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A Novel Nucleoside Prodrug-Activating Enzyme: Substrate Specificity of Biphenyl Hydrolase-like Protein

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Abstract: Biphenyl hydrolase-like protein (BPHL, NCBI accession number NP_004323) is a novel human serine hydrolase recently identified as a human valacyclovirase, catalyzing the hydrolytic activation of the antiviral prodrugs valacyclovir and valganciclovir. The substrate specificity of BPHL was investigated with a series of amino acid ester prodrugs of the therapeutic nucleoside analogues: acyclovir, zidovudine, floxuridine, 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole, and gemcitabine. The hydrolysis of typical esterase and aminopeptidase substrates by BPHL was also investigated. The results indicate that the substrate specificity of BPHL is largely determined by the amino acid acyl promoiety, and is less sensitive to the nucleoside parent drugs. For all nucleoside parent drugs, BPHL preferred the hydrophobic amino acids valine, phenylalanine, and proline over the charged amino acids lysine and aspartic acid. The position and monoester or diester form of the prodrug were also important, with BPHL exhibiting higher affinity for the 5′-esters than for the 3′-esters and the 3′,5′-diesters irrespective of amino acid type. Further, the presence of the 3′-amino acid ester considerably reduced the hydrolysis rate of the 5′-amino acid ester functionality. BPHL exhibited stereoselectivity with an L/D specificity ratio of 32 for 5′-valyl floxuridine and 1.5 for 5′-phenylalanyl floxuridine. The substrate specificity suggests that the substrate-binding pocket of BPHL has a hydrophobic acyl binding site which can accommodate the positively charged α-amino group, while having an alcohol leaving group binding site that can accommodate nucleoside analogues with a relatively generous spatial allowance. In conclusion, BPHL catalyzes the hydrolytic activation of amino acid esters of a broad range of therapeutic nucleoside analogues in addition to valacyclovir and valganciclovir and has considerable potential for utilization as an activation target for design of antiviral and anticancer nucleoside analogue prodrugs.

Keywords: Prodrug; human valacyclovirase; biphenyl hydrolase-like; prodrug-activating enzyme; amino acid esters; nucleoside analogues; drug delivery

Introduction

The biphenyl hydrolase-like protein (BPHL) is a novel human enzyme that has been recently identified as a valacyclovir- and valganciclovir-activating enzyme.[1] While first cloned from human breast carcinoma tissue and exhibiting a sequence similarity to microbial biphenyl hydrolases such as BPHD, BPHL did not exhibit biphenyl hydrolytic activity.[2] BPHL is expressed at a high level in human liver and kidney and lower levels in intestine, heart, and skeletal muscle; however, its endogenous function is unknown. The expression of BPHL in normal human liver, kidney, and intestine suggests that BPHL may play a role in the

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metabolism of xenobiotics.[2] BPHL is a serine hydrolase with the signature Gly-X-Ser-X-Gly motif and the highly conserved catalytic triad of Ser^{122}, His^{255}, and Asp^{227}.[2,3] However, BPHL exhibited only limited activity toward a common serine hydrolase substrate, *p*-nitrophenyl butyrate.[2] The identification of BPHL as a novel prodrug-activating enzyme provides an opportunity to exploit BPHL as a design target for the nucleoside prodrug activation. In this study, we have investigated the substrate specificity of BPHL, a human valacyclovirase (hVACVase), using a series of amino acid ester prodrugs of therapeutic nucleoside analogues. We report the results of investigations into the effect of hydrophobicity, stereochemistry, and the site of esterification of the amino acid promoiety as well as the influence of various nucleoside analogues as leaving groups on the activity of BPHL.

Experimental Section

Materials. Valacyclovir (VACV) and valganciclovir were provided by GlaxoSmithKline Inc. (Research Triangle Park, NC) and Hoffman-La Roche Inc. (Nutley, NJ), respectively. 2-Bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (BDCRB) was kindly provided by Dr. Townsend and Dr. Drach of The University of Michigan. Gemcitabine (2′-deoxy-2′,2′-difluorocytidine) was purchased as Gemzar from Eli Lilly and Co. (Indianapolis, IN). Acyclovir [ACV, 2-amino-1,9-dihydro-9-(2-hydroxyethoxymethyl)-6*H*-purin-6-one], zidovudine (AZT, 3′-azido-3′-deoxythymidine), floxuridine (FUdR, 2′-deoxy-5-fluorouridine), and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Heptafluorobutyric acid was from ICN Biomedicals Inc. (Aurora, OH). Porcine esterase (EC 3.1.1.1, liver) and leucine aminopeptidase (EC 3.4.11.2, bovine kidney) were purchased from Sigma. *p*-Nitrophenyl acetate, *p*-nitrophenyl butyrate, lysine-*p*-nitroanilide, leucine-*p*-nitroanilide, proline-*p*-nitroanilide, Gly-Pro-*p*-nitroanilide, *p*-nitrophenol, and *p*-nitroanilide were purchased from Sigma. The amino acid ester prodrugs, glycyl acyclovir (**5i**) and L-valyl zidovudine (**3a**), were synthesized and identified as described previously[4] (Chart 1). Valine, phenylalanine, and proline ester prodrugs of FUdR were synthesized and analyzed as described previously[5] (Chart 1). The Bio-Scale DEAE5 column, the Bio-Scale CHT2-I column, the Bio-Scale S2 column, and the Bio-Sil SEC 125-5 column were purchased from Bio-Rad (Hercules, CA). The PD-10 column was from Amersham Pharmacia Biotech (Piscataway, NJ). Other chemicals were either analytical- or HPLC-grade.

Overexpression and Purification of Recombinant BPHL. Recombinant BPHL was overexpressed and purified from *Escherichia coli* as described previously.[1] In brief, the expression vector based on pET29b containing BPHL cDNA was constructed and transformed into the BL21(DE) *E. coli* strain, and expression of BPHL was induced by 1 mM (final) isopropyl 1-thio-β-D-galactopyranoside at 25 °C for 6 h. Cells were collected by centrifugation (5000 rpm for 20 min, model JA 10) and lysed with $^1/_{50}$ volume of B-PER II (Pierce, Rockford, IL) containing 0.5 mM EDTA, 1 mg/mL leupeptin, 1 mg/mL pepstatin A, 1 mg/mL E-64, and 2 mg/mL aprotinin. For subsequent procedures, the protease inhibitors were not added. After centrifugation (27 000 rpm for 20 min at 4 °C), BPHL was purified from the supernatant by DEAE, hydroxyapatite, and size exclusion column chromatography. The fraction containing BPHL was judged by 4 to 20% SDS−PAGE stained with Gelcode blue stain reagent (Pierce). The purified BPHL was concentrated and stored at −80 °C until it was used. The protein concentration was determined on the basis of the Bradford method[6] using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

Synthesis of Amino Acid Ester Prodrugs. Amino acids with different characteristics were chosen as a promoiety of prodrugs. Phenylalanine (Phe), valine (Val), *p*-chlorophenylalanine (*p*-Cl-Phe), and proline (Pro) were used as hydrophobic, aromatic, aliphatic, and cyclic promoieties, respectively, while lysine (Lys) and aspartic acid (Asp) as basic and acidic promoieties, respectively. To study the influence of stereochemistry of a promoiety, D-isomers of Val and Phe were also employed. For FUdR and gemcitabine, three types of ester prodrugs, 3′-monoacyl ester, 5′-monoacyl ester, and 3′,5′-diacyl ester, were synthesized depending on the site of esterification, while only 5′-ester prodrugs were synthesized for BDCRB.

(1) Floxuridine Prodrugs. Asp and Lys ester prodrugs of FUdR were prepared following the same procedure described previously.[5] Briefly, *N*-*tert*-butyloxycarbonyl-protected amino acids (Boc-Val-OH, Boc-D-Val-OH, Boc-Phe-OH, Boc-D-Phe-OH, and Boc-Pro-OH) (1 equiv), dicyclohexylcarbodiimide (DCC, 1 equiv), and (dimethylamino)pyridine (DMAP, 0.1 equiv) were allowed to react with FUdR in dry *N*,*N*-dimethylformamide (DMF) for 24 h. The reaction was monitored by TLC. After completion of the reaction, the mixture was filtered, extracted, and dried under vacuum. The three spots observed on TLC were separated using column chromatography. The Boc group was cleaved by treating the residues with 2 mL of a trifluoroacetic acid (TFA, 60%)/dichloromethane (DCM) (35%)/water (5%) mixture for 4 h. After the TFA had evaporated, the residues were reconstituted with water and lyophilized. The amino acid prodrugs of FUdR were obtained as TFA salts. All prodrugs were characterized using mass spectrometry and NMR analysis. The prodrugs were evaluated for their purity by HPLC.

(2) BDCRB Prodrugs. BDCRB prodrugs were synthesized and analyzed as follows.[7] Briefly, the 2′- and 3′-

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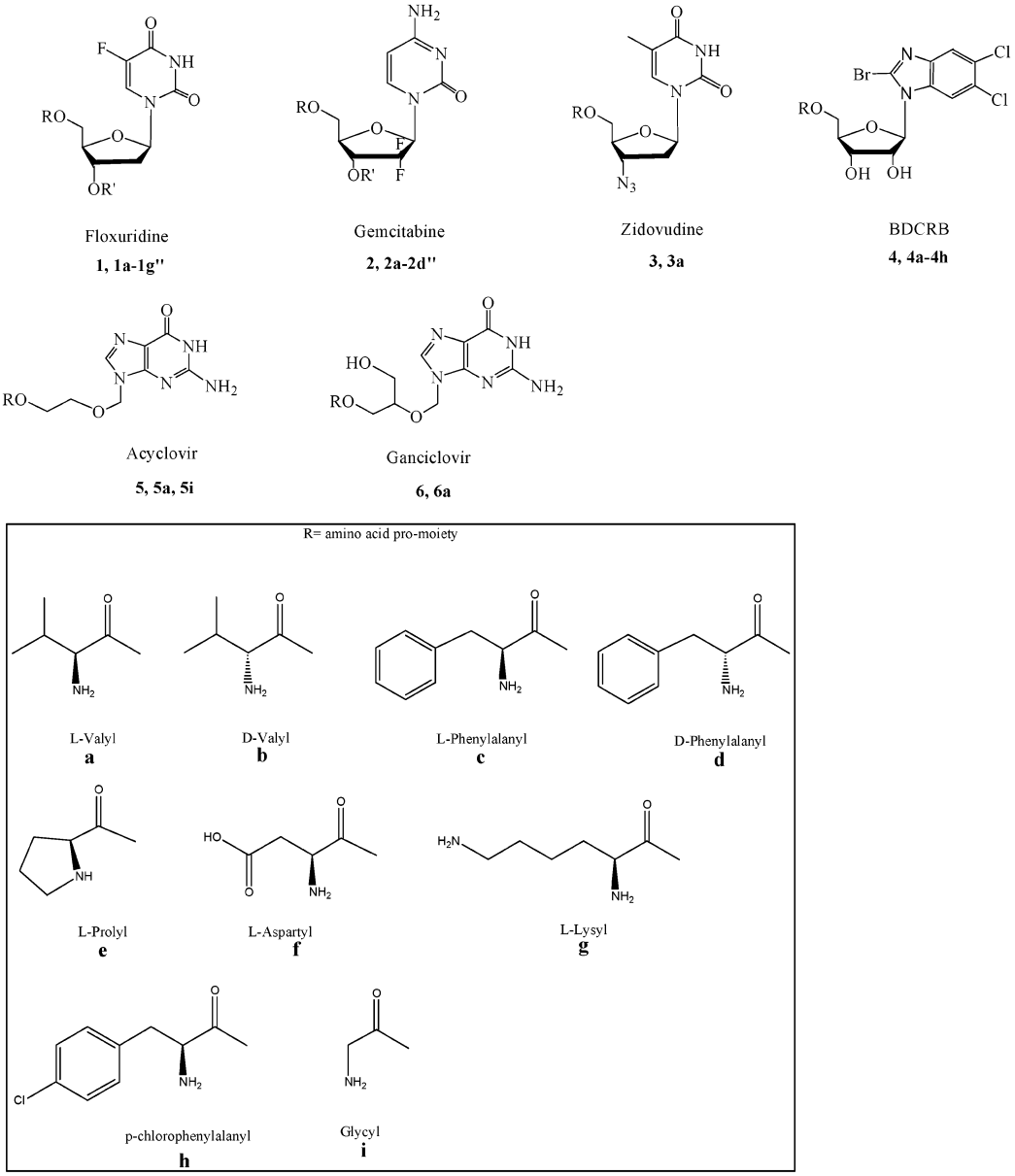
Chart 1. Structures of Therapeutic Nucleoside Analogues and Amino Acid Ester Prodrugs



3',5'-di-ester (1a-1g, 2a-2d): R = a-g, R′= a-g
3'-mono ester (1a′-1g′, 2a′-2d′): R = H, R′ = a-g
5'-mono-ester (1a′′-1g′′, 2a′′-2d′′): R = a-g, R′ = H

hydroxyl groups of BDCRB were protected by allowing them to react with a solution of dry acetone containing *p*-toluenesulfonic acid. The reaction mixture was stirred at room temperature and poured into an ice-cold solution of ammonium hydroxide in water (pH 8.0). The mixture was evaporated *in vacuo*, and the residue was extracted with ethyl acetate and dried over $MgSO_4$. The protected BDCRB was purified by column chromatography. The free 5′-OH was then esterified with N-tBoc-protected amino acids using DCC as a coupling reagent in the presence of a catalytic amount of DMAP for 24 h. The reaction was monitored by TLC. The product was purified by column chromatography. The intermediates obtained in step 2 were simultaneously treated with 50% TFA to remove Boc and 2′,3′-hydroxyl protecting groups. The yield of the purified BDCRB prodrugs was approximately 50%. The prodrugs were evaluated for their purity by HPLC.

(3) Gemcitabine Prodrugs. Prodrugs of gemcitabine (Gem) were synthesized as follows.[8] Briefly, N-tBoc-protected amino acids, DCC, and DMAP were allowed to react with Gem in dry DMF for 24 h. The reaction was monitored by TLC. The mixture was filtered and DMF removed under vacuum. The residue was extracted with ethyl acetate and washed with water, saturated $NaHCO_3$, and

(8) Song, X.; Amidon, G. L. Unpublished results, 2004.

saturated NaCl. The organic layer was dried over $MgSO_4$ and concentrated under vacuum. Attachment of an amino acid to Gem resulted in a mixture of 3′-monoester, 5′-monoester, and 3′,5′-diester prodrugs of Gem. Each individual ester was separated and purified using column chromatography. Fractions from each spot were concentrated under vacuum separately. The Boc group was cleaved by treating the residues with a TFA/DCM (1:1) mixture for 4 h. After TFA had evaporated, the residues were reconstituted with water and lyophilized. The amino acid prodrugs of Gem were obtained as TFA salts. The combined yield of the purified Gem prodrugs was approximately 58%. The prodrugs were evaluated for their purity by HPLC.

Prodrug Hydrolysis Study. Prodrug hydrolysis by BPHL was assayed as follows. BPHL (103 ng/mL) was preincubated in 10 mM phosphate buffer (pH 7.4) at 37 °C for 3 min, and then a prodrug was added to the final concentration of 0.4 mM to initiate the enzymatic reaction. The reaction was quenched with a volume of 10% ice-cold TFA in 10 min, and the generated parent drug and remaining prodrug were simultaneously analyzed by HPLC. Triplicate determinations of the extent of hydrolysis of each prodrug were conducted. The specific activity of BPHL was expressed in units of nanomoles per minute per microgram of protein based on the production of a parent drug. The relative activity was calculated on the basis of the specific activity of BPHL for VACV hydrolysis [68 nmol min^{-1} (μg of protein)$^{-1}$] as 100%. The enzymatic hydrolysis rate was corrected for any chemical hydrolysis observed with the control.

The general aminopeptidase and protease activity of BPHL was tested using commercially available chromogenic substrates. The hydrolytic activities of BPHL toward chromogenic esters, including *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate, and amides such as Lys-*p*-nitroanilide (*p*-NA), Leu-*p*-NA, Pro-*p*-NA, Val-*p*-NA, Phe-*p*-NA, and Gly-Pro-*p*-NA were studied by incubating 0.4 mM chromogenic compounds with BPHL in 10 mM phosphate buffer at 37 °C. The release of *p*-nitroaniline or *p*-nitrophenol after addition of BPHL was assessed continuously for 20 min at 405 or 450 nm with a microplate reader (PowerWaveX340, Bio-tek Instruments, Inc., Winooski, VT). *p*-Nitrophenol and *p*-nitroanilide (Sigma) were used to generate standard curves. The same amount of leucine aminopeptidase (LAP, porcine kidney, EC 3.4.11.2) and esterase (porcine liver, EC 3.1.1.1) served as a control. The protease activity of BPHL was studied using the QuantiCleave Protease Assay Kit (Pierce) following the manufacturer's protocol. In brief, proteolysis of succinylated casein by BPHL (0.6 or 6 μg/mL) was carried out at 37 °C for 30 min, and the change in absorbance at 405 nm after addition of a developing agent was measured after incubation for an additional 30 min. The provided TPCK trypsin served as a positive control.

HPLC Analysis. The concentrations of prodrugs and parent drugs were determined using a Waters HPLC system (Waters Inc., Milford, MA). The HPLC system was comprised of a reversed phase column (Xterra, C-18, 5 mm, 4.6 mm × 250 mm, Waters), a Waters 515 pump, a 996 photodiode array UV detector, and a WiSP model 712 autosampler (Waters). The system was controlled by Waters Milllennium 32 software (version 3.01). The flow rate was 1 mL/min; the injection volume was 50 μL. FUdR prodrugs were analyzed as described previously.[5] In brief, prodrugs and parent drugs except Lys-FUdR and Asp-FUdR were eluted with a linear gradient method and identified by different retention times (details of HPLC methods for BDCRB and Gem prodrugs will be presented elsewhere[7,8]). For all drugs and prodrugs except Gem and its prodrugs, mobile phase A was 0.1% TFA in distilled water and mobile phase B was 0.1% TFA in acetonitrile. For Gem prodrugs, 0.1% heptafluorobutyric acid in distilled water and 0.1% heptafluorobutyric acid in acetonitrile were used as mobile phases A and B, respectively. The gradient of mobile phase A to mobile phase B was run at 2% mobile phase B/min for most of the prodrugs. Lys-BDCRB and Asp-BDCRB were eluted with a gradient of 5% mobile phase B/min, and for Val-Gem, a gradient of mobile phase B of 0 to 8% over 4 min and then from 8 to 40% over 11 min was used. Lys-FUdR and Asp-FUdR were eluted with isocratic 1% and 2% mobile phase B, respectively. The prodrugs of ACV and AZT were eluted with gradients of 2 to 20% mobile phase B (detection at 254 nm) and 12 to 34% mobile phase B (detection at 268 nm), respectively. FUdR prodrugs and BDCRB prodrugs were separated with gradients of 2 to 32% mobile phase B (detection at 268 nm) and 25 to 70% mobile phase B (detection at 261 nm), respectively. Gemcitabine prodrugs were eluted with a 0 to 40% mobile phase B gradient and detected at 274 nm. Standard curves generated for each parent drug were used for quantification of the integrated area under peaks.

Determination of Kinetic Parameters. The kinetic parameters of BPHL for the hydrolysis of Val-, Phe-, and Pro-ester prodrugs of FUdR and BDCRB were determined as follows. Kinetic measurements were carried out in 10 mM potassium phosphate buffer (pH 7.4) at 37.0 ± 0.5 °C. Kinetic parameters were calculated from the initial velocity data at substrate concentrations ranging from 0.04 to 6 mM (from 0.04 to 0.8 mM for Phe-BDCRB). The reaction was initiated by adding 103 ng/mL recombinant BPHL to the preincubated substrate solution. An aliquot was taken at 2 min intervals up to 10 min, and the reaction was quenched with a volume of 10% TFA. Initial velocities were calculated from the linear time course for product formation (three to five time points per concentration). The Michaelis−Menten equation was fitted to the data by the nonlinear least-squares regression analysis in Sigma Plot 8.0 (SPSS Inc., Chicago, IL).

Calculation of the Log Octanol−Water Partition Coefficient. The three-dimensional structure of prodrugs with a protonated α-amino group was modeled by the Molecular Building module in the Molecular Operating Environment (MOE, Chemical Computing Group, Montreal, PQ) and used to calculate the log octanol−water partition coefficient, cLogP, via the QuaSAR descriptor module in MOE.

Table 1. Analytical Data for FUdR Prodrugs

promoiety of prodrugs	short name	purity (%)	ESI-MS $(M + H)^+$ expected	ESI-MS $(M + H)^+$ obsd
3′,5′-di-L-valyl,[a] **1a**	di-Val-FUdR	98	445.4	445.1
3′-L-valyl,[a] **1a′**	3′-Val-FUdR	92	346.3	345.9
5′-L-valyl,[a] **1a″**	Val-FUdR	98	346.3	346.0
3′,5′-L-di-D-valyl,[a] **1b**	di-D-Val-FUdR	98	445.4	445.1
3′-D-valyl,[a] **1b′**	3′-D-Val-FUdR	96	346.3	345.9
5′-D-valyl,[a] **1b″**	D-Val-FUdR	98	346.3	346.0
3′,5′-di-L-phenylalanyl,[a] **1c**	di-Phe-FUdR	94	541.5	541.1
3′-L-phenylalanyl,[a] **1c′**	3′-Phe-FUdR	96	394.4	394.0
5′-L-phenylalanyl,[a] **1c″**	Phe-FUdR	93	394.4	394.0
3′,5′-di-D-phenylalanyl,[a] **1d**	di-D-Phe-FUdR	95	541.5	541.1
3′-D-phenylalanyl,[a] **1d′**	3′-D-Phe-FUdR	94	394.4	394.1
5′-D-phenylalanyl,[a] **1d″**	D-Phe-FUdR	96	394.4	394.0
3′,5′-di-L-prolyl,[a] **1e**	di-Pro-FUdR	94	441.1	441.1
3′-L-prolyl,[a] **1e′**	3′-Pro-FUdR	99	344.3	344.0
5′-L-prolyl,[a] **1e″**	Pro-FUdR	98	344.3	344.1
3′,5′-di-L-aspart-1-yl, **1f**	di-Asp-FUdR	96	476.4	476.4
3′-L-aspart-1-yl, **1f′**	3′-Asp-FUdR	91	362.3	362.3
5′-L-aspart-1-yl, **1f″**	Asp-FUdR	90	362.3	362.3
3′,5′-di-L-lysyl, **1g**	di-Lys-FUdR	90	502.5	502.5
3′-L-lysyl, **1g′**	3′-Lys-FUdR	95	375.4	375.4
5′-L-lysyl, **1g″**	Lys-FUdR	90	375.4	375.4
floxuridine, **1**	FUdR	99	247.2	247.1

[a] From ref 5.

Results

Hydrolysis of Prodrugs of Floxuridine. Twenty-one prodrugs of FUdR (**1a**−**1g″**) were tested for hydrolysis by BPHL (Table 1). The susceptibility of FUdR prodrugs to BPHL was significantly affected by the amino acid promoiety and the site of esterification. BPHL preferentially hydrolyzed the 5′-monoesters of hydrophobic amino acids (Figure 1). Significant hydrolysis by BPHL was observed for the Val-, D-Phe-, Phe-, and Pro-FUdR (**1a″**, **1c″**, **1d″**, and **1e″**), while significantly lower levels of hydrolysis of the corresponding 3′-esters (**1a′**, **1c′**, **1d′**, and **1e′**) and 3′,5′-diesters (**1a**, **1c**, **1d**, and **1e**) and Lys- and Asp-FUdR (**1f″** and **1g″**, respectively) were observed. The ratios of the 5′-ester/3′-ester hydrolysis rates for Val-, Phe-, and Pro-FUdR were 36, 13, and 4, respectively, while those of the 5′-ester/3′,5′-diester hydrolysis rates for Val-, Phe-, and Pro-FUdR were 82, 11, and 5, respectively. BPHL hydrolyzed 5′-Val-, Phe-, and Pro-FUdR at a rate comparable to that of VACV hydrolysis. VACV (**5a**) is taken as our reference prodrug since it is known to undergo rapid hydrolysis *in vivo* in humans. The hydrolysis rates of 5′-Asp- and 5′-Lys-FUdR were only 10% and 3% of the VACV hydrolysis rate, respectively. BPHL exhibited a preference for the L-isomer, hydrolyzing Val-FUdR at a significantly greater rate than D-Val-FUdR (Figure 1). The kinetic parameters indicate that BPHL is much more stereoselective for Val-FUdR than for Phe-FUdR. The ratios of the specificity constant (V_{max}/K_m) for L/D-valyl FUdR and L/D-phenylalanyl FUdR were 98 and 1.6, respectively. This is due to the significantly higher K_m of D-Val-FUdR for BPHL compared to that of L-Val-FUdR (2.78 and 0.2 mM, respectively) and a lower V_{max} (21 and 148 nmol min^{-1} μg^{-1}, respectively), while BPHL exhibited comparable K_m and V_{max} values for D-Phe-FUdR and L-Phe-FUdR (K_m = 0.75 and 0.63 mM and V_{max} = 475 and 643 nmol min^{-1} μg^{-1}, respectively).

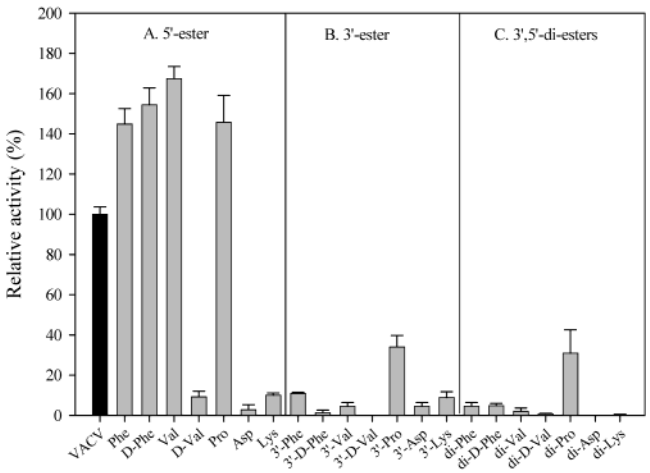


Figure 1. Hydrolysis of amino acid ester prodrugs of floxuridine by BPHL. Twenty-one floxuridine (FUdR) prodrugs with varying amino acid promoieties or sites of esterification were tested for hydrolysis by BPHL. Hydrolysis of FUdR prodrugs was studied by incubating 0.4 mM prodrug with BPHL at 37 °C for 10 min as described in the Experimental Section. After the determination of the specific activity based on the FUdR production, the relative activity was expressed with respect to the rate of VACV hydrolysis (68 nmol min^{-1} μg^{-1}): (A) 5′-amino acid ester FUdR, (B) 3′-amino acid ester FUdR, and (C) 3′,5′-diamino acid ester prodrugs (n = 3).

Table 2. Analytical Data for Gemcitabine and BDCRB Prodrugs

promoiety of prodrugs	short name	purity (%)	ESI-MS $(M + H)^+$ expected	ESI-MS $(M + H)^+$ obsd
gemcitabine prodrugs				
3′,5′-di-L-valyl,[a] **2a**	di-Val-Gem	98	460.2	460.2
3′-L-valyl,[a] **2a′**	3′-Val-Gem	97	362.1	363.1
5′-L-valyl,[a] **2a″**	Val-Gem	95	362.1	363.1
3′,5′-di-D-valyl,[a] **2b**	di-D-Val-Gem	97	460.2	460.2
3′-D-valyl,[a] **2b′**	3′-D-Val-Gem	95	363.1	363.1
5′-D-valyl,[a] **2b″**	D-Val-Gem	96	363.1	363.1
3′,5′-di-L-phenylalanyl,[a] **2c**	di-Phe-Gem	89	558.2	558.1
3′-L-phenylalanyl,[a] **2c′**	3′-Phe-Gem	91	411.1	411.1
5′-L-phenylalanyl,[a] **2c″**	Phe-Gem	97	411.1	411.1
3′,5′-di-D-phenylalanyl,[a] **2d**	di-D-Phe-Gem	88	558.2	558.1
3′-D-phenylalanyl,[a] **2d′**	3′-D-Phe-Gem	90	411.1	411.1
5′-D-phenylalanyl,[a] **2d″**	D-Phe-Gem	96	411.1	411.1
BDCRB prodrugs				
5′-L-valyl,[b] **4a**	Val-BDCRB	97	498.2	498.2
5′-D-valyl,[b] **4b**	D-Val-BDCRB	97	498.2	498.2
5′-L-phenylalanyl,[b] **4c**	Phe-BDCRB	97	546.2	546.2
5′-D-phenylalanyl,[b] **4d**	D-Phe-BDCRB	97	546.0	546.0
5′-L-prolyl,[b] **4e**	Pro-BDCRB	98	496.2	496.2
5′-L-aspart-1-yl,[b] **4f**	Asp-BDCRB	91	514.1	514.1
5′-L-lysyl,[b] **4g**	Lys-BDCRB	96	527.2	527.2
5′-*p*-chloro-L-phenylalanyl,[b] **4h**	*p*-Cl-Phe-BDCRB	96	580.0	580.0
BDCRB, **4**		96	398.1	398.1

[a] From ref 8. [b] From ref 7.

Hydrolysis of BDCRB Prodrugs. The 5′-ester prodrugs (**4a**−**4h**) of 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (BDCRB), a new antiviral agent,[9] were synthesized using Val, D-Val, Phe, D-Phe, Pro, Lys, and *p*-Cl-Phe as promoieties (Table 2). BPHL hydrolyzed the hydrophobic amino acid esters of BDCRB, including Val-, Phe-, D-Phe-, Pro-, and *p*-Cl-Phe-BDCRB (**4a**, **4c**−**4e**, and **4h**, respectively) at rates comparable to that of VACV hydrolysis, while prodrugs with the charged amino acids, Asp-BDCRB and Lys-BDCRB (**4f** and **4g**, respectively), were poor substrates (Figure 2). The specific activity appears to be positively correlated with the hydrophobicity, cLogP, of the promoiety. However, the hydrolysis of *p*-Cl-Phe-BDCRB (**4h**) was slower than that of Phe-BDCRB (**4c**) despite the enhanced hydrophobicity of the promoiety. The specific activity of BPHL for L-Val-BDCRB (**4a**) was ∼10 times higher than for D-Val-BDCRB (**4b**) [41 ± 1.6 and 3.7 ± 0.9 nmol min^{-1} (μg of protein)$^{-1}$, respectively], while the specific activities for L-Phe-BDCRB (**4c**) and D-Phe-BDCRB (**4d**) were similar.

Hydrolysis of Gemcitabine Prodrugs. Twelve prodrugs of gemcitabine (Gem) with varying sites of esterification, using D/L-Val and D/L-Phe, were synthesized (**2a**−**2d″**, Table 2). The preference of BPHL for 5′-esters over 3′-esters and 3′,5′-diesters is similar to that observed for FUdR prodrugs. The ratios of the specific activity for the 5′-ester to 3′-esters of valyl Gem and phenylalanyl Gem were 3.1 and 1.3, respectively, and those of the corresponding 5′-ester to 3′,5′-diesters were 5.1 and 2.7, respectively (Figure 3). However, BPHL exhibited less selectivity for 5′-Phe-Gem (**2c″**) compared to 3′-Phe-Gem (**2c′**) or 3′,5′-di-Phe-Gem (**2c**) than was observed for the FUdR prodrugs. This is due to the fact that the 3′-ester and 3′,5′-diesters of Gem were considerably better substrates for BPHL than the counterpart FUdR

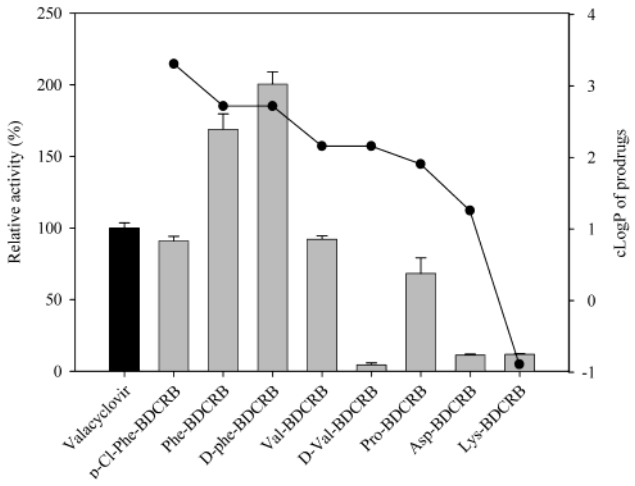


Figure 2. Hydrolysis of BDCRB prodrugs by BPHL. Hydrolysis of FUdR prodrugs was studied by incubating 0.4 mM prodrug with BPHL at 37 °C for 10 min as described in the Experimental Section. After the determination of the specific activity based on BDCRB production, the relative activity was calculated with respect to the rate of VACV hydrolysis (68 nmol min^{-1} μg^{-1}) ($n = 3$).

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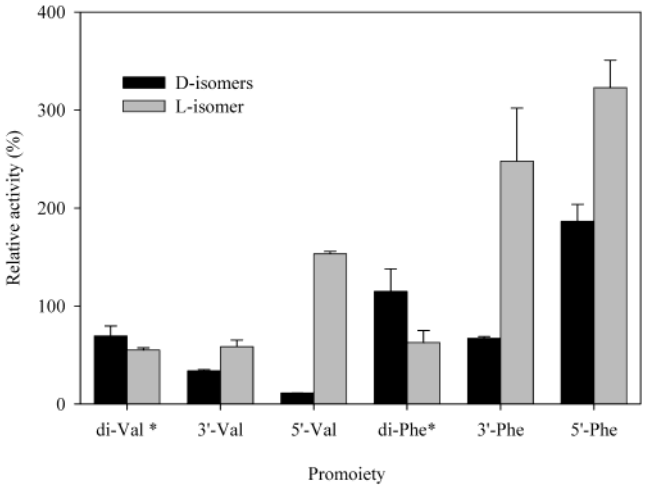

Figure 3. Hydrolysis of gemcitabine prodrugs by BPHL. Hydrolysis of gemcitabine (Gem) prodrugs by BPHL was studied by incubating 0.4 mM prodrug with BPHL at 37 °C for 10 min as described in the Experimental Section. After the determination of the specific activity based on Gem production, the relative activity was calculated with respect to the rate of VACV hydrolysis (68 nmol min^{-1} μg^{-1}). For those marked with an asterisk, the specific activity was determined on the basis of the disappearance of prodrugs ($n = 3$).

prodrugs. BPHL favors the L-isomer over the D-isomer irrespective of the esterification site. The ratios of the specific activity for the 5′-L-isomer to the 5′-D-isomer of valyl and phenylalanyl Gem were 18 and 1.7, respectively, while those of corresponding 3′-L-isomer to the 3′-D-isomer were 1.7 and 3.7, respectively. BPHL is much more selective for Val isomers than for Phe isomers, consistent with the results for the prodrugs of FUdR and BDCRB (Figure 3).

Hydrolysis of Amino Acid Ester Prodrugs of ACV and AZT by BPHL. In our previous study, L-valyl-AZT (**3a**, Val-AZT) and Gly-acyclovir (**5i**, Gly-ACV) were expected to be substrates of BPHL based on the fact that the hydrolysis of Val-AZT and Gly-ACV by Caco-2 cell subcellular fractions was comparable to VACV hydrolysis.[1] The hydrolysis of Val-AZT and Gly-ACV by the purified recombinant BPHL is comparable to that of VACV, confirming this prediction (Figure 4).

Kinetic Parameters for Prodrug Hydrolysis by BPHL and the Effect of Hydrophobicity on Substrate Specificity. The kinetic parameters for BPHL were determined from the linear portion of the V_0 versus [S] plot for several prodrugs of FUdR and BDCRB (Table 3). The specificity constant (V_{max}/K_m) of the prodrugs for BPHL decreased in the following order: Phe-FUdR and Phe-BDCRB > VACV > Val-FUdR and Val-BDCRB > D-Phe-FUdR > Pro-FUdR > valganciclovir > 3′-Val-FUdR > D-Val-FUdR. Notably, the K_m and V_{max} values for prodrugs with the same promoiety at the same modification site are similar. For example, the K_m values of Val-FUdR and Val-BDCRB were 0.2 and 0.34 mM, respectively, while those of Phe-FUdR and Phe-BDCRB were 0.63 and 0.69 mM, respectively. Similarly, the V_{max} values of Val-FUdR and Val-BDCRB were 148 and 149 nmol min^{-1} (μg of protein)$^{-1}$, respectively, while those of Phe-FUdR and Phe-BDCRB were 643 and 685 nmol min^{-1} (μg of protein)$^{-1}$, respectively.

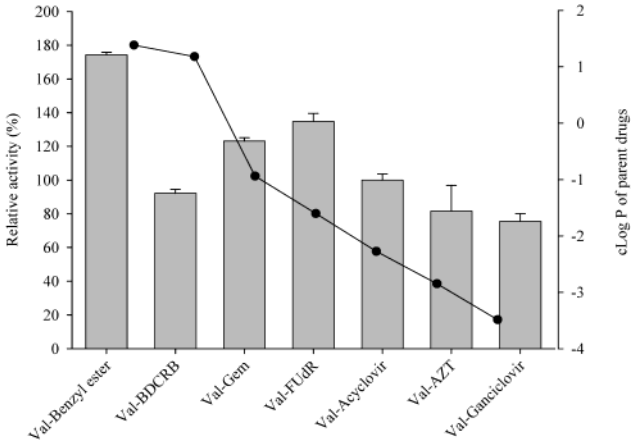

Figure 4. Hydrolysis of BDCRB prodrugs by BPHL. Hydrolysis of FUdR prodrugs was studied by incubating 0.4 mM prodrug with BPHL at 37 °C for 10 min as described in the Experimental Section. After the determination of the specific activity based on the production of each parent drug, the relative activity was calculated with respect to the rate of VACV hydrolysis (68 nmol min^{-1} μg^{-1}) ($n = 3$).

Table 3. Kinetic Parameters for BPHL[a]

prodrug	K_m (mM)	V_{max} (nmol min^{-1} μg^{-1})	V_{max}/K_m	relative V_{max}/K_m
D-Val-FUdR	2.78	21	7.55	0.01
3′-Val-FUdR	0.95	33	34.7	0.04
valganciclovir[b]	1.90	197	104	0.13
Pro-FUdR	1.45	556	383	0.48
Val-BDCRB	0.34	149	438	0.55
D-Phe-FUdR	0.75	475	633	0.79
Val-FUdR	0.20	148	740	0.92
valacyclovir[b]	0.19	152	800	1.00
Phe-BDCRB	0.69	685	993	1.24
Phe-FUdR	0.63	643	1021	1.28

[a] Kinetic parameters were calculated by fitting the Michaelis−Menten equation to the initial velocity data by using the nonlinear least-squares regression analysis module in Sigma Plot 8.0. The rate of hydrolysis was measured by incubating BPHL (103 ng/mL) with various concentrations of a prodrug in 10 mM phosphate buffer (pH 7.4) at 37 °C. The initial velocity was determined during the linear time course at a substrate concentration ranging from 0.04 to 6 mM. The results are means of two independent experiments. $V_0 = V_{max}[S]/(K_m + [S])$. Abbreviations: FUdR, floxuridine; BDCRB, 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole. [b] Previously published by Kim et al.[1]

The significant hydrophobic interaction between BPHL and the promoiety of substrates was suggested by the positive correlation between the estimated log octanol−water partition coefficient, cLogP, and the relative activity of BPHL for the prodrugs (Figure 5A,B). The hydrophobicity of the promoiety has a greater effect on the reactivity of prodrugs with BPHL than that of the parent nucleoside structure. The effect of the hydrophobicity of the nucleoside analogues is illustrated by the valine esters of various parent drugs (Figure 5A). While the cLogP of the parent drug decreased from 1.38 to

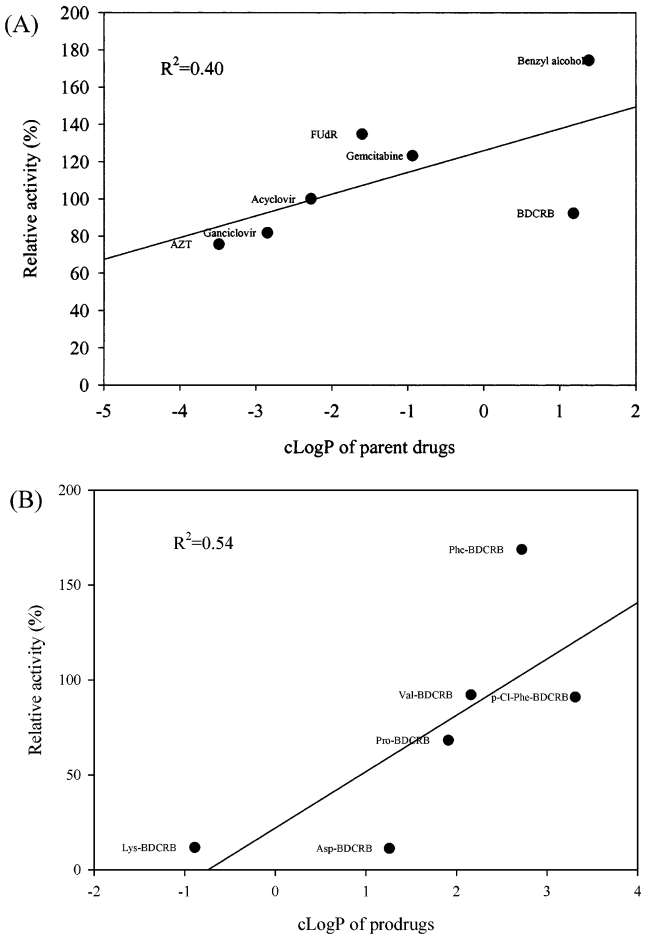

Figure 5. Correlation between cLogP of substrates and BPHL activity. Correlation between (A) cLogP of parent drugs of valine ester prodrugs and (B) cLogP of BDCRB prodrugs and BPHL activity for prodrugs.

−3.49 (from hydrophobic to very hydrophilic), all of the valine esters are good substrates. On the other hand, the cLogP of the BDCRB prodrugs varied from hydrophobic (2.73) to slightly hydrophilic (−0.89) and the relative activity decreased by a factor of 17 for Asp-BDCRB (**4f**) and Lys-BDCRB (**4g**) compared to that for D-Phe-BDCRB (**4b**) (Figure 5B).

Hydrolysis of Chromogenic Ester and Amide Substrates by BPHL. BPHL exhibits a substrate specificity significantly different from those of leucine aminopeptidase (LAP) and porcine liver esterase (ES) shown in Figure 6. BPHL did not exhibit significant hydrolytic activity toward Lys-*p*-NA, Leu-*p*-NA, Pro-*p*-NA, and Gly-Pro-*p*-NA. No significant hydrolysis of Phe-*p*-NA or Val-*p*-NA by BPHL was observed (data not shown). Further, BPHL did not exhibit significant activity toward common esterase substrates *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate. The very limited hydrolytic activity toward *p*-nitrophenyl butyrate is consistent with a previous report.[2] While BPHL hydrolyzes amino acid esters, including valyl benzyl ester (Figure 4), BPHL did not hydrolyze esters with aliphatic acyl groups such as acetate and butyrate in the absence of the α-amino

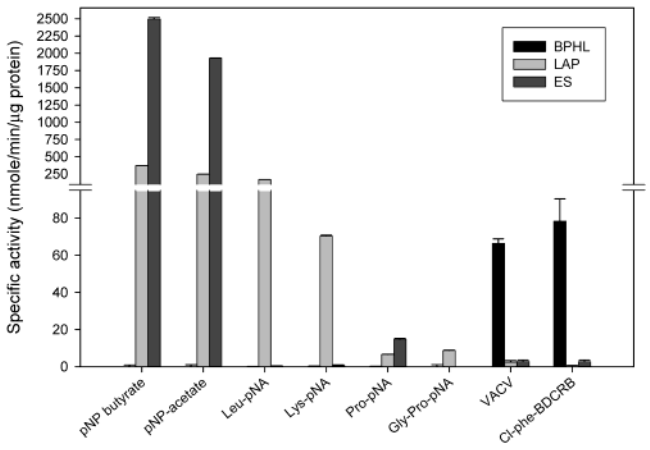

Figure 6. Hydrolysis of chromogenic ester and amide substrates and VACV by BPHL. Hydrolysis of chromogenic substrates was studied by incubating BPHL with 0.4 mM substrate at 37 °C for up to 100 min as described in the Experimental Section. The release of *p*-nitrophenol or *p*-nitroaniline was monitored at 410 or 405 nm, respectively. *p*-Nitrophenol and *p*-nitroaniline from Sigma served as standards. Abbreviations: BPHL, biphenyl hydrolase-like protein; LAP, porcine kidney leucine aminopeptidase; ES, porcine kidney esterase; pNP, *p*-nitrophenyl; *p*NA, *p*-nitroanilide; VACV, valacyclovir.

group, suggesting that the α-amino group may be important for substrate specificity. Interestingly, LAP and ES did not show significant hydrolytic activity toward VACV or *p*-Cl-Phe-BDCRB. Finally, no detectable proteolysis of succinylated casein by BPHL was observed (data not shown).

Discussion

We determined the substrate specificity of BPHL, a human valacyclovirase, to evaluate its potential to serve as a prodrug activating target for amino acid ester prodrugs of nucleoside analogues. In general, the hydrolyses of the prodrugs with the parent drugs FUdR, Gem, and BDCRB were similar for a given amino acid acyl functionality. However, the amino acid promoiety and the site of esterification (3′, 5′, or both) had a significant influence on hydrolysis by BPHL. With both FUdR and BDCRB prodrugs (Figures 2 and 3), the hydrophobic amino acid ester prodrugs were substantially more susceptible to BPHL hydrolysis, while the charged amino acid ester prodrugs were relatively poor substrates, irrespective of the structure of the nucleoside analogue and the site of esterification. The effect of the hydrophobicity of a substrate is also shown by the positive correlation of the specific activity of BPHL with the (estimated) log octanol−water partition coefficients of the substrates. The positive correlation between the hydrophobicity of the promoiety and its reactivity with BPHL suggests that the active site of BPHL has a hydrophobic acyl binding pocket that is sufficiently large to accommodate Phe. On the other hand, the decrease in specific activity for the more hydrophobic *p*-Cl-Phe-BDCRB from Phe-BDCRB suggests that the size of the acyl binding pocket is restricted.

The susceptibility of prodrugs to BPHL hydrolysis was significantly dependent on the site of esterification and the presence of a second amino acyl group (the diesters). The 5′-monoesters are the most preferred among FUdR and Gem prodrugs. However, the reactivity of the less preferred 3′-mono- and 3′,5′-diesters varies significantly, depending on the promoiety (valyl and phenylalanyl vs prolyl FUdR) and the particular nucleoside drug portion (valyl FUdR vs valyl Gem). The ratios of the 5′-ester to 3′-ester hydrolysis rates for valyl FUdR and phenylalanyl FUdR were 33 and 13, respectively, while those for valyl Gem, phenylalanyl Gem, and prolyl FUdR were 3.1, 1.3, and 4.2, respectively. The kinetic parameters suggest that BPHL has lower affinity and catalytic capacity for 3′-Val-FUdR than for 5′-Val-FUdR (Table 3). If we assume BPHL functions in a manner analogous to the classical catalytic mechanism of serine hydrolases, a bulky group close to O_{ester} of the leaving group may interfere with the interaction between the catalytic histidine and O_{ester} of a substrate.[10,11] In addition, the increased steric hindrance around O_{ester} of a secondary alcohol may result in a less favorable binding of the 3′-ester prodrugs. Compared to 3′-Val-FUdR (**1a**′) and 3′-Phe-FUdR (**1c**′), the hydrolyses of 3′-Val-Gem (**2a**′), 3′-Phe-Gem (**2c**′), and 3′-Pro-FUdR (**2e**′) were 12−22 times more rapid. For 3′-ester prodrugs of Gem, the electron withdrawing effect of two very electronegative fluorine at the 2′-position of the ribose of Gem may destabilize the ester bond such that the 3′-ester becomes more susceptible to both enzymatic and chemical hydrolysis.[12] Similarly, the ionized α-imino group of proline in Pro-FUdR is also electron withdrawing, resulting in the ester bond becoming more labile.[13]

The hydrolysis of 5′-esters is significantly influenced by modification at the 3′-position, with hydrolysis being significantly slower for the diesters, at both the 5′- and 3′-positions. The ratios of the hydrolysis rate of 5′-monoester to 3′,5′-diesters of valyl FUdR and phenylalanyl FUdR were 84 and 29, respectively, while those of valyl Gem, phenylalaninyl Gem, and prolyl FUdR were 2.7, 5.1, and 4.7, respectively. This is likely due to the steric hindrance provided by the additional amino acid at the 3′-position. Interestingly, Val-AZT which has an N_3 group at the 3′-position in place of the hydroxyl group was hydrolyzed at a rate comparable to that of VACV hydrolysis (Figure 5). The relatively good reactivity of Val-AZT may be attributed to the smaller size of the N_3 group compared to the 3′-acyl ester group.

BPHL exhibited clear stereoselectivity for L-Val to D-Val prodrugs irrespective of the parent drug while exhibiting comparable hydrolytic activity toward D-Phe and L-Phe esters of all three nucleoside analogues. The influence of the structure of the acyl group on the stereoselectivity has been observed in other enzymes.[14,15] For example, porcine pancreas lipase exhibits a 100-fold higher stereoselectivity toward phenylalanyl methyl ester than toward the threoninyl methyl ester (enantiomeric ratio *E* of 521 and 5, respectively).[15] The stereoselectivity differences observed for the different acyl groups may be due to a better structural complementarity and stronger hydrophobic interaction between Phe and the acyl binding site of BPHL, lowering the activation energy barrier. It is also possible that the less steric *β*-carbon of Phe compared to that of Val may provide a spatial flexibility to the carbonyl carbon and carbonyl oxygen of D-Phe at the active site such that the D-Phe esters can take a more favorable position for further enzymatic catalysis. Further insights into D/L specificity observed for BPHL will require a three-dimensional model of the active site. We are currently developing a homology model which may provide further insight into the observed D/L specificity differences.

Recently, Vig et al.[5] reported that the 5′-amino acid ester prodrugs of FUdR were significantly less stable in Caco-2 cell homogenates than the 3′-esters or the diester prodrugs. Further, the differential stereoselectivity for valyl and phenylalanyl FUdR is consistent with that of recombinant BPHL. The fact that BPHL was identified from Caco-2 cells[1] and the profile of hydrolysis of FUdR prodrugs by Caco-2 cell homogenates and by recombinant BPHL is similar suggests that BPHL may be the major enzyme responsible for the hydrolytic activation of amino acid ester prodrugs of FUdR in the Caco-2 human intestinal cell line.

Knowledge of enzyme systems in mammals such as mouse, rat, and monkey is very useful in the selection of animal models for use in drug development. According to Burnette et al.,[16] VACV was completely converted to ACV in mouse, which implies the presence of a mouse enzyme(s) function similar to that of BPHL. In fact, potential mouse orthologs of BPHL whose sequences are 86% identical are found in a homology search.[1] The complete and rapid conversion of VACV to ACV in cynomolgus monkey and rats[17,18] also indicates the presence of enzyme(s) for VACV activation in these species. Previously, a rat valacyclovir hydrolase (rVACVase), purified from rat liver by Burnette et al.,[16] catalyzed the hydrolysis of a series of amino acid ester prodrugs of ACV in the following order of specificity: Met-ACV > Leu-ACV > Ala-ACV > VACV > Ile-ACV > D-Val-ACV. The rVACVase was suggested to be a rat ortholog of BPHL on the basis of its similarity with BPHL

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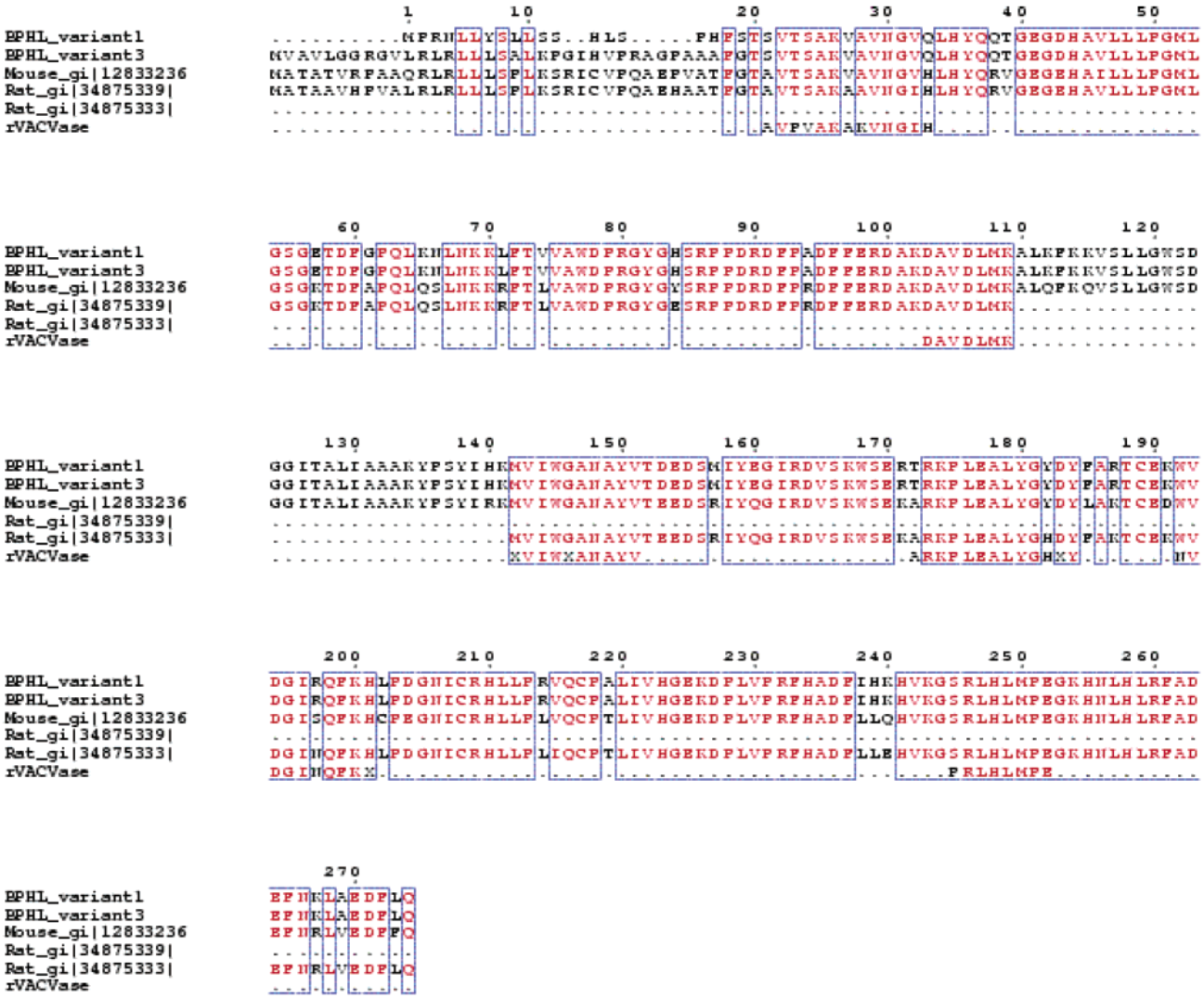

Figure 7. Sequence alignment among BPHL and potential mammalian orthologs. The sequence alignment was conducted with ClustalW (version 1.82)[21] at the European Bioinformatics Institute server (http://www.ebi.ac.uk/clustalw/) with default parameters and manually adjusted. The alignment was printed with ESPript[22] (http://prodes.toulouse.inra.fr/ESPript/cgi-bin/ESPript.cgi): BPHL_variant1 (NP_004323), BPHL_variant3 (CAD70626), Mouse_gi|12833236 (BAB22447, RIKEN cDNA 2010012D11), Rat_gi|34875339 (XP_341522, similar to RIKEN cDNA 2010012D11), Rat_gi|34875333 (XP_341521, RIKEN cDNA 2010012D11), and rVACVase (rat liver valacyclovir hydrolase[16]). For Rat_gi:34875339, 150 N-terminal amino acids of 196 are aligned.

in biochemical characteristics and the protein sequence.[1] Consistent with BPHL, rVACVase showed a stereopreference for VACV over D-valyl acyclovir ester and no hydrolytic activity on *p*-nitrophenyl acetate. rVACVase or BPHL did not exhibit aminopeptidase activity for several di- and tripeptides or amino acid derivatives of *p*-nitroanilide, respectively. In addition, the importance of the α-amino group in substrate recognition by rVACVase was suggested by its hydrolytic activity for 2-aminovalery ester ACV but not for valeryl ester ACV.[16] However, the identity of rVACVase is still elusive. As of December 2003, two putative rat proteins (XP_341522 and XP_341521) highly homologous to the N- and C-termini, respectively, of BPHL are found in the nonredundant protein database. The putative protein "similar to RIKEN cDNA 2010012D11" (XP_341522) is 196 amino acids long, and its N-terminal 150-amino acid sequence is 86% identical to the N-terminal region of BPHL; on the other hand, the protein "similar to RIKEN cDNA 2010012D11" (XP_341521) is 86% identical to the C-terminal region of BPHL. In addition, of six peptide fragments of the sequence of rVACVase,[16] two fragments that include the N-terminus are homologous to the putative protein (XP_341522) and four fragments are homologous to the putative protein (XP_341521) (Figure 7). However, neither sequence spans the whole length of BPHL.

Comparison of the chromosomal location of the genes encoding BPHL and mammalian homologues (Genome Map Viewer via NCBI server, http://www.ncbi.nlm.nih.gov/genomes/static/euk_g.html) revealed a conservation of synteny among chromosome regions encoding BPHL and the

homologous proteins in rat and mouse. The *BPHL* gene is located on the human 6p25 chromosome[19] neighbored by 5′-flanking RIPK1 [receptor (TNFRSF)-interacting serine-threonine kinase 1] and 3′-flanking TUBB (tubulin β-polypeptide).[20] Two rat proteins similar to BPHL are encoded on the rat chromosome 17p2 region where the putative protein similar to the N-terminal half of BPHL (XP_341522) is encoded at the upstream of the 5′-end of the gene corresponding to the C-terminal half of BPHL (XP_341521), and the region encoding two rat sequences is flanked by LOC306886 encoding a putative protein "similar to RIP" at the upstream of the 5′-end and by LOC291081 encoding a putative protein "similar to tubulin, beta" in the downstream of the 3′-end. In a similar way, the mouse homologue of BPHL (BAB22447) is located on the mouse chromosome 13A3 region neighbored by 5′-flanking RIPK1 and by 3′-flanking TUBB2. This strongly suggests that rVACVase is very likely encoded on rat chromosome 17p2, and the 17p2 region deserves further analysis for identification of a gene encoding full-length rVACVase. The identification of potential mouse and rat homologues of BPHL strongly supports the uses of these animal models for further investigation of amino acid ester prodrugs of nucleoside analogues in drug development.

In summary, BPHL catalyzes the hydrolytic activation of amino acid esters of several nucleoside prodrugs in addition to the valacyclovir and valganciclovir. BPHL shows carboxylic acid ester hydrolase activity and exhibited neither aminopeptidase nor protease activity. BPHL catalyzes the hydrolysis of acyl amino acid ester nucleoside analogues with a general preference for 5′-esters and hydrophobic amino acids. The hydrolytic activity is significantly affected by the site and the number of ester functionalities as well as the stereochemistry of a promoiety. The contribution of the parent drug to the substrate specificity of BPHL was less significant than that of the amino acid acyl group. The stereoselectivity of BPHL depends on the acyl amino acid, and the α-amino group is important for substrate recognition by BPHL. These results suggest that the substrate binding site of BPHL is a hydrophobic acyl binding pocket, possessing a charge−charge interaction site for the α-amino group, and a relatively generous spatial binding site for the nucleoside leaving group with relatively limited space around the site for the cleavable ester group.

In conclusion, we have described an initial structure−activity relationship for hydrolysis of ester prodrugs of nucleoside analogue drugs by BPHL and determined the general structural requirements for BPHL substrates. These results suggest that a prodrug design strategy for antiviral and anticancer nucleoside analogues, based on BPHL specificity, provides an exciting new target for the design of more effective antiviral and anticancer nucleoside drugs.

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