The Role of Nucleoside Transporters in the Erythrocyte Disposition and Oral Absorption of Ribavirin in the Wild-Type and Equilibrative Nucleoside Transporter 1(–/–) Mice

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ABSTRACT

Ribavirin [1-(β -D-ribofuranosyl)-1*H*-1,2,4-triazole-3-carboxamide] is the treatment of choice for hepatitis C virus infection. Ribavirin is a substrate of several nucleoside transporters, including the equilibrative nucleoside transporter (Ent) and the concentrative nucleoside transporter 2. To determine the role of Ent1 in ribavirin absorption and erythrocyte distribution, we examined its pharmacokinetics in Ent1-null mice. After intravenous administration, we found that the erythrocyte area under the curve ($AUC_{0-12\text{ h}}$) was reduced 3.05-fold along with 2.63-fold reduction of erythrocyte versus plasma AUC ratio in the Ent1(–/–) mice, whereas there was no significant difference in the plasma $AUC_{0-12\text{ h}}$ between Ent1(+/+) and Ent1(–/–) mice. After 48 h, we found a similar fraction of ribavirin or total radioactivity excreted in the urine between the Ent1(+/+) and Ent1(–/–) mice. After oral administration of three different

doses, 0.024, 0.24, and 6.1 mg/kg, we found that the dose-normalized plasma $AUC_{0-12\text{ h}}$ of ribavirin was 69.7 ± 12.0 , 20.7 ± 1.5 , and $18.3 \pm 2.7\text{ min/l}$, respectively, in the Ent1(+/+) mice and 18.9 ± 2.8 , 13.0 ± 0.5 , and $12.2 \pm 1.0\text{ min/l}$, respectively, in the Ent1(–/–) mice. It is interesting that at the highest dose, the dose-normalized plasma $AUC_{0-30\text{ min}}$, $AUC_{0-12\text{ h}}$, and C_{max} in the Ent1(+/+) mice were decreased 4.0-, 3.8-, and 3.4-fold, respectively, compared with the lowest dose, suggesting absorption was saturated at the highest dose we used. The dose-normalized plasma $AUC_{0-12\text{ h}}$ was 3.7- and 1.5-fold lower at the lowest and the highest dose, respectively, in the Ent1(–/–) mice compared with those of the Ent1(+/+) mice. Our findings indicate that Ent1 plays a significant role in the oral absorption and erythrocyte distribution of ribavirin.

Ribavirin [1-(β -D-ribofuranosyl)-1*H*-1,2,4-triazole-3-carboxamide], with polyethylene glycol-conjugated interferon- α , is currently the standard of treatment for chronic hepatitis C virus infection. Worldwide, approximately 200 million people (of which 3.2 million are in the United States) are chronically infected with the hepatitis C virus (Sherlock, 1995; Armstrong et al., 2006). Even at its usual recommended dose,

treatment with ribavirin is limited by its major toxicity, hemolytic anemia (Fried, 2002). In 10 to 13% of patients, this anemia is profound enough to result in either dose reduction or discontinuation of therapy (Fried, 2002), leading to lack of effective treatment of the infection. Ribavirin is a prodrug and exhibits its broad antiviral activity through its active phosphorylated metabolites (Parker, 2005). There is mounting evidence that the hematological toxicity of ribavirin is due to the significant accumulation and lack of dephosphorylation of the active phosphorylated metabolites in the erythrocytes (Parker, 2005). Multiple studies have observed significant correlations between the intracellular ribavirin concentration and the magnitude of hemolytic anemia observed clinically (Homma et al., 2004; Inoue et al., 2006).

Because ribavirin is hydrophilic (log *P* approximately –2.0 to –2.5), it needs to be transported into cells to produce its efficacy. This transport is mediated by the nucleoside trans-

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ABBREVIATIONS: ENT/Ent, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; RMP, ribavirin 5'-monophosphate; RTP, ribavirin triphosphate; AUC, area under the curve; dH₂O, deionized water; RDP, ribavirin 5'-diphosphate; HPLC, high-performance liquid chromatography; RTCOOH, 1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxylic acid; TCONH₂, 1,2,4-triazole-3-carboxamide; TCOOH, 1,2,4-triazole-3-carboxylic acid; D, dose normalized.

porters. There are two families of the nucleoside transporters, the equilibrative nucleoside transporters (ENTs) and the sodium-dependent concentrative nucleoside transporters (CNTs). Ribavirin is a substrate of the human nucleoside transporters ENT1 (Jarvis et al., 1998), ENT2 (Yamamoto et al., 2007), CNT2 (Yamamoto et al., 2007), and CNT3 (Hu et al., 2006). The active metabolites of ribavirin, ribavirin 5'-mono and 5'-triphosphate (RMP and RTP), are formed intracellularly (Russmann et al., 2006); thus, this metabolic activation is in part dependent on the transport of ribavirin into the cell. In addition, these metabolites are polar, and once formed inside the cell, they are unable to diffuse out (Canonica et al., 1984).

Ribavirin is transported into human erythrocytes by the human ENT1 (Jarvis et al., 1998). Based on *in vitro* transport data, we have shown that ENT1 is the rate-limiting step in erythrocyte accumulation (as phosphates) of ribavirin (Endres et al., 2009). However, because ENT1 is an equilibrative transporter, it is not clear whether this is true *in vivo*. Here, we report a study that tests this hypothesis using mice in which *Ent1* has been genetically ablated.

For efficacy, ribavirin must be well absorbed after oral administration. The usual oral dose of ribavirin is 600 mg b.i.d. The oral bioavailability of ribavirin is variable, from 33 to 64% (Paroni et al., 1989; Connor et al., 1993; Preston et al., 1999). Saturation of ribavirin absorption has been observed in humans, as well as in mice. For example, the maximal plasma concentration ($C_{\max}$) of ribavirin does not increase proportionally with the ribavirin oral dose (600–1200 or 2400 mg). In addition, two independent preclinical oral dose-ranging studies in mice (20, 40, and 75 mg/kg or 10, 50, and 100 mg/kg) showed that the ribavirin plasma AUC and $C_{\max}$ increases less than proportionally with dose (FDA, 2007a,b). Using brush-border membrane vesicles isolated from the human intestine, we have demonstrated that the saturation of ribavirin absorption is probably due to saturation of CNT2 expressed on the apical membrane of the enterocytes (Patil and Unadkat, 1997). In addition, our preliminary studies, using the *in situ* perfused mouse intestine, have demonstrated that the egress of ribavirin from the basolateral membrane of enterocytes is mediated primarily by ENT1 (Moss et al., 2007). Such spatial arrangement would allow vectorial transport of ribavirin from the intestinal lumen to the blood.

Previously, we measured and modeled the *in vitro* uptake and metabolism of ribavirin into erythrocytes in *Ent1*(+/+) and *Ent1*(–/–) mice to predict the contribution of *Ent1* to the *in vivo* distribution and accumulation of ribavirin (Endres et al., 2009). We predicted that the accumulation of the active phosphorylated metabolites of ribavirin would be substantially greater (~15-fold) in the presence of ENT1/*Ent1* than in the absence of ENT1/*Ent1*. In experiments presented here, we have tested this prediction as well as the hypothesis that nucleoside transporters are important in the intestinal absorption of oral ribavirin.

Materials and Methods

Mouse Husbandry. All animal procedures were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee. *Ent1*(+/+) and *Ent1*(–/–) colonies were maintained as described previously (Endres et al., 2009).

Pharmacokinetic Study. [^3H]Ribavirin (in 0.9% saline) was administered intravenously (in the retro-orbital sinus; 3.0 mg/kg; 0.5

mCi/kg) under ketamine/xylazine (130 and 8.8 mg/kg, respectively) anesthesia or orally (by gavage; 0.024, 0.244 and 6.1 mg/kg; 0.5 mCi/kg, 5 ml/kg) to both male and female *Ent1*(+/+) and *Ent1*(–/–) mice. Serial blood samples (~20 μl) were obtained ($n = 3$) from each animal in heparinized microhematocrit tubes at the following time points (5, 15, 30, 60, 120, 240, 480, 720, and 1440 min) by sampling from the saphenous vein. The total blood volume sampled from each animal was less than 1% of the total body weight. The blood samples were immediately centrifuged for 1 min at 5000g. The hematocrit was measured, and the hematocrit tube was scored at the buffy-coat/plasma interface. The plasma or packed erythrocytes (5–10 μl , leaving behind the interface) were transferred to preweighed microhematocrit tubes containing 100 μl of deionized water (dH_2O). These samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Urinary Excretion Study. [^3H]Ribavirin (in 0.9% saline) was administered intravenously (in the retro-orbital sinus; 3 mg/kg; 1.1 $\mu\text{Ci/g}$) under ketamine/xylazine (130 and 8.8 mg/kg, respectively) anesthesia to male and female *Ent1*(+/+) and *Ent1*(–/–) mice. The animals were immediately placed in metabolic cages to collect urine. After 48 h, the animals were sacrificed. The contents of the bladder collected using a syringe with a 26-gauge needle and pooled with the urine collected from the metabolic cages. The cages were washed with dH_2O , and the cage-wash was stored and analyzed separately from the urine. All samples were stored at -80°C until further analysis.

Analysis of Total [^3H]Radioactivity in Plasma, Erythrocytes, and Urine. Twenty microliters of each diluted plasma, erythrocyte, or urine sample was added to 100 μl of dH_2O in 7-ml scintillation vials. The samples were decolorized with 300 μl of 30% H_2O_2 , agitated for 20 min on a plate shaker, and 5 ml of scintillation fluid was added to each sample. The ^3H radioactivity (microcuries per milliliter) concentrations were determined using liquid scintillation counting.

Dephosphorylation of Phosphorylated Metabolites. The phosphorylated nucleotides [^3H]RMP and [^3H]RTP (Moravek, Brea, CA) exhibited spontaneous degradation to ribavirin in the presence of erythrocyte lysate. Pilot study data confirmed that RMP and RTP were not detected in the plasma at either 15 min or ~3 h after intravenous dosing, which was consistent with previous observations in humans (Homma et al., 2004). Thus, the erythrocyte samples were incubated for 30 min at 37°C with 10 U of alkaline phosphatase to dephosphorylate the ribavirin nucleotides (RMP, RDP, and RTP) to ribavirin.

Sample Workup and HPLC Analysis to Determine Ribavirin Composition. The protein in each sample was precipitated by the addition of 60 μl of 6% perchloric acid and vortexed 1 min. The samples were neutralized by the addition of 20 μl of 2 M K_2HPO_4 and then centrifuged at 20,000g for 10 min at 4°C . Twenty microliters of the supernatant was added to a 7-ml scintillation vial containing 100 μl of dH_2O and counted using scintillation counting to determine sample workup recovery. One-hundred twenty microliters of the supernatant was analyzed by HPLC using a method that resolved ribavirin from RTCOOH , TCONH_2 , TCOOH (kindly provided by Valeant Pharmaceuticals, Costa Mesa, CA), and RMP and RTP (Moravek Biochemicals, Brea, CA) metabolites. The radioactivity coeluting at the retention time of the unlabeled ribavirin standard (as determined by UV detection) was expressed as a percentage of the total radioactivity injected to determine the percentage of ribavirin composition.

Data Analysis. The ribavirin blood concentration-time profile was derived from the plasma and erythrocyte concentration time-profile and the measured hematocrit at each time point. Pharmacokinetic parameters were calculated using noncompartmental analysis using linear interpolation between concentration-time data points using WinNonlin (Pharsight, Mountain View, CA). In the plasma and blood samples, C_0 was determined by linear extrapolation of the first two plasma concentration time points. In addition,

the intravenous plasma concentration-time profiles were analyzed using compartmental analysis using a standard two-compartment model. Significant differences between the pharmacokinetic parameters in Ent1(+/+) and Ent1(-/-) mice were tested using the two-sided Student's *t* test for continuous parameters (e.g., AUC and $C_{\max}$) and the Mann-Whitney U test for discrete parameters (e.g., $T_{\max}$).

Simulation of in Vivo Transport and Metabolism of Ribavirin after Intravenous Dosing. In vivo ribavirin transport and metabolism was simulated using the following equations:

$$\frac{dC_{RBV}^{in}}{dt} = C_{RBV}^{ex} \cdot \frac{(CL_{dif} + CL_{ent})}{V_2} - C_{RBV}^{in} \cdot \frac{(CL_{dif} + CL_{ent} + CL_{phosp})}{V_2} \quad (1)$$

$$\frac{dC_{phosp}^{in}}{dt} = C_{RBV}^{in} \cdot \frac{CL_{phosp}}{V_2} - C_{phosp}^{in} \cdot K_{deg} \quad (2)$$

where C_{RBV}^{in} is the intracellular erythrocyte concentration of ribavirin; CL_{dif} and CL_{ent} are the diffusional and Ent1-mediated transmembrane distributional clearances of ribavirin, respectively; V_1 and V_2 are the extracellular and intracellular distributional volumes of ribavirin, respectively; C_{phosp}^{in} is the intracellular phosphorylated metabolite concentration (RMP, RDP, and RTP; pooled); CL_{phosp} is the phosphorylated metabolite formation clearance; and K_{deg} is the in vivo elimination rate constant of the phosphorylated metabolite. For simplicity, in this model the volume distribution of the intracellular phosphorylated metabolite was assumed to be equal to V_2 (Fig. 1). The predicted ribavirin plasma concentrations as determined by compartmental modeling of the observed data (described above) were used to drive the plasma ribavirin concentrations (C_{RBV}^{ex}) in this model. The values of CL_{dif} (0.204 $\mu\text{L}/10^9$ cells/min), CL_{ent} (167.1 $\mu\text{L}/10^9$ cells/min), CL_{phosp} [2.89 and 6.06 $\mu\text{L}/10^9$ cells/min for Ent1(+/+) and Ent1(-/-) mice, respectively], and V_2 (43.0 $\mu\text{L}/10^9$ cells) were fixed using parameters determined previously (Endres et al., 2009). In addition, for simplicity, the nonphosphorylated pathway of erythrocyte metabolism was eliminated from this model, because it played a relatively minor role in the total intracellular metabolism of ribavirin in the erythrocytes. K_{deg} was calculated from the observed erythrocyte ribavirin terminal half-life after intravenous dosing, and fixed at 0.000605 and 0.000894 min^{-1} for Ent1(+/+) and Ent1(-/-) animals, respectively. Additional simula-

tions were performed using values of 1.0 and 0.5 $\mu\text{L}/10^9$ cells/min for CL_{phosp} . The data obtained from Ent1(+/+) and Ent1(-/-) animals were simultaneously modeled using two sets of differential equations, one with and one without the CL_{ent} parameter, to predict the plasma ribavirin, and erythrocyte ribavirin and phosphorylated metabolite concentrations after multiple dosing of ribavirin to steady state with a dosing interval of 12 h.

Results

Assay Validation, Sample Workup Recovery, and Parent Compound Composition. Ribavirin was stable in phosphate-buffered saline at 37°C for up to 24 h (data not shown). In addition, the assay recovery of ribavirin spiked in plasma and erythrocytes was expressed as a percentage of the total radioactivity recovered when spiked into water and then processed. The total recovery of ribavirin from plasma and erythrocytes was 99.5 ± 1.7 and $96.7 \pm 1.7\%$, respectively ($n = 3$).

Ribavirin was separated from its metabolites TCOOH, RT-COOH, TCONH₂, RMP, and RTP using ion-paired reversed-phase HPLC assay described previously (Endres et al., 2009). We did not have a standard for RDP, but it is predicted to elute between RMP and RTP. In the presence of alkaline phosphatase, there was no detectable RMP after 15 min and no detectable RTP after 30 min of incubation (data not shown).

Intravenous Ribavirin Pharmacokinetics. As mentioned above, because the phosphorylated metabolites of ribavirin were dephosphorylated, all reference to ribavirin erythrocyte concentration represents both ribavirin and its phosphorylated metabolites. The ribavirin plasma concentration-time profile was remarkably similar between the Ent1(+/+) and the Ent1(-/-) mice (Fig. 2A). The profile was biexponential through 12 h, with a rapid distributional (α)-phase and a longer terminal (β)-phase. There was no significant difference in the plasma ribavirin AUC_{0-12 h}, C_0 , or terminal half-life ($t_{1/2\beta}$) between the Ent1(+/+) and the Ent1(-/-) animals (Fig. 2A; Table 1).

The ribavirin (and phosphorylated metabolite) erythrocyte concentrations in the Ent1(-/-) animals were significantly lower than those in the Ent1(+/+) animals (Fig. 2B). The ribavirin erythrocyte AUC_{0-12 h} was significantly decreased, by 3.05-fold, in the Ent1(-/-) mice (Table 1). When normalized to the ribavirin plasma AUC, the ribavirin erythrocyte to plasma AUC ratio was significantly decreased, by 2.63-fold, in the Ent1(-/-) versus the Ent1(+/+) mice (Table 1). In addition, the erythrocyte ribavirin $C_{\max}$ was significantly decreased 3.78-fold in the Ent1(-/-) mice, whereas there was no difference in the erythrocyte ribavirin $t_{1/2\beta}$. Similar to our observations in the erythrocytes, the blood AUC_{0-12 h} was decreased 2.36-fold in the Ent1(-/-) versus the Ent1(+/+) mice (Fig. 2C).

Modeling and Simulation of Accumulation of Erythrocyte Ribavirin and Phosphorylated Metabolites. We expanded the ex vivo model we developed previously (Endres et al., 2009) to predict the accumulation of ribavirin and phosphorylated metabolites in the erythrocyte using the in vivo ribavirin pharmacokinetic data obtained above. The ribavirin plasma concentration-time profiles were first modeled using a two-compartment model (Table 2). The concentration-time profile predicted by these parameters was used as a "forcing function" to drive the erythrocyte distribution of

Fig. 1. Model of ribavirin transport and metabolism. Compartmental model describing the disposition of ribavirin and metabolites (phosphorylated and nonphosphorylated) for the distribution and metabolism of ribavirin in erythrocytes. Ribavirin enters the erythrocyte by a diffusional (CL_{dif}) and ENT1 (CL_{ent})-mediated clearance. Intracellular ribavirin is metabolized by phosphorylation (CL_{phosp}) and nonphosphorylation ($CL_{nonphosp}$) pathways. Extracellular ribavirin is eliminated by systemic elimination (K_{sys}), whereas intracellular phosphorylated metabolite is eliminated by degradation (K_{deg}).

ribavirin as described in eqs. 1 and 2. In addition, K_{deg} was fixed at the observed half-life of ribavirin in the erythrocytes after intravenous administration. Generally, erythrocyte ribavirin and phosphorylated metabolite to plasma ribavirin concentration ratio (accumulation ratio) and the difference in this ratio between Ent1(+/+) and Ent1(-/-) mice decreased with decreasing CL_{phosp} (Table 3). In addition, although the predicted accumulation after a single dose in Ent1(+/+) mice was similar to the observed accumulation ratio when CL_{phosp} was fixed at $1.0 \mu\text{l}/10^9$ cells/min, the predicted accumulation in Ent1(-/-) underpredicted the observed by approximately one third at all values of CL_{phosp} (Table 3).

Ribavirin Urinary Excretion. The 0- to 48-h urinary excretion of ribavirin and its metabolites after intravenous administration was examined in Ent1(+/+) and Ent1(-/-) mice. After 48 h, there was no significant difference in the fraction excreted of the total radioactivity or ribavirin (both expressed as a percentage of the dose) between the Ent1(+/+) and Ent1(-/-) animals (Table 4). In addition, there was no significant difference in the composition of the urine (i.e., percentage of composition of each analyte) with respect to ribavirin, TCOOH, RTCOOH, and RMP. The composition (percentage) of TCONH₂ in the urine was significantly increased 1.68-fold in the Ent1(-/-) versus the Ent1(+/+) mice. In addition, although RTP was undetected in the urine, approximately 20 and 24% of the total radioactivity [in the Ent1(+/+) and Ent1(-/-) mice, respectively] could not be attributed to any of the known ribavirin metabolites.

Oral Ribavirin Pharmacokinetics. [³H]Ribavirin was administered orally to Ent1(+/+) and Ent1(-/-) mice at doses of 0.024, 0.244, and 6.1 mg/kg in ~125 µl, resulting in dosing solution concentrations of 20, 200, and 5000 µM, respectively. At all three doses, the plasma, erythrocyte and blood ribavirin concentrations observed in the Ent1(-/-) animals were lower than those in the Ent1(+/+) animals (Fig. 3). In addition, at all three doses, and in both Ent1(+/+) and Ent1(-/-) animals, ribavirin was rapidly absorbed, with plasma and erythrocyte T_{max} less than 65 min (Table 5). In addition, both the plasma and erythrocyte terminal half-life ($t_{1/2\beta}$) and T_{max} were not significantly different with increasing dose, nor were they different between Ent1(+/+) and Ent1(-/-) mice (Table 6).

The plasma, erythrocyte, and blood $t_{1/2\beta}$ at all oral doses in the Ent1(-/-) mice were not significantly different compared with the intravenous dose. In addition, compared with the intravenous dose, the plasma $t_{1/2\beta}$ at all oral doses in the Ent1(+/+) mice were not significantly different, whereas the erythrocyte $t_{1/2\beta}$ was significantly decreased at both 0.244 and 6.1 mg/kg doses and the blood $t_{1/2\beta}$ was significantly decreased at all doses.

In the Ent1(+/+) or Ent1(-/-) mice, the erythrocyte to plasma $AUC_{0-12\text{ h}}$ ratio did not significantly differ with dose (Fig. 3B). In the Ent1(+/+) mice, the ranges of these values after oral dosing (3.38–4.82-fold) were similar to those ob-

Fig. 2. Plasma, erythrocytes, and blood concentration-time profile after intravenous administration of ribavirin to Ent1(+/+) or Ent1(-/-) mice. Ribavirin plasma (A), erythrocytes (B), and blood (C) concentrations in Ent1(+/+) (●) and Ent1(-/-) (○) mice after intravenous [³H]ribavirin (3 mg/kg) administration. Insets represent data on a linear scale. Values represent mean ± S.D. from three independent experiments.

TABLE 1
Summary of ribavirin pharmacokinetic parameters after intravenous administration of ribavirin (3 mg/kg) to Ent1(+/+) or Ent1(-/-) mice
Values represent mean $\pm$ S.D., $n = 3$. Values in parentheses represent -fold Ent1(+/-).

Ent1	Plasma AUC _{0-12 h}	Erythrocyte AUC _{0-12 h}	Blood AUC _{0-12 h}	Erythrocyte:Plasma AUC Ratio	Plasma C ₀	Erythrocyte C _{max}	Plasma t _{1/2β}	Erythrocyte t _{1/2β}
		$\mu\text{g} \cdot \text{min}/\text{ml}$				$\mu\text{g}/\text{ml}$	h	
+/+	220.1 $\pm$ 39.9	919.4 $\pm$ 120.5	574.6 $\pm$ 43.0	4.35 $\pm$ 1.44	4.15 $\pm$ 1.62	3.13 $\pm$ 0.18	16.0 $\pm$ 12.2	19.1 $\pm$ 6.36
-/-	210.7 $\pm$ 97.2(1.04) ↓	301.4* $\pm$ 59.2 (3.05) ↓	243.2* $\pm$ 43.4(2.36) ↓	1.66* $\pm$ 0.74(2.63) ↓	4.97 $\pm$ 3.58(1.20) ↑	0.82* $\pm$ 0.29(3.78) ↓	4.20 $\pm$ 0.66(3.81) ↓	12.9 $\pm$ 8.67(1.48) ↓

* $P < 0.05$ compared with Ent1(+/-).

TABLE 2

Summary of pharmacokinetic parameters (based on plasma concentrations) after intravenous administration of ribavirin (3 mg/kg) to Ent1(+/+) or Ent1(-/-) mice

Values represent mean $\pm$ relative S.E. of parameter estimates.

	V ₁	K ₁₂	K ₂₁	K ₁₀
	ml/kg		min^{-1}	
Ent1 (+/+)	0.679 $\pm$ 0.054	0.109 $\pm$ 0.013	0.015 $\pm$ 0.003	0.014 $\pm$ 0.004
Ent1 (-/-)	0.974 $\pm$ 0.072	0.039 $\pm$ 0.008	0.012 $\pm$ 0.005	0.015 $\pm$ 0.004

served after intravenous dosing (4.35-fold). Likewise, in the Ent1(-/-) mice, the ranges of these values after oral dosing (1.44–2.13-fold) were also similar to those observed after intravenous dosing (1.66).

The erythrocyte and blood ribavirin AUC_{0-30 min} were significantly reduced in the Ent1(-/-) mice at all doses compared with the Ent1(+/+) mice (Table 5). In the plasma, the AUC_{0-30 min} was reduced in the Ent1(-/-) mice (compared with the Ent1(+/+) mice) at the lowest dose, whereas the intermediate and high doses were not significantly different (Table 5). In contrast, the plasma, erythrocyte, and blood ribavirin AUC_{0-12 h} were all significantly reduced in the Ent1(-/-) mice at all doses compared with the Ent1(+/+) mice (Table 6).

To better examine any nonlinearity in the oral pharmacokinetics of ribavirin, the dose-normalized C_{max} (C_{max}/D) was calculated for plasma, erythrocyte, and blood in Ent1(+/+) and Ent1(-/-) mice (Table 5). The erythrocyte and blood ribavirin C_{max}/D were significantly reduced in the Ent1(-/-) mice at all doses compared with the Ent1(+/+) mice (Table 5). In the plasma, the C_{max}/D was reduced in the Ent1(-/-) mice [compared with the Ent1(+/+) mice] at the lowest dose, whereas the intermediate and high doses were not significantly different (Table 5). In contrast to Ent1(-/-) mice, in the Ent1(+/+) mice, the plasma, erythrocyte and blood C_{max}/D was significantly reduced at the 0.244 and 6.1 mg/kg doses compared with the lowest (0.024 mg/kg) dose (Table 5).

The dose-normalized AUC (AUC/D) was calculated between both 0 and 30 min (absorption phase) and 0 and 12 h. In both the Ent1(+/+) and Ent1(-/-) mice, the plasma, erythrocyte, and blood dose-normalized AUC_{0-30 min} and AUC_{0-12 h} decreased with increasing dose (Figs. 4 and 5). In the Ent1(+/+) mice, the plasma, erythrocyte, and blood AUC_{0-30 min}/D and AUC_{0-12 h}/D were significantly reduced at the 0.244 and 6.1 mg/kg doses compared with the lowest (0.024 mg/kg) dose (Figs. 4 and 5). In the Ent1(-/-) mice, the plasma AUC_{0-12 h}/D was significantly reduced at the 0.244 and 6.1 mg/kg doses compared with the lowest (0.024 mg/kg) dose (Fig. 5A).

The oral bioavailability of ribavirin calculated from the blood AUC_{0-12 h} in Ent1(+/+) mice was 57.9, 33.0, and 34.1% for ribavirin doses of 0.024, 0.244, and 6.1 mg/kg, respectively. The oral bioavailability of ribavirin calculated from the blood AUC_{0-12 h} in Ent1(-/-) mice was 38.5, 24.8, and 18.2% for ribavirin doses of 0.024, 0.244, and 6.1 mg/kg, respectively. The oral bioavailability of ribavirin calculated from the plasma AUC_{0-12 h} in Ent1(+/+) mice was 96.5, 31.5, and 25.0% for ribavirin doses of 0.024, 0.244, and 6.1 mg/kg, respectively. The oral bioavailability of ribavirin calculated from the plasma AUC_{0-12 h} in Ent1(-/-) mice was 27.3, 18.4,

TABLE 3

Predicted and observed ratio of ribavirin phosphorylated metabolite to ribavirin plasma concentration in mouse Ent1(+/+) and Ent1(-/-) erythrocytes

		Steady-State or Single-Dose AUC _{0-t} Ratio ^a		
		Observed	Predicted CL _{phosp}	
			$\mu\text{L}/\text{min}/10^9\text{cells}$	
RBC ^c :plasma	Ent1 (+/+)	(4.35)	Ex vivo ^b	1.0
	Ent1 (-/-)	(1.66)	8.1 (10.0)	3.2 (4.1)
Ent1(+/-):Ent1(-/-)	Ent1 (+/+)	(2.62)	0.3 (0.5)	0.3 (0.6)
	Ent1 (-/-)	(2.62)	27.2 (19.8)	9.7 (7.3)

^a Values represent steady-state erythrocyte phosphorylated metabolite (RBC) to plasma ribavirin AUC_{0-t} ratio or Ent1(+/+) to Ent1(-/-) ratio. Values in parentheses represent the same ratios after a single dose.

^b Values were 2.89 and 6.06 $\mu\text{L}/10^9\text{cells}/\text{min}$ for erythrocytes from Ent1(+/+) and Ent1(-/-) mice, respectively.

^c RBC represents both erythrocyte ribavirin and erythrocyte-phosphorylated metabolite concentrations.

and 17.4% for ribavirin doses of 0.024, 0.244, and 6.1 mg/kg, respectively. We also attempted to characterize the saturation of ribavirin absorption by compartmental modeling, but were unable to adequately determine K_a given the rapid absorption of ribavirin and our blood sampling scheme.

Discussion

After intravenous administration, the plasma concentration time-profile of ribavirin in both Ent1(+/+) and Ent1(-/-) mice was biphasic, with a relatively rapid α -phase and a relatively long β -phase. This was similar to the observations in humans, in which ribavirin exhibits triexponential behavior (Laskin et al., 1987; Paroni et al., 1989; Preston et al., 1999). The rapid α -distribution phase is attributed to the distribution of ribavirin in the tissues such as the skeletal muscle and erythrocytes, and the long terminal half-life is attributed to the slow redistribution of ribavirin (after dephosphorylation) of these peripheral compartment(s). There was no significant difference in the plasma concentration-time profile between the Ent1(+/+) and the Ent1(-/-) mice. This suggests that although Ent1 plays a substantial role in the distribution of ribavirin into the erythrocytes, the total mass distributing there is not enough to significantly modulate the plasma exposure of ribavirin. In humans, ribavirin is administered orally at doses between 800 and 1200 mg/day (~6–7 mg/kg b.i.d.) (FDA, 2007a,b), and after a single 1200-mg oral dose, ribavirin has a C_{max} of $9.9 \pm 0.9 \mu\text{M}$ (~2.5 $\mu\text{g}/\text{mL}$) (Laskin et al., 1987). For mice, we chose an intravenous dose of 3 mg/kg, which resulted in a plasma C_0 of ~4 to 5 $\mu\text{g}/\text{mL}$, similar to the plasma concentrations observed in humans.

As noted under *Materials and Methods*, all reference to erythrocyte ribavirin concentration should be read as the concentration of intracellular ribavirin plus its phosphorylated metabolites. There was a significant decrease in the ribavirin erythrocyte to plasma AUC ratio and C_{max} in the Ent1(-/-) mice after both intravenous and oral dosing. For both Ent1(+/+) and Ent1(-/-) mice, the ratio was similar between intravenous and oral doses. Surprisingly, the magnitude of the ratio in vivo (3.1- and 3.8-fold for intravenous AUC ratio and C_{max} , respectively) was relatively small compared with the corresponding ratio observed after 10 s of uptake ex vivo (27-fold). This is because Ent1 is an equilibrative transporter and therefore increases the rate of entry of ribavirin into the cells, but not its extent. Ex vivo and in vivo, Ent1 mediated distribution of ribavirin into the erythrocyte occurs very rapidly (within 60 s), and it is then trapped there

intracellular phosphorylation (Endres et al., 2009). In our previously published ex vivo study (Endres et al., 2009), we predicted that, after a single intravenous dose, the difference in the erythrocyte to plasma AUC ratio between Ent1(+/+) and Ent1(-/-) mice would be approximately 27-fold. In contrast, as reported here, we observed a difference of only approximately 3-fold (Table 3). This overprediction from ex vivo to in vivo may be due to several factors. First, we may have overestimated the intracellular phosphorylation activity of ribavirin. We previously modeled the intracellular phosphorylation of ribavirin as a “linear” first-order process. This process is probably not first-order in vivo, because the enzymes responsible for this pathway (adenosine kinase, and other nucleotide kinases) may have competition from the natural nucleosides and nucleotides. Thus, the activity in vivo is probably lower than that which we estimated ex vivo. Second, it is probable that, in vivo, there is intracellular depletion of ATP and therefore reduction in enzyme activity, as the ribavirin nucleotides accumulate in the cells. Finally, underestimation of the actual in vivo K_{deg} or overestimation of the actual in vivo CL_{diff} could also have led to the overprediction based on ex vivo data.

Because of the above-mentioned ex vivo in vivo discrepancy, we refined the model of ribavirin distribution into the erythrocytes after intravenous administration to better predict the magnitude of ribavirin accumulation in the erythrocyte. First, we used the observed ribavirin plasma concentration-time profile to drive the distribution of ribavirin into the erythrocytes. Second, we used the observed half-life of ribavirin in the erythrocytes to drive the elimination of the erythrocyte phosphorylated metabolites (K_{deg}). Finally, we simulated the accumulation ratio [and the difference in this ratio between Ent1(+/+) and Ent1(-/-) mice] after both a single dose, and at steady-state, at various values of the phosphorylated metabolic clearance (CL_{phosp}). Our simulations suggest that we may have overpredicted the magnitude of CL_{phosp} in the ex vivo studies, because values of CL_{phosp} of 1.0 and 0.5 $\mu\text{L}/10^9\text{cells}/\text{min}$ [in the Ent1(+/+) and Ent1(-/-) mice, respectively] better predicted the ribavirin erythrocyte to plasma concentration ratio. Despite these changes, the predicted accumulation ratios in Ent1(-/-) erythrocytes were different from those observed in vivo. For example, the single-dose accumulation ratio in erythrocytes from the Ent1(-/-) mice were underpredicted for all values of CL_{phosp} . In these erythrocytes, the predicted accumulation was ~0.5- to 0.6-fold after a single dose, whereas the observed accumulation was ~1.66-fold. This discrepancy may

TABLE 4
Urinary excretion (0–48 h) after intravenous administration of ribavirin to Ent1(+/+) and Ent1(–/–) mice
Values represent mean ± S.D., n = 3. Values in parentheses represent -fold Ent1(+/+).

Ent1	F _e (0–48 h)					Urine Composition				
	Total-μ Ci	RBV	RBV	RBV	TCOOH	RTCOOH	TCONH ₂	RMP	RTP	UNK ^a

+/+	42.2 ± 6.0	24.4 ± 4.0	58.0 ± 5.0	1.2 ± 0.5	1.7 ± 0.5	0.5 ± 0.1	18.2 ± 2.5	N.D.	N.D.	20.4 ± 4.5
–/–	44.6 ± 10.9(1.06 ↑)	18.8 ± 9.9(1.30 ↓)	40.2 ± 12.1(1.44 ↓)	1.3 ± 0.4(1.07 ↑)	2.2 ± 0.9(1.27 ↑)	0.7 ± 0.2(1.57 ↑)	30.5* ± 5.3(1.68 ↑)	N.D.	N.D.	25.1 ± 9.1(1.23 ↑)

N.D., not detected; RBV, ribavirin.

*P < 0.05 compared with Ent1(+/+).

^a Unknown metabolite.

Fig. 3. Plasma, erythrocytes, and blood plasma concentration-time profile after oral administration of ribavirin to Ent1(+/+) or Ent1(–/–) mice. Ribavirin plasma (A), erythrocytes (B), and blood (C) concentrations in Ent1(+/+) (filled symbols) and Ent1(–/–) (open symbols) mice after [³H]ribavirin [0.024 (circles), 0.244 (squares), and 6.1 mg/kg (triangles)] was administered orally. Values represent mean ± S.D. from three independent experiments.

be due to differences in CL_{phosp} between the Ent1(–/–) and Ent1(+/+) animals. This is not unreasonable, given the possibility of differences in the intracellular nucleoside pools between Ent1(+/+) and Ent1(–/–) animals and highlights

TABLE 5
Summary of ribavirin pharmacokinetic parameters (0–30 min) after oral administration to Ent1(+/+) or Ent1(−/−) mice
Values represent mean ± S.D., $n = 3$. Values in parentheses represent -fold Ent1(+/+) at equivalent dose.

Ent1	Dose mg/kg	Plasma AUC _{0-30 min}	Erythrocyte AUC _{0-30 min} μg min/ml	Blood AUC _{0-30 min}	Plasma C _{max} /D	Erythrocyte C _{max} /D μg/ml/mg/kg	Blood C _{max} /D	Plasma T _{max}	Erythrocyte T _{max}
<i>min</i>									
+/+	0.024	0.150 ± 0.035	0.281 ± 0.029	0.220 ± 0.029	0.252 ± 0.047	0.494 ± 0.072	0.372 ± 0.033	11.7 ± 5.8	65.0 ± 52.7
	0.244	0.800 ± 0.360	1.49 ± 0.611	1.2 ± 0.50	0.107 [†] ± 0.016	0.249 [†] ± 0.052	0.174 [†] ± 0.028	11.7 ± 5.8	60.0 ± 0.0
	6.1	9.39 ± 1.67	20.8 ± 2.1	16.0 ± 1.6	0.075 [†] ± 0.018	0.181 [†] ± 0.029	0.134 [†] ± 0.015	30.0 ± 26.0	50.0 ± 17.3
−/−	0.024	0.055* ± 0.026(2.72 ↓)	0.061* ± 0.015(4.63 ↓)	0.059* ± 0.015(3.71 ↓)	0.096* ± 0.041	0.128* ± 0.046(3.88 ↓)	0.105* ± 0.031(3.53 ↓)	26.7 ± 29.3	50.0 ± 17.3
	0.244	0.499 ± 0.139(1.60 ↓)	0.307* ± 0.061(4.87 ↓)	0.386* ± 0.093(3.13 ↓)	0.093 ± 0.017	0.068* ± 0.003(3.66 ↓)	0.071* ± 0.013(2.45 ↓)	15.0 ± 0.0	60.0 ± 0.0
	6.1	11.7 ± 2.6 (1.25 ↑)	5.44* ± 1.75 (3.82 ↓)	8.27* ± 1.9 (1.93 ↓)	0.102 ± 0.023 (1.35)	0.054* ± 0.006(3.36 ↓)	0.068* ± 0.013(1.96 ↓)	20.0 ± 8.7	50.0 ± 17.3

* $P < 0.05$ compared with Ent1(+/+) at equivalent dose determined by Student's t test (AUC and C_{max}/D) or Mann-Whitney U test (T_{max}).

[†] $P < 0.05$ compared with lowest dose at equivalent genotype.

TABLE 6
Summary of ribavirin pharmacokinetic parameters (0–12 h) after oral administration of the drug to Ent1(+/+) or Ent1(−/−) mice
Values represent mean ± S.D., $n = 3$. Values in parentheses represent -fold Ent1(+/+) at equivalent dose.

Ent1	Dose mg/kg	Plasma AUC _{0-12 h}	Erythrocyte AUC _{0-12 h} mg min/ml	Blood AUC _{0-12 h}	Erythrocyte:Plasma AUC Ratio	Plasma t _{1/2β}	Erythrocyte t _{1/2β}
<i>h</i>							
+/+	0.024	1.70 ± 0.29	5.78 ± 1.22	2.66 ± 0.75	3.38 ± 0.13	6.71 ± 1.63	7.92 ± 3.47
	0.244	5.64 ± 1.18	24.1 ± 2.15	15.4 ± 0.88	4.82 ± 0.47	4.96 ± 1.03	6.25 ± 2.67
	6.1	111.9 ± 16.4	398.6 ± 53.7	269.9 ± 39.8	3.59 ± 0.45	6.20 ± 2.65	5.95 ± 1.18
−/−	0.024	0.46* ± 0.07(3.70 ↓)	0.99* ± 0.49(5.86 ↓)	0.75* ± 0.29(3.52 ↓)	2.13 ± 1.00(1.58 ↓)	5.37 ± 1.00(1.25 ↓)	6.48 ± 0.86(1.22 ↓)
	0.244	3.16* ± 0.18(1.78 ↓)	6.24* ± 0.09(3.87 ↓)	4.9* ± 0.05(3.13 ↓)	1.96* ± 0.10(2.46 ↓)	4.56 ± 0.04(1.09 ↓)	5.12 ± 0.37(1.22 ↓)
	6.1	74.5* ± 5.9 (1.50 ↓)	106.6* ± 4.4 (3.74 ↓)	90.1* ± 5.9 (3.00 ↓)	1.44* ± 0.06(2.50 ↓)	5.11 ± 1.52(1.21 ↓)	8.15 ± 5.69(1.37 ↑)

* $P < 0.05$ compared with Ent1(+/+) at equivalent dose.

Fig. 4. Summary of the dose-normalized oral plasma, erythrocytes, and blood $AUC_{0-30 \text{ min}}$ after oral administration of ribavirin to Ent1(+/+) or Ent1(-/-) mice. Dose-normalized ribavirin plasma (A), erythrocytes (B), and blood (C) $AUC_{0-30 \text{ min}}$ in Ent1(+/+) (filled bars) and Ent1(-/-) (open bars) mice after oral [^3H]ribavirin administration (0.024, 0.244, and 6.1 mg/kg). Values represent mean $\pm$ S.D. from three independent experiments. *, $P < 0.05$ compared with the value of the equivalent genotype at the 0.024 mg/kg dose. †, $P < 0.05$ compared with Ent1(+/+) mice.

Fig. 5. Summary of the dose-normalized oral plasma, erythrocytes, and blood $AUC_{0-12 \text{ h}}$ after oral administration of ribavirin to Ent1(+/+) or Ent1(-/-) mice. Dose-normalized ribavirin in plasma (A), erythrocytes (B), and blood (C) $AUC_{0-12 \text{ h}}$ in Ent1(+/+) (filled bars) and Ent1(-/-) (open bars) mice after oral [^3H]ribavirin administration (0.024, 0.244, and 6.1 mg/kg). Values represent mean $\pm$ S.D. from three independent experiments. *, $P < 0.05$ compared with the value of the equivalent genotype at the 0.024 mg/kg dose. †, $P < 0.05$ compared with Ent1(+/+) mice.

the importance of the magnitude of CL_{phosp} in determining the accumulation ratio in vivo.

In humans, renal excretion is a major elimination mechanism of ribavirin (Laskin et al., 1987; Paroni et al., 1989). The systemic and renal clearance of ribavirin is 280 to 400 and 99 ml/min, respectively (Laskin et al., 1987; Paroni et al., 1989), so renal elimination is approximately one third of the total systemic clearance. Although, in humans, the net renal clearance of ribavirin is approximately equal to that of the glomerular-filtration rate, it is possible that ribavirin undergoes both secretion and reabsorption, possibly mediated by the expression of ENTs and CNTs on the basolateral and brush-border membranes of the renal epithelial cells (Lai et al., 2002; Govindarajan et al., 2007). Thus, we investigated whether there were any differences in the renal excretion of ribavirin between the Ent1(+/+) and Ent1(-/-) mice. We did not observe any difference in the fraction of ribavirin or the fraction of total radioactivity excreted in the urine between the Ent1(+/+) and Ent1(-/-) mice up to 48 h after intravenous dosing. Given our observations of no difference in the $AUC_{0-12 \text{ h}}$ and $t_{1/2\beta}$ between the Ent1(+/+) and Ent1(-/-) mice, this suggests that Ent1 does not significantly contribute to the renal clearance of ribavirin. In addition, the fraction of ribavirin eliminated unchanged in the urine and the metabolite composition (i.e., primary urinary metabolite TCONH_2) was similar to that observed in humans (Austin et al., 1983; Paroni et al., 1989), monkeys, and rats (Lin et al., 2003).

In addition to ENT1 expressed on the basolateral membrane of the enterocytes, CNT2 and CNT3 are expressed on the brush-border membrane and have been suggested to play

role in the absorption of ribavirin (Chandrasena et al., 1997). For example, using brush-border membrane vesicles of the human intestine, we have shown that CNT2 transports ribavirin into the enterocytes (Patil et al., 1998). We administered ribavirin orally at three doses, 0.024, 0.244, and 6.1 mg/kg, as solutions of 20 μM , 200 μM , and 5 mM, respectively. These concentrations were chosen to investigate the role of both the CNTs and ENTs on the intestinal absorption of ribavirin. In humans, the K_m of CNT2 for ribavirin is approximately 18 μM (Yamamoto et al., 2007), whereas the K_m of CNT3 is ~ 14 to 61 μM (Hu et al., 2006; Yamamoto et al., 2007). We previously found that the K_m of mouse Ent1 for ribavirin is 382 μM (Endres et al., 2009). By administering ribavirin orally in solutions at concentrations approximately equal to (20 μM) and substantially greater than the K_m of CNT2/3 (200 μM) or K_m of Ent1 (5 mM) in both Ent1(+/+) and Ent1(-/-) mice, we were able to examine the role of both the CNTs and Ent1 in the absorption of ribavirin. In addition, the volume of the dosing solution was relatively large and constant across the doses (125 μL), and we expected minimal dilution of this solution in the gastrointestinal tract of the mouse. Compared with the Ent1(+/+) mice, the oral $AUC_{0-12 \text{ h}}$ was significantly decreased in the Ent1(-/-) animals at all three doses in both plasma (from ~ 2 - to 4-fold) and blood (from ~ 3 - to 5-fold). Generally, in both the Ent1(+/+) and Ent1(-/-) mice, the dose-normalized ribavirin AUC (both $AUC_{0-30 \text{ min}}$ and $AUC_{0-12 \text{ h}}$) and C_{max}/D decreased upon increasing ribavirin dose, suggesting saturation of absorption. In addition, this decrease in dose-normalized AUC was greatest between the lowest ($\sim 20 \mu\text{M}$ luminal concentration) and intermediate ($\sim 200 \mu\text{M}$ luminal concen-

tration) dose, whereas the decrease in dose-normalized AUC was relatively minor between the intermediate and highest (~5 mM luminal concentration) doses. This suggests that saturation of absorption between 20 and 200 μ M, which is consistent with saturation of intestinal Cnt2 and/or Cnt3. It is interesting that this dose dependence in the Ent1(-/-) mice was much less pronounced and in most cases not statistically significant. This observation suggests that the absence of Ent1 is extremely important in the absorption of ribavirin. This is further supported by the substantial decrease in the AUC₀₋₃₀, AUC_{0-12 h}, and C_{max} between the Ent1(+/+) and Ent1(-/-). Collectively, these data suggest that, although ribavirin is absorbed into the enterocyte by Cnt2 and/or Cnt3, in the absence of Ent1, it is unable to egress out of the intestinal tissue. It is interesting that, in the presence of Ent1, the saturation of ribavirin absorption by the CNTs with increasing ribavirin dose resulted in a 3.8-fold decrease in the plasma AUC, which was similar to the magnitude of the decrease (3.7-fold) observed in the absence of Ent1 at the lowest dose. These data suggest that both the ENTs and the CNTs are equally important in the oral absorption of ribavirin. However, these data also highlight the importance of the rate-limiting step in the mediated absorption of a drug. Despite the fact that CNTs are important in the influx of ribavirin into the intestine, the level of expression of ENT1 in the intestine may be the rate-limiting step in the overall bioavailability of the drug. Based on these data, we predict that during the absorption period, ribavirin will significantly accumulate in the intestinal tissues in the Ent1(-/-) mice. Preliminary validation of this hypothesis has been achieved by conducting in situ intestinal perfusion studies in both Ent1(+/+) and Ent1(-/-) mice in the absence and presence of sodium (Moss et al., 2007).

These data suggest that Ent1 and the CNTs play an important role in the oral absorption and erythrocyte distribution of ribavirin in vivo. Specifically, in the Ent1(-/-) mice, the erythrocyte ribavirin exposure was decreased by approximately 3-fold after intravenous dosing, and the oral exposure was decreased approximately 3- to 4-fold. In addition, mice replicate the saturation of absorption of ribavirin that was observed in humans, and this was a result of both Ent1 and most likely Cnt2 and/or Cnt3 playing a critical role in the absorption of ribavirin. Additional experiments (e.g., intestinal perfusion studies) are needed to elucidate the specific role of the CNTs (either Cnt2 or Cnt3) in the oral absorption of ribavirin. For example, it would be important to determine whether hepatic extraction of ribavirin is mediated by both ENTs and CNTs and whether such extraction is saturated at clinically relevant doses.

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