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Specificity of a Prodrug-Activating Enzyme hVACVase: The Leaving Group Effect

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Abstract: Human valacyclovirase (hVACVase) is a prodrug-activating enzyme for amino acid prodrugs including the antiviral drugs valacyclovir and valganciclovir. In hVACVase-catalyzed reactions, the leaving group of the substrate corresponds to the drug moiety of the prodrug, making the leaving group effect essential for the rational design of new prodrugs targeting hVACVase activation. In this study, a series of valine esters, phenylalanine esters, and a valine amide were characterized for the effect of the leaving group on the efficiency of hVACVase-mediated prodrug activation. Except for phenylalanine methyl and ethyl esters, all of the ester substrates exhibited a relatively high specificity constant (k_{cat}/K_m), ranging from 850 to 9490 $mM^{-1}\cdot s^{-1}$. The valine amide Val-3-APG exhibited significantly higher K_m and lower k_{cat} values compared to the corresponding ester Val-3-HPG, indicating poor specificity for hVACVase. In conclusion, the substrate leaving group has been shown to affect both binding and specific activity of hVACVase-catalyzed activation. It is proposed that hVACVase is an ideal target for α-amino acid ester prodrugs with relatively labile leaving groups while it is relatively inactivate toward amide prodrugs.

Keywords: hVACVase; prodrug

Introduction

The prodrug strategy has proven to be a successful approach for improving oral absorption of low-permeability drugs by various mechanisms.[1−4] Following absorption, the prodrug must be activated, either chemically or enzymatically, to the active parent drug. Historically, the activation mechanism was often not investigated. A more modern approach, on the other hand, would exploit the mechanism behind the activation, enabling targeting of the location and control over the extent of this essential process.[5−8] We have recently identified an amino acid ester prodrug-activating

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enzyme, human valacyclovirase, hVACVase,[9] which has been shown to be one of the major enzymes for the activation of the valine ester prodrugs valacyclovir and valganciclovir. We have also shown that hVACVase effectively hydrolyzes many other amino acid esters as well.[10−12] Its potential to serve as a prodrug-activating enzyme for amino acid ester and amide prodrugs depends on its leaving group (drug) specificity.

hVACVase is a serine hydrolase with a catalytic triad S122-H255-D227. The very specific preference for amino acid analogues can be attributed to the electrostatic interaction between the critical residue D123, adjacent to the active S122, and the free amino group of the substrate.[11] As revealed by its crystal structure, hVACVase contains a large leaving group-accommodating groove, which explains the diversity of its substrate leaving groups (potential drugs), including nucleoside analogues acyclovir, ganciclovir, floxuridine, gemcitabine, zidovudine, 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole, and other alcohols such as methanol, ethanol, benzyl alcohol, and [3-(hydroxymethyl)phenyl]guanidine (3-HPG).[10−12] Because the leaving group corresponds to the drug moiety of the prodrug, hVACVase, due to its expression in the intestinal epithelial cells, is an ideal target for oral delivery and activation of amino acid ester prodrugs and perhaps amide prodrugs as well. While 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.[11] In addition, the valine 5′-floxuridine has a higher V_{max} value compared with valine 3′-floxuridine ester (148 and 33 nmol/min/μg, respectively),[10] thus indicating that the alcohol leaving groups do have an effect on hVACVase catalytic activity. Additionally, hVACVase does not exhibit significant hydrolytic activity toward 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.[10] This may be due to the much more stable amide bond compared with the ester bond. The 10-fold difference in K_m values of the prodrugs valacyclovir and valganciclovir (0.19 and 1.90 mM, respectively) indicates that the leaving groups may also affect the binding affinity.[9] To better understand the hVACVase-catalyzed prodrug activation and guide future prodrug design, an investigation of the leaving group effect is essential.

The purpose of this study was to investigate the effect of leaving groups on the hVACVase-catalyzed prodrug activation in a systematic fashion. 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 1). This approach allows us to determine the rate-limiting step of hVACVase-catalyzed hydrolysis and the effect of leaving groups on the binding affinity and specific activity. This will allow a more rational design of successful amino acid ester and amide prodrugs.

Experimental Section

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 *p*-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, 2 M 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. 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-[[Bis[[(1,1-dimethylethoxy)carbonyl]amino]methylene]-amino]benzonitrile (2). To a stirred suspension of 3-aminobenzonitrile (**1**, 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

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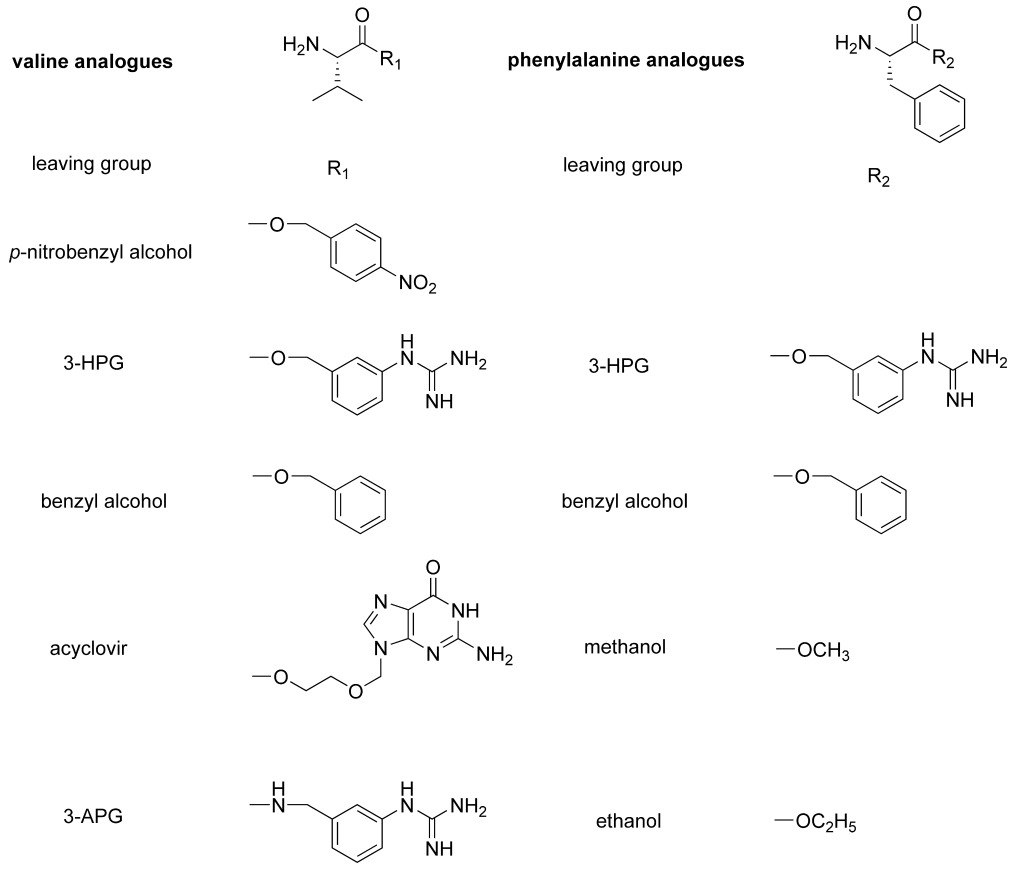
Scheme 1. Structures of Valine and Phenylalanine Analogues



tetrahydrofuran (THF), 405 mg (4.0 mmol) of triethylamine was added dropwise under argon. After 2 h, 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 **2** 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 (3). A mixture of **2** (150 mg, 0.416 mmol) and Pd/C (10 wt % palladium on activated carbon, 150 mg) in 100 mL of 2 M 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, 4). Compound **3** (24.8 mg) was dissolved in 2.2 mL of trifluoroacetic acid (TFA):CH_2Cl_2 (1:1.2) and stirred at room temperature for 2 h. Then solvents were removed and the residue was dissolved in 0.1% TFA, filtered and lyophilized. The raw product was further purified by semiprep 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 $(M + H)^+$.

(2*S*)-2-Amino-3-methyl-*N*-[3-[(aminoiminomethyl)amino]phenyl]methylbutanamide (Val-3-APG, 6). To a stirred solution of **3** (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 **5** 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 $(M + H)^+$.

Compound **5** was treated with 2.2 mL of TFA:CH_2Cl_2 (1:1.2) for 2 h. Solvents were removed and residue was dissolved in 0.1% TFA, filtered, and lyophilized to give 12.86 mg of **6** 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 °C. 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 of 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, the quenching plate was filtered (2000 rpm, 4 °C, 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 slope of this plot equals to negative rate constant (k). The degradation half-life was 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 of pH 7.4 HEPES buffer and incubated at 37 °C. 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 (2000 rpm, 4 °C, 10 min), and the filtrate was immediately assayed by HPLC.

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:

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

hVACVase-Mediated Hydrolysis. hVACVase was overexpressed and purified from *Escherichia coli* as described previously[11] and used for all the test compounds except Val-3-APG. 6×His-tagged hVACVase (M−S tag−PDLGTLVPRGSMGM−hVACVase−AAALE−His tag) 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 toward 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-squares 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) consisting of two Waters pumps (model 515), a Waters autosampler (WISP model 712), and a Waters UV detector (model 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 mm × 150 mm) equipped with a guard column and the flow rate was 1.0 mL/min. For all the test compounds except for valine *p*-nitrobenzyl ester, 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% to 30% at a rate of 2%/min during a 25 min run. The retention times for 3-HPG, Val-3-HPG, Phe-3-HPG, acyclovir, valacyclovir, 3-APG, Val-3-APG, benzyl alcohol, Val-OBn, phenylalanine, Phe-OMe, Phe-OEt, and Phe-OBn were 6.7, 9.7, 12.1, 4.9, 7.9, 4.2, 8.4, 11.3, 15.7, 8.2, 10.7, 13.0, and 18.9 min, respectively. For valine *p*-nitrobenzyl ester, the mobile phase consisted of 70:30 (v/v) 0.1% trifluoroacetic acid in milli-Q water: 0.1% trifluoroacetic acid in methanol. The retention times were 8.5 min for *p*-nitrobenzyl alcohol and 16.4 min for valine *p*-nitrobenzyl ester. 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 *p*-nitrobenzyl alcohol and L-valine *p*-nitrobenzyl ester, and 256 nm for phenylalanine, benzyl alcohol, Val-OBn, and all the phenylalanine esters except Phe-3-HPG.

Statistical Analysis. All the experiments were performed in triplicate unless stated otherwise. The data are presented as mean ± SEM. Statistical difference between groups with equal sample size was determined using the Tukey test ($p < 0.01$). Statistical difference between groups with unequal sample size was determined using the Tukey−Kramer test ($p < 0.05$). Letters designate groups with statistical significance from one another.

Results

Synthesis of 3-APG and Val-3-APG. The synthesis of 3-APG and its valine amide is summarized in Scheme 2. 3-Aminobenzonitrile was treated with 1,3-bis(*tert*-butoxy-

Scheme 2. Synthesis of 3-APG and Its Valine Amide

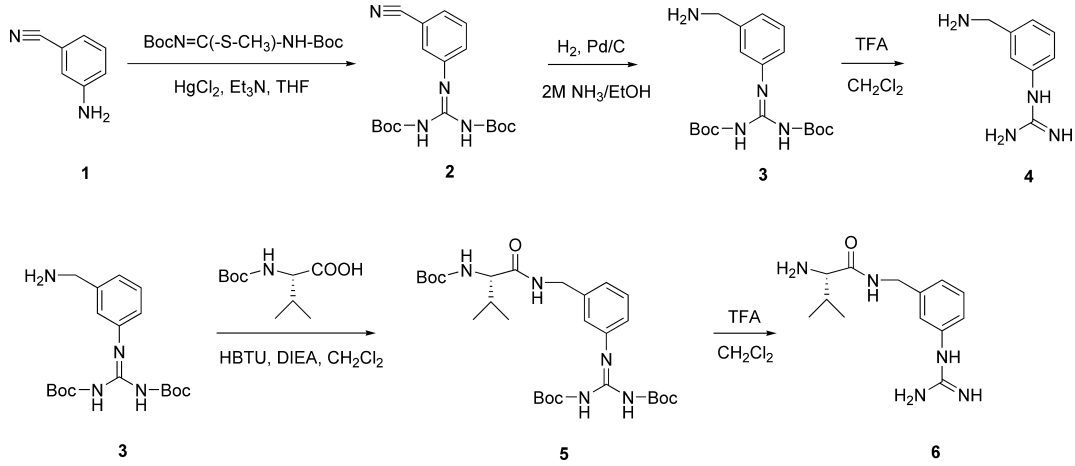


Table 1. Estimated Half-Life Values of Valine and Phenylalanine Analogues in pH 7.4 HEPES Buffer (Mean ± SEM; Esters, $n = 3$; Val-3-APG, $n = 1$)

valine analogues	$t_{1/2}$ (min)[a]	phenylalanine analogues	$t_{1/2}$ (min)[a]
valine *p*-nitrobenzyl ester	412.3 ± 11.9	Phe-3-HPG	230.0 ± 6.5
Val-3-HPG	486.9 ± 6.6	Phe-OBn	365.0 ± 9.4
valacyclovir	742.1 ± 5.5	Phe-OMe	470.6 ± 12.6
Val-OBn	857.8 ± 7.6	Phe-OEt	1006.4 ± 26.0
Val-3-APG	6.2×10^7		

[a] All the groups are statistically different.

carbonyl)-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 **3**. Deprotection of **3** gives 3-APG. Valine-3-APG was synthesized by coupling intermediate **3** and Boc-valine followed by deprotection.

Hydrolysis in Buffer and Cell Homogenates. The half-life values of valine and phenylalanine analogues in pH 7.4 HEPES buffer are shown in Table 1. Valacyclovir and valine benzyl ester exhibited approximately 2-fold longer $t_{1/2}$ than valine *p*-nitrobenzyl ester and Val-3-HPG. Compared with Val-3-HPG, the hydrolysis of Val-3-APG was 5 orders of magnitude slower. For the phenylalanine esters, the half-life values ranked in the following order: Phe-3-HPG < Phe-OBn < Phe-OMe < PheOEt.

In contrast to the fast hydrolysis of Val-3-HPG ($t_{1/2} < 5$ min),[12] 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.

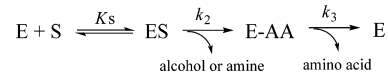
hVACVase-Mediated Hydrolysis. The Michaelis−Menten kinetic parameters of valine derivatives for hVACVase-mediated hydrolysis are listed in Table 2. All the valine esters showed relatively low K_m values ranging from 14 to 68 μM. The k_{cat} values of valine *p*-nitrobenzyl ester and Val-3-HPG were about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

Phe-3-HPG and phenylalanine benzyl ester exhibited high specificity constants as shown in Table 2, indicating that they are good hVACVase substrates. However, the specificity constants of phenylalanine methyl and ethyl esters were much lower, which was mainly attributable to the higher K_m value.

Discussion

Rational design of prodrugs includes a thorough investigation of the activation process, which releases the free active drug. hVACVase, a novel prodrug-activating enzyme, has been shown to mediate the hydrolytic activation of various amino acid ester prodrugs including the significant antiviral drugs valacyclovir and valganciclovir. In the hVACVase-mediated prodrug activation process, the leaving group corresponds to the drug moiety of the prodrug molecule, hence, investigating the leaving group effect is essential for designing new prodrugs targeting this enzyme for activation.

As a serine hydrolase, the hydrolysis process catalyzed by hVACVase is presumably a three-step reaction as shown in the following scheme, where E stands for enzyme, S stands for substrate, and AA stands for amino acid:[13]



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

$$v = [\mathrm{E}]_0[\mathrm{S}]\frac{\dfrac{k_2k_3}{k_2 + k_3}}{\dfrac{K_sk_3}{k_2 + k_3} + [\mathrm{S}]}$$

in which

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Table 2. Michaelis−Menten Kinetic Parameters of Valine and Phenylalanine Derivatives for hVACVase-Mediated Hydrolysis (Mean ± SEM; Phe-OMe, $n = 2$; others, $n = 3$)

valine analogues	K_m (μM)[b]	V_{max} (nmol/min/μg)	k_{cat} (s^{-1})[c]	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
valine *p*-nitrobenzyl ester	14 ± 1	272 ± 3	130 ± 2	9490 ± 580
Val-3-HPG[a]	46 ± 5	321 ± 9	154 ± 4	3370 ± 460
valacyclovir[a]	68 ± 4	120 ± 2	58 ± 1	850 ± 66
Val-OBn	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}
phenylalanine analogues	K_m (μM)[d]	V_{max} (nmol/min/μg)	k_{cat} (s^{-1})[e]	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
Phe-3-HPG[a]	207 ± 16	718 ± 16	345 ± 8	1660 ± 160
Phe-OBn	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

[a] Previously published by Sun et al.[12] [b] Statistically different groups: (A) valine *p*-nitrobenzyl ester, Val-3-HPG, valacyclovir, Val-OBn; (B) Val-3-APG. [c] Statistically different groups: (A) valine *p*-nitrobenzyl ester; (B) Val-3-HPG; (C) valacyclovir, Val-OBn; (D) Val-3-APG. [d] Statistically different groups: (A) Phe-3-HPG, Phe-OBn; (B) Phe-OMe, Phe-OEt. [e] Statistically different groups: (A) Phe-3-HPG, Phe-OBn; (B) Phe-OMe; (C) Phe-OEt.

$$K_m = K_s \frac{k_3}{k_2 + k_3}$$

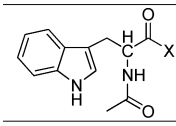
and

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

It can be seen that the effect of the leaving groups depends on the rate-limiting step, which can be 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 ($k_2 \ll k_3$), then the k_{cat} value will be affected by the leaving group ($k_{cat} \approx k_2$) and the K_m value will approach K_s. On the other hand, if the deacylation step is rate-limiting ($k_2 \gg k_3$), the k_{cat} value will be independent of the leaving group ($k_{cat} \approx k_3$) and K_m value will be a function of K_s, k_2, and k_3. For a series of esters or amides with the 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 of the substrates. Thus, the rate-limiting step can be determined by comparing k_{cat} (or V_{max}) values for the ester and amide substrates. A classical example of this strategy is the case of chymotrypsin, where the amide exhibits a ~3 orders of magnitude lower k_{cat} value compared with the corresponding esters, clearly indicating that the acylation step is rate-limiting for the amide substrate (Table 3).[14] Further, for chymotrypsin-mediated hydrolysis of esters, while *p*-nitrophenol is a much better leaving group than methanol and ethanol, the very similar k_{cat} values suggested that the deacylation step was rate-limiting (Table 3). In the present study, we have applied this classical strategy to investigate hVACVase-mediated prodrug activation.

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 2-fold faster than valine benzyl ester, consistent with the electron withdrawing effect of the nitro and guanidino groups (Table 1). The order of phenylalanine esters hydrolysis rate was also in correspondence to the leaving group lability (Table 1). The very low acidity of 3-APG makes it a poor leaving group, explaining the 5 orders of magnitude slower buffer hydrolysis of Val-3-APG compared with Val-3-HPG.

Table 3. Kinetic Parameters for *N*-Acetyltryptophanyl Substrates of Chymotrypsin (Adapted from Bender and Kezdy[14])

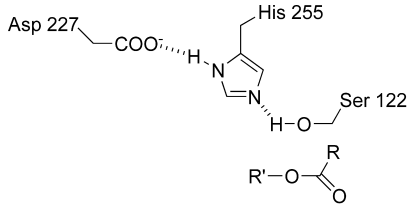
	X	K_m (mM)	k_{cat} (s^{-1})
	-OEt	0.097	0.448
	-OMe	0.095	0.462
	-OPNP	0.002	0.508
	-NH_2	5	0.0006

The 4−5 orders of magnitude difference between the k_{cat} values of Val-3-HPG and Val-3-APG indicates that for the amide Val-3-APG, the acylation step (k_2) was rate-limiting, similar to chymotrypsin-catalyzed hydrolysis (Table 2). For the valine esters, the k_{cat} values were also different and matched the trend of buffer hydrolysis rate. This observation suggests that the lability of the leaving groups did affect the k_{cat} and, unlike chymotrypsin, the acylation step (k_2) is rate-limiting. Similar phenomenon was observed for the rat liver carboxylesterase-catalyzed hydrolysis of acetate esters, where the chain length of the alkyl alcohol leaving group significantly affects the reaction rate.[15] However, because the k_{cat} differences in the current study were relatively small (up to 2.5-fold), more compounds with a broader range of labilities will be necessary to conclusively confirm this analysis. For the phenylalanine benzyl, methyl, and ethyl esters, the k_{cat} value also matched the buffer hydrolysis rate, suggesting that

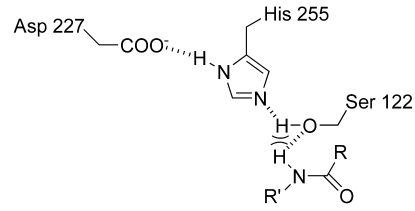
(14) Bender, M. L.; Kezdy, J. Mechanism Of Action Of Proteolytic Enzymes. *Annu. Rev. Biochem.* **1965**, *34*, 49–76.

(15) Arndt, R.; Krisch, K. Catalytic properties of an unspecific carboxylesterase (E1) from rat-liver microsomes. *Eur. J. Biochem.* **1973**, *36* (1), 129–134.

Scheme 3. Ester and Amide Substrates Binding to hVACVase



ester



amide

the acylation step is rate-limiting. Despite the faster chemical hydrolysis of Phe-3-HPG, the k_{cat} values of Phe-3-HPG and phenylalanine benzyl ester were similar. It may indicate that the deacylation rate becomes similar to acylation rate, but because k_2 depends on multiple factors other than the leaving group lability, this hypothesis needs further evidence.

Assuming acylation as the rate-limiting step, the K_m value should be close to the binding affinity K_s. As shown in Table 2, all the amino acid esters showed similarly high affinity to hVACVase except phenylalanine methyl and ethyl esters, likely 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 (Table 2). This may be due to the water molecules in the leaving group-accommodating groove interfering with expected electrostatic interaction. The relatively similar K_m values of amino acid ester substrates suggest that the requirement of the alcohol leaving groups for the binding is not very stringent, in accordance with the large open leaving group-accommodating groove. Although structurally similar to Val-3-HPG, the amide Val-3-APG showed much lower affinity to hVACVase (Table 2). The difference of ester and amide could be due to the unfavorable interaction between the amide hydrogen and the hydroxyl hydrogen of S122 (Scheme 3). The high K_m combined with the low k_{cat} value explains why amides are poor substrates of hVACVase.[10]

In summary, these results indicate that the leaving groups of substrates have a significant effect on both the binding and reaction rate of hVACVase-catalyzed hydrolysis. A good hVACVase substrate should have a leaving group labile enough for a reasonable activation rate but not too labile to be chemically unstable. Alcohol leaving groups with reasonable sizes and labilities are preferred by hVACVase. On the other hand, the amino acid amide is a very poor substrate of hVACVase due to both low affinity and the much stronger amide bond. Therefore, amide prodrugs are more likely to circumvent hVACVase-catalyzed hydrolysis. This can be further exploited in rational prodrug design: if comparatively quick intraenterocyte activation is desired, an amino acid ester prodrug would be a better choice. However, if stability of the prodrug in the enterocyte and activation at a later step with a different mechanism is desired, amino acid amide may be more suitable.

Abbreviations

hVACVase, human valacyclovirase; 3-HPG, [3-(hydroxymethyl)phenyl] guanidine; *p*-NA, *p*-nitroanilide; 3-APG, [3-(aminomethyl)phenyl]guanidine; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; PNP, *p*-nitrophenyl.

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