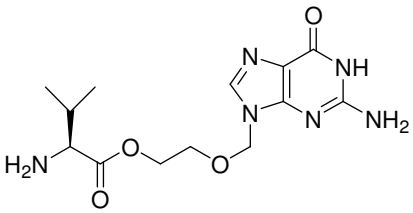
Scheme 1.1. Examples of successful prodrugs and their corresponding parent drugs.



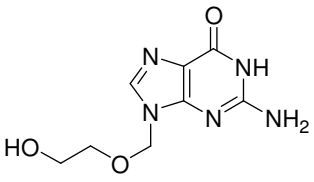
oseltamivir



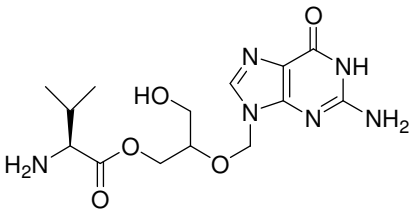
oseltamivir carboxylate



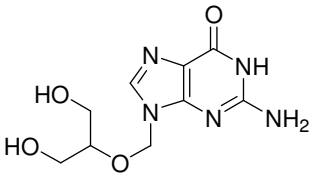
valacyclovir



acyclovir

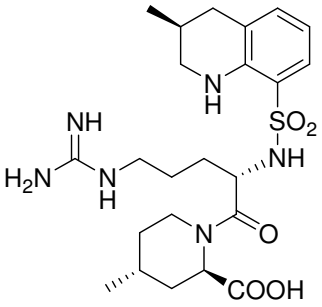


valganciclovir

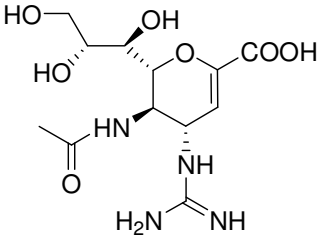


ganciclovir

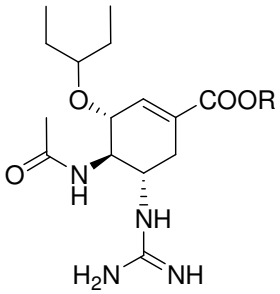
Scheme 1.2. Structures of argatroban, zanamivir, GOCarb and its ethyl ester.



argatroban



zanamivir



GS 4116 (**GOCarb**) R = H
GS 4109 R = CH_2CH_3

Scheme 1.3. Proposed catalytic mechanism of hVACVase.

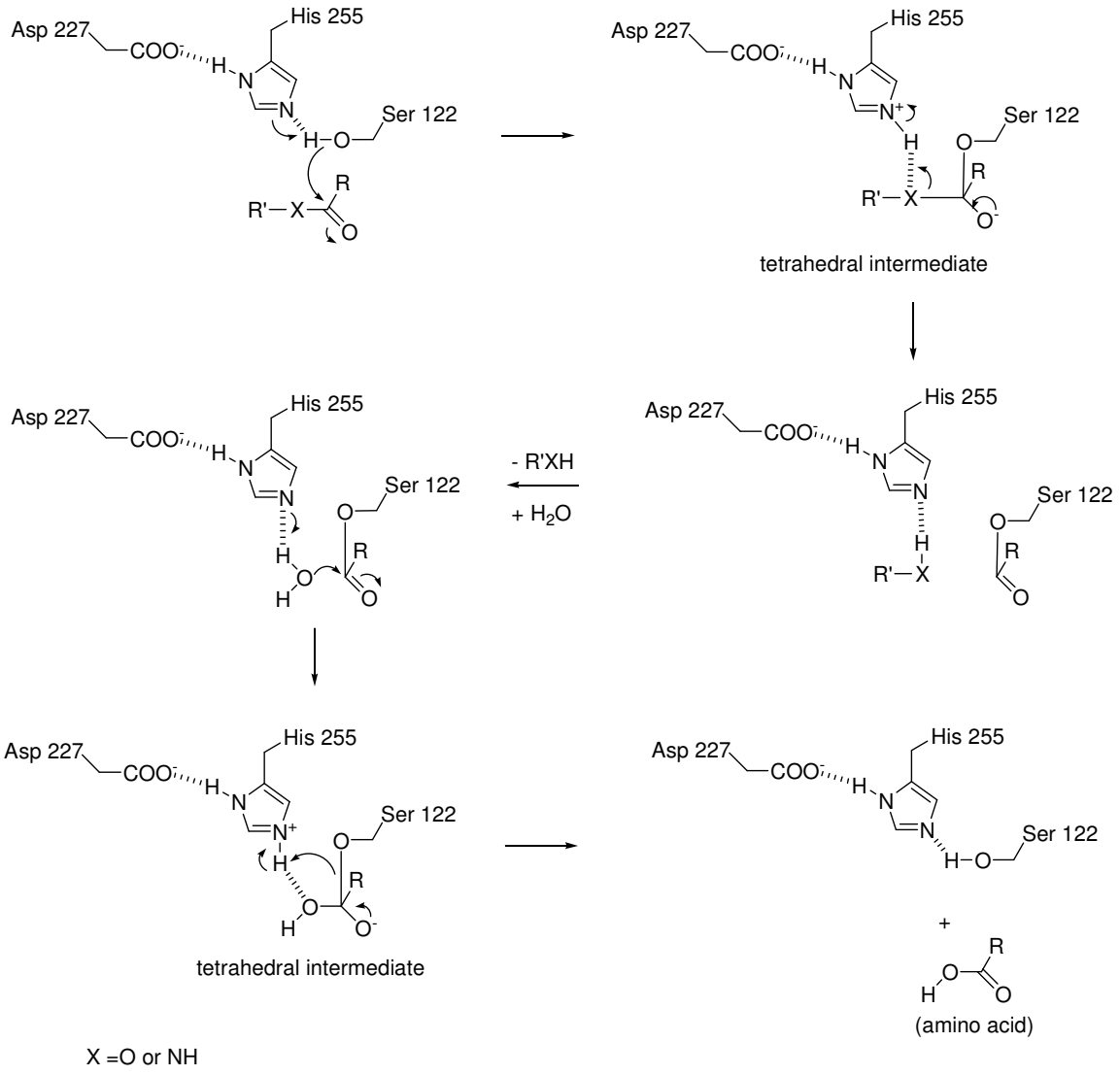


Table 1.1. Plasma membrane transporters that accept arginine as a substrate (adopted from reference [25]).

Protein	Gene	Transport system
CAT-1	SLC7A1	y^+
CAT-2A	SLC7A2	Not defined
CAT-2B	SLC7A2	y^+
CAT-3	SLC7A3	y^+
y^+LAT1 + 4F2hc	SLC7A7 + SLC3A2	y^+L
y^+LAT2 + 4F2hc	SLC7A6 + SLC3A2	y^+L
$b^{0,+}$AT + rBAT	SLC7A9 + SLC3A1	$b^{0,+}$
$ATB^{0,+}$	SLC6A14	$B^{0,+}$

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Chapter 2

A prodrug strategy for molecules containing the polar guanidino functionality

Introduction

Human peptide transporter 1 (hPEPT1), the oligopeptide transporter expressed in the apical brush border membrane of the small intestine, mediates the transport of di- and tripeptides and a variety of peptidomimetics across the intestinal wall.[1, 2] The broad substrate specificity of hPEPT1 makes it an attractive target for prodrug design. By conjugating to an amino acid promoiety, a low permeability drug can be recognized by hPEPT1 and obtain significantly higher oral absorption. As examples, valacyclovir and valganciclovir, the valine ester prodrugs of poorly absorbed antiviral drugs acyclovir and ganciclovir, respectively, exhibit more than 50% oral bioavailability by hPEPT1-mediated membrane transport and extensive activation to their corresponding parent drugs.[3-6] The rapid activation of valacyclovir and valganciclovir is due to the cooperation of chemical hydrolysis and multiple activating enzymes. However, some particular esterase(s), such as a specific amino acid ester prodrug-activating enzyme we identified recently, human valacyclovirase (hVACVase), may be primarily responsible for this activation process.[7] Encouraged by the success of valacyclovir and valganciclovir, the amino acid ester prodrug strategy has also been applied to other poorly absorbed therapeutic agents, especially nucleosides[8] such as zidovudine,[9]

floxuridine, [10-13] gemcitabine,[14] levovirin,[15] and cytarabine.[16] Results suggested that the membrane permeability was increased greatly by targeting hPEPT1, and many of the prodrugs could be activated by hVACVase.[17]

Frequently required for the high potency of drugs and drug candidates, the guanidino functionality is also an obstacle for oral absorption due to polarity and positive charge. Unless paracellular or active transport mechanisms exist, guanidino-containing drugs or drug candidates are expected to have very limited oral absorption, such as thrombin inhibitor argatroban as well as influenza neuraminidase inhibitors zanamivir and guanidine oseltamivir carboxylate (GOCarb). It is well-known that the guanidino group occurs in arginine-containing small peptides, which are natural substrates of hPEPT1 transporter. Although hPEPT1 appears to prefer neutral side chains instead of the charged ones,[1, 2, 18, 19] many arginine-containing small peptides still have at least medium affinity to hPEPT1 transporter and show evidence of transport.[18-21] Therefore, it will be interesting to see if other compounds with the guanidino functionality can also be tolerated by hPEPT1. If successful, the amino acid ester prodrug strategy may be applied to guanidino-containing drugs as long as an ester bond can be made through a hydroxyl group in the drug structure or a linker (Scheme 2.1). For this amino acid ester prodrug strategy, some questions that may arise are: (1) whether the guanidino functionality on the parent drug structure affects recognition and transport by hPEPT1, and (2) if the guanidino functionality plays a negative role, whether it can be compensated by a good amino acid promoiety so the prodrug will be transported.

Another challenge for prodrug design is the activation process; the prodrug form should be retained prior to absorption and be quickly metabolized to the parent

compound following absorption. The classic prodrug strategy suffered from the unpredictable activation of the shielded guanidino functionality. The targeted prodrug strategy, however, circumvents this problem by leaving the guanidino group intact and at the same time, introducing a well-studied and relatively predictable amino acid ester bond. We have found that one of the primary amino acid ester prodrug-activating enzymes is hVACVase,[7] a serine hydrolase containing a catalytic triad S122-H255-D227. The very specific preference for amino acid ester substrates is attributed to the critical residue D123 forming electrostatic interaction with the α-amino group of substrate. hVACVase contains a large leaving group-accommodating groove, which accommodates various leaving groups including nucleoside analogues acyclovir, ganciclovir, floxuridine, gemcitabine, zidovudine and BDCRB, as well as simple alcohols such as methanol, ethanol and benzyl alcohol.[7, 17, 22] In addition, the crystal structure of hVACVase revealed negative electrostatic potential in the groove, which may prefer a positively charged leaving group. Therefore, hVACVase may be a good target for guanidino-containing prodrugs in order to control the activation process and secure the quick release of parent drug *in vivo*.

The specific aim of this chapter was to apply a transporter- and enzyme-targeted (hPEPT1 and hVACVase) prodrug strategy for guanidino-containing molecules. We have designed and synthesized L-valine, L-isoleucine and L-phenylalanine esters of a model parent compound, [3-(hydroxymethyl)phenyl]guanidine (3-HPG, Scheme 2.2). These promoieties were chosen based on affinity to hPEPT1 transporter and stability profiles learned from our previous studies of amino acid nucleoside prodrugs. D-valine ester of 3-HPG was synthesized and used as a control to help determine the transport mechanism.

The different model compounds were tested for hPEPT1-mediated uptake and transport across HeLa/hPEPT1 and Caco-2 cells, intestinal permeability in the rats, and activation by hVACVase. This setup allowed us to evaluate the feasibility of the suggested strategy to improve oral absorption of low permeable guanidino-containing drugs.

Experimental procedures

Materials.

The *tert*-butyloxycarbonyl (Boc) protected amino acids, Boc-L-valine, Boc-L-isoleucine, Boc-L-phenylalanine and Boc-D-valine were obtained from Calbiochem-Novabiochem (San Diego, CA). Valacyclovir was a gift from GlaxoSmithKline, Inc. (Research Triangle Park, NC). High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Fisher Scientific (St. Louis, MO). L-Isoleucine benzyl ester 4-toluenesulfonate salt was obtained from Chem-Impex International, Inc. (Wood Dale, IL). 3-Amino benzyl alcohol, 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl), 4-(dimethylamino)pyridine (DMAP), trifluoroacetic acid (TFA), L-valine benzyl ester hydrochloride, L-phenylalanine benzyl ester hydrochloride, metoprolol, phenol red and all other reagents and solvents were purchased from Sigma-Aldrich Co. (St. Louis, MO). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA), and cell culture supplies were obtained from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). All chemicals were either analytical or HPLC grade.

Synthesis.

NMR spectra were obtained on a Bruker AVANCE DRX500 NMR spectrometer. Electronspray ionization mass spectra were obtained on a Micromass LCT Time-of-Flight mass spectrometer. The purity of all synthesized test compounds was at least 95% as determined by HPLC.

[3-(Hydroxymethyl)phenyl]guanidine (3-HPG, 3). To a stirred solution of 3-amino benzyl alcohol (154 mg, 1.25 mmol) in 1 mL of dry tetrahydrofuran (THF), 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (145 mg, 0.5 mmol) in 1.5 mL of THF was added dropwise, and then temperature was increased to 50°C. After 2 hours, the reaction was stopped and solvents were removed. The residue was dissolved in 30 mL of dichloromethane and washed twice with 10 mL of brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The product **2** was purified by column chromatography (CH_2Cl_2 : MeOH, 60 : 1) as colorless oil. Yield: 82.4%. 119.2 mg of **2** was dissolved in 4 mL of trifluoroacetic acid (TFA):CH_2Cl_2 (1:1) and stirred at room temperature for 5 hours. Then solvents were removed and the residue was dissolved in 0.1% TFA, filtered and lyophilized. The raw product was further purified by semi-prep HPLC to give a colorless oil. Yield: 69.1%. ^{1}H NMR (D_2O) δ 7.40 (1H, dd, J = 7.8 Hz, 7.8 Hz, H-5), 7.29 (1H, d, J = 7.8 Hz, H-4 or H-6), 7.22 (1H, s, H-2), 7.17 (1H, d, J = 7.8 Hz, H-4 or H-6), 4.56 (2H, s, -CH_2OH); ESI-MS: 166.0 $(M+H)^+$.

L-Isoleucine [3-[(aminoiminomethyl)amino]phenyl]methyl ester (Ile-3-HPG, 5b). 33.1 mg of **2** (0.09 mmol), 35.6 mg of Boc-L-isoleucine (0.154 mmol) and 20.5 mg of 4-(dimethylamino)pyridine were dissolved in 1 mL of dry CH_2Cl_2 and stirred in ice bath. 30.5 mg of *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC·HCl,

0.159 mmol) was slowly added to the above solution. The reaction mixture was stirred overnight at room temperature until reaction was complete. The solvents were removed and residue was dissolved in 30 mL of CH_2Cl_2 and washed with 10% (w/v) citric acid, saturated $NaHCO_3$ and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture was then chromatographed on silica gel to obtain **4b** as colorless oil. **4b** was treated with 2.2 mL of TFA:CH_2Cl_2 (1:1.2) under argon for 2 hours. Solvents were removed and residue was dissolved in 0.1% TFA, filtered and lyophilized to give **5b** as white solid. **5a**, **5c** and **5d** were synthesized similarly. Yield of two steps: 70.9%. ^{1}H NMR (methanol-d4) δ 7.53 (1H, dd, J=7.8 Hz, 7.8 Hz, H-5), 7.44 (1H, d, J=7.8 Hz, H-4 or H-6), 7.38 (1H, s, H-2), 7.33 (1H, d, J=7.8 Hz, H-4 or H-6), 5.35 (2H, m, -*CH*$_2$-O-), 4.10 (1H, d, J=3.9 Hz, H-α), 2.02 (1H, m, H-β), 1.51 (1H, m, CH_2- γ), 1.35 (1H, m, CH_2- γ), 0.99 (6H, m, CH_3-γ, CH_3-δ); ESI-MS: 279.1 $(M+H)^+$.

L-Valine [3-[(aminoiminomethyl)amino]phenyl]methyl ester (Val-3-HPG, 5a). Yield of two steps: 39.5%. ^{1}H NMR (methanol-d4) δ 7.53 (1H, dd, J=7.8 Hz, 7.8 Hz, H-5), 7.45 (1H, d, J=7.8 Hz, H-4 or H-6), 7.38 (1H, s, H-2), 7.33 (1H, d, J=7.8 Hz, H-4 or H-6), 5.36 (2H, m, -*CH*$_2$-O-), 4.02 (1H, d, J=4.5 Hz, H-α), 2.32 (1H, m, H-β), 1.07 (6H, m, H-γ); ESI-MS: 265.1 $(M+H)^+$.

L-Phenylalnine [3-[(aminoiminomethyl)amino]phenyl]methyl ester (Phe-3-HPG, 5c). Yield of two steps: 79.7%. ^{1}H NMR (methanol-d4) δ 7.49 (1H, dd, J=7.9 Hz, 7.8 Hz, H-5), 7.20-7.38 (8H, m, aromatic protons), 5.27 (2H, s, -*CH*$_2$-O-), 4.41 (1H, t, J=7.0 Hz, H-α), 3.25 (2H, m, H-β); ESI-MS: 313.1 $(M+H)^+$.

D-Valine [3-[(aminoiminomethyl)amino]phenyl]methyl ester (D-Val-3-HPG, 5d). Yield of two steps: 86.6%. ^{1}H NMR (methanol-d4) δ 7.53 (1H, dd, J=7.8 Hz, 7.8 Hz),

7.44 (1H, d, J=7.8 Hz), 7.38 (1H, s), 7.32 (1H, d, J=7.8 Hz), 5.35 (2H, m), 4.02 (1H, d, J=4.5 Hz), 2.32 (1H, m), 1.06 (6H, m); ESI-MS: 265.2 $(M+H)^+$.

Cell culture.

HeLa cells (passage 17-31) and Caco-2 cells (passage 15-32) from American Type Culture Collection (Rockvile, MD) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1 mmol/L sodium pyruvate and 1% L-glutamine. Cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Hydrolysis in HeLa and Caco-2 cell homogenates.

HeLa cells or Caco-2 cells were washed with 0.15 M NaCl solution and then collected with 10 mM phosphate buffer (pH 7.4). The cell suspension was ultrasonicated in ice bath to extract enzymes. The suspension was spun at 10,000 rpm for 10 minutes at 4°C. Supernatant was placed in a new tube. The concentration of enzyme was determined as protein amount with the Bio-Rad DC assay (Hercules, CA) and adjusted to 500 μg/mL.

Hydrolysis in cell homogenates was determined at 37 ℃. The hydrolysis reaction was initiated by adding 0.75 μL of test compound solution (200 mM in DMSO) to a reaction tube containing 749.25 μL cell homogenates. At various time points (0, 5, 10, 30, 60 and 120 min), 100 μL of the reaction mixture was removed and added to a quenching plate containing 100 μL of 10% TFA (in water) and stored in ice. Following

the collection of all samples, quenching plate was filtered (2,000 rpm, 4 ºC, 10 min). The filtrate was removed and assayed by HPLC.

The apparent first-order degradation rate constants were determined by plotting the natural logarithm of test compound remaining as a function of time. The slopes of these plots equal to negative rate constant (k). The degradation half-lives were then estimated by the equation:

$t_{1/2} = 0.693/k$

Hydrolysis in pH 6.0 uptake buffer and pH 7.4 phosphate buffer.

The hydrolysis in buffer was carried out the same way as above except each reaction tube contains 749.25 μL of uptake buffer (pH 6.0, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaC1_2$, 0.8 mM $MgSO_4$, 5 mM D-glucose, and 25 mM MES) or 10 mM phosphate buffer (pH 7.4), and quenching plate contains 100 μL of 0.1% TFA (in water).

hVACVase-mediated hydrolysis.

hVACVase was overexpressed and purified from *Escherichia coli* as described previously[22]. The protein concentration was determined by Bio-Rad DC assay (Hercules, CA) with bovine serum albumin as a standard. The kinetic parameters of hVACVase-catalyzed hydrolysis were determined as follows. Kinetic measurements were carried out in 50 mM HEPES (pH 7.4) buffer at 37°C. After preincubation of the buffer for 5 min, hVACVase was added, and then the reaction was initiated by the addition of substrate. Aliquots were taken at different time points and quenched by adding to same volume of 10% (v/v) trifluoroacetic acid. Initial velocities were

calculated from the linear time course for the product formation. The kinetic parameters K_m and V_{max} were determined by fitting the initial velocity data to the Michaelis-Menten equation by the nonlinear least-square regression analysis in GraphPad Prism software version 4.01. The k_{cat} value was calculated from V_{max}/[enzyme]$_0$ based on the 28.83-kDa molecular mass of hVACVase. Specific activity of valacyclovir was routinely monitored to normalize active protein concentration.

[^{3}H]Gly-Sar uptake inhibition.

The affinity of test compounds to hPEPT1 transporter was evaluated by measuring their ability to inhibit the uptake of [^{3}H]Gly-Sar, a standard hPEPT1 substrate, in HeLa/hPEPT1 cells. Prior to experiment, HeLa cells were grown in 12-well plates for 5 days and infected with adenovirus containing hPEPT1 3 days before experiment.[23] Cells were washed twice with uptake buffer (pH 6.0, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaC1_2$, 0.8 mM $MgSO_4$, 5 mM D-glucose, and 25 mM MES) and incubated with 10 μM Gly-Sar (9.9 μM Gly-Sar and 0.1 μM [^{3}H]Gly-Sar) and various concentrations (0.05-5 mM) of test compounds in 0.3 mL uptake buffer for 30 min in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C. After 30 min, the donor solutions were aspirated and the cells were washed three times with ice-cold uptake buffer and solubilized with 0.1% SDS. Aliquots of the suspensions were used for scintillation counting (Beckman LS 6000SC). IC_{50} values were determined by fitting the data to the one site competition equation (GraphPad Prism software version 4.01).

Direct uptake.

The carrier-mediated uptake of the test compounds was screened in HeLa/hPEPT1 cells and nomal HeLa cells. Prior to experiment, HeLa cells were grown in 12-well plates for 5 days, and infected with adenovirus containing hPEPT1 3 days before experiment.[23] Cells were washed with uptake buffer (pH 6.0, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaC1_2$, 0.8 mM $MgSO_4$, 5 mM D-glucose, and 25 mM MES) and incubated with 1 mL/well of fresh uptake buffer in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C. After 15 min, uptake buffer was removed and 0.5 mL of freshly prepared test compound solution (1 mM) in uptake buffer was added to each well. The cell plate was incubated at 37°C for 60 min. Then the cells were washed twice with ice-cold Dulbecco's Phosphate Buffered Saline, and collected in 10 mM phosphate buffer (pH 7.4) with cell scrapers. The cell suspension was ultrasonicated in ice bath, and cell lysate was treated with ice-cold trifluoroacetic acid (final concentration of 7%), vortexed, and centrifuged for 10 min at 10,000 rpm. The supernatant was then filtered (0.22 μm) and analyzed by HPLC. Control experiments were performed in normal HeLa cells. The protein amount of each sample was determined with the Bio-Rad DC Protein Assay using bovine serum albumin as a standard.

Caco-2 monolayer permeability studies.

Caco-2 cells were grown on 6-well collagen-coated transwell inserts (Corning, 3.0 μm pore size; area 4.67 cm^2) and permeability studies were performed 22 to 23 days post-seeding. Transepithelial electrical resistance (TEER) was monitored before and after experiment. Each well was rinsed with 1.5 mL of MES buffer (pH 6.0, 140 mM NaCl,

5.4 mM KCl, 1.8 mM $CaC1_2$, 0.8 mM $MgSO_4$, 5 mM D-glucose, and 25 mM MES) at the apical side and 2.5 mL of HEPES buffer (pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaC1_2$, 0.8 mM $MgSO_4$, 5 mM D-glucose, and 25 mM HEPES) at the basolateral side. Then 1.5 mL of MES buffer and 2.5 mL of HEPES buffer were added to the apical side and basolateral side of each well respectively and the plate was incubated in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C for 15 min. Experiments were initiated by replacing the apical buffer with 1.5 mL of 200 μM test compound solution in MES buffer and the basolateral buffer with 2.5 mL of fresh HEPES buffer and incubating the plate at 37 °C. 200 μL aliquots of the basolateral receiver solution were withdrawn at predetermined intervals and replaced with fresh HEPES buffer. The concentrations were determined by HPLC.

The apparent permeability ($P_{app;}$ cm/s) across Caco-2 cell monolayers was calculated using the following equation:

$$P_{app} = (1/C_0A)\ (dQ/dt)$$

Where C_0 is the initial test compound concentration in the donor solution, A is the surface area of the exposed monolayer, and dQ/dt is the steady-state appearance rate of the test compound in the receiver solution.

Rat perfusion studies.

Male albino Wistar rats (Charles River, IN) weighing 250-280 g were used for all perfusion studies. Prior to each experiment, the rats were fasted over night (12-18 h) with free access to water. Animals were randomly assigned to the different experimental groups.

The procedure for the in situ single-pass intestinal perfusion followed previously published reports.[24, 25] Briefly, rats were anesthetized with an intra-muscular injection of 1 mL/kg of ketamine-xylazine solution (9%:1%, respectively) and placed on a heated surface maintained at 37°C (Harvard Apparatus Inc., Holliston, MA). The abdomen was opened by a midline incision of 3-4 cm. A jejunal segment of approximately 10 cm was carefully exposed and cannulated on two ends with flexible PVC tubing (2.29 mm i.d., inlet tube 40 cm, outlet tube 20 cm, Fisher Scientific Inc., Pittsburgh, PA). Care was taken to avoid disturbance of the circulatory system, and the exposed segment was kept moist with 37°C normal saline solution (Hospira, Lake Forest, IL). The isolated segment was rinsed with saline solution in order to clean out any residual debris.

The perfusion buffer (pH 6.5, 10 mM MES, 135 mM NaCl, 5 mM KCl, 0.1 mg/mL phenol red, 0.4 mg/mL metoprolol, and 0.1 mM test compound) was incubated in a 37°C water bath. At the start of the study, perfusion buffer was pumped through the jejunal segment at a flow rate of 0.2 mL/min (Watson Marlow Pumps 323S, Watson-Marlow Bredel Inc, Wilmington, MA). Phenol red was added to the perfusion buffer as a nonabsorbable marker for measuring water flux. Metoprolol was co-perfused as a compound with known permeability that serves as a marker for the integrity of the experiment and as a reference standard for permeability in close proximity to the low/high permeability class boundary.[26] The perfusion buffer was first perfused for 1 h in order to ensure steady state conditions (as also assessed by the inlet over outlet concentration ratio of phenol red which approaches 1 at steady state). Following reaching steady state, samples were taken in 10 min intervals for 1 h (10, 20, 30, 40, 50, and 60 min). All samples including perfusion samples at different time points, original drug

solution, and inlet solution taken at the exit of the syringe were immediately assayed by HPLC. Following the termination of the experiment, the length of the perfused intestinal segment was accurately measured.

The effective permeability (P_{eff}) in the *in situ* rat perfusion experiments was calculated using the following equation:

$$P_{eff} = [-Q \ln (C_{out}/C_{in})'] / 2\pi RL$$

Where Q is the perfusion flow rate (0.2 mL/min), R is radius of the intestine, L is the perfused intestinal lenth, C_{in} and C_{out} are the inlet and outlet solution concentrations, respectively. The latter was corrected by multiplying the outlet concentration with $[\text{phenol red}]_{in}/[\text{phenol red}]_{out}$ in order to account for water flux.

HPLC analysis.

The concentrations of test compounds were determined on a Waters HPLC system (Waters Inc., Milford, MA). The HPLC system consisted of two Waters pumps (Model 515), a Waters auto-sampler (WISP model 712) and a Waters UV detector (996 Photodiode Array Detector). The system was controlled by Waters Millennium 32 software (Version 3.0.1). Samples were resolved in an Agilent ZORBAX Eclipse XDB-C18 column (3.5 µm, 4.6 × 150 mm) equipped with a guard column. The mobile phase consisted of 0.1% (v/v) TFA in milli-Q water (solvent A) and 0.1% (v/v) TFA in acetonitrile (solvent B) with the solvent B gradient changing from 2-30% at a rate of 2%/min during a 20 min run. The retention times for 3-HPG, Val-3-HPG, Ile-3-HPG, Phe-3-HPG, D-Val-3-HPG, acyclovir and valacyclovir were 6.7, 9.7, 11.2, 12.1, 9.7, 4.9

and 7.9 minutes, respectively. The detection wavelength was 235 nm for 3-HPG and its amino acid esters and 254 nm for acyclovir and valacyclovir.

Statistical analysis.

All the [^{3}H]Gly-Sar uptake inhibition and animal experiments were n=4 unless stated otherwise, and all the hydrolysis, direct uptake and Caco-2 permeability experiments were performed in triplicate. The data are presented as mean ± SEM. To determine statistically significant differences among the experimental groups, the two-tailed nonparametric Mann-Whitney U test was used for two-group comparison and one-way analysis of variance followed by Dunnett's test was performed for comparison of several groups against one control group. The difference was termed significant when p-value is smaller than 0.05.

Results

Synthesis of 3-HPG and its valine, isoleucine and phenylalanine esters.

To synthesize 3-HPG, 3-amino benzyl alcohol was treated with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea to convert the amino group to the Boc-protected guanidino group. Followed by cleavage of the Boc group, 3-HPG was obtained as TFA salt (Scheme 2.3).

Amino acid esters were synthesized by coupling intermediate **2** and Boc-protected amino acids followed by deprotection (Scheme 2.3).

Hydrolysis in buffer and cell homogenates.

Hydrolysis studies of 3-HPG and its L-amino acid esters were conducted in buffer and cell homogenates and the estimated half-lives ($t_{1/2}$) obtained from linear regression of pseudo-first-order plots are shown in Table 2.1. The half-life of the reference amino acid ester prodrug valacyclovir is also listed for comparison. In all buffers and cell homogenates, the parent compound 3-HPG exhibited good stability, which makes it an excellent model compound for subsequent studies. All the L-amino acid esters were also stable in pH 6 uptake buffer, and relatively stable in pH 7.4 phosphate buffer. In both HeLa and Caco-2 cell homogenates, the estimated half-lives of L-amino acid esters were much shorter than in pH 7.4 buffer, suggesting predominant contribution of enzymatic hydrolysis.

hVACVase-mediated hydrolysis.

The estimated Michaelis-Menten kinetic parameters of 3-HPG esters as well as valacyclovir are listed in Table 2.2. All L-amino acid esters of 3-HPG showed low K_m values ranging from 9 to 207 μM. Interestingly, the k_{cat} values followed the same order as K_m values (Phe-3-HPG > Val-3-HPG > Ile-3-HPG), so the specificity constant, k_{cat}/K_m, did not differ as much as K_m or k_{cat} value itself. When compared with valacyclovir, Val-3-HPG exhibited lower, but not significantly different K_m value. All the 3-HPG esters showed higher specificity constant than valacyclovir, clearly indicating that they are good substrates of hVACVase.

[^{3}H]Gly-Sar uptake inhibition.

The IC_{50} values of 3-HPG, its valine, isoleucine and phenylalanine esters to inhibit [^{3}H]Gly-Sar uptake in HeLa/hPEPT1 cells are listed in Table 2.3. The amino acid benzyl esters were used for comparison to obtain the contribution of the guanidino group. Valacyclovir, a known substrate of hPEPT1, was used as positive control.

The parent compound 3-HPG showed high IC_{50} value in [^{3}H]Gly-Sar uptake inhibition study. Valine and isoleucine esters of 3-HPG, however, exhibited lower IC_{50} values than valacyclovir, indicating good affinity to the hPEPT1 transporter. Phe-3-HPG also showed some affinity to hPEPT1, although it was not as good as valacyclovir. Comparing with the corresponding benzyl esters, all the 3-HPG esters showed similar or higher affinity to hPEPT1.

Direct uptake.

The results of direct uptake study in HeLa/hPEPT1 cells and normal HeLa cells are summarized in Table 2.4. The parent compound 3-HPG, its valine and isoleucine esters and valacyclovir were very stable in donor solution (> 95% remaining after 1 h). Phe-3-HPG was also relatively stable (> 80% remaining after 1h). Valacyclovir, a known substrate of hPEPT1 transporter, showed more than 25-fold higher uptake in HeLa/hPEPT1 cells compared to that in normal HeLa cells. The 3-HPG esters displayed 8- to more than 25-fold increased uptake in HeLa/hPEPT1 cells compared with in normal HeLa cells, whereas 3-HPG only showed little enhancement. In HeLa/hPEPT1 cells, the uptake of the amino acid esters was 2.6- to 9-fold higher than 3-HPG.

Caco-2 monolayer permeability studies.

The permeability of 3-HPG and its amino acid esters across Caco-2 monolayers is shown in Figure 2.1. Val-3-HPG and Ile-3-HPG exhibited higher permeability than the parent compound 3-HPG and the permeability of Val-3-HPG was comparable to valacyclovir. Because both Val-3-HPG and Ile-3-HPG were stable in the donor solution (Table 2.5), and only the parent compound was detected in the receiver solution, most of the esters appeared to be metabolized in the cells prior to penetration of the basolateral membrane. Phe-3-HPG did not show significantly different permeability than 3-HPG. However, due to its low stability in the donor solution (Table 2.5), the permeability may be underestimated. The stability issues, the lower affinity to hPEPT1 transporter and uptake in HeLa/hPEPT1 cells indicated that Phe-3-HPG was not a favorable candidate for *in vivo* studies.

In contrast to Val-3-HPG, no transport of D-Val-3-HPG was detected. Since D-Val-3-HPG and Val-3-HPG are enantiomers, they should have similar permeability if the transport mechanism is predominantly passive diffusion. The much higher permeability of Val-3-HPG suggested that the transport was carrier-mediated. To identify the transporter(s) involved in Val-3-HPG transport, we also investigated the effect of inhibitors on Val-3-HPG permeability, including hPEPT1 inhibitors Gly-Sar and cephalexin, amino acid transporter inhibitors L-valine and L-arginine, and organic cation transporter (OCT) inhibitor tetraethylammonium (TEA). Only hPEPT1 inhibitors reduced Val-3-HPG permeability, suggesting hPEPT1 playing a dominant role in Val-3-HPG transport (Figure 2.2).

Rat perfusion studies.

The effective permeability (P_{eff}) of 3-HPG, Val-3-HPG and Ile-3-HPG in rat jejunum perfusion study is shown in Figure 2.3. Metoprolol was co-perfused with the test compounds as a reference standard for permeability in close proximity to the low/high permeability class boundary. It can be seen that Val-3-HPG and Ile-3-HPG exhibited higher or similar permeability compared to metoprolol, respectively, whereas the permeability of 3-HPG was very low. Hence, the amino acid ester prodrug approach significantly improved the rat jejunal permeability of 3-HPG, bringing it to high-permeability level.

Discussion

The positively charged guanidino functionality provides favorable interactions with important drug targets such as thrombin and influenza neuraminidase. However, the permanent positive charge at all relevant pHs can also cause low membrane permeability and poor oral bioavailability. To overcome this drug delivery barrier, we have utilized a targeted prodrug strategy, aiming to exploit hPEPT1 as the prodrug transport carrier and hVACVase as the predominant activating enzyme. This prodrug approach may have the advantages of higher transport accompanied by more predictable activation, leading to favorable properties for oral delivery.

In order to increase oral bioavailability of low permeability drugs, different classes of amino acids, including acidic, basic, hydrophobic and aromatic, were used as promoieties targeting drugs to oligopeptide transporters such as hPEPT1 and intracellular activating enzymes such as hVACVase.[9-17] Among all the amino acid ester prodrugs,

valine, isoleucine and phenylalanine derivatives were frequently studied. Valine and isoleucine ester prodrugs showed consistently high affinity to hPEPT1 transporter and enhanced drug permeability in both cell culture and animal models. In addition, the success of valacyclovir and valganciclovir suggested that valine may be one of the safest choices as promoiety for a new compound. Phenylalanine esters of floxuridine and gemcitabine also showed good affinity to hPEPT1 transporter and significantly increased the uptake in hPEPT1 overexpressed HeLa cells or transport in Caco-2 cells compared with the parent drugs. However, the phenylalanine esters of levovirin and cytarabine didn't exhibit substantial improvement for Caco-2 permeability and absorption in rats. Most of the valine, isoleucine and phenylalanine ester prodrugs showed good chemical stability and rapid enzymatic activation, and both valine and phenylalanine promoieties were proven to be preferred by hVACVase. Isoleucine esters were not studied for hVACVase-catalysed activation; however, the effective activation of isoleucine ester prodrugs suggested that it may also be a good hVACVase substrate. In order to rule out the negative effects of amino acid side chains, only the well-studied and most promising amino acid promoieties, valine, isoleucine and phenylalanine, were used in this study.

A primary concern of this research was whether the guanidino functionality can be tolerated and transported by hPEPT1 transporter. In [^{3}H]Gly-Sar uptake inhibition studies, both the valine and isoleucine esters of 3-HPG showed high affinity to hPEPT1, while the comparison between benzyl esters and 3-HPG esters did not show significant difference. Therefore, at least in the framework of this research, there was no evidence that the guanidino functionality hindered the binding to hPEPT1 transporter. The results in direct uptake, Caco-2 monolayer permeability and rat perfusion studies further support

our hypothesis that the amino acid esters of 3-HPG can be transported by hPEPT1 for improved permeability. In normal HeLa cells, the uptake of the amino acid esters was even lower than the parent compound 3-HPG. However, in HeLa/hPEPT1 cells, the amino acid esters showed 2.6- to 9-fold higher uptake than 3-HPG, which can be attributed to hPEPT1-mediated transport. In the same fashion, both valine and isoleucine esters of 3-HPG exhibited considerably higher permeability across Caco-2 monolayers than the parent compound. The low permeability of D-Val-3-HPG and the studies with inhibitors clearly demonstrate that this enhancement can be attributed to hPEPT1. In rat perfusion studies, the effective permeability of Val-3-HPG and Ile-3-HPG exceeded or matched the high-permeability drug metoprolol, indicating high intestinal permeability. Taken together, these results demonstrate that it is feasible to target guanidino-containing compounds to hPEPT1 transporter for enhanced oral absorption. Up to now, the amino acid ester prodrug strategy has mainly been limited to nucleoside analogues. The positive results with the guanidino functionality illustrated great promise to expand this strategy to other polar and charged low-permeability drugs.

In addition to the guanidino functionality, this study provided more insights into the influence of amino acid promoieties on the hPEPT1-mediated transport. Among the three amino acid esters, valine ester exhibited the highest permeability in both cell culture and rat perfusion model, which is consistent with 5'-amino acid ester prodrugs of floxuridine,[10] gemcitabine,[14] levovirin,[15] and cytarabine.[16] The phenylalanine ester exhibited the lowest affinity to hPEPT1, smallest uptake enhancement and no significant improvement in permeability studies. However, the poor stability in the donor solution of Caco-2 monolayer (Table 2.5) inevitably leads to underestimation of the

permeablility. This is also the case with 5'-phenylalanine ester of levovirin,[15] where the low Caco-2 permeability accompanied low apical stability. Indeed, the 5'-phenylalanine ester prodrugs of floxuridine and gemcitabine exhibited enhanced uptake and permeability compared to corresponding parent drugs. Therefore, phenylalanine should not be excluded as a promising promoiety for hPEPT1 targeting. However, when phenylalanine ester prodrugs are designed, attention must be paid to make sure the prodrug is relatively stable in the brush border membrane to realize their full potential.

When utilizing the prodrug approach to increase oral absorption, the activation mechanism is often overlooked. As long as the parent drug can be regenerated, the activation will be considered successful, and the enzymes responsible for the prodrug activation may not capture much further attention. However, the activation process is actually the unique and one of the most critical steps for a prodrug to exert therapeutic effect. If the possible activating enzymes are identified, prodrugs can be designed to target these enzymes, which greatly increase the chances for the effective production of the parent drug. Although the amino acid ester prodrug strategy has been applied to many nucleoside analogues and improved the oral absorption by targeting hPEPT1, the activation was not well-studied and was considered nonspecific until the identification of the amino acid ester prodrug-activating enzyme hVACVase.[7] hVACVase is at least one of the primary enzymes activating valacyclovir *in vivo* and is capable of activating many other amino acid ester prodrugs of nucleoside analogues.[17] The large leaving group-accommodating groove of hVACVase makes it an ideal target for prodrug design. The data presented in this research demonstrate that a positively charged leaving group, 3-HPG, can be well tolerated by hVACVase, although whether the guanidino functionality

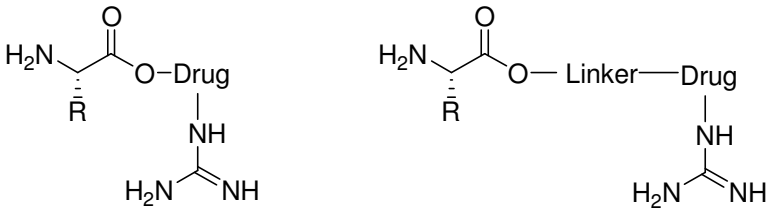
helped the binding is still unclear. The high specificity constant (k_{cat}/K_m) of all the L-amino acid esters of 3-HPG is consistent with their fast activation in Caco-2 cell homogenates as well as Caco-2 monolayer permeability studies. Therefore, hVACVase can be exploited as the target for activating amino acid ester prodrugs of guanidino-containing drugs. At the same time, the possibility that other enzymes can also contribute to the activation of amino acid ester prodrugs cannot be ruled out. We have estimated the hydrolysis rate of 3-HPG esters in Caco-2 cell homogenates with the kinetic data in hVACVase, based on the assumption that hVACVase was the predominant enzyme activating valacyclovir. After comparison with the hydrolysis data in Caco-2 homogenates, it turns out that hVACVase was also the primary enzyme responsible for Val-3-HPG and Ile-3-HPG hydrolysis, whereas the hydrolysis of Phe-3-HPG may involve other enzymes.

Because of the success with the model compound 3-HPG, Sheeba Varghese Gupta and Deepak Gupta *et al* applied this prodrug approach to the poorly absorbed anti-influenza agents zanamivir and guanidine oseltamivir carboxylate (GOCarb). By using an acyloxyester linker, valine and isoleucine were covalently connected to zanamivir and GOCarb (Scheme 2.4). In Caco-2 cells, all the prodrugs showed affinity to hPEPT1 transporter by inhibiting the uptake of hPEPT1 substrate [^{3}H]Gly-Sar, and the IC_{50} values of GOC-Val and GOC-Ile were lower than valacyclovir. On the other hand, the parent drugs zanamivir and GOCarb didn't show much inhibition. In HeLa/hPEPT1 cells, Zan-Val, GOC-Val and GOC-Ile exhibited elevated uptake (2-6 fold) than in normal HeLa cells. In Caco-2 cell transport study, the permeability of all the prodrugs were higher than corresponding parent drugs (2-12 fold). Most importantly, Zan-Val and GOC-Val showed

at least similar rat jejunal permeability to metoprolol, suggesting high oral absorption. All the prodrugs were rapidly metabolized in Caco-2 cell homogenates presumably by hVACVase and other activating enzymes. These results further confirmed the feasibility of this transporter- and enzyme-targeted prodrug approach for improved oral absorption of guanidino-containing drugs. (All the data in this paragraph came from unpublished manuscripts prepared by Sheeba Varghese Gupta and Deepak Gupta *et al*.)

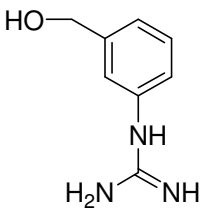
In summary, the amino acid esters of a guanidino-containing model compound exhibited high affinity to hPEPT1 transporter, leading to enhanced intestinal permeability accompanied by hVACVase-mediated activation to the parent compound. Combined with the study of zanamivir and GOCarb prodrugs, the results suggested that it is feasible to apply the same strategy to other poorly absorbed guanidino analogues. This transporter- and enzyme-targeted approach makes the prodrug design more rational and less empirical, with the ability to mechanistically control the two most important aspects governing prodrug success.

Scheme 2.1. Proposed prodrug structures of guanidino-containing drugs.

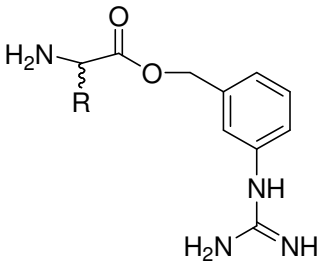


R = amino acid side chain

Scheme 2.2. Structures of 3-HPG and its L-valine, L-isoleucine, L-phenylalanine and D-valine esters.

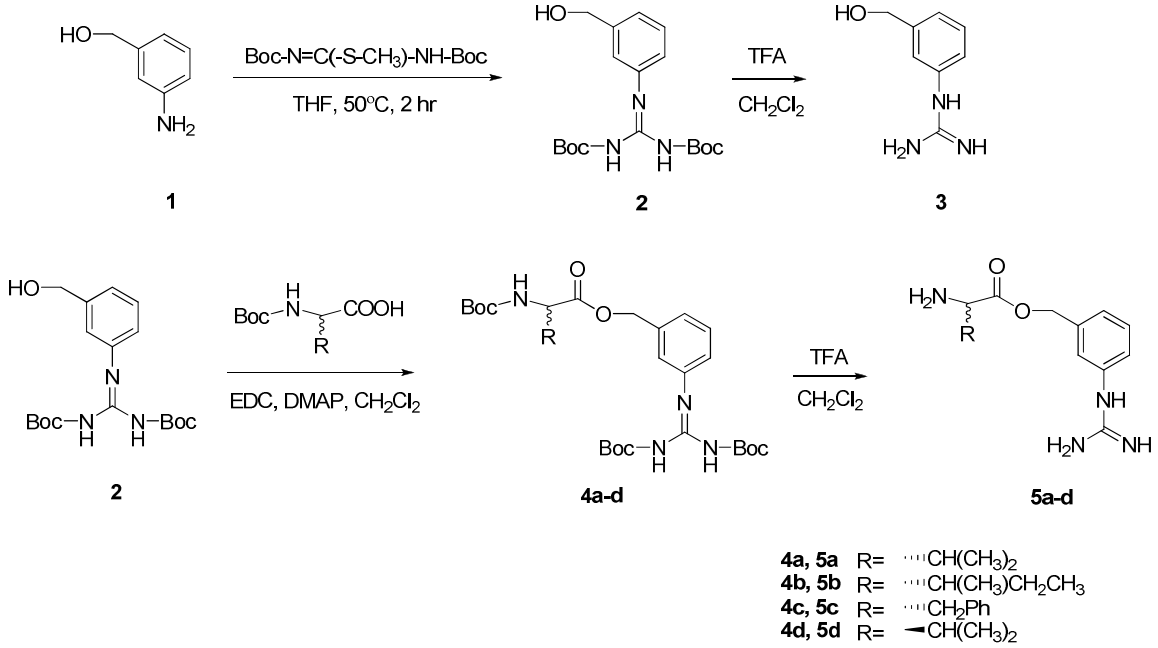


3-HPG (**3**)

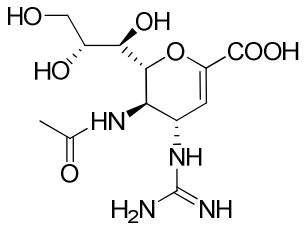


		R
Val-3-HPG	(**5a**)	····CH(CH$_3$)$_2$
Ile-3-HPG	(**5b**)	····CH(CH$_3$)CH$_2$CH$_3$
Phe-3-HPG	(**5c**)	····CH$_2$Ph
D-Val-3-HPG	(**5d**)	◄CH(CH$_3$)$_2$

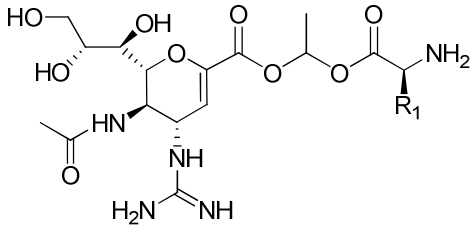
Scheme 2.3. Synthesis of 3-HPG and its valine, isoleucine, phenylalanine and D-valine esters.



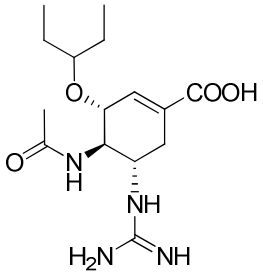
Scheme 2.4. Structures of zanamivir, GOCarb and their prodrugs.



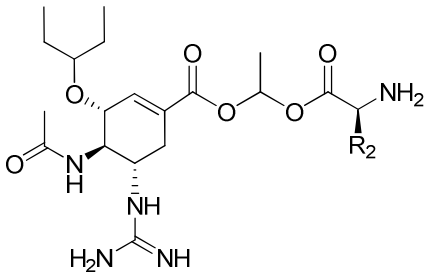
zanamivir



Zan-Val $R_1 =$ $-CH_2(CH_3)_2$
Zan-Ile $R_1 =$ $-CH(CH_3)CH_2CH_3$



GOCarb



GOC-Val $R_2 =$ $-CH_2(CH_3)_2$
GOC-Ile $R_2 =$ $-CH(CH_3)CH_2CH_3$

Table 2.1. Estimated half-lives in pH 6 uptake buffer, pH 7.4 phosphate buffer, HeLa cell homogenates and Caco-2 cell homogenates (mean ± SEM; n=3).

	Half-lives (min)			
	pH 6 buffer	pH 7.4 buffer	HeLa cell homogenates	Caco-2 cell homogenates
3-HPG	stable*	stable*	stable*	stable*
Val-3-HPG	stable*	493.6 ± 10.4	20.3 ± 0.7	4.8 ± 0.2
Ile-3-HPG	stable*	704.8 ± 9.7	137.2 ± 3.6	59.6 ± 2.3
Phe-3-HPG	stable**	261.8 ± 5.0	<< 5	<< 1
valacyclovir	stable*	829.4 ± 25.0	142.2 ± 1.2	31.3 ± 0.8

**no area decrease detected in HPLC after 2 h **less than 5% decrease after 2 h*

Table 2.2. Michaelis-Menten kinetic parameters of 3-HPG esters and valacyclovir for hVACVase-mediated hydrolysis (mean ± SEM; n=3).

	K_m (μM)	V_{max} (nmol/min/μg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Val-3-HPG	46 ± 5	321 ± 9	154 ± 4	3370 ± 460
Ile-3-HPG	9.0 ± 1.2	30 ± 1	14 ± 1	1580 ± 260
Phe-3-HPG	207 ± 16	718 ± 16	345 ± 8	1660 ± 160
valacyclovir	68 ± 4	120 ± 2	58 ± 1	850 ± 66

Table 2.3. [^{3}H]Gly-Sar uptake inhibition in HeLa/hPEPT1 cells (mean ± SEM; valacyclovir, n=6; others n=4).

	IC_{50} (mM)
3-HPG	>3
Val-3-HPG	0.65 ± 0.04
Val-OBz	0.39 ± 0.04
Ile-3-HPG	0.63 ± 0.04
Ile-OBz	1.05 ± 0.13
Phe-3-HPG	2.33 ± 0.45
Phe-OBz	> 3
valacyclovir	1.42 ± 0.18

Table 2.4. Direct uptake and stability in HeLa/hPEPT1 and normal HeLa cells (mean ± SEM; n = 3).

	HeLa/hPEPT1 cells		HeLa cells		
	uptake (nmol/mg in 60 min)	stability* (%)	uptake (nmol/mg in 60 min)	stability* (%)	hPEPT1/control
3-HPG	5.5 ± 0.6	-	3.8 ± 0.1	-	1.4 ± 0.2
Val-3-HPG	50.2 ± 1.6	96.9 ± 0.0	< 2	98.0 ± 0.1	> 25
Ile-3-HPG	27.7 ± 1.4	98.7 ± 0.0	< 2	99.0 ± 0.1	>13
Phe-3-HPG	14.8 ± 1.7	83.8 ± 0.3	1.7 ± 0.2	87.2 ± 0.9	8.8 ± 2.0
valacyclovir	5.5 ± 0.3	96.8 ± 0.1	< 0.2	99.2 ± 0.0	> 25

**percentage of ester form in donor solution at 60 min*

Table 2.5. Stability in apical side during permeability study across Caco-2 monolayers (mean ± SEM; n = 3).

	Stability* (%)
3-HPG	-
Val-3-HPG	86.5 ± 0.3
Ile-3-HPG	96.6 ± 0.6
Phe-3-HPG	3.9 ± 2.0
D-Val-3-HPG	98.8 ± 0.1
valacyclovir	94.7 ± 0.3

** percentage of ester form in donor solution at 120 min*

Figure 2.1. Apparent apical-to-basolateral permeability coefficient (P_{app}) across Caco-2 monolayers (mean ± SEM; n=3; *difference is statistically significant).

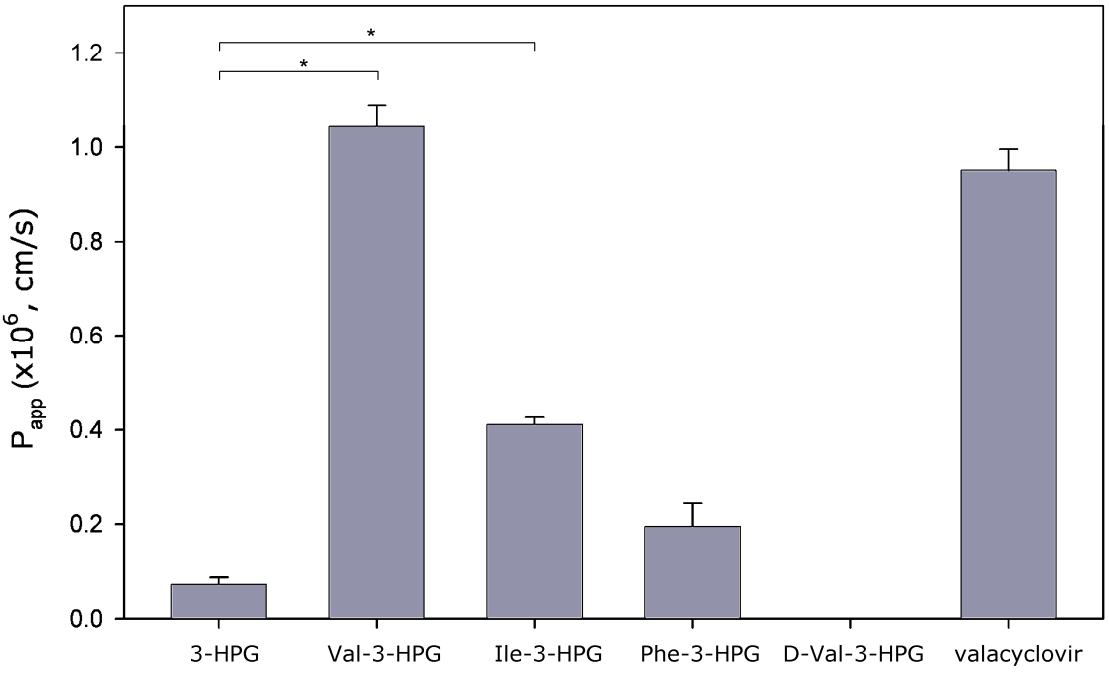


Figure 2.2. Apparent apical-to-basolateral permeability coefficient (P_{app}) of Val-3-HPG without or with 5 mM inhibitors across Caco-2 monolayers (mean ± SEM; n=3; * difference is statistically significant).

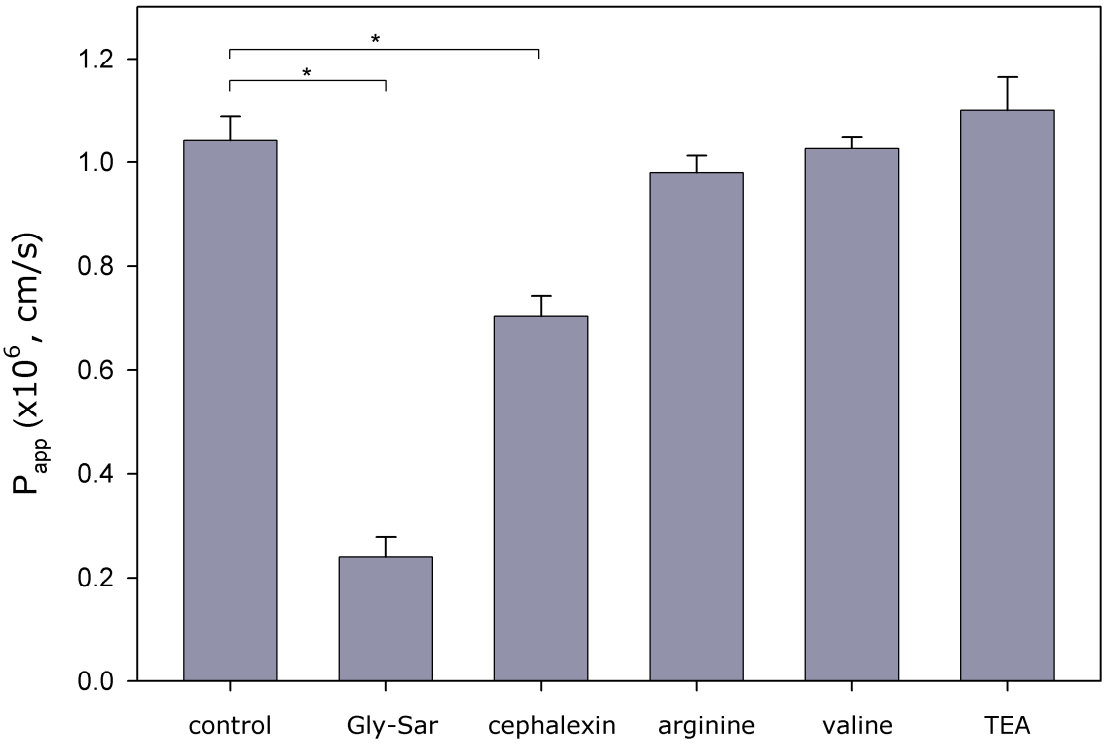
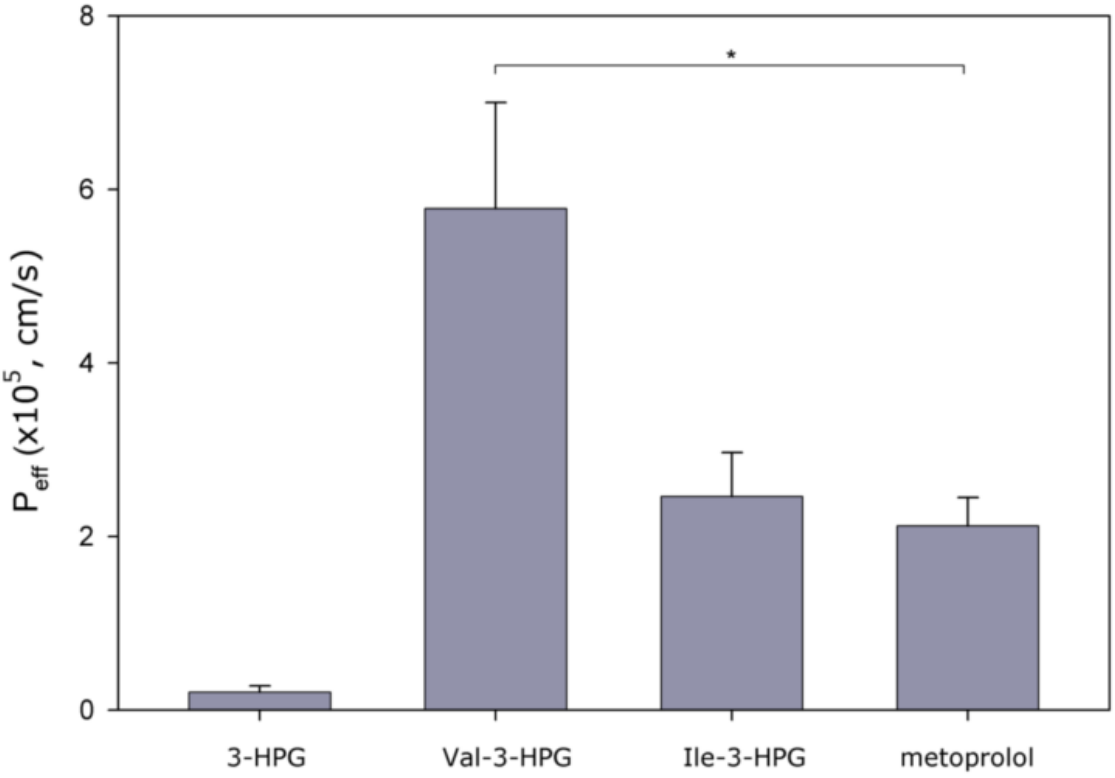


Figure 2.3. Rat jejunal membrane permeability (mean ± SEM; metoprolol, n=12; others, n=4; *difference is statistically significant).



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Notes:

Majority of the work in this chapter has been published entitled "Enhancing the intestinal absorption of molecules containing the polar guanidino functionality: a double-targeted prodrug approach". (Sun, J., Dahan, A., Amidon, GL. J Med Chem, 2010. **53**(2), p. 624-632.)

Chapter 3

The effect of leaving groups on hVACVase-catalyzed prodrug activation

Introduction

Amino acid ester or amide prodrug strategy is a successful approach to improve oral absorption of low-permeability drugs by targeting oligopeptide transporters.[1-6] Following absorption, the prodrug needs to be metabolized chemically or enzymatically to the active parent drug. We have identified an amino acid ester prodrug-activating enzyme, hVACVase,[7] which is believed to be one of the major enzymes for the activation of valine ester prodrugs valacyclovir and valganciclovir. hVACVase also effectively hydrolyzes many other amino acid esters of nucleoside analogues and simple alcohols.[8, 9] Its potential to serve as a universal prodrug-activating enzyme for amino acid ester and amide prodrugs made hVACVase an interesting research subject.

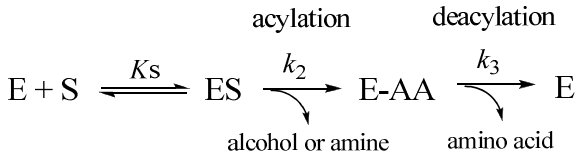
hVACVase is a serine hydrolase with a catalytic triad S122-H255-D227. The very specific preference for amino acid analogues is due to the electrostatic interaction between the critical residue D123 and the free amino group of the substrate. Based on our previous kinetic studies, the amino acid side chain of the substrate has a significant effect on both the K_m and V_{max} values. As an example, the K_m values (in mM) and V_{max} values (in nmol/min/μg) of a series of floxuridine (FUdR) prodrugs are proline-FUdR 1.45, 556, valine-FUdR 0.20, 148 and phenylalanine-FUdR 0.63, 643, respectively.[8] In a study of

9 different amino acid benzyl esters, the specific activities of hVACVase-catalyzed activation range from 0 to 5148 units/mg.[9] The effect of amino acid promoieties on the hVACVase-catalyzed hydrolysis is able to aid the prediction of the prodrug activation and the rational design of new prodrugs.

Apart from the free amino group and amino acid side chain, the effect of leaving groups is also essential. Currently many amino acid ester prodrugs were proved to be hVACVase substrates and the leaving groups include nucleoside analogues acyclovir, ganciclovir, floxuridine, gemcitabine, zidovudine and BDCRB, as well as simple alcohols such as methanol, ethanol and benzyl alcohol.[8, 9] This is consistent with the large leaving group-accommodating groove revealed by the crystal structure. However, although the phenylalanine benzyl and ethyl esters are hVACVase substrates (specific activities are 358.3 and 75.3 units/mg, respectively), phenylalanine *t*-butyl ester is not a substrate.[9] Also, the valine 5'-floxuridine has a higher V_{max} value compared with valine 3'-floxuridine ester (V_{max} values in nmol/min/μg are 148 and 33, respectively).[8] Those results suggested that the alcohol leaving groups may have an effect on hVACVase specificity. Also, hVACVase did not exhibit significant hydrolytic activity towards a series of amides, including Lys-*p*-NA, Leu-*p*-NA, Pro-*p*-NA, Phe-*p*-NA, Val-*p*-NA and Gly-Pro-*p*-NA.[8] This may be due to the much more stable amide bond compared with the ester bond, suggesting hVACVase may not be a good activating enzyme for amide prodrugs. The ten-fold different K_m values of prodrugs valacyclovir and valganciclovir (0.19 and 1.90 mM, respectively) indicated that the leaving group may also affect binding affinity.[7] To better understand the hVACVase-catalyzed prodrug activation and guide

future prodrug design, a more careful investigation of the leaving group effect will be crucial.

As a serine hydrolase, the hydrolysis catalyzed by hVACVase is presumably a three-step reaction as shown in the following scheme:[10]



For this kinetic scheme the Michaelis-Menten equation can be expressed as:

$$v = [\mathrm{E}]_0\,[\mathrm{S}]\frac{\dfrac{k_2 k_3}{k_2 + k_3}}{\dfrac{K_\mathrm{s} k_3}{k_2 + k_3} + [S]}$$

in which

$$K_\mathrm{m} = K_\mathrm{s}\frac{k_3}{k_2 + k_3}$$

and

$$k_\mathrm{cat} = \frac{k_2 k_3}{k_2 + k_3}$$

It can be seen that the effect of the leaving groups relies on the rate-limiting step, which is either acylation or deacylation. Because the leaving group (alcohol or amine) is released from the enzyme in the acylation step, it will only have an effect on the k_2 value. If the acylation step is rate-limiting ($k_2 << k_3$), the k_cat value will be affected by the leaving group, and K_m value will approach K_s. If the deacylation step is rate-limiting ($k_2 >> k_3$), the k_cat value will be independent of the leaving group, and K_m value will be a function of K_s, k_2 and k_3.

The specific aim of this chapter was to investigate the effect of leaving groups on the hVACVase-catalysed prodrug activation. We have determined the kinetic parameters of a series of valine esters (Val-3-HPG, valacyclovir, valine benzyl ester, valine *p*-nitrobenzyl ester), phenylalanine esters (Phe-3-HPG, phenylalanine benzyl ester, phenylalanine ethyl ester, phenylalanine methyl ester) and valine amide of [3-(aminomethyl)phenyl]guanidine, Val-3-APG (Scheme 3.1 and 3.2). This approach allowed us to determine the rate-limiting step of hVACVase-catalyzed hydrolysis and the effect of leaving groups on the binding affinity and specific activity, and further lead to the more rational design of the amino acid ester and amide prodrugs.

Experimental procedures

Materials.

Boc-L-valine and *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were obtained from Calbiochem-Novabiochem (San Diego, CA). Valacyclovir was a gift from GlaxoSmithKline, Inc. (Research Triangle Park, NC). L-Valine 4-nitrobenzyl ester hydrobromide was obtained from Chem-Impex International, Inc. (Wood Dale, IL). High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Fisher Scientific (St. Louis, MO). 3-Aminobenzonitrile, 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea, mercury(II) chloride, Pd/C, 2M NH_3/EtOH, trifluoroacetic acid (TFA), *N,N*-diisopropylethylamine, L-valine benzyl ester hydrochloride, L-phenylalanine benzyl ester hydrochloride, L-phenylalanine methyl ester hydrochloride, L-phenylalanine ethyl ester hydrochloride, and all other reagents and solvents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Cell culture reagents were obtained from Invitrogen (Carlsbad, CA), and cell culture supplies were obtained from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). All chemicals were either analytical or HPLC grade.

Synthesis.

3-[[Bis[[(1,1-dimethylethoxy)carbonyl]amino]methylene]amino]benzonitrile (7).

To a stirred suspension of 3-aminobenzonitrile (118 mg, 1.0 mmol), 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (290 mg, 1 mmol) and mercury(II) chloride (353 mg, 1.3 mmol) in 5 mL of dry tetrahydrofuran (THF), 405 mg (4.0 mmol) of triethylamine was added dropwise under argon. After 2 hours, the reaction was stopped and reaction mixture was diluted with ethyl acetate and filtered through Celite®. Solvents were removed and the residue was dissolved in 40 mL of ethyl acetate and washed with 10% w/v citric acid, saturated $NaHCO_3$ and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Product **7** was purified by column chromatography (hexanes : ethyl acetate, 20 : 1) to give 327.9 mg white powder. Yield: 91.0%. ^{1}H NMR ($CDCl_3$) δ 11.65 (1H, br), 10.55 (1H, br), 8.11 (1H, s), 7.77 (1H, d, J = 7.8 Hz), 7.42 (2H, m), 1.56 (9H, s), 1.54 (9H, s); ESI-MS: 383.1 $(M+Na)^+$.

3-[[Bis[[(1,1-dimethylethoxy)carbonyl]amino]methylene]amino]benzylamine (8).

A mixture of **7** (150 mg, 0.416 mmol) and Pd/C (10 wt % palladium on activated carbon, 150 mg) in 100 mL of 2M NH_3/EtOH was hydrogenated in a Parr apparatus at 45 psi and room temperature for 9 h. The reaction mixture was filtered through Celite®. The solvents were removed and product was purified by column chromatography (dichloromethane : methanol, 15 : 1) to give 80.0 mg light yellow gum. Yield: 52.7%. ^{1}H NMR ($CDCl_3$) δ

11.65 (1H, br), 10.37 (1H, br), 7.56 (1H, s), 7.52 (1H, d, J = 7.8 Hz), 7.32 (1H, dd, J = 7.8 Hz, 7.8 Hz), 7.10 (1H, d, J = 7.8 Hz), 3.90 (2H, s), 1.56 (9H, s), 1.53 (9H, s); ESI-MS: 365.2 $(M+H)^+$.

[3-(Aminomethyl)phenyl]guanidine (3-APG, 9).

24.8 mg of **8** was dissolved in 2.2 mL of trifluoroacetic acid (TFA):CH_2Cl_2 (1:1.2) and stirred at room temperature for 2 hours. Then solvents were removed and the residue was dissolved in 0.1 % TFA, filtered and lyophilized. The raw product was further purified by semi-prep HPLC to give 15.4 mg white solid. Yield: 57.7%. ^{1}H NMR (CD3OD) δ 7.57 (1H, dd, J = 7.9 Hz, 7.9 Hz), 7.44 (2H, m), 7.36 (1H, d, J = 7.9 Hz), 4.18 (2H, s); ESI-MS: 165.1 $(M+H)^+$.

(2S)-2-Amino-3-methyl-*N*-[3-[(aminoiminomethyl)amino]phenyl]methylbutanamide (Val-3-APG, 11).

To a stirred solution of **8** (12.6 mg, 0.035 mmol), Boc-L-valine (9.1 mg, 0.042 mmol) and *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 16.0 mg, 0.042 mmol) in 1 mL of anhydrous CH_2Cl_2, 18 µl of *N,N*-diisopropylethylamine were added dropwise under argon. The reaction mixture was stirred at ambient temperature for 3 h and then the solvents were removed. The residue was dissolved in 30 mL of CH_2Cl_2 and washed with 10% w/v citric acid, saturated $NaHCO_3$ and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The mixture was then chromatographed on silica gel (hexanes : ethyl acetate, 5 : 1) to obtain **10** as colorless oil. ^{1}H NMR ($CDCl_3$) δ 11.65 (1H, br), 10.35 (1H, br), 7.52 (1H, d, J=7.8 Hz), 7.47 (1H, s), 7.28 (1H, dd, J=7.8 Hz, 7.8 Hz), 7.03 (1H, d, J=7.8 Hz), 6.37 (1H, br), 5.06

(1H, m), 4.43 (2H, d, J=5.6 Hz), 3.92 (1H, m), 2.19 (1H, m), 1.42-1.53 (27 H, m), 0.96 (3H, d, J=6.8 Hz), 0.91 (3H, d, J=6.8 Hz); ESI-MS: 564.2 $(M+H)^+$.

10 was treated with 2.2 mL of TFA : CH_2Cl_2 (1:1.2) for 2 hours. Solvents were removed and residue was dissolved in 0.1 % TFA, filtered and lyophilized to give 12.86 mg of **11** as light brown syrup. Yield of two steps: 74.8%. ^{1}H NMR (CD_3OD) δ 7.48 (1H, dd, J=7.9 Hz, 7.8 Hz), 7.35 (1H, d, J=7.9 Hz), 7.28 (1H, s), 7.23 (1H, d, J=7.8 Hz), 4.49 (2H, m), 3.70 (1H, d, J=5.7 Hz), 2.21 (1H, m), 1.06 (6H, m); ESI-MS: 264.1 $(M+H)^+$.

Hydrolysis of valine and phenylalanine esters in pH 7.4 HEPES buffer.

Hydrolysis in pH 7.4 HEPES buffer was determined at 37 ºC. The hydrolysis reaction was initiated by adding 0.75 µL of test compound solution (200 mM in DMSO) to a reaction tube containing 749.25 µL pH 7.4 HEPES buffer. At various time points, 100 µL of the reaction mixture was removed and added to a quenching plate containing 100 µL of 1% TFA (in water) and stored in ice. Following the collection of all samples, quenching plate was filtered (2,000 rpm, 4 ºC, 10 min). The filtrate was removed and assayed by HPLC.

The apparent first-order degradation rate constants were determined by plotting the natural logarithm of test compound remaining as a function of time. The slopes of these plots equal to negative rate constant (k). The degradation half-lives were then estimated by the equation:

$$t_{1/2} = 0.693/k$$

Hydrolysis of Val-3-APG in pH 7.4 HEPES buffer.

The hydrolysis reaction was initiated by adding 20 μL of Val-3-APG solution (200 mM in DMSO) to a reaction tube containing 380 μL pH 7.4 HEPES buffer and incubated at 37 ºC. At various time points (up to 800 h), 85 μL of the reaction mixture was removed and added to a quenching tube containing 85 μL of 10% TFA (in water), filtered (2,000 rpm, 4 ºC, 10 min), and the filtrate was assayed by HPLC immediately.

The apparent first-order degradation rate constants were determined by plotting the natural logarithm of Val-3-APG percentage (1-[3-APG]/([3-HPG]+[Val-3-APG])) as a function of time. The slope equals to negative rate constant (k). The degradation half-lives were then estimated by the equation:

$t_{1/2} = 0.693/k$

Cell culture.

Caco-2 cells (passage 23) from American Type Culture Collection (Rockvile, MD) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1 mmol/L sodium pyruvate and 1% L-glutamine. Cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37°C.

Val-3-APG hydrolysis in Caco-2 cell homogenates.

Caco-2 cells were washed with 0.15 M NaCl solution and then collected with 10 mM phosphate buffer (pH 7.4). The cell suspension was ultrasonicated in ice bath to extract enzymes. The suspension was spun at 10,000 rpm for 10 minutes at 4°C.

Supernatant was placed in a new tube. The concentration of enzyme was determined as protein amount with the Bio-Rad DC assay (Hercules, CA) and adjusted to 500 µg/mL.

Hydrolysis in cell homogenates was determined at 37 ºC. The hydrolysis reaction was initiated by adding 0.75 µL of Val-3-APG (200 mM in DMSO) to a reaction tube containing 749.25 µL cell homogenates. At various time points (0, 5, 10, 30, 60 and 120 min), 100 µL of the reaction mixture was removed and added to a quenching plate containing 100 µL of 10% TFA (in water) and stored in ice. Following the collection of all samples, quenching plate was filtered (2,000 rpm, 4 ºC, 10 min). The filtrate was removed and assayed by HPLC.

hVACVase-mediated hydrolysis.

hVACVase was overexpressed and purified from *Escherichia coli* as described previously[9] and used for all the test compounds except Val-3-APG. 6×His-tagged hVACVase (M - S tag – PDLGTLVPRGSMGM – hVACVase – AAALE - His tag), provided by Dr. Zachary Walls, was produced in bacteria, purified by affinity chromatography and used for Val-3-APG. The results from the two proteins are considered to be comparable due to their very similar specific activity towards valacyclovir activation. The protein concentration was determined by Bio-Rad DC assay (Hercules, CA) with bovine serum albumin as a standard. The kinetic parameters of hVACVase-catalyzed hydrolysis were determined as follows. Kinetic measurements were carried out in 50 mM HEPES (pH 7.4) buffer at 37°C. After preincubation of the buffer for 5 min, hVACVase was added, and then the reaction was initiated by the addition of substrate. Aliquots were taken at different time points, and quenched by

adding to same volume of 10% (v/v) trifluoroacetic acid. Initial velocities were calculated from the linear time course for the product formation. The kinetic parameters K_m and V_{max} were determined by fitting the initial velocity data to the Michaelis-Menten equation by the nonlinear least-square regression analysis in GraphPad Prism software version 4.01. The k_{cat} value was calculated from V_{max}/[enzyme]$_0$ based on the 28.83-kDa molecular mass of hVACVase and 33.38-kDa molecular mass of 6×His-tagged hVACVase. Specific activity of valacyclovir was routinely monitored to normalize active protein concentration.

HPLC analysis

The concentrations of test compounds were determined on a Waters HPLC system (Waters Inc., Milford, MA). The HPLC system consisted of two Waters pumps (Model 515), a Waters auto-sampler (WISP model 712) and a Waters UV detector (996 Photodiode Array Detector). The system was controlled by Waters Millennium 32 software (Version 3.0.1). Samples were resolved in an Agilent ZORBAX Eclipse XDB-C18 column (3.5 µm, 4.6 × 150 mm) or an Agilent ZORBAX SB-Aq column (3.5µm, 4.6 × 150 mm) equipped with a guard column. The mobile phase consisted of 0.1% (v/v) TFA in milli-Q water (solvent A) and 0.1% (v/v) TFA in acetonitrile or 0.1% (v/v) TFA in methanol (solvent B). The detection wavelength was 235 nm for 3-HPG, 3-APG, Val-3-HPG, Phe-3-HPG and Val-3-APG, 254 nm for acyclovir and valacyclovir, 275 nm for L-valine 4-nitrobenzyl ester and 256 nm for valine benzyl ester and all the phenylalanine esters.

Statistical analysis

All the experiments were performed in triplicate unless stated otherwise. The data are presented as mean ± SEM.

Results

Synthesis of 3-APG and its valine amide.

The synthesis of 3-APG and its valine amide was summarized in Scheme 3.3. 3-Aminobenzonitrile was treated with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea to convert the free amino group to the Boc-protected guanidino group. Then the cyano group was reduced by hydrogenation to afford intermediate **8**. Deprotection of **8** gives 3-APG. Valine-3-APG was synthesized by coupling intermediate **8** and Boc-valine followed by deprotection.

Hydrolysis in buffer and cell homogenates.

The half-lives in pH 7.4 HEPES buffer of valine and phenylalanine analogues were shown in Table 3.1 and 3.2. Valacyclovir and valine benzyl ester exhibited about two-fold longer half-lives than valine *p*-nitrobenzyl ester and Val-3-HPG. Compared with Val-3-HPG, the hydrolysis of Val-3-APG was five orders of magnitude slower. For the phenylalanine esters, the half-lives increased in the following order: Phe-3-HPG < Phe-OBz < Phe-OMe < PheOEt.

In contrast to the fast hydrolysis of Val-3-HPG (Table 2.1), the valine amide Val-3-APG was fairly stable in Caco-2 cell homogenates. During the 2 h incubation time no degradation of Val-3-APG was detected.

hVACVase-mediated hydrolysis.

The Michaelis-Menten kinetic parameters of valine derivatives for hVACVase-mediated hydrolysis were listed in Table 3.3. All the valine esters showed relatively low K_m values ranging from 14 to 68 μM. The k_{cat} values of valine *p*-nitrobenzyl ester and Val-3-HPG were about 2-fold higher than valacyclovir and valine benzyl ester, following the same trend as buffer hydrolysis rate. The valine amide Val-3-APG exhibited much higher K_m and lower k_{cat} values compared with the esters, suggesting that it is a very poor substrate of hVACVase.

Phe-3-HPG and phenylalanine benzyl ester exhibited high specificity constants as shown in Table 3.4, suggesting that they are good hVACVase substrates. However, the specificity constants of phenylalanine methyl and ethyl esters were much lower, which was mainly due to the higher K_m value.

Discussion

To investigate the effects of leaving groups, the rate-limiting step of hVACVase-catalyzed reactions must be determined. According to the mechanism of serine hydrolases, for a series of esters or amides with same acyl group but different leaving groups with diverse lability, k_2 values should be different but k_3 values should be the same because the deacylation step is identical for all the substrates. Therefore, the rate-limiting step could be determined by comparing k_{cat} (or V_{max}) values for the ester and amide substrates. A classic example of this strategy was the case of chymotrypsin.[11] As shown in Table 3.5, the amide showed about three orders of magnitude lower k_{cat} value compared with the esters, clearly indicating that the acylation step is rate-limiting for the

amide. However, for the esters, although *p*-nitrophenol is a much better leaving group than methanol and ethanol, the very similar k_{cat} value suggested that the deacylation step was rate-limiting. In the present study we are using a similar strategy for hVACVase by comparing the k_{cat} values of a series of valine and phenylalanine analogs.

The buffer hydrolysis rate reflects the lability of the leaving groups and therefore the k_2 value of the hVACVase-catalyzed hydrolysis. The buffer hydrolysis rates of valine *p*-nitrobenzyl ester and Val-3-HPG were two-fold faster than valine benzyl ester, consistent with the electron withdrawing effect of the nitro and guanidino groups. The order of phenylalanine esters hydrolysis rate was also in correspondence to the leaving group lability. The very low acidity of 3-APG makes it a poor leaving group, explaining the five orders of magnitude slower buffer hydrolysis of Val-3-APG compared with Val-3-HPG.

According to the four to five orders of magnitude difference between the k_{cat} values of Val-3-HPG and Val-3-APG, it becomes very clear that for the amide Val-3-APG, the acylation step (k_2) was rate-limiting, which was the same case as chymotrypsin. For the valine esters, the k_{cat} values were also different and matched the trend of buffer hydrolysis rate, therefore the relationship between the first order rate constant k of buffer hydrolysis and k_{cat} was linear (Figure 3.1). It suggested that the lability of leaving groups did affect the k_{cat} and opposite to that of chymotrypsin, the acylation step (k_2) should be rate-limiting. This is more similar to the case of rat liver carboxylesterase-catalyzed hydrolysis of acetate esters, where the chain length of the alkyl alcohol leaving group significantly affects the reaction rate.[12] However, we cannot rule out the possibility that the k_{cat} differences in the current study was due to experimental error because they are

relatively small (up to 2.5-fold). Therefore, more compounds with a broader range of labilities need to be tested to prove our hypothesis.

For the phenylalanine benzyl, methyl and ethyl esters, the k_{cat} value also matched the buffer hydrolysis rate, suggesting that the acylation step is rate-limiting. The poor linearity between the first order rate constant k of buffer hydrolysis and k_{cat} shown in Figure 3.2 was mainly due to the similar k_{cat} of Phe-3-HPG and phenylalanine benzyl ester, despite the faster chemical hydrolysis of Phe-3-HPG. It may be an indication that at this point the deacylation rate is similar to acylation rate or even smaller, although it may also be a coincidence because lability is not the only factor determining k_2.

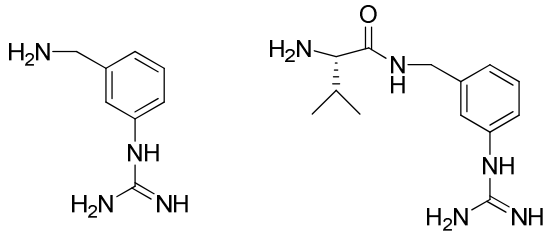
The leaving groups of hVACVase-catalyzed reactions are of interest because they correspond to the drug part of the prodrug molecule. The kinetic studies with a series of valine and phenylalanine analogues provided insight into the effect of leaving groups on hVACVase-catalysed hydrolysis. The comparison of k_{cat} values between Val-3-HPG and Val-3-APG suggested that the acylation step was rate-limiting for the amine leaving group, which explains why amides are poor substrates of hVACVase.[8] In contrast to esters, amide prodrugs are more likely to circumvent hVACVase-catalyzed hydrolysis, which may be beneficial for prodrug targeting purposes. The different k_{cat} values of valine and phenylalanine esters presented in this study suggested that the acylation step was also rate-limiting for most ester substrates, especially the ones with less labile leaving groups. Therefore, a good hVACVase substrate should have a leaving group labile enough for a reasonable activation rate, but not too liable to be chemically unstable.

Assuming acylation as the rate-limiting step, the K_m value should be the same as binding affinity K_s. Although the leaving groups do affect the K_m values, all the amino

acid esters showed similarly high affinity to hVACVase except phenylalanine methyl and ethyl esters, probably because methyl and ethyl groups are too small to have favorable interactions such as hydrophobic interactions with the leaving group binding pocket. Despite the negative electrostatic potential in the leaving group-accommodating groove, Val-3-HPG and Phe-3-HPG with the positively charged leaving group exhibited similar binding affinity to valine and phenylalanine benzyl esters, respectively. This may be due to the water molecules in the leaving group-accommodating groove, which interfere with the proposed favorite electrostatic interaction. Although structurally similar to Val-3-HPG, the amide Val-3-APG showed much lower affinity to hVACVase. The difference of ester and amide could be due to (1) the hydrogen bond[13, 14] between the amide hydrogen and the hydroxyl oxygen of S122 and (2) the unfavorable interaction between the amide hydrogen and the hydroxyl hydrogen of S122 (Scheme 3.4).

In summary, the results suggested that the leaving groups of amino acid esters had an effect on both the binding and reaction rate of hVACVase-catalysed hydrolysis. However, the requirement for the leaving groups should be relatively flexible due to the large open leaving group-accommodating groove. Therefore, hVACVase may serve as an amino acid ester prodrug-activating enzyme target for a broad range of parent drugs with reasonable sizes and labilities. On the other hand, the amino acid amides are very poor substrates of hVACVase due to both low affinity and the much stronger amide bond.

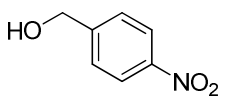
Scheme 3.1. Structures of 3-APG and Val-3-APG.



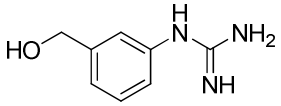
3-APG (**9**) Val-3-APG (**11**)

Scheme 3.2. Leaving groups of valine and phenylalanine analogues.

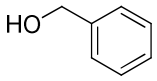
Leaving groups of valine analogues:



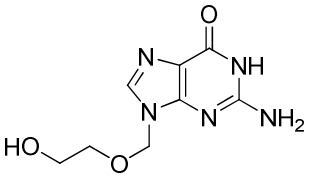
***p*-nitrobenzyl alcohol**



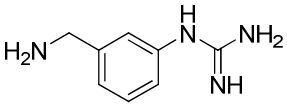
3-HPG



benzyl alcohol

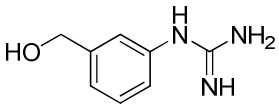


acyclovir

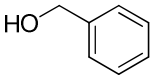


3-APG

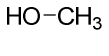
Leaving groups of phenylalanine analogues:



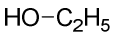
3-HPG



benzyl alcohol

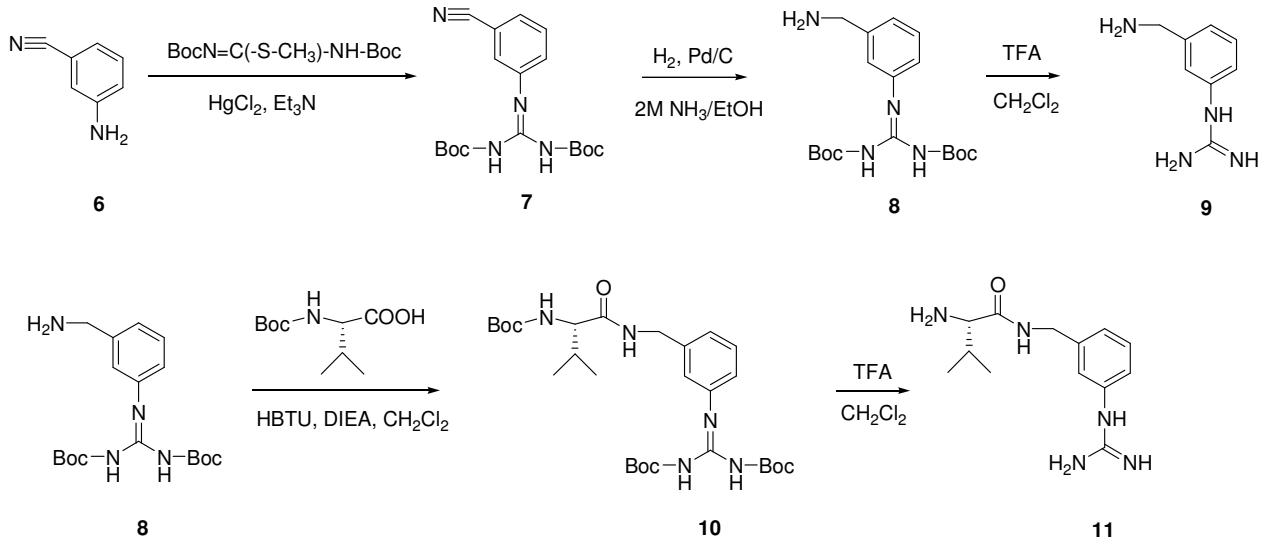


methanol

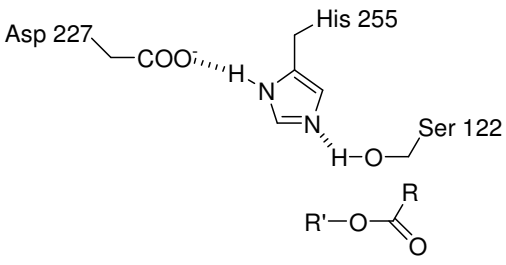


ethanol

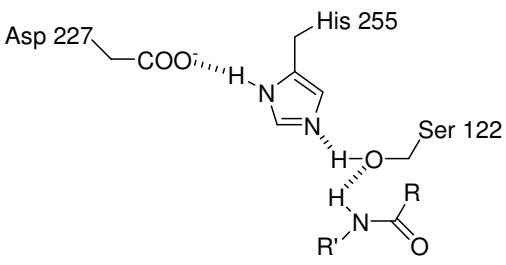
Scheme 3.3. Synthesis of 3-APG and its valine amide.



Scheme 3.4. Ester and amide substrates binding to hVACVase.



ester



amide

Table 3.1. Estimated half-lives of valine analogues in pH 7.4 HEPES buffer (esters, mean ± SEM, n=3; Val-3-APG, n=1).

	Half-lives (min)
Valine p-nitrobenzyl ester	412.3 ± 11.9
Val-3-HPG	486.9 ± 6.6
valacyclovir	742.1 ± 5.5
Val-OBz	857.8 ± 7.6
Val-3-APG	6.2×10^7

Table 3.2. Estimated half-lives of phenylalanine analogues in pH 7.4 HEPES buffer (mean ± SEM, n=3).

	Half-lives (min)
Phe-3-HPG	230.0 ± 6.5
Phe-OBz	365.0 ± 9.4
Phe-OMe	470.6 ± 12.6
Phe-OEt	1006.4 ± 26.0

Table 3.3. Michaelis-Menten kinetic parameters of valine derivatives for hVACVase-mediated hydrolysis (mean ± SEM; n=3).

	K_m (μM)	V_{max} (nmol/min/μg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Valine *p*-nitrobenzyl ester	14 ± 1	272 ± 3	130 ± 2	9490 ± 580
Val-3-HPG	46 ± 5	321 ± 9	154 ± 4	3370 ± 460
valacyclovir	68 ± 4	120 ± 2	58 ± 1	850 ± 66
Val-OBz	60 ± 15	125 ± 7	60 ± 3	992 ± 300
Val-3-APG	1810 ± 80	(4.12 ± 0.08) × 10^{-3}	(2.29 ± 0.04) × 10^{-3}	(1.26 ± 0.08) × 10^{-3}

Table 3.4. Michaelis-Menten kinetic parameters of phenylanine derivatives for hVACVase-mediated hydrolysis (mean ± SEM; Phe-OMe, n=2; others, n=3).

	K_m (μM)	V_{max} (nmol/min/μg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Phe-3-HPG	207 ± 16	718 ± 16	345 ± 8	1660 ± 160
Phe-OBz	99 ± 26	822 ± 54	395 ± 26	3980 ± 1310
Phe-OMe	5140 ± 1120	474 ± 49	228 ± 24	44 ± 14
Phe-OEt	6700 ± 560	193 ± 8	93 ± 4	14 ± 2

Table 3.5 Kinetic parameters for N-acetyltryptophanyl substrates of chymotrypsin (adopted from reference [11]).

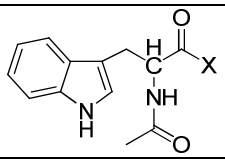
	X	K_m (mM)	k_{cat} (s^{-1})
	-OEt	0.097	0.448
	-OMe	0.095	0.462
	-OPNP	0.002	0.508
	$-NH_2$	5	0.0006

Figure 3.1. Correlation between the turnover number k_{cat} of hVACVase-catalyzed hydrolysis and first order rate constant k of buffer hydrolysis for valine esters of different leaving groups.

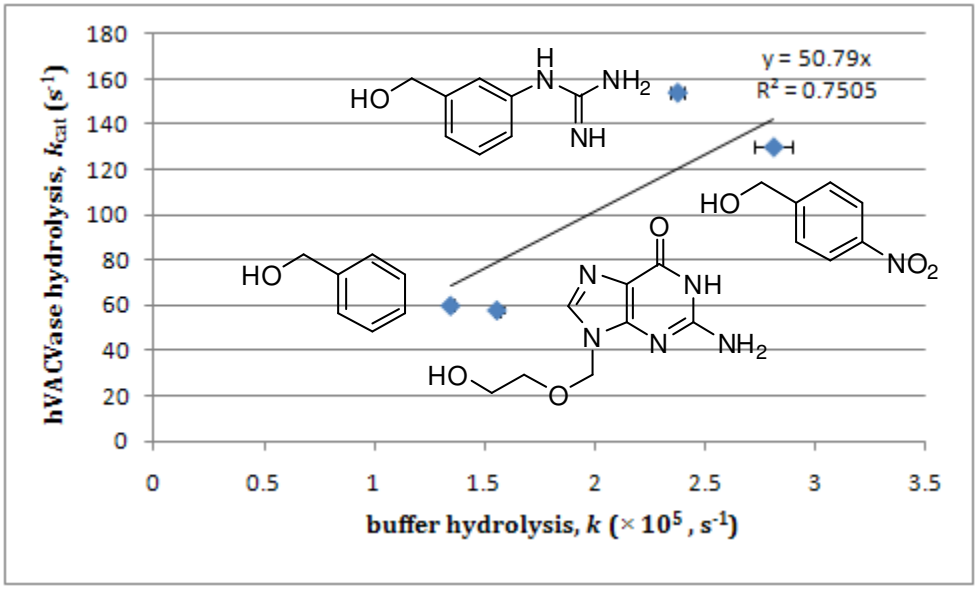
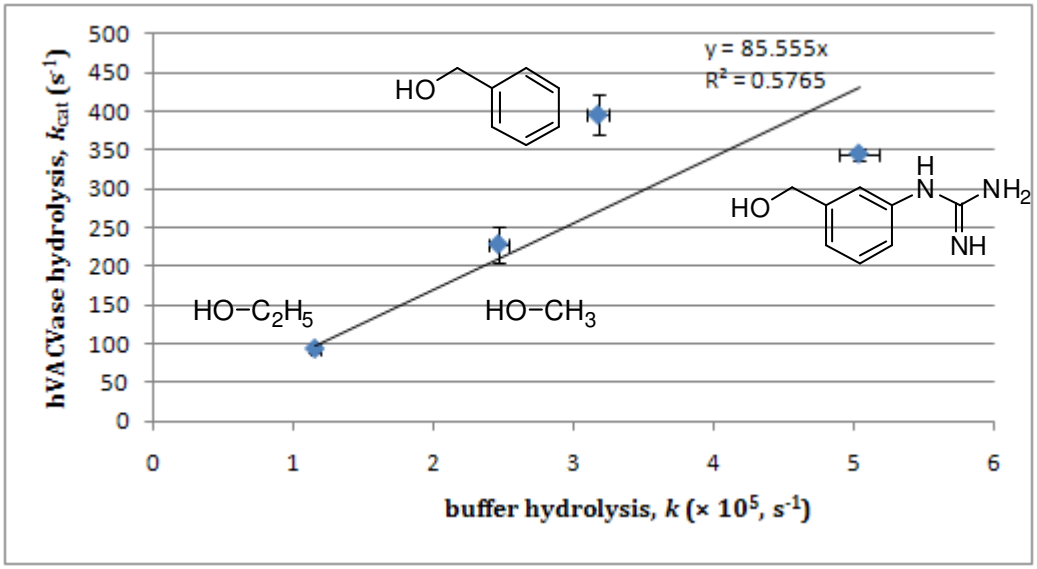


Figure 3.2. Correlation between the turnover number k_{cat} of hVACVase-catalyzed hydrolysis and first order rate constant k of buffer hydrolysis for phenylalanine esters of different leaving groups.



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Chapter 4

Summary

One of the dilemmas in drug development is that the most potent therapeutic agents may not have favorable biopharmaceutical properties, such as high oral absorption. Ironically sometimes the most important functional groups for the optimal binding to the drug target are actually the barriers for oral delivery. As an example, the positively charged guanidino group of anti-influenza agent zanamivir is not only essential for its high affinity to influenza neuraminidase, but also one of the reasons for its low oral bioavailability. The solution of this problem normally involves an analogue approach, screening for new compounds to balance the potency and biopharmaceutical properties. However, this approach may not always provide the required drug profile, especially when the drug binding pocket is hydrophilic. Another approach to improve oral absorption is the prodrug approach. Prodrugs are inactive drug analogues which can be metabolized to the active drug *in vivo* after the pharmaceutical, pharmacokinetic or pharmacodynamic barriers are overcome. The classic prodrug strategy is most commonly used to shield the charges and decrease the polarity of a poorly absorbed drug and facilitate passive diffusion following the usually non-specific biotransformation to the active parent drug. The transporter- and enzyme-targeted prodrug approach, however, is

aiming at targeting the prodrugs to membrane transporters and intracellular activating enzymes, which makes the prodrug design more rational and less empirical.

The first specific aim of this research was to apply a transporter- and enzyme-targeted prodrug approach to guanidino-containing drugs (Figure 4.1). Among the membrane transporters expressed in the brush border membrane of the small intestine that may tolerate the guanidino functionality, hPEPT1 is one of the most well-studied and widely used for prodrug targeting purposes. hPEPT1 mediates the intestinal absorption of di- and tripeptides including guanidino-containing arginine analogues as well as peptidomimetics. Despite its broad substrate specificity, the preference of hydrophobic side chains for hPEPT1 may be a problem for guanidino-containing parent drugs. Therefore, a model parent compound, [3-(hydroxymethyl)phenyl]guanidine (3-HPG), and its L-valine, L-isoleucine and L-phenylalanine esters were used to test the extent to which the guanidino group can be tolerated by hPEPT1. Although the amino acid ester hydrolysis can be catalyzed either chemically or by multiple esterases or proteases, a specific amino acid ester prodrug-activating enzyme hVACVase was chosen as the activation target to ensure the quick *in vivo* biotransformation .

At first the buffer and enzymatic hydrolysis of 3-HPG and its amino acid esters were evaluated. The parent compound 3-HPG was very stable at all conditions, making it an excellent model parent compound. The amino acid esters exhibited relatively good stability in pH 6.0 and 7.4 buffers but much faster hydrolysis in HeLa and Caco-2 cell homogenates, which are the favorable properties for prodrugs. Then, the kinetic parameters of hVACVase-mediated hydrolysis were determined. All of the 3-HPG esters showed higher specificity constant (k_{cat}/K_m) than the reference substrate valacyclovir,

suggesting that they are good substrates of hVACVase (k_{cat}/K_m values in $mM^{-1}\cdot s^{-1}$ were 3370 for Val-3-HPG, 1580 for Ile-3-HPG, 1660 for Phe-3-HPG and 850 for valacyclovir). The [^{3}H]Gly-Sar uptake inhibition was conducted to determine the affinity to hPEPT1. Both Val-3-HPG and Ile-3-HPG exhibited higher affinity than hPEPT1 substrate valacyclovir (IC_{50} values: Val-3-HPG 0.65 mM, Ile-3-HPG 0.63 mM, valacyclovir 1.42 mM) and the comparison with corresponding benzyl ester suggested that the guanidino functionality did not hinder the binding to hPEPT1 transporter. In the direct uptake studies, all the three L-amino acid esters showed higher uptake (2.6- to 9-fold) than the parent compound 3-HPG in HeLa/hPEPT1 cells. Compared with normal HeLa cells, the 3-HPG esters displayed 8- to more than 25-fold increased uptake, whereas 3-HPG only showed little enhancement. It suggested that the L-amino acid 3-HPG esters were not only inhibitors of hPEPT1 but also substrates. In Caco-2 cells, Val-3-HPG and Ile-3-HPG demonstrated remarkable permeability enhancement and Val-3-HPG exhibited comparable permeability to valacyclovir. The low permeability of D-Val-3-HPG indicated that Val-3-HPG transport was carrier-mediated and the studies with inhibitors suggested that hPEPT1 was the predominant transporter. In rat perfusion studies, Val-3-HPG and Ile-3-HPG permeabilities were significantly higher than 3-HPG and also exceeded/matched the high-permeability standard metoprolol, respectively. Combined with the subsequent prodrug studies with the guanidino-containing parent compounds zanamivir and GOCarb, the results indicated that the prodrug strategy is effective at increasing the intestinal permeability of polar guanidino analogues via targeting hPEPT1 for transport and hVACVase for activation.

The second specific aim was to investigate the effect of leavings group on the hVACVase-catalyzed prodrug activation. hVACVase is a serine hydrolase specific for the hydrolysis of α-amino acid esters. The broad leaving group-accomodating groove makes it a potentially universal prodrug-activating target for amino acid analogues. To determine the effect of leaving groups on the binding affinity and specific activity, the kinetic studies of a series of valine esters (Val-3-HPG, valacyclovir, valine benzyl ester, valine *p*-nitrobenzyl ester), phenylalanine esters (Phe-3-HPG, phenylalanine benzyl ester, phenylalanine ethyl ester, phenylalanine methyl ester) and a valine amide Val-3-APG were conducted.

Compared with Val-3-HPG, the amide Val-3-APG exhibited more than four orders of magnitude lower k_{cat} value, which clearly indicated that the acylation step was rate-limiting. Combined with the high K_m value, the specificity constant (k_{cat}/K_m) of Val-3-APG was negligible. This is consistent with the previous result that hVACVase cannot activate amides. Therefore, amide prodrugs are more likely to circumvent the ubiquitously expressed hVACVase, which may be beneficial for prodrug-targeting purposes. For the amino acid esters, the correlation between the k_{cat} values of hVACVase-catalyzed hydrolysis and the first order rate constant k of buffer hydrolysis suggested that the acylation step may still be rate-limiting for some of the labile primary alcohol leaving groups. Therefore, a good hVACVase substrate should have a leaving group labile enough for a reasonable activation rate, but not too liable to be chemically unstable. The leaving groups seem to affect binding to hVACVase as well, although the favorable features for binding need to be further identified. However, with the broad

leaving group-accommodating groove, the requirement for the leaving group binding should not be stringent.

In conclusion, the transporter- and enzyme-targeted approach is successful to enhance the oral absorption of a guanidino-containing model compound, indicating its feasibility for other poorly-absorbed guanidino analogs. The results in this study suggested that the leaving groups had an effect on both the binding and specific activity of hVACVase-catalyzed prodrug activation, and the high specificity constant (k_{cat}/K_m) of 3-HPG esters presumably resulted from high binding affinity to hVACVase as well as favorable lability of the leaving group. hVACVase is an ideal target for activating α-amino acid ester prodrugs with relatively labile leaving groups but is unable to activate amide prodrugs.

Figure 4.1. The transporter- and enzyme-targeted approach.