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Effect of Biphenyl Hydrolase-Like (BPHL) Gene Disruption on the Intestinal Stability, Permeability and Absorption of Valacyclovir in Wildtype and *Bphl* Knockout Mice

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Abstract

Biphenyl hydrolase-like protein (BPHL) is a novel human serine hydrolase that was originally cloned from a breast carcinoma cDNA library and shown to convert valacyclovir to acyclovir and valganciclovir to ganciclovir. However, the exclusivity of this process has not been determined and, indeed, it is possible that a number of esterases/proteases may mediate the hydrolysis of valacyclovir and similar prodrugs. The objectives of the present study were to evaluate the *in situ* intestinal permeability and stability of valacyclovir in wildtype (WT) and *Bphl* knockout (KO) mice, as well as the *in vivo* oral absorption and intravenous disposition of valacyclovir and acyclovir in the two mouse genotypes. We found that *Bphl* knockout mice had no obvious phenotype and that *Bphl* ablation did not alter the jejunal permeability of valacyclovir during *in situ* perfusions (i.e., 0.54×10^{-4} in WT vs. 0.53×10^{-4} cm/sec in KO). Whereas no meaningful changes occurred between genotypes in the gene expression of proton-coupled oligopeptide transporters (i.e., PepT1, PepT2, PhT1, PhT2), enzymatic upregulation of Cyp3a11, Cyp3a16, Abhd14a and Abhd14b was observed in some tissues of *Bphl* knockout mice. Most importantly, we found that valacyclovir was rapidly and efficiently hydrolyzed to acyclovir in the absence of BPHL, and that hydrolysis was more extensive after the oral vs. intravenous route of administration (for both genotypes). Taken as a whole, BPHL is not obligatory for the conversion of valacyclovir to acyclovir either presystemically or systemically.

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Conflict of Interest

The authors declare no conflicts of interest, financial or otherwise.

Graphical abstract



Keywords

Biphenyl hydrolase-like protein; *Bphl* knockout mice; Valacyclovir; Intestinal stability and permeability; Oral absorption

1. Introduction

By targeting transporters and enzymes, prodrugs have proven to be a valuable strategy in overcoming pharmaceutical, pharmacokinetic and pharmacodynamic barriers. Such barriers include poor solubility, permeability and stability, and unacceptable toxicity of the active parent drug [1–7]. Once entering the human body, prodrugs can be activated chemically such as through pH sensitivity [8–10], or enzymatically such as through paraoxonase, serine hydrolase, carboxylesterase, and acetylcholinesterase [11–14]. One notable example is the antiviral ester prodrug valacyclovir, which is actively transported by the proton-coupled oligopeptide transporter PepT1 (SLC15A1) from intestinal lumen. Previous studies have suggested that this prodrug is converted to the active parent drug acyclovir by the serine hydrolase biphenyl hydrolase-like (BPHL) protein [15–18]. As illustrated by this prototypic prodrug, a comprehensive understanding of all possible transporters and activating enzymes involved in valacyclovir's intestinal oral absorption, activation and systemic availability can aid in the rational design of future drug candidates.

Along with other prodrug targeted transporters, such as the organic anion transporter (OAT) family and the organic cation transporter (OCT) family, the PepT1 transporter is predominately expressed on the apical membrane of enterocytes and its biochemistry, biology, pharmacokinetics and substrate specificity have been well characterized [19–23]. As such, PepT1 is a potential drug design target for improving intestinal absorption and the systemic availability of poorly absorbed drug molecules [4,24,25]. In addition to increasing oral drug absorption, an essential step is the conversion of prodrug to its active therapeutic moiety. Recent advances in biochemistry and biology concerning the structural and functional characteristics of enzymes have provided abundant information to benefit prodrug design. More sophisticated strategies have been developed involving the covalent binding of

special pro-moieties to relevant parent drugs. These prodrugs can then be activated by one or more enzymes presystemically, systemically or at the site of action [12,26,27].

BPHL, also called human valacyclovirase, is an α -amino acid ester hydrolase that was initially cloned from a human breast carcinoma cDNA library and found to be highly expressed in human liver and kidney [28]. BPHL protein consists of 274 amino acids containing the Gly-X-Ser-X-Gly motif, which is characteristic of serine hydrolases involved in the degradation of aromatic compounds by cleavage of carbon-carbon bonds [29]. In 2008, a study on the crystal structure and biochemical analysis of recombinant BPHL identified the key determinants for substrate recognition [30]. Specifically, these authors reported that the protein had a unique active site defined by a flexible and mostly hydrophobic acyl pocket, a localized negative electrostatic potential, and a large open leaving group-accommodating groove. Most importantly, BPHL protein also had a pivotal acidic residue, Asp-123, located after the nucleophile Ser-122, whose side chain was directed into the substrate binding pocket, thereby, playing a critical role in the substrate discriminating ability of serine hydrolases [30]. Moreover, the recombinant BPHL enzyme (originally from Caco2 cells) was shown to effectively hydrolyze many amino acid ester nucleoside prodrugs, converting compounds such as valacyclovir to acyclovir [18] and valganciclovir to ganciclovir [31]. Physiologically, BPHL was reported as a likely candidate for protecting against homocysteine thiolactone toxicity [32].

Despite the fact that BPHL is a major enzyme for the activation of valine ester prodrugs, the *in vivo* impact of BPHL on prodrug stability, permeability and activation has yet to be investigated. It is also uncertain as to whether valacyclovir (and similar prodrugs) are hydrolyzed exclusively by BPHL or if other enzymes contribute to the *in vivo* hydrolysis (activation) of valacyclovir to acyclovir. With this in mind, the objectives of this study were to evaluate the *in situ* intestinal permeability and stability of valacyclovir in wildtype and *Bphl* knockout mice, and to determine the *in vivo* oral absorption and intravenous disposition of valacyclovir and acyclovir in the two mouse genotypes. This is the first time that a phenotypic, biochemical, molecular and functional assessment of BPHL ablation has been reported under both *in situ* and *in vivo* study conditions.

2. Materials and methods

2.1. Chemicals

Anti-mouse BPHL antiserum was generated by Lampire Biological Laboratories, Inc (Pipersville, PA USA) using the C-terminal 20 amino acid residues (HNLHLRFADFNRLVEDFLQ, amino acids 272–291) of the protein. Valacyclovir and acyclovir were purchased from Sigma-Aldrich (St. Louis, MO USA). Valacyclovir-D4 and acyclovir-D4 internal standards were purchased from Toronto Research Chemicals (Toronto, Ontario Canada). Other chemicals and reagents were from standard sources.

2.2. Animals

Bphl knockout mice on a pure C57BL/6N background were generously provided by the Institut Clinique de la Souris (Illkirch Cedex, France). Animals were bred and housed in a

temperature-controlled environment with 12-hour light and dark cycles. They received a standard diet and water *ad libitum*, as provided by the Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI. Gender-matched mice (8–10 weeks) were used in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. All relevant mouse strains were validated by genotyping.

2.3. Initial phenotypic analysis

Wildtype and *Bphl* knockout mice were evaluated for viability, fertility, serum clinical chemistry and histology, as described previously [23].

2.4. Quantitative real-time PCR (qRT-PCT) and immunoblot analysis

Gene transcripts of *Bphl*, *PepT1*, *PepT2*, *PhT1*, *PhT2*, *Ces1*, *Ces2*, *Cyp3a11*, *Cyp3a16*, *Cyp3a41* and *Cyp3a44* were evaluated in the small intestine, colon, kidney, spleen and liver of wildtype and *Bphl* knockout mice using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA), as described previously [21]. The primers are listed in Table 1. Briefly, all reactions were performed in triplicate, where each reaction included 30 ng of cDNA that was reverse-transcribed using the Omniscript RT kit (Qiagen, Valencia, CA) and 16-mer random primers from RNA isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The qRT-PCR thermal conditions were one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and forty cycles at 95°C for 15 sec followed by 60°C for 1 min. Relative levels of the target gene transcripts in mice were calculated using the 2^{-CT} method, where the ratio of target gene to *Gapdh* was equal to 2^{-CT} , $CT = CT(\text{gene}) - CT(\text{Gapdh})$. The mouse *Gapdh* gene was set as an internal control of cDNA quality and quantity.

Protein levels of BPHL were determined in the liver, jejunum, spleen and kidney of wildtype and *Bphl* knockout mice by immunoblot analysis, as described previously [21,33,34]. In brief, after processing the tissues, membrane proteins were denatured at 40°C for 45 min, resolved by 7.5% SDS-PAGE, transferred to a PVDF membrane (Millipore, Billerica, MA), and then blotted for one hour with rabbit anti-mouse BPHL antisera (1:5000). The membrane was washed three times with TBST and then incubated with the secondary antibody, goat anti-rabbit IgG-HRP (1:3000) (Bio-Rad, Hercules, CA). For β -actin, the membrane was blotted with a mouse monoclonal antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by the secondary antibody, goat anti-mouse IgG-HRP (1:1000) (Santa Cruz Biotechnology). The membrane was then washed five times with TBST, the UCL substrate (Millipore) added, and the x-ray film exposed and developed.

2.5. In situ single-pass intestinal perfusion studies

Prodrug design can be used to increase the permeability and stability of the parent drug [35]. The effects of *Bphl* gene deletion on valacyclovir permeability and stability were evaluated using *in situ* single-pass jejunal perfusion studies, as described previously with wildtype mice as the control group [20,21,23]. In brief, following an overnight fast, wildtype and *Bphl* knockout mice were anesthetized with sodium pentobarbital (40–60 mg/kg ip) and an 8-cm jejunal segment was isolated. The segment was then rinsed with 0.9% isotonic saline solution and a glass cannula (0.2 mm OD) was inserted at each end of the intestinal segment.

Perfusion buffer (135 mM NaCl, 5 mM KCl and 10 mM MES/Tris, pH 6.5) containing 50 μ M valacyclovir was perfused through the jejunal segment at a flow rate of 0.1 mL/min and the outlet perfusate was collected every 10 min for 90 min. Water flux during the perfusion was corrected by mass balance using a gravimetric method.

Hydrolysis studies were also performed in which, following jejunal perfusions of 1.0 mM valacyclovir, plasma samples were collected from the portal vein at 5 and 30 min after initiating the perfusion.

2.6. Ultra performance liquid chromatography with photodiode array detector assay conditions for in situ perfusion studies

Following centrifugation of the 100- μ L perfusate samples, a 25- μ L aliquot was injected into the ultra performance liquid chromatography (UPLC) system, as previously described with minor modification [36]. Specifically, the method employed a UPLC Acquity H-Class System (Waters Corp, Milford, MA) coupled to a Photodiode Array Detector set at 254 nm. A 100-cm Acquity HSS T3 column (1.8 μ m) was maintained at 40°C with gradient elution at a flow rate of 0.5 mL/min. The mobile phase started with 100% water (0.1% TFA), and increased linearly to 10% acetonitrile and 90% water (0.1% TFA) over 5 min. This level was maintained for 2 min, then reverted back to 100% water (0.1% TFA) at 7 min, which was maintained until the end of the run (12 min). The lower limits of quantitation for acyclovir and valacyclovir in perfusate buffer were 50 and 10 nM, respectively.

2.7. Oral and intravenous pharmacokinetic studies

The effects of *Bphl* gene deletion on the oral absorption and disposition of valacyclovir were evaluated, as described previously for valacyclovir in wildtype mice [17]. For oral studies, 25 nmol/g of valacyclovir (in 0.2 mL of water per 20 g body weight) was administered by oral gavage. For intravenous bolus studies, 25 nmol/g of valacyclovir (in 0.1 mL of saline per 20 g body weight) was administered by tail vein injection. Blood samples (~ 20 μ L aliquots), after both dosing routes, were obtained by tail biopsy at 2, 5, 15, 30, 60, 90, 120 and 180 min post-injection, with an additional sample taken at 45 min for the oral studies. The blood samples were then mixed with 1 μ L EDTA-K3 and centrifuged at 3,000 g for 5 min at 4°C. Following centrifugation, the plasma was harvested and a 10- μ L aliquot was mixed with 40 μ L of acetonitrile containing 200 nM of the internal standards VACV-d4 and ACV-d4. Samples were then centrifuged at 17,000 g for 10 min at 4°C to precipitate the proteins, the supernatant transferred carefully into a new tube, and frozen at -80°C for subsequent analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.8. LC-MS/MS assay conditions for in vivo pharmacokinetic studies

Plasma supernatant (10 μ L aliquot), obtained from the *in vivo* pharmacokinetic studies (see above), was analyzed by LC-MS/MS, as previously described [37]. In brief, the LC-MS/MS analysis consisted of a Shimadzu HPLC system (Shimadzu, Tokyo, Japan) coupled to an API 4000 triple quadrupole/linear ion trap (QTRAP) mass spectrometer (AB Sciex, Foster City, CA, USA). Analytes were separated on a Waters Atlantis T3 C18 column (5 μ m, 150 \times 2.1 mm, Dublin, Ireland). The mobile phase consisted of water containing 2 mM ammonium acetate and 0.2% formic acid (v/v) (Phase A) and acetonitrile containing 0.2% formic acid

(v/v) (Phase B), which was delivered at a flow rate of 0.2 ml/min. Gradient elution was applied for compound separation and the program was timed as follows: Phase B was started at 2% for 2 min, then increased linearly to 4% over the next 2 min, further increased to 50% over the next 2 min, and then returned to 2% at 6.5 min, which was maintained until the end of run (9 min). The mass spectrometer was operated in positive ion mode using electrospray ionization. The following transitions were monitored in a multiple reaction monitoring mode: VACV, 325.2 > 152.1; ACV, 226.2 > 152.1; VACV-d4, 329.2 > 152.1; ACV-d4, 230.2 > 152.1. The lower limit of quantification was 2 nM for both valacyclovir and acyclovir.

2.9. Data analysis

Data were reported as mean \pm SE, unless otherwise noted. Statistical differences between two groups were determined by an unpaired t-test using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). A value of $p < 0.05$ was considered statistically significant. Pharmacokinetic analyses were carried out by noncompartmental analysis (NCA) using Phoenix WinNonlin 8.0 (Certara, St. Louis, MO USA).

3. Results

3.1. Initial phenotypic analysis

Bphl knockout mice appeared normal with no obvious behavioral abnormalities as compared to wildtype mice. They also had normal fertility, litter size, gender distribution, viability, and body weight. As shown in Table 2, no significant differences were observed in serum clinical chemistry between wildtype and *Bphl* knockout mice, although a statistical difference was noted for sodium (i.e., 147 vs 144 mM, respectively). Histological evaluation, as judged by hematoxylin and eosin staining, demonstrated normal morphology in the small and large intestines, liver, kidney and spleen of wildtype and *Bphl* knockout mice (Fig. 1).

3.2. Confirmation of BPHL ablation

Even though knockout mice were identified by genotyping, select tissues were further tested by qRT-PCR for *Bphl* gene expression and by immunoblotting for the presence of BPHL protein. As shown in Fig. 2A, *Bphl* transcripts were barely detectable in the duodenum, jejunum, ileum, colon, liver, kidney and spleen of *Bphl* knockout mice. In wildtype mice, *Bphl* mRNA expression was greatest in the kidney and liver, moderate in the colon and ileum, and low in the small intestine and spleen. This result was confirmed in Fig. 2B, where abundant expression of BPHL protein was observed in the kidney and liver of wildtype mice, but faint expression of protein in the jejunum and spleen. In contrast, no BPHL protein expression was observed in the counterpart tissues of *Bphl* knockout mice.

3.3. Gene expression of select transporters and enzymes in tissues from wildtype and *Bphl* knockout mice

It is important to know whether *Bphl* gene ablation resulted in a compensatory response for relevant transporters and/or enzymes of mice. The four mammalian proton-coupled oligopeptide transporters were examined first in several tissues of wildtype and *Bphl* knockout mice. The intestinal PepT1 transporter was of particular interest because of its strategic value as a target for improving drug (and prodrug) oral absorption. As shown in

Fig. 3A, no significant differences were observed between the two genotypes in the gene expression of PepT1 in tissues, with the exception of colon in which PepT1 mRNA was increased. In regard to PepT2, no differences in gene expression were noted between wildtype and *Bphl* knockout mice in any of the tissues tested (Fig. 3B). In contrast, PhT1 gene expression was increased in the duodenum, ileum, colon, kidney and spleen of *Bphl* knockout mice (Fig. 3C). Finally, PhT2 gene expression was increased only in the ileum during *Bphl* ablation (Fig. 3D).

Enzymes that might impact the hydrolysis of valacyclovir were examined next. As shown in Fig. 3E and 3F, no differences were observed between wildtype and *Bphl* knockout mice in the gene expression of Ces1 or Ces2, respectively. Although Cyp3a11 transcripts were increased in the liver during *Bphl* ablation (Fig. 3G), as were Cyp3a16 transcripts in the duodenum and liver (Fig. 3H), no differences in Cyp3a41 (Fig. 3I) and Cyp3a44 (Fig. 3J) gene expression were discerned in any of the tissues tested between wildtype and *Bphl* knockout mice. Nonetheless, significant increases in Abhd14a gene expression were observed in the duodenum, kidney and spleen of *Bphl* knockout mice (Fig. 3K). Likewise, during *Bphl* ablation, Abhd14b transcripts were significantly increased in the duodenum, ileum, colon, liver, kidney and spleen of these mice (Fig. 3L).

3.4. Intestinal permeability and stability of valacyclovir during in situ perfusion of prodrug in wildtype and *Bphl* knockout mice

Single-pass jejunal perfusion studies were performed in order to evaluate the effect of *Bphl* gene ablation on the intestinal permeability, stability, and presystemic metabolism of valacyclovir. As shown in Fig. 4A, there was no significant difference in the effective permeability of valacyclovir in the jejunum of wildtype and *Bphl* knockout mice (i.e., 0.54 ± 0.10 and $0.53 \pm 0.05 \times 10^{-4}$ cm/sec, respectively). Moreover, valacyclovir was stable during the jejunal perfusion, as assessed by the very low appearance of acyclovir in outlet perfusate of both genotypes during the experiment, that is about 5% through 80 min and 5–10% at 90 min (Fig. 4B). Presystemic metabolism was determined by sampling the portal vein after 5 and 30 min of jejunal perfusion of valacyclovir. As shown in Fig. 4C, after 5 min of perfusion, prodrug hydrolysis was significantly greater in wildtype mice as compared to *Bphl* knockout mice (i.e., 8.0 ± 1.0 vs. $23.3 \pm 4.4\%$ valacyclovir remaining intact, respectively). However, after 30 min of perfusion, there was no significant difference between genotypes in the proportion of valacyclovir found intact in portal vein blood ($6.8 \pm 2.3\%$ in wildtype vs. $11.7 \pm 6.7\%$ in *Bphl* knockout mice).

3.5. Oral and intravenous pharmacokinetic studies of valacyclovir in wildtype and *Bphl* knockout mice

The effect of *Bphl* ablation on valacyclovir absorption, disposition and conversion to acyclovir was evaluated *in vivo* after both oral and intravenous administrations. The pharmacokinetics of valacyclovir (and acyclovir) were considered first after intravenous dosing of prodrug since disposition and conversion could be evaluated in the absence of absorption. As shown in Fig. 5A, similar (but not superimposable) plasma concentration - time profiles were observed between wildtype and *Bphl* knockout mice for prodrug and active parent drug. On average, the C_{\max} and AUC_{0-180} were about 30–40% greater for both

drug species, whereas the T_{max} for acyclovir was about 2.5-fold greater in *Bphl* knockout mice (Table 3). The changes in valacyclovir C_{max} and AUC_{0-180} , though most were not statistically significant, reflect the reduced conversion of valacyclovir to acyclovir in the absence of BPHL. The log-linear terminal half-life ($T_{1/2}$) showed only a modest difference between genotypes (i.e., 10% larger for prodrug and 20% smaller for active parent drug in *Bphl* knockout mice).

After oral dosing of valacyclovir, the plasma concentration - time profiles of acyclovir were quite similar between the two genotypes (Fig. 5B). Accordingly, the C_{max} of acyclovir was about 20% greater but the AUC_{0-180} only 4% greater in *Bphl* knockout mice (Table 3). Small (20%) reductions were also observed for the T_{max} and $T_{1/2}$ values of acyclovir during *Bphl* ablation. On the other hand, larger differences were noted between genotypes for valacyclovir in which the C_{max} and AUC_{0-180} were about 50 and 70% greater, respectively, in *Bphl* knockout mice as compared to wildtype animals. Although no change was found in the T_{max} of valacyclovir, a small 25% reduction was observed in the $T_{1/2}$ of prodrug during *Bphl* ablation. Interestingly, the ratio of AUC_{0-180} for prodrug to active parent drug (i.e., VACV/ACV) was 70% greater after oral dosing in *Bphl* knockout mice. In contrast, the AUC ratio differed by only 8% between genotypes after intravenous dosing.

Collectively, these findings demonstrate that, in the absence of BPHL, other enzymes are available to convert valacyclovir to acyclovir *in vivo* and that the conversion is more efficient after oral than intravenous administration of prodrug.

4. Discussion

Biphenyl hydrolase-like protein (BPHL) is a novel human serine hydrolase that was originally cloned from a breast carcinoma cDNA library [28] and, because of its ability to catalyze the hydrolytic activation of valacyclovir (to acyclovir), referred to as valacyclovirase (VACVase). VACVase, initially purified from rat liver, was reported to be a basic protein that associated intracellularly with mitochondria [38]. This enzyme was further characterized by studying its hydrolysis kinetics for six amino acid esters of acyclovir, including that of valacyclovir. A human valacyclovirase was later identified from the human colon carcinoma cell line, Caco-2 [18], after which the human gene was cloned and a crystal structure characterized [30]. Although several studies investigated the hydrolytic activation for a broad range of amino acid ester nucleoside prodrugs by BPHL [18,31,39], no study has yet evaluated the *in vivo* exclusivity of this process. Indeed, a number of esterases/proteases may mediate the hydrolysis of valacyclovir and similar prodrugs.

In this study, the *in situ* intestinal permeability and stability of valacyclovir were evaluated during jejunal perfusions, and the *in vivo* absorption and disposition of valacyclovir (and acyclovir) evaluated during oral and intravenous bolus dosing of prodrug in wildtype and *Bphl* knockout mice. The use of transgenic mice, in which the *Bphl* gene was ablated, offers a unique opportunity to test the relative importance of BPHL in hydrolyzing valacyclovir, as compared to other possible enzymes. Major findings of this study revealed, for the first time, that: 1) no obvious phenotype was observed for *Bphl* knockout mice; 2) enzymatic upregulation of Cyp3a11, Cyp3a16, Abhd14a and Abhd14b was observed in some tissues of

Bphl knockout mice; 3) *Bphl* ablation had no effect on the jejunal permeability of valacyclovir in mice; 4) valacyclovir was stable during intestinal perfusions and, following absorption, was found mostly as acyclovir (>75%) in portal vein blood; 5) valacyclovir was rapidly and efficiently hydrolyzed to acyclovir *in vivo* in the absence of BPHL; and 6) hydrolysis was more extensive after the oral vs. intravenous route of administration (for both genotypes), as judged by the 10- to 20-fold higher AUC₀₋₁₈₀ values of acyclovir to valacyclovir after oral dosing as compared to the only 2- to 3-fold higher AUC₀₋₁₈₀ values of acyclovir to valacyclovir after intravenous dosing. Taken as a whole, BPHL is not obligatory for the *in vivo* conversion of valacyclovir prodrug to its active parent drug acyclovir.

Assuming that BPHL was solely responsible for valacyclovir to acyclovir hydrolysis, one would have expected substantial differences between wildtype and *Bphl* knockout mice in the systemic exposure of valacyclovir and acyclovir following oral and intravenous administrations of prodrug. Although some differences in AUC₀₋₁₈₀ were noted, none of the changes achieved a level of statistical significance. For example, after oral administration of prodrug, the AUC₀₋₁₈₀ of valacyclovir was increased 70%, the AUC₀₋₁₈₀ of acyclovir was essentially unchanged, and the VACV/ACV ratio was increased 70% in *Bphl* knockout mice. In contrast, after intravenous administration of prodrug, the AUC₀₋₁₈₀ of valacyclovir was increased 30%, the AUC₀₋₁₈₀ of acyclovir was increased 40%, and the VACV/ACV ratio was essentially unchanged in *Bphl* knockout mice. Thus, during BPHL ablation, the hydrolysis of valacyclovir to acyclovir was less extensive following oral dosing, suggesting that presystemic metabolism of valacyclovir was more important than systemic metabolism of prodrug. This finding is corroborated by our *in situ* jejunal perfusion results in mice showing that, as early as 5 min, < 10% of valacyclovir remains intact in portal vein blood, and by studies in humans showing that valacyclovir is nearly completely converted to acyclovir by first-pass intestinal and/or hepatic metabolism [40].

Ces1 and Ces2 are mouse carboxylesterases that are expressed in the liver (CES1 predominates in human) and small intestine (contains CES2 alone in human), and hydrolyze ester prodrugs, including the antiviral substrates valacyclovir and oseltamivir [41]. Cyp3a11, Cyp3a16, Cyp3a41 and Cyp3a44 are mouse orthologs of the human CPY3A4/5 genes that are highly expressed in both small intestine and liver, and account for the metabolism in over 50% of clinically used drugs [42]. Abhd14a and Abhd14b are mouse orthologs of the human hydrolases (ABHD14A/B) that are expressed about equally in most human tissues, with lowest expressions in brain, testis and placenta [12]. They are believed to have some role in transcriptional initiation and have been shown to hydrolyze the model substrate p-nitrophenyl butyrate. Although the present study design did not allow us to identify which additional enzymes were involved in the hydrolysis of valacyclovir during BPHL ablation, some compensatory (upregulatory) mechanisms were noted in *Bphl* knockout mice. For instance, gene expression was significantly increased for Cyp3a16 in the duodenum and liver, and for Abhd14a in the duodenum, ileum and kidney of mice lacking the *Bphl* gene. More prevalent changes were observed for Abhd14b in which gene expression was significantly increased in the duodenum, ileum, colon, liver, kidney and spleen of *Bphl* knockout mice. Although speculative, the increased exposure of ACV in *Bphl* knockout

mice, after intravenous administration of VACV, might be the result of significant upregulation of Abhd14a/b in the liver, kidney and spleen (Figs 3K and 3L).

In regard to the proton-coupled oligopeptide transporters, PepT1 was upregulated in the colon of *Bphl* knockout mice. However, this change is unlikely to have a significant impact on the *in vivo* pharmacokinetic results because valacyclovir is almost entirely absorbed in the small intestine [43]. Significant changes in the gene expression of PhT1 and PhT2 are also unlikely to impact the absorption of valacyclovir since these transporters are not expressed at the apical membrane of small intestinal epithelia but are, instead, located intracellularly [44].

In conclusion, the *in situ* intestinal permeability and stability of valacyclovir, and *in vivo* absorption and disposition of valacyclovir (and acyclovir) were evaluated after oral and intravenous administrations in wildtype and *Bphl* knockout mice. Whereas the jejunal permeability of valacyclovir was unchanged between the two genotypes, rapid and extensive hydrolysis of prodrug was still evident in mice even during *Bphl* gene deletion. Thus, BPHL is not obligatory for the *in vivo* conversion of valacyclovir to acyclovir either presystemically or systemically. The relevance of the upregulation of mouse Abhd14a and/or Abhd14b hydrolases in small intestinal segments, liver, kidney and spleen is currently unknown but deserves further attention.

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Abbreviations:

ABHD14A	alpha/beta hydrolase domain containing 14a
ABHD14B	alpha/beta hydrolase domain containing 14b
ACV	acyclovir
Bphl	biphenyl hydrolase-like protein
KO	knockout
PepT1	peptide transporter 1 (SLC15a1)
PepT2	peptide transporter 2 (SLC15a2)
PhT1	peptide/histidine transporter 1 (SLC15a4)
PhT2	peptide/histidine transporter 2 (SLC15a3)
POT	proton-couple oligopeptide transporter
SLC	solute carrier family
VACV	valacyclovir

WT wildtype

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Figure 1. Histological evaluation, using hematoxylin and eosin staining, of the duodenum, jejunum, ileum, cecum, colon, liver, kidney and spleen from wildtype and *Bphl* knockout (KO) mice. One representative example is shown of six individual stains. Magnification 20 \times .

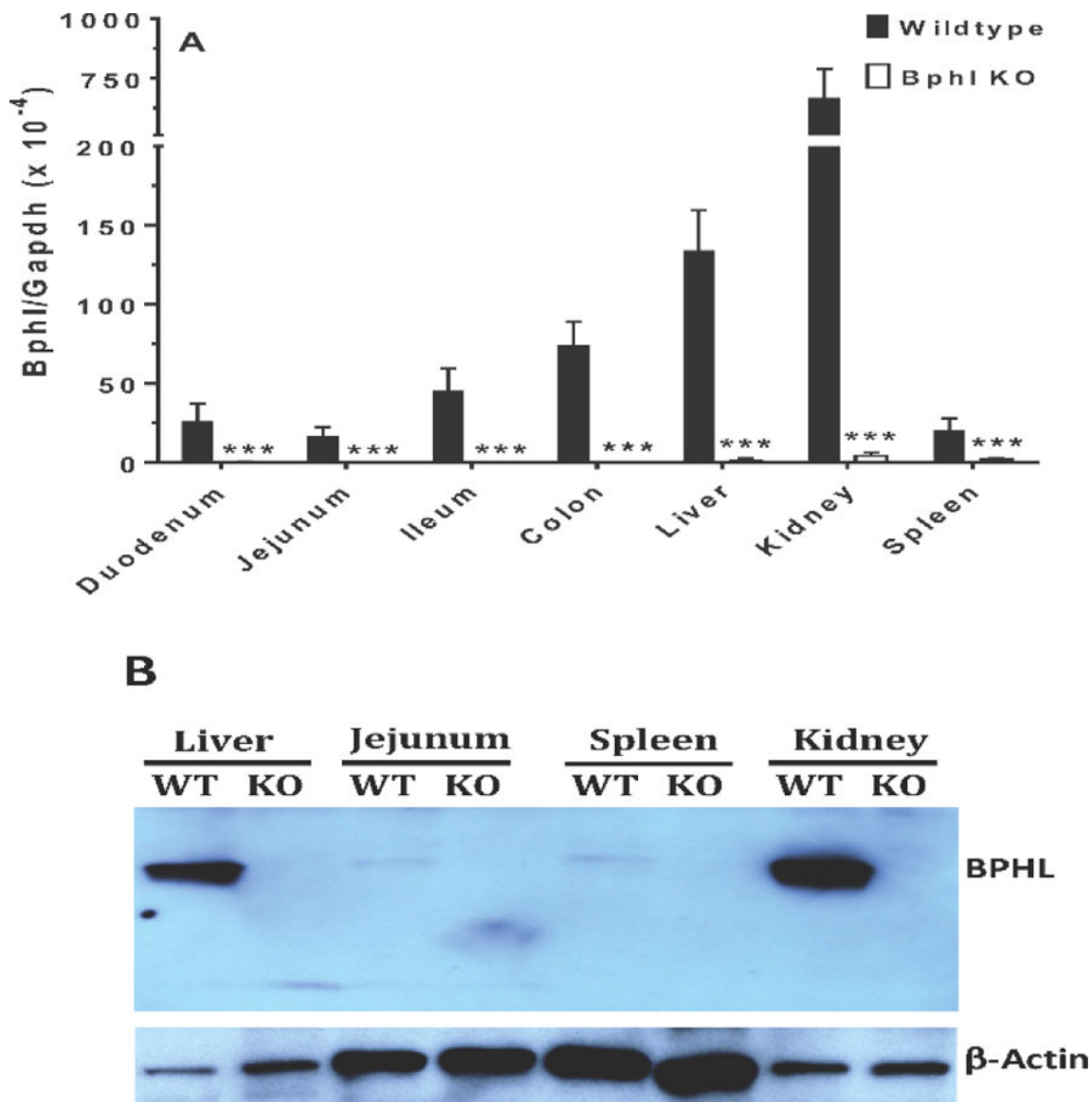


Figure 2. BPHL gene and protein expression in select tissues from wildtype and *Bphl* knockout mice. (A) *Bphl* transcripts were measured by quantitative real-time PCR and the data expressed as mean \pm SE (n=6, each sample run in triplicate). ***p < 0.001, as compared to wildtype, using an unpaired t-test. (B) BPHL protein was measured by immunoblotting in which 10 μ g of protein lysate was loaded for liver and kidney, and 40 μ g of protein lysate loaded for jejunum and spleen. One representative example is shown of three individual immunoblots. WT, wildtype mice; KO, *Bphl* knockout mice.

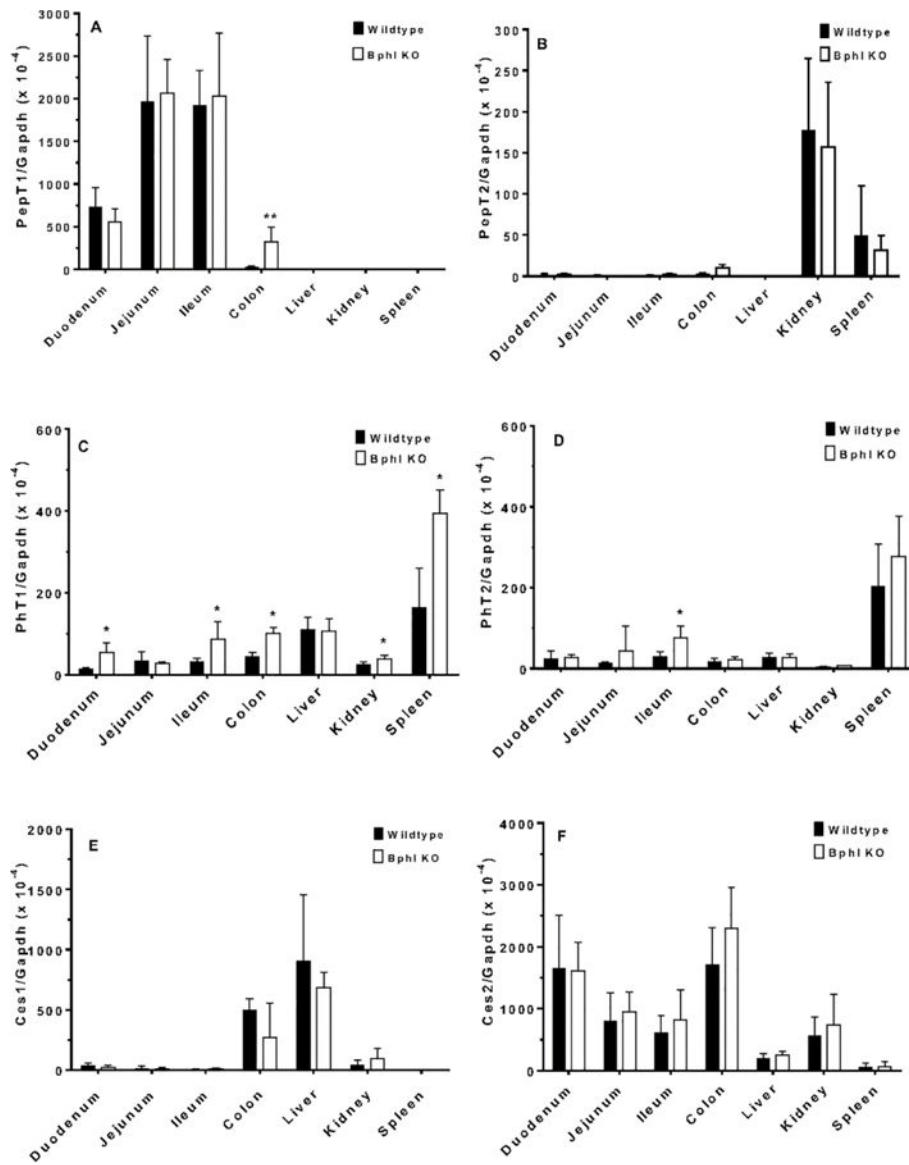


Figure 3. Quantitative real-time PCR analysis of relevant transporters and enzymes in select tissues from wildtype and *BphI* knockout (KO) mice. Data are expressed as mean \pm SE (n=6, each sample run in triplicate). *p < 0.05, **p < 0.01 and ***p < 0.001, as compared to wildtype mice, using an unpaired t-test. Refer to Table 1 for gene identification.

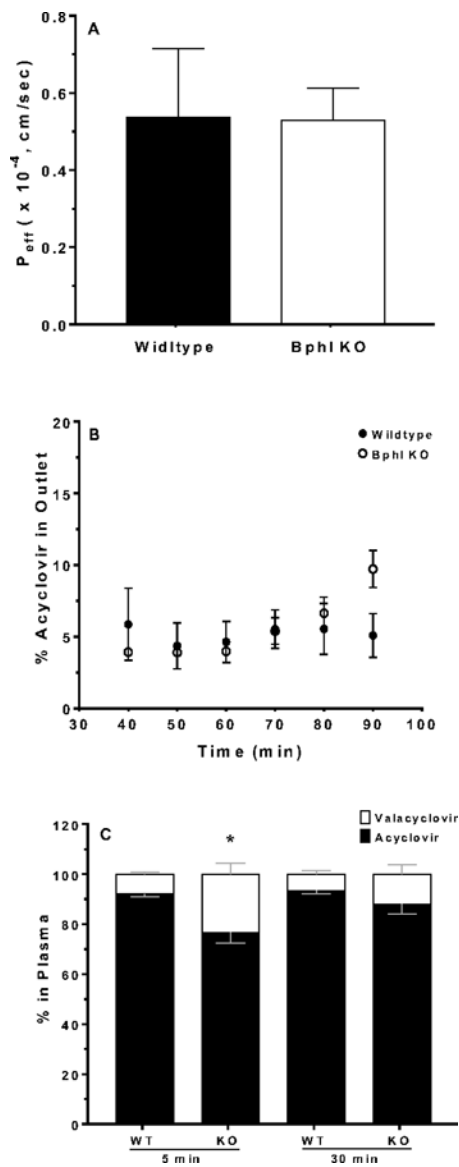


Figure 4. Intestinal permeability and stability of valacyclovir during *in situ* jejunal perfusions of prodrug in wildtype and *Bphl* knockout (KO) mice. (A) Effective permeability of 50 μ M valacyclovir in both genotypes, and (B) acyclovir concentrations in the outlet perfusate of both genotypes as a function of time. Data are expressed as mean \pm SE (n=3–4). (C) Percent of valacyclovir and acyclovir found in the portal vein blood, at 5 and 30 min, following jejunal perfusions of 50 μ M valacyclovir. Data are expressed as mean \pm SE (n=3). *p < 0.05, as compared to wildtype mice, using an unpaired t-test.

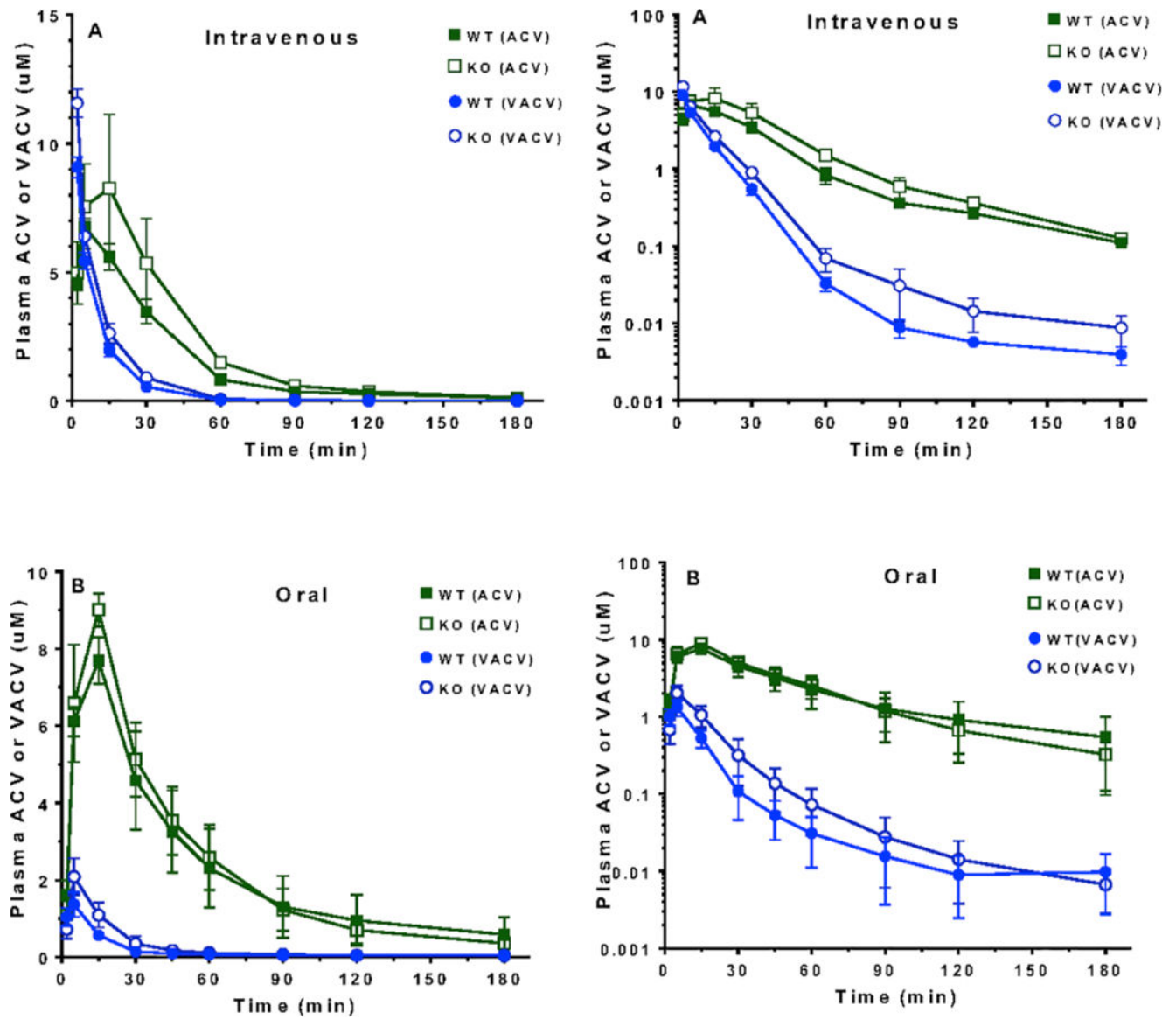


Figure 5. Plasma concentration-time profile of acyclovir (ACV) and valacyclovir (VACV) after (A) intravenous and (B) oral administrations of 25 nmol/g valacyclovir in wildtype (WT) and *Bph1* knockout (KO) mice. Data are expressed as mean \pm SE (n=3) in which the y-axis is displayed on a linear scale (left-sided panels) and on a logarithmic scale (right-sided panels).

Table 1

Primers used in qRT-PCR

Gene	Forward Primer	Reverse Primer
Gapdh (EC1.2.1.12)	5'-GAGACAGCCGCATCTTCTTGT-3'	5'-CACACCGACCTTCACCATTTT-3'
Bph1 (EC3.1.-.-)	5'-CCCTGAAATCCCGGATCTG -3'	5'-AAGTTACTGCAGTGCCGAAGGT-3'
PepT1 (Slc15a1)	5'-CCACGGCCATTTACCATACG-3'	5'-TGCGATCAGAGCTCCAAGAA-3'
PepT2 (Slc15a2)	5'-TGCAGAGGCACGGACTAGATAC-3'	5'-GGGTGTGATGAACGTAGAAATCAA-3'
PhT1 (Slc15a4)	5'-GCTGCCACCTGCATTACTACTTC-3'	5'-CGTACTTCACAGACACAATGAGGAA-3'
PhT2 (Slc15a3)	5'-GCTGAAGCTTGC GTTCAA-3'	5'-AACAGGTGGGCAC TTTTCAGAGT-3'
Ces1 (EC3.1.1.-)	5'-GCCCTGGAGCTTCGTGAA -3'	5'-TCAATGCTGCCTCTGGGTTT -3'
Ces2 (EC3.1.1.-)	5'-GCTGAAGGCTGCAGTTCTG -3'	5'-GTGCTTGTCTGAGAAGCCTTT -3'
Cyp3a11 (EC1.14.99.38)	5'-AACTGCAGGATGAGATCGATGAG -3'	5'-TTCATTAAGCACCATATCCAGGTATT -3'
Cyp3a16 (EC1.14.99.-)	5'-CTGCAGGAGGAGATCGATGAG -3'	5'-CCATCGCCATCACGGTATC -3'
Cyp3a41 (EC1.14.-.-)	5'-CTGACAGACAAGCAGGGATGAA -3'	5'-GTAGAGGAGCACCAGGATGATTG -3'
Cyp3a44 (EC1.14.-.-)	5'-CAGCTCTCTACTGGATACATTGG -3'	5'-GTACGGGTCCCATATCGGTAGA -3'
Abhd14a (EC3.-.-.-)	5'-CCAGAGGCAACTCTCGAATCTT-3'	5'-CACGACTTCTGCCCTGCAT -3'
Abhd14b (EC3.-.-.-)	5'-GGCTCCTCCAGCTTCAACA -3'	5'-CCCTGCCCTTCCATGAC -3'

Table 2Serum clinical chemistry of wildtype and *Bph1* knockout mice

	Wildtype	<i>Bph1</i> Knockout
Sodium (mmol/L)	144 ± 1 (10)	147 ± 1* (8)
Potassium (mmol/L)	9.1 ± 0.4 (10)	7.7 ± 0.7 (8)
Chloride (mmol/L)	109 ± 1 (10)	109 ± 1 (8)
Creatine (mg/dL)	0.32 ± 0.05 (9)	0.27 ± 0.03 (8)
TRIG (mg/dL)	64 ± 6 (10)	76 ± 5 (8)
CHOL (mg/dL)	79 ± 10 (10)	67 ± 3 (8)
AST (U/L)	121 ± 12 (10)	226 ± 57 (8)
Glucose (mg/dL)	250 ± 79 (9)	242 ± 13 (8)
Albumin (g/dL)	2.5 ± 0.3 (10)	2.4 ± 0.3 (8)
Protein (g/dL)	5.1 ± 0.1 (9)	5.2 ± 0.1 (8)
Calcium (mg/dL)	9.2 ± 0.4 (9)	7.0 ± 1.0 (8)
ALT (U/L)	79 ± 41 (9)	132 ± 31 (8)
CPK (U/L)	1629 ± 197 (9)	1562 ± 344 (6)
Bilirubin (mg/dL)	0.12 ± 0.01 (9)	0.10 ± 0.00 (7)
BUN (mg/dL)	42 ± 5 (9)	31 ± 2 (8)
ALP (U/L)	86 ± 11 (9)	105 ± 5 (8)

Data are expressed as mean ± SE (number of mice). TRIG, triglycerides; CHOL, Cholesterol; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CPK, creatine phosphokinase; BUN, blood urea nitrogen; ALT, alanine aminotransferase.

* p < 0.05, as compared to wildtype mice, using an unpaired t-test.

Table 3.

Pharmacokinetic Parameters of Valacyclovir and Acyclovir in Wildtype and *Bph1* Knockout Mice after the Oral and Intravenous Administrations of 25 nmol/g Valacyclovir

Oral	C _{max} (μM)	T _{max} (min)	AUC ₀₋₁₈₀ ($\mu\text{M}\cdot\text{min}$)	T _{1/2} (min)
Wildtype				
VACV	1.3 \pm 0.6	5 \pm 0	22.1 \pm 6.5	48.9 \pm 18.4
ACV	7.6 \pm 0.6	15 \pm 0	402 \pm 126	47.5 \pm 10.1
VACV/ACV (%)	17 \pm 6	33 \pm 0	5.5 \pm 2.4	103 \pm 1
<i>Bph1</i> Knockout				
VACV	2.0 \pm 0.5	5 \pm 0	38.3 \pm 10.3	37.2 \pm 2.6
ACV	9.4 \pm 0.2 *	12 \pm 3	419 \pm 81	37.0 \pm 6.6
VACV/ACV(%)	22 \pm 2	43 \pm 2 *	9.1 \pm 3.0	100 \pm 1
Intravenous				
Intravenous	C _{max} (μM)	T _{max} (min)	AUC ₀₋₁₈₀ ($\mu\text{M}\cdot\text{min}$)	T _{1/2} (min)
Wildtype				
VACV	9.1 \pm 0.4	2 \pm 0	109 \pm 8	50.2 \pm 15.4
ACV	6.8 \pm 0.3	5 \pm 0	259 \pm 18	45.9 \pm 6.0
VACV/ACV(%)	134 \pm 1	40 \pm 0	37.3 \pm 3.9	109 \pm 1
<i>Bph1</i> Knockout				
VACV	11.6 \pm 0.5 *	2 \pm 0	145 \pm 11	53.7 \pm 19.8
ACV	8.8 \pm 2.6	12 \pm 3	373 \pm 97	37.3 \pm 6.2
VACV/ACV(%)	131 \pm 3	17 \pm 2 ***	34.2 \pm 9.3	144 \pm 1 ***

* p 0.05

*** p 0.001, as compared to wildtype mice for the same route of administration, using an unpaired t-test.