JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726

JPET #92726

Nucleoside Ester Prodrug Substrate Specificity of Liver Carboxylesterase

Christopher P. Landowski, Philip L. Lorenzi, Xueqin Song, and Gordon L. Amidon

Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109

JPET #92726

Running Title: Ester Prodrug Specificity of Liver Carboxylesterase

Corresponding author: Gordon L. Amidon, Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, MI 48109. Tel: 734-764-2464, Fax: 734-763-6423, email: glamidon@umich.edu

Number of Text pages: 24

Number of Tables: 5

Number of Figures: 4

Number of References: 27

Number of words in Abstract: 240

Number of words in Introduction: 685

Number of words in Discussion: 1425

Recommended Section: Absorption, Distribution, Metabolism & Excretion

ABBREVIATIONS: CES1, carboxylesterase 1; CES2, carboxylesterase 2; BDCRB, 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole; pNPA, *p*-nitrophenyl acetate; pNPB, *p*-nitrophenyl butyrate; PEE, phenylalanine ethyl ester; cinn, cinnamic acid; AcPEE, N-acetyl phenylalanine ethyl ester; PBE, phenylalanine benzyl ester; CPT-11, irinotecan; VACV, valacyclovir; flox, floxuridine; gem, gemcitabine; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid.

ABSTRACT

Carboxylesterases are among the best characterized prodrug hydrolyzing enzymes involved in the activation of several therapeutic carbamate and ester prodrugs. The broad specificity of these enzymes makes them amenable for designing prodrugs. Porcine liver carboxylesterase 1 specificity for amino acid esters of three nucleoside analogs (floxuridine, gemcitabine, and 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl) benzimidazole) was evaluated to assess optimal structural preferences for prodrug design. The amino acid promoiety and the esterification site influenced carboxylesterase hydrolysis rates up to 1164-fold and the binding affinity up to 26-fold. CES1 exhibited high catalytic efficiency hydrolyzing prodrugs containing a phenylalanyl moiety, but was over 100-fold less efficient with valyl or isoleucyl prodrugs, regardless of the nucleoside or esterification site. CES1 catalytic efficiency was two-fold higher with 5′ phenylalanyl monoesters than the corresponding 3′ esters of floxuridine. This preference was reversed with phenylalanyl gemcitabine prodrugs, evident from a two-fold preference for 3′ monoesters over 5′ esters. The newly characterized esterase VACVase was several-hundred fold more efficient (up to 19,000-fold) than carboxylesterase in hydrolyzing amino acid esters, but similar in apparent binding affinity. The specific activities of the two enzymes with several amino acid ester prodrugs clearly suggest that initial hydrolysis rates are relatively low for prodrugs with isoleucyl, aspartyl, and lysyl promoieties for both enzymes compared to those with phenylalanyl, valyl, prolyl, and leucyl progroups. The low relative hydrolysis rates of isoleucyl, aspartyl, and lysyl prodrugs may facilitate prolonged systemic disposition of the nucleoside analogs for improved therapeutic action.

INTRODUCTION

Many therapeutic agents exhibit undesirable pharmaceutical or pharmacological properties that limit their potential clinical utility. These include low bioavailability due to metabolism, narrow therapeutic indices, excessive damage to normal cells, lack of selectivity for target cells, and multidrug resistance upon chronic treatment (Rooseboom et al., 2004). Prodrug strategies afford oral bioavailability enhancement of poorly absorbed drugs, facilitate selective activation in target tissues or organs, and may also provide protection from undesirable metabolic conversion of the active agent. Of these, prodrugs designed for selective activation in target tissues are by far the most efficient and attractive option. However, a prerequisite for such selective targeting is an enzyme or transporter that is exclusively or preferentially expressed in these tissues or organs. We previously reported on the design of melphalan prodrugs that are selectively activated by prolidase, an over-expressed enzyme in melanomas (Mittal et al., 2005).

Valacyclovir represents a prototype prodrug for enhancing the oral bioavailability of a poorly absorbed drug, acyclovir. The enhancement in oral absorption of this amino acid ester prodrug has since been attributed to carrier-mediated transport by intestinal oligopeptide transporters followed by rapid bioconversion to acyclovir. The successful clinical utility of oral valacyclovir indicated the potential for amino acids as promoieties for other potent agents. The synthesis, *in vitro* activation, and uptake characterization of various amino acid prodrugs of floxuridine (Vig et al., 2003; Landowski et al., 2005b), gemcitabine (Song et al., 2005a), and BDCRB (Song et al., 2005b) in cell systems have been previously reported. We also reported the identification and characterization of a novel enzyme, valacyclovirase (VACVase), that appears to be primarily responsible for

valacyclovir ester hydrolysis and demonstrates activity toward amino acid prodrugs of floxuridine, gemcitabine, and BDCRB (Kim et al., 2003; Kim et al., 2004a; Kim et al., 2004b).

Although rapid activation following transport is desirable for valacyclovir, extensive intestinal activation of anticancer prodrugs and subsequent release of agents such as floxuridine or gemcitabine would lead to severe intestinal toxicity. These side-effects may be reduced with intravenous administration, however, rapid activation and/or metabolism in the liver may cause toxicity and lower drug efficacy due to formation of inactive metabolites. Thus, gemcitabine deamination by cytidine deaminase, glycosidic bond cleavage of floxuridine by thymidine phosphorylase, and BDCRB cleavage by glycosylases are major detriments that need to be overcome to assure improved systemic disposition of these drugs. In this regard, we had demonstrated that amino acid ester prodrugs provided resistance to deamination of gemcitabine (Song et al., 2005a), to glycosidic bond cleavage of BDCRB (Lorenzi et al., 2005), and to floxuridine cleavage (Landowski et al., 2005a). These studies also revealed that ester bond hydrolysis of the amino acid ester prodrugs was the rate determining step in their metabolism. Thus, the resistance to drug metabolism was directly related to the enzymatic stability of the ester bond. Prodrug activation therefore determines the pharmacological efficacy of the parent drug.

In addition to VACVase, several other enzymes may be involved in the hydrolysis of these amino acid ester prodrugs. Of these, carboxylesterases are among the most widely studied prodrug hydrolyzing enzymes and are involved in the activation of various antiviral, anticancer, and antibiotic prodrugs (Campbell et al., 1987; Annaert et al., 1997;

Takai et al., 1997; Miwa et al., 1998; Rooseboom et al., 2004). Carboxylesterases hydrolyze a wide variety of ester, amide, and thioester linkages and metabolize various analgesic and narcotic compounds such as cocaine, heroin, and meperidine (Kamendulis et al., 1996; Zhang et al., 1999). The two major liver carboxylesterase isoforms in humans are carboxylesterase 1 (CES1) and carboxylesterase 2 (CES2), whereas in the intestine CES2 is mainly expressed (Satoh et al., 2002).

Carboxylesterase and valacyclovirase isoforms represent two major serine hydrolases that are widely distributed in the human body and are capable of activating amino acid ester prodrugs. In this report we describe the hydrolysis kinetics of various amino acid ester prodrugs of three potent nucleoside analogs, floxuridine, gemcitabine, and 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole, with porcine CES1. These results were compared to those obtained with VACVase in an effort to determine the effects of the promoieties as well as that of the parent drug on ester bond hydrolysis.

METHODS

Materials

HPLC-grade acetonitrile was purchased from Fisher Scientific (St. Louis, MO.) Trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were purchased from Aldrich Chemical Company (Milwaukee, WI). Valacyclovir was a gift from GlaxoSmithKline, Inc. (Research Triangle Park, NC). Porcine liver carboxylesterase, *p*-nitrophenyl acetate (pNPA) and *p*-nitrophenyl butyrate (pNPB) were purchased from Sigma (St. Louis, MO). All chemicals were either analytical or HPLC grade.

Amino acid prodrugs

The synthesis and characterization of the floxuridine, gemcitabine, and BDCRB amino acid ester derivatives have been reported previously (Vig et al., 2003; Landowski et al., 2005b; Song et al., 2005a; Song et al., 2005b). Figure 1 shows the prodrug structures.

HPLC conditions

Prodrug and parent drug concentrations were determined using a Waters HPLC system (Waters, Inc., Milford, MA). The HPLC system includes 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 injected onto a Waters Xterra C_{18} reversed-phase column (5 μm, 4.6 × 250 mm) equipped with a guard column. HPLC conditions have been reported previously (Vig et al., 2003; Landowski et al., 2005b; Song et al., 2005a; Song et al., 2005b).

Enzymatic studies

Carboxylesterase activity was measured by incubating porcine liver carboxylesterase (E.C. 3.1.1.1) with 200–400 μM prodrug in 10 mM KH_2PO_4, pH 7.4 (300 μl total volume) at 37°C. The prodrug stock solutions (100 mM) were prepared in dimethyl sulfoxide. To determine initial reaction rates, aliquots were sampled every two minutes up to 10 minutes and quenched with TFA (1% final v/v) before being analyzed by HPLC. The time course of product formation was linear throughout the 10 minutes of the assay ($r^2 \geq 0.98$). Specific activity is expressed as nmol product formed per min per μg protein. While the specific activity for monoester bond hydrolysis was reported as the product appearance rate, the specific activity for the diester prodrugs was reported as the substrate disappearance rate. CES hydrolytic activity toward pNPA and pNPB were studied in a manner similar to that for prodrugs except that *p*-nitrophenol release was measured at 405 nm for 0–20 min with a Power Wave X340 (Bio-Tek Instruments, Inc., Winooski, VT).

The kinetic parameters for the standard substrates, pNPA and pNPB, were determined by incubation with 1.38 ng/μl enzyme for 5 minutes over a concentration range of 0.003–6 mM substrate at 37°C. To determine K_m and k_{cat} parameters for the amino acid prodrugs, irinotecan, and phenylalanine esters, the enzyme reactions were typically run for 5 minutes, however, 10 and 15 minute incubations were used in a few cases (i.e. irinotecan) with substrate concentrations ranging from 0.078–5 mM. Typically 44 ng/μl of enzyme produced linear hydrolysis rates through 5 minutes. Lower enzyme concentrations were used for the 5′ cinnamic acid ester of floxuridine (11 ng/μl), 3′ gemcitabine prodrugs (2.75 ng/μl), phenylalanine benzyl ester (1.38 ng/μl), and

phenylalanine ethyl ester (0.69 ng/µl). VACVase specific activity was measured as previously described (Kim et al., 2003). The kinetic parameters were calculated by non-linear data fitting using Graph Pad Prism version 4.

Prodrug hydrolysis in Caco-2 cell extract

Confluent Caco-2 cells were washed with phosphate buffered saline (pH 7.4) and then harvested with 0.05% trypsin/EDTA at 37°C for 5-10 min. Trypsin was neutralized by adding Dulbecco's modified Eagle's medium. The cells were washed off the plate and spun down by centrifugation. The pelleted cells were washed twice with phosphate buffer (10 mM, pH 7.4), and resuspended in phosphate buffer (10 mM, pH 7.4) to obtain a final concentration of 4.7 x 10^6 cells/ml. The cells were then lysed by gentle sonication and spun down at 10,000 rpm for 10 min at 4°C. The supernatant was collected and total protein quantified with the BioRad DC Protein Assay using bovine serum albumin as a standard. The hydrolysis reactions were carried out in 96-well plates (Applied Biosciences). Caco-2 cytosolic extract (230 µl) was placed in triplicate wells; the reactions were started with the addition of substrate (40 µl) and incubated at 37°C for 0-30 min. The final prodrug concentration in the mixture was 400 µM. At each time point, 40 µl aliquot sample was removed and added to two volumes of 2% ice-cold TFA. The mixtures were centrifuged for 10 min at 1,800 rpm and 4°C. The supernatant was then filtered with a 0.45 µm filter and analyzed via reverse-phase HPLC. The kinetic parameters were calculated by non-linear data fitting using Graph Pad Prism version 4.

Molecular docking of floxuridine prodrugs into the carboxylesterase active site

Prodrug structures were modeled using the MOE-Molecule Builder (Chemical Computing Group, Montreal, PQ). The initial structures were minimized using molecular

mechanics with the MMFF94 force field (Halgren, 1996). The hCES1 crystal structure (PDB code 1MX5) used for the docking study contained the cocaine analog homatropine positioned in the active site, which was used to position the floxuridine prodrugs. The 5′-Phe-floxuridine and 5′-Val-floxuridine were docked using MOE-DOCK into (rigid body) hCES1 by simulated annealing with the MMFF94 force field containing a solvation term (distance dependent dielectric constant). A docking box was designated 7.5 Å around the manually positioned prodrug with $50 \times 50 \times 50$ grid points and a 0.375 Å grid spacing.

RESULTS

CES1 activity against floxuridine prodrugs

The kinetic parameters K_m and k_{cat} determined for 18 floxuridine monoester prodrugs are listed in Table 1. Of the uncharged amino acids tested, valyl esters exhibited the highest K_m values while the lowest values were observed with phenylalanine esters. Also, the apparent affinity increased as the length of the side chain increased. Thus, the trend was as follows: valyl esters < prolyl esters < isoleucyl esters < leucyl esters < phenylalanyl esters. The negatively charged aspartyl esters, on average, exhibited lower K_m values than the positively charged lysyl esters. Besides the amino acid promoiety, the esterification site also affected apparent affinity for the enzyme. K_m values were calculated for both the 3′ and 5′ monoester prodrugs, but not for the 3′,5′ diester prodrugs. For Phe, Leu, Pro, and D-Val prodrugs, the 5′ esters exhibited lower K_m values compared to the 3′ esters; 5′/3′ K_m ratios for Phe, D-Phe, Pro, Leu, and D-Val were 0.62, 0.31, 0.39, 0.66, and 0.37, respectively. On the other hand, 3′ monoester prodrugs of the charged amino acids Asp and Lys as well as the aliphatic amino acids, L-Val and Ile, exhibited lower K_m values compared to the 5′ analogs. Thus, 5′/3′ K_m ratios for Lys, Asp, L-Val, and Ile were 2.02, 3.23, 1.25, and 2.65, respectively.

The stereochemistry of the amino acid promoiety appeared to affect K_m only for the 3′ monoester prodrugs. Thus, there was little difference in apparent affinity of 5′-L- and 5′-D-phenylalanyl floxuridine or of 5′-L- and 5′-D-valyl floxuridine; however, the K_m for 3′-L-phenylalanyl floxuridine was almost half that of its D-analog and that of 3′-L-valyl floxuridine was 4-fold lower than its D-ester.

JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Catalytic activity, k_{cat}, was also affected by the amino acid promoiety. Among the amino acids evaluated, Phe and Pro were the most rapidly hydrolyzed esters, while Ile and Val were the least rapidly hydrolyzed. Thus, the 5′ Phe ester k_{cat} was 12-fold higher than the corresponding Ile ester. The negatively charged aspartyl esters exhibited around 4-fold lower k_{cat} values than the positively charged lysine esters.

The effect of esterification site on catalytic rate, k_{cat}, appears to be dependent on the amino acid promoiety. Thus, for Phe, Leu and Val prodrugs, k_{cat} values for 5′ and 3′ esters were not very different; 5′/3′ ratios of 1.17, 1.30, and 1.13, respectively. However, for the charged Asp and Lys and for Ile prodrugs, the catalytic rates for 5′ esters were significantly higher compared to the 3′ analog; 5′/3′ k_{cat} ratios for Lys, Asp, and Ile prodrugs were 1.87, 3.24, and 5.85, respectively. The 3′ esters hydrolyzed faster than the 5′ esters for D-Phe and Pro prodrugs with 5′/3′ ratios of 0.40 and 0.44, respectively. The promoiety stereochemistry also affected catalytic rates. Accordingly, the D-analogs of 3′-L-Phe, 3′-L-Val, and 5′-L-Val esters, exhibited slightly higher k_{cat} values with D/L k_{cat} ratios being 2.61, 1.45, and 1.41, respectively. Though, stereochemistry did not affect 5′ Phe esters significantly, where the D/L k_{cat} ratio was 0.90.

For the amino acid ester prodrugs examined, catalytic efficiency was in the order: Phe-prodrugs > Pro-prodrugs > Leu-prodrugs > Lys-prodrugs > Asp-prodrugs > Val-prodrugs ≥ Ile-prodrugs. The catalytic efficiency toward Ile-prodrugs were on average 90-fold lower than that observed with Phe-prodrugs of floxuridine and quite similar to that obtained with the carbamate prodrug, irinotecan. Interestingly, prodrugs that exhibited preferential affinity for the 3′ ester over the 5′ analog were also found to exhibit low catalytic efficiency with CES1. The k_{cat}/K_m values for the amino acid ester prodrugs

of floxuridine were, however, 28- to 4900-fold lower than that observed with the pNPA ester.

Hydrolysis in Caco-2 cytosolic extracts

Eleven select amino acid prodrugs were also analyzed in cytosolic extracts from in human intestinal cell line, Caco-2 to evaluate the activity of the human CES1, which is highly expressed in these cells (Sun et al., 2002;Imai et al., 2005). The prodrug hydrolysis rates in the extracts were well correlated (r^2=0.87) to those seen in experiments with porcine enzyme suggesting hCES1 has a similar promoeity preference, especially for phenylalanine esters (Figure 2).

CES activity against gemcitabine and BDCRB prodrugs

The influence of parent drug structure on carboxylesterase substrate specificity was examined using amino acid ester prodrugs of gemcitabine, as well as a pyrimidine nucleoside analog. Table 2 shows the kinetic parameters K_m and k_{cat} determined for 10 gemcitabine monoester prodrugs. As with floxuridine prodrugs, phenylalanyl gemcitabine prodrugs exhibited the lowest K_m values, while valyl and isoleucyl prodrugs exhibited much higher values. The esterification site also influenced apparent affinity. With the exception of L-Val, the 5′ monoesters appear to exhibit higher affinity resulting in the 5′/3′ K_m ratios: 0.33, 0.23, 0.81, and 0.90 for Phe, D-Phe, D-Val, and Ile, respectively. The promoiety stereochemistry appeared to influence K_m for gemcitabine prodrugs in a manner similar to that observed with floxuridine prodrugs. Thus, in general L-monoesters exhibited higher apparent affinity for carboxylesterase compared to their D-monoesters; the L-/D- K_m ratios were 0.77, 0.83, 0.53, and 0.63 for 5′ Phe, 5′ Val, 3′ Phe, and 3′ Val, respectively.

The different amino acid promoieties influence the catalytic rates of the gemcitabine esters similarly as seen with floxuridine esters. The most readily hydrolyzed esters contained a phenylalanine promoiety. Valine provided the least rapidly hydrolyzable ester. The site of esterification affected catalytic activity towards gemcitabine prodrugs in a profound manner. The 3′/5′ k_{cat} ratios were 5.45, 7.98, 16.20, 12.33, and 2.54 for Phe, D-Phe, Val, D-Val, and Ile, respectively.

In general, the CES1 catalytic efficiency, k_{cat}/K_m, with amino acid ester prodrugs of gemcitabine was consistently higher than that observed with the corresponding floxuridine prodrug. Except for valyl prodrugs, CES1 exhibited a 2- to 17-fold higher catalytic efficiency with gemcitabine prodrugs compared to its floxuridine analog. The CES1 catalytic efficiency with 5′-L-valyl and 5′-D-valyl esters of gemcitabine was lower than the corresponding floxuridine esters and similar to the poorest floxuridine substrate, 3′-L-Ile-floxuridine.

The kinetic parameters for 5′-L-phenylalanyl and 5′-D-phenylalanyl BDCRB listed in Table 2 indicate that their K_m values are significantly higher than the corresponding gemcitabine or floxuridine prodrugs (K_m of 0.90 mM compared to ~0.20 mM and 0.45 mM for gemcitabine and floxuridine prodrugs, respectively). Catalytic rates, k_{cat}, and catalytic efficiencies, k_{cat}/K_m, were similar for 5′ phenylalanyl monoesters of BDCRB and floxuridine, but dramatically lower than that observed with gemcitabine.

CES activity against phenylalanine esters

The overwhelming preference for phenylalanine promoieties exhibited by CES1 suggested that evaluating simple phenylalanine esters may provide additional structural information. The results of kinetic studies with a few simple esters are shown in Table 3

along with data from the standard substrates pNPA and pNPB, and the 5′-L-phenylalanyl prodrugs of floxuridine, gemcitabine, and BDCRB. CES1 exhibited the highest hydrolytic rate and catalytic efficiency with phenylalanine ethyl ester (PHEE), despite a low apparent binding affinity. PHBE, the benzyl ester, on the other hand enhanced binding affinity about 15-fold; however, catalytic rate and efficiency were severely diminished (>50-fold). Alteration of the amino group *via* N-acetylation (AcPHEE) also decreased catalytic rate and efficiency significantly. The catalytic efficiencies for PHBE and AcPHEE were similar to those obtained with 5′-L-phenylalanyl floxuridine and BDCRB prodrugs. 5′ cinnamic acid floxuridine ester, similar to 5′-L-phenylalanyl floxuridine except for the alpha amino group, exhibited a slightly lower apparent binding affinity for carboxylesterase compared to 5′-L-phenylalanyl floxuridine, but the catalytic rate and efficiency were enhanced 1.5- to 2-fold.

Molecular docking

Molecular docking was performed with a rapidly hydrolyzed substrate, 5′ Phe-FUdR, and with a slowly hydrolyzed substrate, 5′ Val-FUdR, using homatropine as a guide to position the prodrugs into the CES1 active site. Both amino acid ester substrates were able to bind to the active site in the expected orientation. The majority of binding conformations were consistent with that those reported for previously studied substrates (Bencharit et al., 2002; Bencharit et al., 2003a; Bencharit et al., 2003b). The amino acid ester groups bound oriented toward the large, flexible pocket of the enzyme, while the floxuridine group was facing the small, rigid pocket (Figure 3 and 4). The phenylalanine ester group was positioned between 3.53-5.15 Å of Leu255, Leu304, Leu318, Leu388, Thr252, Met425, and Phe426. The ridged pocket amino acid residues, Leu96, Leu97,

Phe101, Leu358, are positioned 3.30-4.36 Å from floxuridine. The best scoring conformations are docked with their carbonyl carbon of the ester linkage aligned for nucleophilic attack by Ser221 (2.64 to 4.09 Å away). The valyl ester group was positioned between 2.10-4.45 Å from Leu255, Leu304, Leu318, Leu388, Thr252, Met425, and Phe426. The ridged pocket amino acid residues, Leu96, Leu97, Phe101, Leu358, are positioned 1.64-5.75 Å from floxuridine. The best scoring confirmation is docked with the carbonyl carbon of the ester linkage aligned for nucleophilic attack by Ser221 (3.48 Å away).

The best scoring binding conformations were observed with carboxylesterase binding to the 5′ phenylalanine ester (Figure 3), which produced more energetically favored binding interactions compared to carboxylesterase complexed with the valine ester (Figure 4). Carboxylesterase bound to 5′ Phe-floxuridine generated a total energy of -114 kcal/mol, while 5′ Val-floxuridine bound with 24 kcal/mol of total energy. The benzyl ring of the phenylalanine residue Phe426 in the flexible binding pocket interacts closely (3.74-5.66 Å) with the phenylalanine ester of the floxuridine prodrug. The added strength of this hydrophobic interaction may be responsible for the increased total binding energy observed. In the kinetic studies performed the apparent affinity (K_m = 0.46 mM) for the phenylalanine 5′ ester was higher and much less with the 5′ valine prodrug (K_m = 3.58 mM). Therefore the calculated binding energies from the docking study are consistent with the apparent affinity in which the substrates bind the enzyme in experimental studies.

Comparison of CES1 and VACVase hydrolysis of floxuridine prodrugs

The CES1 hydrolysis kinetics for floxuridine prodrugs in this study were compared with their hydrolysis by VACVase, previously published (Kim et al., 2003; Kim et al., 2004b). Table 4 lists K_m and k_{cat} values obtained with the two enzymes for a few select prodrugs and the reference prodrug valacyclovir. It is clear from Table 4 that the affinity, catalytic activity, and catalytic efficiency of the two enzymes are dramatically different for 5′-L-valyl esters. It is also evident that VACVase exhibits higher selectivity for 5′-monoesters compared to 3′-monoesters.

VACVase and CES1 specific activities for several monoester floxuridine prodrugs and with valacyclovir are listed in Table 5. The VACVase specific activity for valacylovir was similar to that previously reported (68.0 nmol min^{-1} μg^{-1}, (Kim et al., 2003). The specific activity of VACVase with 5′-L-valyl floxuridine was roughly similar to that with valacyclovir, but was significantly lower with lysyl, isoleucyl, and aspartyl prodrugs. In contrast, CES1 specific activity against valacylovir and valyl floxuridine was low relative to phenylalanyl, prolyl, and lysyl floxuridine prodrugs. Interestingly, the specific activity for both VACVase and CES1 were lowest with the isoleucyl and aspartyl prodrugs of floxuridine.

The specific activities for the two enzymes with diester prodrugs of floxuridine are also shown in Table 5. The CES1 initial hydrolysis rates with the diester prodrugs were higher than either the 3′ or 5′ monoesters, regardless of the amino acid promoiety. The significantly higher specific activity with phenylalanine diester indicates the preference of CES1 for aromatic residues. In comparison, the specific activity of VACVase for diester floxuridine prodrugs was generally lower than that observed with

the corresponding 3′ or 5′ monoester prodrug. It is noteworthy that there was no detectable VACVase hydrolysis of the lysyl, isoleucyl, and aspartyl diester prodrugs.

Initial hydrolysis rates for a few 5′ monoester amino acid prodrugs of BDCRB with CES1 and VACVase are also listed in Table 5. Phenylalanine-containing BDCRB monoester prodrugs exhibited initial rates that are nearly the same, regardless of stereochemistry or substitutions on the phenyl group (with the possible exception of the *p*-chloro derivative). Further, these rates were similar to that observed with 5′-L-phenylalanyl floxuridine. For valyl BDCRB monoesters, the promoiety stereochemistry influenced initial rates in a profound manner. Thus, initial hydrolysis rate of 5′-L-valyl-BDCRB was roughly 30-fold higher than that observed with 5′-D-valyl-BDCRB.

DISCUSSION

Previous studies with amino acid ester prodrugs of the potent nucleoside agents floxuridine and gemcitabine have indicated that it is possible to enhance their uptake into cell systems that are surrogate models of the human intestine *via* PEPT1-mediated transport. It was also clear from such studies that besides transport, enzymatic stability of the ester linkage determined the resistance of the parent drug to metabolizing enzymes and is a key factor affecting prodrug disposition and efficacy. In this study, we describe the ester hydrolysis kinetics of various amino acid ester prodrugs catalyzed by porcine CES1, a widely distributed serine hydrolase.

The carboxylesterase-catalyzed hydrolysis rates for the floxuridine, gemcitabine, and BDCRB prodrugs varied over 1000-fold depending on the amino acid promoiety and the esterification site. The binding affinity of these prodrugs for carboxylesterase varied 26-fold depending on promoiety and esterification site. Catalytic efficiencies were found to be highest with phenylalanyl prodrugs and were over 1500-fold less efficient with valyl and isoleucyl prodrugs regardless of the parent nucleoside or esterification site. The preference for prodrugs containing aromatic promoieties was especially evident with phenylalanyl diester prodrugs of all three nucleosides examined.

Besides the amino acid promoiety, the esterification site also influenced carboxylesterase catalytic activity. Distinct preferences for either the 3′ or 5′ monoesters were evident and were dependent on the parent nucleoside analog structure. Thus, with floxuridine prodrugs carboxylesterase catalytic activity with 5′ monoesters were in general slightly higher than that with the corresponding 3′ monoester (Table 1). This preference is reversed for gemcitabine prodrugs; catalytic activity with 3′ esters was

significantly higher (3- to 16-fold) than that with the corresponding 5′ monoester (Table 2). The preferences for the two sets of prodrugs were maintained regardless of the promoiety stereochemistry. The preference reversal with the gemcitabine esters is likely influenced by the two fluorine groups attached to the 2′ position of the furanose. The highly electronegative fluorines may exert electron-withdrawing effects on the 3′ ester bond and destabilize it to enzymatic attack (Gray, 1971). The slight preference for 5′ monesters with floxuridine prodrugs suggests steric effects around the ester bond depending on esterification site. The 5′ hydroxyl is a primary alcohol, while the 3′ hydroxyl is a secondary alcohol. Thus, the ester bond in 3′ monoesters is closer proximity to the furanose moiety and may hinder the catalytic action of the enzyme to some extent.

The *p*-nitrophenyl ester standard substrates were among the most rapidly hydrolyzed substrates investigated (Table 3). Porcine carboxylesterase exhibited 4-fold greater binding affinity and a 1.4-fold higher catalytic rate with pNPB compared to pNPA, which is consistent with the roughly 1.5-fold higher specific activity of hCES1 reported for pNPB compared to that for pNPA (Xie et al., 2002). These authors also reported that hCES2 exhibits a 4-fold higher specific activity with pNPA compared to hCES1 and hydrolyzes pNPA about 7-fold faster than pNPB (Xie et al., 2002). The low catalytic efficiency of pCES1 with irinotecan is in agreement with the low catalytic efficiencies reported for hCES1A1 (Sanghani et al., 2004) and for hCES1 (Humerickhouse et al., 2000). Structural studies have revealed that the hCES1 active site contains both specific and nonspecific compartments, which allow it to hydrolyze an array of structurally distinct compounds (Bencharit et al., 2003). In the active site, hCES1 has been proposed

to recognize a large acyl moiety that is held as the acyl-enzyme intermediate and a small alcohol group that is released as the alcohol product in the ester hydrolysis reaction (Satoh and Hosokawa, 1998; Satoh et al., 2002). Thus, the hCES1 isoform prefers bulkier acyl groups (acid side) and smaller alcohol groups whereas the hCES2 isoform shows preference for smaller acyl groups and larger alcohol groups (Satoh et al., 2002). The dramatic decrease in catalytic activity as well as catalytic efficiency upon increasing the alcohol group size from ethyl to benzyl (PHEE *vs* PHBE) is consistent with hCES1 structural preferences (Table 3). However, acetylation of the PHEE amino group also resulted in significantly reduced catalytic efficiencies. The results suggest that despite the overwhelming preference for phenylalanine-containing acyl groups exhibited by pCES1, minor alterations in either the acyl group or the alcohol group affect overall catalytic efficiencies significantly. It is interesting to note that the catalytic efficiencies of PHBE and AcPHEE are similar to those obtained with 5′-phenylalanyl prodrugs of floxuridine and BDCRB.

Computational studies using the human CES1 structure to simulate docking of two amino acid esters revealed structural insights into potential floxuridine prodrug binding conformations. The Phe426 in the flexible binding pocket seems to strongly interact with the phenylalanine ester of the prodrug. The binding energy calculated for the 5′ Phe ester substrate suggests favorable substrate binding. The potential for strong hydrophobic interactions between the two benzyl groups may explain the superior apparent affinity of the phenylalanyl esters compared to the valyl esters.

Having similar substrate hydrolytic preferences (Figure 2) for these prodrugs and the observation that the porcine liver enzyme and the human CES1 share high sequence

homology (78% sequence identity and 88% similarity) suggest that pCES1 is a reasonably good substitute for hCES1. However, there are a few significant differences between the two enzymes, particularly with respect to the composition of the amino acid residues in the binding pocket. The porcine CES1 enzyme contains two phenylalanine residues (Phe_{318} and Phe_{426}) in the flexible binding pocket that may provide favorable binding for the phenylalanine ester of the prodrugs due to strong hydrophobic interactions between the aromatic groups and may explain the superior affinity of the Phe esters compared to the Val esters. The biggest difference in the human enzyme relates to the presence of Leu_{318} in the flexible binding pocket instead of Phe_{318}. This additional Phe residue in the porcine isoform may alter the binding affinity of aromatic substrates relative to the human enzyme. With one less phenylalanine in the flexible binding pocket, the human CES1 isoform might be expected to show relatively less binding affinity for aromatic substrates as the porcine isoform.

Esterases, including VACVase and CES, share a common α/β hydrolase fold framework, a similar active site, and a two step serine hydrolysis mechanism (Ollis et al., 1992). Overall, VACVase appears to be more catalytically active than carboxylesterase in hydrolyzing the amino acid ester prodrugs (Table 4). The affinity of pCES1 was higher than that of VACVase for phenylalanyl prodrugs; however, VACVase exhibited higher affinity with valyl prodrugs. This may be a result of the Phe residues present in the carboxylesterase acyl binding site. The proposed hydrophobic acyl-binding site of VACVase lacks phenylalanine residues, but instead contains residues Ile_{158}, Gly_{161}, Ile_{162}, and Leu_{229} (Kim et al., 2004a). This VACVase binding pocket may exhibit the most affinity for L-valyl esters such as valacylovir and 5′-L-valyl floxuridine, while

carboxylesterase binds these valyl esters with 18- and 46-fold less affinity, respectively. Catalytic activity and catalytic efficiency on the other hand were dramatically higher for VACVase compared to pCES1.

A few important differences in structural requirements for the two enzymes were also evident. First, it appears that a free alpha amino group is necessary for VACVase activity but not for pCES1. Thus, substrates such as 5′ cinnamic acid ester, pNPA, and pNPB that exhibited high catalytic activity with pCES1 were poorly hydrolyzed by VACVase (Kim et al., 2004b). Second, pCES1 exhibited negligible dependence on promoiety stereochemistry. Thus, k_{cat}/K_m ratios for L- and D- monoesters of valyl and phenylalanyl prodrugs of floxuridine, gemcitabine, and BDCRB were similar. In contrast, VACVase exhibited almost a 100-fold preference for the L-valyl floxuridine ester over the D-analog. The specific activities of pCES1 and VACVase for several floxuridine prodrugs shown in Table 5 also clearly reflect these structural aspects.

The hydrolysis kinetics indicate that prodrugs with promoieties that exhibit relatively low ester hydrolysis with either of these two representative serine hydrolases may provide prolonged systemic disposition of the prodrug and minimize metabolic degradation of the parent drug in the liver and other tissues. In this regard, lysyl, aspartyl, and isoleucyl prodrugs appear to be promising candidates. Our recent findings that oral administration of 5′-L-aspartyl BDCRB to mice resulted in highly favorable pharmacokinetic disposition of BDCRB and also provided a reservoir of prodrug provides some support to the hypothesis (Lorenzi et al., 2005).

In conclusion, we have investigated the structure activity relationships between porcine liver carboxylesterase and several nucleoside amino acid esters. Several factors

including choice of amino acid progroup, esterification site, and nucleoside structure were key factors determining carboxylesterase activity. Liver carboxylesterase substrate preferences show distinct differences compared to VACVase and can potentially be exploited by prodrug strategies to improve the pharmacokinetic disposition of nucleoside agents.

REFERENCES

Annaert P, Kinget R, Naesens L, de Clercq E and Augustijns P (1997) Transport, uptake, and metabolism of the bis(pivaloyloxymethyl)-ester prodrug of 9-(2-phosphonylmethoxyethyl)adenine in an in vitro cell culture system of the intestinal mucosa (Caco-2). *Pharm Res* **14**:492-496.

Bencharit S, Morton CL, Hyatt JL, Kuhn P, Danks MK, Potter PM and Redinbo MR (2003) Crystal structure of human carboxylesterase 1 complexed with the Alzheimer's drug tacrine. From binding promiscuity to selective inhibition. *Chem Biol* **10**:341-349.

Campbell CJ, Chantrell LJ and Eastmond R (1987) Purification and partial characterization of rat intestinal cefuroxime axetil esterase. *Biochem Pharmacol* **36**:2317-2324.

Gray CJ (1971) *Enzyme-catalyzed reactions*. Van Nostrand Reinhold Company, London.

Humerickhouse R, Lohrbach K, Li L, Bosron WF and Dolan ME (2000) Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res* **60**:1189-1192.

Imai T, Imoto M, Sakamoto H, and Hashimoto M (2005) Identification of esterases expressed in Caco-2 cells and effects of their hydrolyzing activity in predicting human intestinal absorption. *Drug Metab Dispos.* 33:1185-1190.

Kamendulis LM, Brzezinski MR, Pindel EV, Bosron WF and Dean RA (1996) Metabolism of cocaine and heroin is catalyzed by the same human liver carboxylesterases. *J Pharmacol Exp Ther* **279**:713-717.

Kim I, Chu XY, Kim S, Provoda CJ, Lee KD and Amidon GL (2003) Identification of a human valacyclovirase: biphenyl hydrolase-like protein as valacyclovir hydrolase. *J Biol Chem* **278**:25348-25356.

Kim I, Crippen GI and Amidon GL (2004a) Structure and specificity of a human valacyclovir activating enzyme: a homology model of BPHL. *Molecular Pharmaceutics* **1**:434-446.

Kim I, Xueqin S, Vig BS, Mittal S, Shin HC, Lorenzi PJ and Amidon GL (2004b) A novel nucleoside prodrug-activating enzyme: substrate specificity of biphenyl hydrolase-like protein. *Mol Pharm* **1**:117-127.

Landowski CP, Song X, Lorenzi PL, Hilfinger JM and Amidon GL (2005a) Floxuridine Amino Acid Ester Prodrugs: Enhancing Caco-2 Permeability and Resistance to Glycosidic Bond Metabolism. *Pharm Res* **22**:1510-8.

Landowski CP, Vig BS, Song X and Amidon GL (2005b) Targeted delivery to PEPT1-overexpressing cells: acidic, basic, and secondary floxuridine amino acid ester prodrugs. *Mol Cancer Ther* **4**:659-667.

Lorenzi PL, Landowski CP, Song X, Borysko KZ, Breitenbach JM, Kim JS, Hilfinger JM, Townsend LB, Drach JC and Amidon GL (2005) Amino Acid Ester Prodrugs of 2-Bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole Enhance Metabolic Stability *In Vitro* and *In Vivo. J Pharmacol Exp Ther* **314**:883-90.

Mittal S, Song X, Vig BS, Landowski CP, Kim I, Hilfinger JM and Amidon GL (2005) Prolidase, a potential enzyme target for melanoma: design of proline-containing dipeptide-like prodrugs. *Mol Pharm* **2**:37-46.

Miwa M, Ura M, Nishida M, Sawada N, Ishikawa T, Mori K, Shimma N, Umeda I and Ishitsuka H (1998) Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. *Eur J Cancer* **34**:1274-1281.

Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, Franken SM, Harel M, Remington SJ, Silman I, Schrag J and et al. (1992) The alpha/beta hydrolase fold. *Protein Eng* **5**:197-211.

Rooseboom M, Commandeur JN and Vermeulen NP (2004) Enzyme-catalyzed activation of anticancer prodrugs. *Pharmacol Rev* **56**:53-102.

Sanghani SP, Quinney SK, Fredenburg TB, Davis WI, Murry DJ and Bosron WF (2004) Hydrolysis of irinotecan and its oxidative metabolites, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin, by human carboxylesterases CES1A1, CES2, and a newly expressed carboxylesterase isoenzyme, CES3. *Drug Metab Dispos* **32**:505-511.

Satoh T and Hosokawa M (1998) The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* **38**:257-288.

Satoh T, Taylor P, Bosron WF, Sanghani SP, Hosokawa M and La Du BN (2002) Current progress on esterases: from molecular structure to function. *Drug Metab Dispos* **30**:488-493.

Song X, Lorenzi PL, Landowski CP, Vig BS, Hilfinger JM and Amidon GL (2005a) Amino acid ester prodrugs of the anticancer agent gemcitabine: synthesis,

bioconversion, metabolic bioevasion, and hPEPT1-mediated transport. *Mol Pharm* **2**:157-167.

Song X, Vig BS, Lorenzi PL, Drach JC, Townsend LB and Amidon GL (2005b) Amino acid ester prodrugs of the antiviral agent 2-bromo-5,6-dichloro-1-(beta-D-ribofuranosyl)benzimidazole as potential substrates of hPEPT1 transporter. *J Med Chem* **48**:1274-1277.

Sun D, Lennernas H, Welage LS, Barnett JL, Landowski CP, Foster D, Fleisher D, Lee KD, and Amidon GL (2002) Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. *Pharm.Res.* 19:1400-1416.

Takai S, Matsuda A, Usami Y, Adachi T, Sugiyama T, Katagiri Y, Tatematsu M and Hirano K (1997) Hydrolytic profile for ester- or amide-linkage by carboxylesterases pI 5.3 and 4.5 from human liver. *Biol Pharm Bull* **20**:869-873.

Vig BS, Lorenzi PJ, Mittal S, Landowski CP, Shin HC, Mosberg HI, Hilfinger JM and Amidon GL (2003) Amino acid ester prodrugs of floxuridine: synthesis and effects of structure, stereochemistry, and site of esterification on the rate of hydrolysis. *Pharm Res* **20**:1381-1388.

Xie M, Yang D, Liu L, Xue B and Yan B (2002) Human and rodent carboxylesterases: immunorelatedness, overlapping substrate specificity, differential sensitivity to serine enzyme inhibitors, and tumor-related expression. *Drug Metab Dispos* **30**:541-547.

Zhang J, Burnell JC, Dumaual N and Bosron WF (1999) Binding and hydrolysis of meperidine by human liver carboxylesterase hCE-1. *J Pharmacol Exp Ther* **290**:314-318.

FOOTNOTES

This investigation was supported by NIH Grant R01-GM37188.

Reprint requests to: Gordon L. Amidon, College of Pharmacy, The University of Michigan, 428 Church Street, Ann Arbor, MI 48109-1065. Email: glamidon@umich.edu

FIGURE LEGENDS

Figure 1. Structure of nucleoside analogs and amino acid progroups.

Figure 2. Correlation of hydrolysis of amino acid ester prodrugs with porcine CES1 and in Caco-2 cell extracts.

Figure 3. 5′-L-phenylalanyl floxuridine docked into substrate binding site of hCES1. White residues are the catalytic triad, yellow are the rigid pocket residues, and blue are the flexible pocket residues.

Figure 4. 5′-L-valyl floxuridine docked into active site of hCES1. White residues are the catalytic triad, yellow are the rigid pocket residues, and blue are the flexible pocket residues.

JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Kinetic parameters for carboxylesterase catalyzed hydrolysis of floxuridine prodrugs.

Substrate	**V_{max} (nmol/min/μg)**	**K_m (mM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_m**
5'-L-phenylalanyl-floxuridine	1.82 ± 0.05	0.46 ± 0.05	37.4 ± 1.1	81.3
5'-D-phenylalanyl-floxuridine	1.63 ± 0.04	0.44 ± 0.04	33.6 ± 0.9	77.2
3'-D-phenylalanyl-floxuridine	4.05 ± 0.12	1.40 ± 0.13	83.5 ± 2.4	59.5
3'-L-phenylalanyl-floxuridine	1.55 ± 0.06	0.74 ± 0.10	32.0 ± 1.1	42.9
5'-L-prolyl-floxuridine	1.97 ± 0.06	1.87 ± 0.15	40.6 ± 1.1	21.7
5'-L-leucyl-floxuridine	0.92 ± 0.04	0.97 ± 0.13	18.9 ± 0.7	19.4
3'-L-prolyl-floxuridine	4.53 ± 0.15	4.85 ± 0.34	93.3 ± 3.0	19.2
3'-L-leucyl-floxuridine	0.70 ± 0.03	1.46 ± 0.22	14.5 ± 0.7	9.9
3'-L-lysyl-floxuridine	0.59 ± 0.03	3.08 ± 0.37	12.2 ± 0.6	4.0
5'-L-lysyl-floxuridine	1.11 ± 0.03	6.25 ± 0.34	22.9 ± 0.6	3.7
3'-L-isoleucyl-floxuridine	0.16 ± 0.01	1.11 ± 0.14	3.3 ± 0.1	3.0
5'-L-aspartyl-floxuridine	0.33 ± 0.01	3.00 ± 0.17	6.9 ± 0.2	2.3
3'-L-aspartyl-floxuridine	0.10 ± 0.00	0.93 ± 0.13	2.1 ± 0.1	2.3
5'-D-valyl-floxuridine	0.30 ± 0.01	4.12 ± 0.20	6.1 ± 0.1	1.5
3'-L-valyl-floxuridine	0.19 ± 0.01	2.87 ± 0.23	3.9 ± 0.1	1.3
5'-L-valyl-floxuridine	0.21 ± 0.01	3.58 ± 0.22	4.3 ± 0.1	1.2
5'-L-isoleucyl-floxuridine	0.15 ± 0.00	2.94 ± 0.23	3.0 ± 0.1	1.0
3'-D-valyl-floxuridine	0.27 ± 0.05	11.23 ± 2.99	5.6 ± 0.9	0.5

JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2. Kinetic parameters for carboxylesterase catalyzed hydrolysis of gemcitabine and BDCRB prodrugs.

Substrate	**V_{max} (nmol/min/μg)**	**K_m (mM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_m**
3'-L-phenylalanyl-gemcitabine	283.52 ± 0.41	0.51 ± 0.04	365.0 ± 8.2	718.3
3'-D-phenylalanyl-gemcitabine	406.92 ± 0.72	0.97 ± 0.08	523.9 ± 14.4	541.3
5'-L-phenylalanyl-gemcitabine	3.25 ± 0.03	0.17 ± 0.03	67.0 ± 0.6	386.7
5'-D-phenylalanyl-gemcitabine	3.19 ± 0.03	0.22 ± 0.04	65.7 ± 0.7	301.6
3'-L-valyl-gemcitabine	0.35 ± 0.01	0.90 ± 0.11	7.3 ± 0.3	8.1
3'-L-isoleucyl-gemcitabine	0.24 ± 0.01	0.94 ± 0.08	5.0 ± 0.1	5.3
3'-D-valyl-gemcitabine	0.32 ± 0.01	1.43 ± 0.13	6.7 ± 0.2	4.7
5'-L-isoleucyl-gemcitabine	0.09 ± 0.00	0.85 ± 0.05	2.0 ± 0.0	2.3
5'-D-valyl-gemcitabine	0.13 ± 0.00	1.16 ± 0.07	0.5 ± 0.1	0.5
5'-L-valyl-gemcitabine	0.12 ± 0.00	0.96 ± 0.11	0.5 ± 0.1	0.5
5'-L-phenylalanyl-BDCRB	2.33 ± 0.04	0.91 ± 0.02	48.0 ± 0.8	52.7
5'-D-phenylalanyl-BDCRB	1.60 ± 0.21	0.90 ± 0.28	33.0 ± 4.2	36.5

JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3. Kinetic parameters for carboxylesterase catalyzed hydrolysis phenylalanine esters.

Substrate	**V_{max} (nmol/min/μg)**	**K_m (mM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_m**
pNPB	82.25 ± 2.35	0.14 ± 0.02	1645.1 ± 47.0	11750.7
pNPA	60.45 ± 2.12	0.52 ± 0.06	1209.0 ± 42.3	2319.7
PHEE	11859.78 ± 2.72	1.18 ± 0.06	3817.4 ± 54.5	3235.1
PHBE	7.24 ± 0.02	0.08 ± 0.03	4.7 ± 0.5	59.9
AcPHEE	1.29 ± 0.05	0.40 ± 0.06	26.7 ± 1.1	67.0
5'cinn-floxuridine	4.00 ± 0.12	0.63 ± 0.07	82.5 ± 2.4	131.6
5'-L-phenylalanyl-floxuridine	1.82 ± 0.05	0.46 ± 0.05	37.4 ± 1.1	81.3
5'-L-phenylalanyl-gemcitabine	3.25 ± 0.03	0.17 ± 0.03	67.0 ± 0.6	386.7
5'-L-phenylalanyl-BDCRB	2.33 ± 0.04	0.91 ± 0.02	48.0 ± 0.8	52.7
CPT-11	0.03 ± 0.00	4.21 ± 0.37	0.55 ± 0.02	0.13

pNPB, *p*-nitrophenyl butyrate; pNPA, *p*-nitrophenyl acetate; PHEE, phenylalanine ethyl ester; cinn, cinnamic acid; AcPEE, N-acetyl phenylalanine ethyl ester; PHBE, phenylalanine benzyl ester; CPT-11, irinotecan.

JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Table 4. Comparison of kinetic parameters between VACVase and pCES1

Prodrug	K_m (mM)		k_{cat} (min^{-1})		k_{cat}/K_m (mM^{-1} min^{-1})		k_{cat}/K_m ratio
	VACVase	CES	VACVase	CES	VACVase	CES	VACVase/CES
5'-D-valyl-floxuridine	2.78	4.12	663.2	6.1	238.6	1.5	160
5'-L-valyl-floxuridine	0.20	3.58	4673.7	4.3	23368.4	1.2	19288
3'-L-valyl-floxuridine	0.95	2.87	1042.1	3.9	1097.0	1.3	819
5'-D-phenylalanyl-floxuridine	0.75	0.44	15000.0	33.6	20000.0	77.2	259
5'-L-phenylalanyl-floxuridine	0.63	0.46	20305.3	37.4	32230.6	81.3	396
5'-L-prolyl-floxuridine	1.45	1.87	17557.9	40.6	12108.9	21.7	557
5'-L-phenylalanyl-BDCRB	0.69	0.91	21631.6	48.0	31350.1	52.7	595
VACV	0.19	8.81	4800.0	17.1	25263.2	1.9	13052

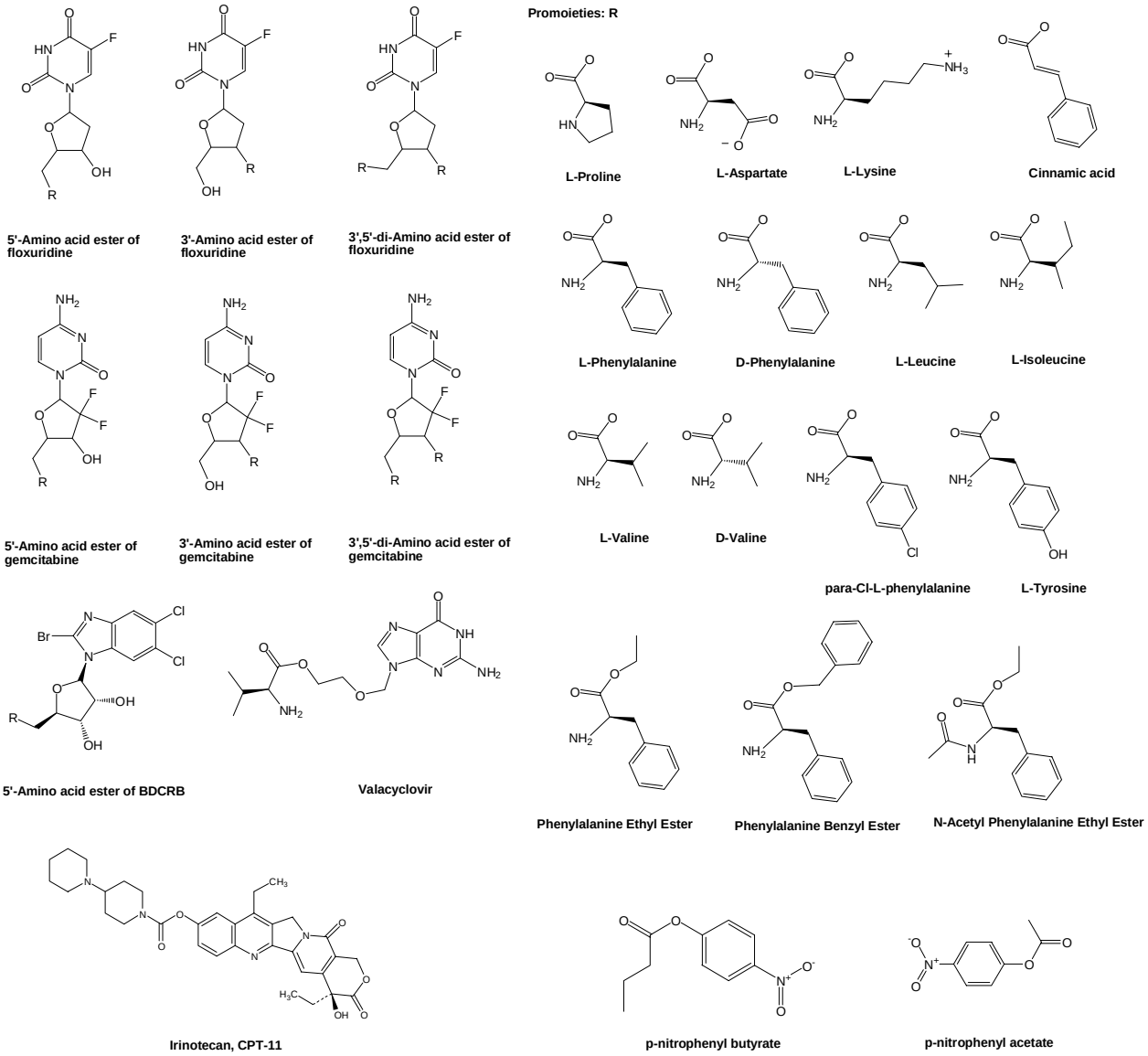
JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Table 5: Specific activity of floxuridine prodrugs with pCES1 and VACVase (expressed as nmol/min/mg protein; mean ± SD, n = 3).

prodrug	pCES1	VACVase
Valacyclovir reference (VACV)	**0.013 ± 0.004**	**64.28 ± 7.61**
Floxuridine prodrugs		
5′-L-valyl	0.053 ± 0.023	80.16 ± 0.79
5′-L-phenylalanyl	0.534 ± 0.010	46.28 ± 13.03
3′-L-phenylalanyl	0.466 ± 0.032	21.35 ± 13.32
5′-L-leucyl	0.238 ± 0.023	20.55 ± 2.01
3′-L-leucyl	0.113 ± 0.014	13.39 ± 0.80
5′-L-lysyl	0.044 ± 0.001	6.66 ± 0.76
3′-L-lysyl	0.052 ± 0.007	5.90 ± 1.94
5′-L-isoleucyl	0.011 ± 0.000	4.59 ± 0.62
3′-L-isoleucyl	0.010 ± 0.000	2.87 ± 0.35
3′-L-valyl	0.019 ± 0.001	2.76 ± 0.55
5′-L-aspartyl	0.026 ± 0.001	1.88 ± 1.61
3′-L-aspartyl	0.026 ± 0.003	1.76 ± 0.86
5′-D-valyl	0.014 ± 0.000	5.94 ± 1.78
3′-D-valyl	0.010 ± 0.000	0.00 ± 0.00
5′-D-phenylalanyl	0.508 ± 0.023	102.44 ± 5.58
3′-D-phenylalanyl	0.455 ± 0.013	0.86 ± 0.83
3′,5′-di-L-leucyl	0.729 ± 0.025	18.38 ± 2.31
3′,5′-di-L-phenylalanyl	3.580 ± 0.257	8.15 ± 3.19
3′,5′-di-D-phenylalanyl	5.592 ± 0.318	3.07 ± 0.77
3′,5′-di-L-valyl	0.073 ± 0.007	1.35 ± 1.10
3′,5′-di-D-valyl	0.054 ± 0.005	0.49 ± 0.19
3′,5′-di-L-lysyl	0.217 ± 0.041	0.00 ± 0.00
3′,5′-di-L-isoleucyl	0.152 ± 0.011	0.00 ± 0.00
3′,5′-di-L-aspartyl	0.091 ± 0.028	0.00 ± 0.00
BDCRB prodrugs		
5'-L-phenylalanyl	0.83 ± 0.30	85.12 ± 1.94
5'-L-tyrosyl	0.71 ± 0.02	nd
5'-D-phenylalanyl	0.67 ± 0.03	nd
5'-*p*-Cl-L-phenylalanyl	0.52 ± 0.01	31.83 ± 4.88
5'-L-valyl	0.06 ± 0.02	40.88 ± 1.57
5'-D-valyl	0.04 ± 0.00	3.69 ± 0.91

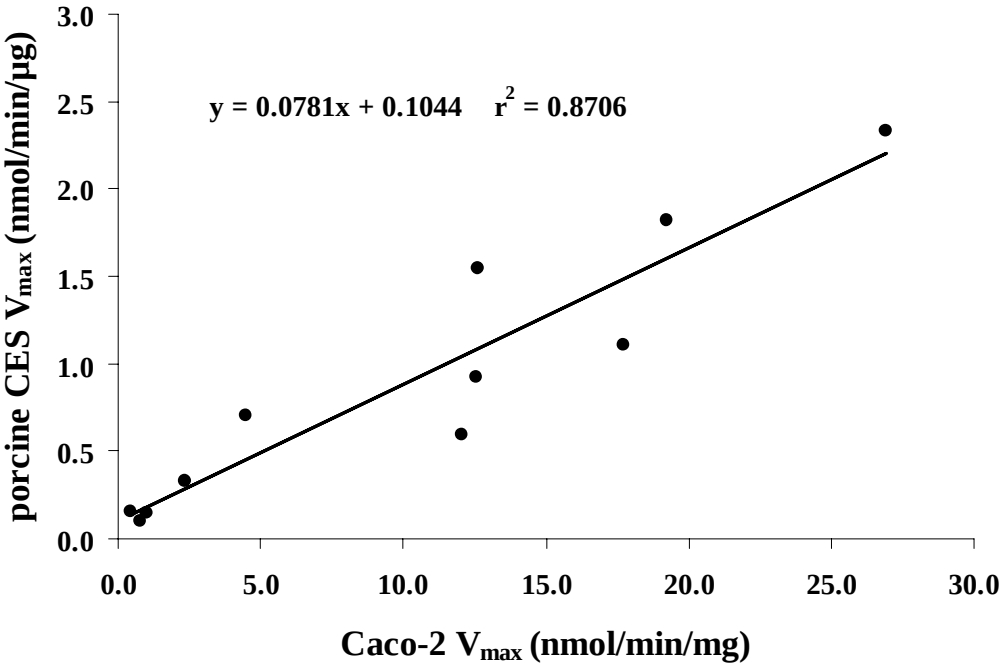
JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1.



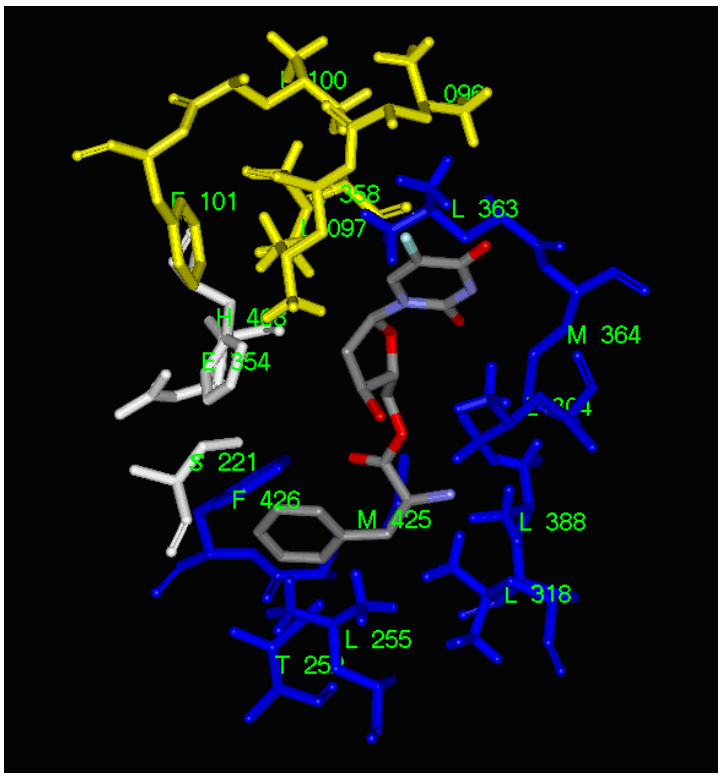
JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2.



JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3.



JPET Fast Forward. Published on October 13, 2005 as DOI: 10.1124/jpet.105.092726
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4.