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 is the treatment of choice for hepatitis C virus infection. Ribavirin is a substrate of several nucleoside transporters, including the equilibrative nucleoside transporter 1 (Ent1) 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 AUC₀⁻¹₂hr 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 AUC₀⁻¹₂hr between Ent1(+/+) and Ent1(-/-) mice. After 48 hours, 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 AUC₀⁻¹₂hr of ribavirin was 69.7±12.0, 20.7±1.5 and 18.3±2.7 min/L respectively in the Ent1(+/+) mice and 18.9±2.8, 13.0±0.5 and 12.2±1.0 min/L respectively in the Ent1(-/-) mice. Interestingly, at the highest dose, the dose-normalized plasma AUC₀⁻¹₂hr, AUC₀⁻¹₂hr and Cmax 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 AUC₀⁻¹₂hr was 3.7 and 1.5-fold lower at the lowest and the highest dose respectively in the Ent1(-/-) mice compared to those of the Ent1(+/+) mice. Our findings indicate that Ent1 plays a significant role in the oral absorption and erythrocyte distribution of ribavirin.
**Introduction**

Ribavirin (1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide), with PEGylated interferon-α, is currently the standard of treatment for chronic hepatitis C virus (HCV) infection. Worldwide, about 200 million people (of which 3.2 million are in the USA) are chronically infected with the HCV (Armstrong et al., 2006)(Sherlock, 1995). Even at its usual recommended dose, treatment with ribavirin is limited by its major toxicity, hemolytic anemia (Fried, 2002). In 10-13% 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 pro-drug 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 ~-2.0 to -2.5), it needs to be transported into cells to produce its efficacy. This transport is mediated by the nucleoside transporters. 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), and thus this metabolic activation is in part dependent on the transport of ribavirin into
the cell. Additionally, these metabolites are polar, and once formed inside the cell, are unable to diffuse out (Canonico et al., 1984).

Ribavirin is transported into human erythrocytes by the human ENT1 (hENT1; (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, since ENT1 is an equilibrative transporter, it is not clear if 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 bid. The oral bioavailability of ribavirin is variable, 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*<sub>max</sub>) of ribavirin does not increase proportionally with the ribavirin oral dose (600 to 1200 or 2400 mg). Additionally, two independent pre-clinical 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*<sub>max</sub> increases less than proportionally with dose (FDA, 2007b; FDA, 2007a). Using brush-border membrane vesicles isolated from the human intestine, we have demonstrated that the saturation of ribavirin absorption is most likely 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* purfused 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 \textit{in vitro} uptake and metabolism of ribavirin into erythrocytes in Ent1(+/+) and Ent1(-/-) mice to predict the contribution of Ent1 to the \textit{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 studies presented here, we have tested this prediction as well as the hypothesis that nucleoside transporters are important in the intestinal absorption of oral ribavirin.

\section*{Methods}

\textit{Mouse Husbandry}

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

\textit{Pharmacokinetic Study}

$[^3]$H-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 \mu L) 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 minutes) 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 minute at 5000 × g. The hematocrit was measured, and the hematocrit tube scored at the buffy-coat/plasma interface. The plasma or packed erythrocytes (5-10 μL, leaving behind the interface) were transferred to pre-weighed microhematocrit tubes containing 100 μL deionized water (dH₂O). 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 μCi/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 hours, the animals were sacrificed. The contents of the bladder collected using a syringe with a 26g needle and pooled with the urine collected from the metabolic cages. The cages were washed with dH₂O, and the cage-wash 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 μL of each diluted plasma, erythrocyte or urine sample was added to 100 μL of dH₂O in 7 mL scintillation vials. The samples were decolorized with 300 μL of 30% H₂O₂, agitated for 20 minutes on a plate shaker, and 5 mL of scintillation fluid added to each sample.[^3H]-radioactivity (µCi/mL) concentrations were determined using liquid scintillation counting.
Dephosphorylation of Phosphorylated Metabolites

The phosphorylated nucleotides \([\text{3H}]\)-RMP and \([\text{3H}]\)-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 minutes or ~3 hours after intravenous dosing, which was consistent with previous observations in humans (Homma et al., 2004). Because of this, the erythrocyte samples were incubated for 30 minutes at 37 °C with 10 U 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 (PCA) and vortexed 1 minute. The samples were neutralized by the addition of 20 μL 2 M K₂HPO₄, and then centrifuged at 20,000 × g for 10 minutes at 4 °C. Twenty μL of the supernatant was added to a 7 mL scintillation vial containing 100 μL dH₂O, and counted using scintillation counting to determine sample work-up recovery. One-hundred twenty μL of the supernatant was analyzed by HPLC using a method that resolved ribavirin from RTCOOH, TCONH₂, TCOOH (kindly provided by Valeant Pharmaceuticals International) and RMP and RTP (Moravek) metabolites. The radioactivity co-eluting 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 percent 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 non-compartmental analysis using linear interpolation between concentration-time data points using WinNonlin®. In the plasma and blood samples, $C_0$ was determined by linear extrapolation of the first two plasma concentration time-points.

Additionally, the intravenous plasma concentration-time profiles were analyzed using compartmental analysis using a standard 2-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, $C_{\text{max}}$) and the Mann-Whitney U-Test for discrete parameters (e.g. $T_{\text{max}}$).

**Simulation of In Vivo Transport and Metabolism of Ribavirin after Intravenous Dosing**

In vivo ribavirin transport and metabolism was simulated using the following equations

\[
\frac{dC_{\text{RBV}}^{\text{in}}}{dt} = C_{\text{RBV}}^{\text{ex}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_2} - C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}} + CL_{\text{phosp}})}{V_2}
\]

Equation 1

\[
\frac{dC_{\text{phosp}}^{\text{in}}}{dt} = C_{\text{RBV}}^{\text{in}} \cdot \frac{CL_{\text{phosp}}}{V_2} - C_{\text{phosp}}^{\text{in}} \cdot K_{\text{deg}}
\]

Equation 2

where $C_{\text{RBV}}^{\text{in}}$ is the intracellular erythrocyte concentration of ribavirin, $CL_{\text{dif}}$ and $CL_{\text{ent}}$ 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, $C_{\text{phosp}}^{\text{in}}$ is the intracellular phosphorylated metabolite concentration (RMP, RDP and RTP).
pooled), $\text{CL}_{\text{phosph}}$ is the phosphorylated metabolite formation clearance and $K_{\text{deg}}$ 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 $V_2$ (Figure 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 ($C_{\text{RBV}}^p$) in this model. The values of $\text{CL}_{\text{dif}}$ (0.204 $\mu$L/10$^9$ cells/min), $\text{CL}_{\text{ent}}$ (167.1 $\mu$L/10$^9$ cells/min), $\text{CL}_{\text{phosph}}$ (2.89 and 6.06 $\mu$L/10$^9$ cells/min for Ent1(+/+) and Ent1(-/-) mice), and $V_2$ (43.0 $\mu$L/10$^9$ cells) were fixed using parameters previously determined (Endres et al., 2009). Additionally, for simplicity, the non-phosphorylated pathway of erythrocyte metabolism was eliminated from this model, as it played a relatively minor role in the total intracellular metabolism of ribavirin in the erythrocytes. $K_{\text{deg}}$ 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 simulations were performed using values of 1.0 and 0.5 $\mu$L/10$^9$cells/min for $\text{CL}_{\text{phosph}}$. The data obtained from Ent1(+/+) and Ent1(-/-) animals were simultaneously modeled using two sets of differential equations, one with and one without the $\text{CL}_{\text{ent}}$ 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 hours.
Results

Assay Validation, Sample Work-Up Recovery and Parent Compound Composition

Ribavirin was stable in PBS at 37°C for up to 24 hours (data not shown). Additionally, 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 ±1.7 % respectively (n=3).

Ribavirin was separated from its metabolites TCOOH, RTCOOH, TCONH₂, RMP and RTP using ion-paired reversed-phase HPLC assay previously described (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 minutes and no detectable RTP after 30 minutes 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 (Figure 2A). The profile was bi-exponential through 12 hours, with a rapid distributional (α) phase and a longer terminal (β) phase. There was no significant difference in the plasma ribavirin AUC₀–₁₂ hr, C₀ or terminal half-life (t₁/₂β) between the Ent1(+/+) and the Ent1(-/-) animals (Figure 2A and Table 1).
The ribavirin (and phosphorylated metabolite) erythrocyte concentrations in the Ent1(-/-) animals were significantly lower than those in the Ent1(+/+) animals (Figure 2B). The ribavirin erythrocyte AUC$_{0-12\ hr}$ 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). Additionally, the erythrocyte ribavirin C$_{\text{max}}$ 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$_{0-12\ hr}$ was decreased 2.36-fold in the Ent1(-/-) versus the Ent1(+/+) mice (Figure 2C).

**Modeling and Simulation of Accumulation of Erythrocyte Ribavirin and Phosphorylated Metabolites**

We expanded the *ex vivo* model we previously developed (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 ribavirin as described in Equation 1 and 2. Additionally, $K_{\text{deg}}$ 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 $CL_{\text{phosp}}$ (Table 3). Additionally, while 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 μL/10^9 cells/min, the predicted accumulation in Ent1(-/-) under 
predicted the observed by approximately one-third at all values of CL_phosp (Table 3).

**Ribavirin Urinary Excretion**

The 0 to 48 hours urinary excretion of ribavirin and its metabolites after intravenous 
administration was examined in Ent1(+/+) and Ent1(-/-) mice. After 48 hours, 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). 
Additionally, there was no significant difference in the composition of the urine (i.e. percent 
composition of each analyte) with respect to ribavirin, TCOOH, RTCOOH and RMP. The 
composition (percent) of TCONH2 in the urine was significantly increased 1.68-fold in the 
Ent1(-/-) versus the Ent1(+/+) mice. Additionally, while 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 (Figure 3). Also, at all three doses, and in both Ent1(+/+) and Ent(-/-) animals, ribavirin was rapidly absorbed, with plasma and erythrocyte $T_{\text{max}}$ less than 65 minutes (Table 5). Additionally, both the plasma and erythrocyte terminal half-life ($t_{1/2\beta}$) and $T_{\text{max}}$ 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 when compared to the intravenous dose. Additionally, when compared to 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 $AUC_{0-12\text{ hr}}$ ratio did not significantly differ with dose (Figure 3B). In the Ent1(+/+) mice, the range of these values after oral dosing (3.38 to 4.82-fold) were similar to those observed after intravenous dosing (4.35-fold).

Similarly, in the Ent1(-/-) mice, the range of these values after oral dosing (1.44 to 2.13-fold) were also similar to those observed after intravenous dosing (1.66).

The erythrocyte and blood ribavirin $AUC_{0-30\text{ min}}$ were significantly reduced in the Ent1(-/-) mice at all doses when compared to the Ent1(+/+) mice (Table 5). In the plasma, the $AUC_{0-30\text{ min}}$ was
reduced in the Ent1(-/-) mice (when compared to the Ent1(+/-) mice) at the lowest dose, whereas the intermediate and high doses were not significantly different (Table 5). On the other hand, the plasma, erythrocyte and blood ribavirin AUC\textsubscript{0-12 hr} were all significantly reduced in the Ent1(-/-) mice at all doses when compared to the Ent1(+/-) mice (Table 6).

To better examine any non-linearity in the oral pharmacokinetics of ribavirin, the dose-normalized C\textsubscript{max} (C\textsubscript{max}/D) was calculated for plasma, erythrocyte and blood in Ent1(+/-) and Ent1(-/-) mice (Table 5). The erythrocyte and blood ribavirin C\textsubscript{max}/D were significantly reduced in the Ent1(-/-) mice at all doses when compared to the Ent1(+/-) mice (Table 5). In the plasma, the C\textsubscript{max}/D was reduced in the Ent1(-/-) mice (when compared to 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\textsubscript{max}/D was significantly reduced at the 0.244 and 6.1 mg/kg doses when compared to the lowest (0.024 mg/kg) dose (Table 5).

The dose-normalized AUC (AUC/D) was calculated between both 0 and 30 minutes (absorption phase) and 0 and 12 hours. In both the Ent1(+/-) and Ent1(-/-) mice the plasma, erythrocyte and blood dose-normalized AUC\textsubscript{0-30 min} and AUC\textsubscript{0-12 hr} decreased with increasing dose (Figures 4 and 5). In the Ent1(+/-) mice, the plasma, erythrocyte and blood AUC\textsubscript{0-30 min}/D and AUC\textsubscript{0-12 hr}/D was significantly reduced at the 0.244 and 6.1 mg/kg doses when compared to the lowest (0.024 mg/kg) dose (Figures 4 and 5). In the Ent1(-/-) mice, the plasma AUC\textsubscript{0-12 hr}/D was significantly reduced at the 0.244 and 6.1 mg/kg doses when compared to the lowest (0.024 mg/kg) dose (Figure 5A).
The oral bioavailability of ribavirin calculated from the blood AUC_{0-12\ hr} 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\ hr} 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\ hr} 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\ hr} in Ent1(-/-) mice was 27.3, 18.4 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 ribvarin and our blood sampling scheme.
Discussion

After intravenous administration, the plasma concentration time-profile of ribavirin in both Ent1(+/+) and Ent1(-/-) mice was bi-phasic, with a relatively rapid $\alpha$-phase and a relatively long $\beta$-phase. This was similar to the observations in humans, where ribavirin exhibits tri-exponential behavior (Laskin et al., 1987; Paroni et al., 1989; Preston et al., 1999). The rapid $\alpha$-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) out 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 while 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/bid) (FDA, 2007b; FDA, 2007a), and after a single 1200 mg oral dose, ribavirin has a $C_{\text{max}}$ of 9.9 ± 0.9 μM (~2.5 μg/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 μg/ml, similar to the plasma concentrations observed in humans.

As noted in the methods section, 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 $C_{\text{max}}$ 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\textsubscript{max} respectively) was relatively small when compared to the corresponding ratio observed after 10 seconds 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 seconds) 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 a number of 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 most likely not-first order in vivo, as the enzymes responsible for this pathway (adenosine kinase, and other nucleotide kinases) may have competition from the natural nucleosides and nucleotides. Because of this, the activity in vivo is most likely 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\textsubscript{deg} or overestimation of the actual in vivo CL\textsubscript{diff} could also have led to the overprediction based on ex-vivo data.

Because of the above 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 over-predicted the magnitude of CL_{phosp} in the ex vivo studies, as values of CL_{phosp} of 1.0 and 0.5 μL/10^9 cells/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 under-predicted 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 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 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 are 280-400 mL/min 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. While, in humans, the net renal clearance of ribavirin is approximately equal to that of the glomerular-filtration rate (GFR), 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). Because of this, 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 hours after intravenous dosing. Given our observations of no difference in the AUC$_{0-12}$ hr, 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. Additionally, 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 solution of 20 \(\mu\)M, 200 \(\mu\)M and 5 mM respectively. These concentrations were chosen to investigate the role of both the CNTs and ENTs on the intestinal absorption or ribavirin. In humans, the \(K_m\) of CNT2 for ribavirin is approximately 18 \(\mu\)M (Yamamoto et al., 2007), whereas the \(K_m\) of CNT3 is ~14 to 61 \(\mu\)M (Yamamoto et al., 2007) (Hu et al., 2006). We previously found that the \(K_m\) of mouse Ent1 for ribavirin is 382 \(\mu\)M (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. Additionally, 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. When compared with the Ent1(+/-) mice, the oral AUC$_{0-12hr}$ was significantly decreased in the Ent1(-/-) animals at all three doses in both plasma (~2 to 4-fold) and blood (~3 to 5 fold). Generally, in both the Ent1(+/-) and Ent1(-/-) mice, the dose-normalized ribavirin AUC (both AUC$_{0-30 min}$ and AUC$_{0-12 hr}$) and $C_{\text{max}}/D$ decreased upon increasing ribavirin dose, suggesting saturation of absorption. Additionally, this decrease in dose-normalized AUC was greatest between the lowest (~20 μM luminal concentration) and intermediate (~200 μM luminal concentration) 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 μM, which is consistent with saturation of intestinal Cnt2 and/or Cnt3. Interestingly, this dose dependency 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 AUC$_{0-30}$, AUC$_{0-12hr}$ and $C_{\text{max}}$ 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. Interestingly, 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 EN\n
ts 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 \textit{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 \textit{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. Additionally, 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 if hepatic extraction of ribavirin is mediated by both EN\nts and CNTs and if such extraction is saturated at clinically relevant doses.
Acknowledgements

We would like to thank Drs. Li-Tian Yeh and Chin-Chung Lin of Valeant Pharmaceuticals International for the ribavirin metabolites (RTCOOH, TCONH₂ and TCOOH).
References


FDA (2007a) Copegus® (Ribavirin, USP) Tablets, in *FDA Revised Label - NDA 21-511/S-014*.


Footnotes
a) This work was supported by National Institute of Health [Grants GM54447 (to J.D.U.), and AA015164 (to D.-S. C)], and in part by the Eli Lilly Foundation (to C.J.E.).

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Legends for Figures

Figure 1. Model of Ribavirin Transport and Metabolism

Compartmental model describing the disposition of ribavirin and metabolites (phosphorylated and non-phosphorylated) 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_{phosph}) and non-phosphorylation (CL_{non-phosph}) pathways. Extracellular ribavirin is eliminated by systemic elimination (K_{sys}), whereas intracellular phosphorylated metabolite is eliminated by degradation (K_{deg}).

Figure 2. Plasma, Erythrocyte 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 [^{3}H]-ribavirin (3 mg/kg) administration. Insets represent data on a linear scale. Values represent mean ± S.D. from 3 independent experiments.

Figure 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 [^{3}H]-ribavirin (0.024 (circles), 0.244 (squares) and 6.1 (triangles) mg/kg) was administered orally. Values represent mean ± S.D. from 3 independent experiments.
Figure 4. Summary of the Dose-Normalized Oral Plasma, Erythrocyte and Blood AUC_{0-30\text{ min}} After Oral Administration of Ribavirin to Ent1(+/+) or Ent(-/-) 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 ± S.D. from 3 independent experiments. *P<0.05 when compared to the value of the equivalent genotype at the 0.024 mg/kg dose. †P<0.05 when compared to Ent1(+/+) mice.

Figure 5. Summary of the Dose-Normalized Oral Plasma, Erythrocyte and Blood AUC_{0-12\text{ hr}} After Oral Administration of Ribavirin to Ent1(+/+) or Ent(-/-) Mice

Dose-normalized ribavirin in plasma (A), erythrocytes (B) and blood (C) AUC_{0-12\text{ hr}} 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 ± S.D. from 3 independent experiments. *P<0.05 when compared to the value of the equivalent genotype at the 0.024 mg/kg dose. †P<0.05 when compared to Ent1(+/+) mice.
Table 1. Summary of Ribavirin Pharmacokinetic Parameter After Intravenous Administration of Ribavirin (3mg/kg) to Ent1(+/+) or Ent1(-/-) Mice

<table>
<thead>
<tr>
<th>Ent1</th>
<th>Plasma (μg min/mL)</th>
<th>Erythrocyte (μg/mL)</th>
<th>Blood (μg/mL)</th>
<th>Erythrocyte : Plasma</th>
<th>Plasma (μg/mL)</th>
<th>Erythrocyte (μg/mL)</th>
<th>Plasma (hr)</th>
<th>Erythrocyte (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC0-12hr</td>
<td>AUC-Ratio</td>
<td></td>
<td></td>
<td>C₀</td>
<td>Cₘ₅</td>
<td>t₁/₂β</td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>220.1 ±39.9</td>
<td>919.4 ±120.5</td>
<td>574.6 ±43.0</td>
<td>4.35 ±1.44</td>
<td>4.15 ±1.62</td>
<td>3.13 ±0.18</td>
<td>16.0 ±12.2</td>
<td>19.1 ±6.36</td>
</tr>
<tr>
<td>-/-</td>
<td>210.7 ±97.2</td>
<td>301.4* ±59.2</td>
<td>243.2* ±43.4</td>
<td>1.66* ±0.74</td>
<td>4.97 ±3.58</td>
<td>0.82* ±0.29</td>
<td>4.20 ±0.66</td>
<td>12.9 ±8.67</td>
</tr>
</tbody>
</table>

*P<0.05 when compared to Ent1(+/+) Values represent mean ±S.D.; n=3. Values in parenthesis represent fold-Ent1(+/+).
Table 2. Summary of Pharmacokinetic Parameters (Based on Plasma Concentrations) After Intravenous Administration of Ribavirin (3 mg/kg) to Ent1(+/-) or Ent1(-/-) Mice

<table>
<thead>
<tr>
<th></th>
<th>V₁ (mL/kg)</th>
<th>K₁₂ (min⁻¹)</th>
<th>K₂₁ (min⁻¹)</th>
<th>K₁₀ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ent1(+/-)</td>
<td>0.679 ± 0.054</td>
<td>0.109 ± 0.013</td>
<td>0.015 ± 0.003</td>
<td>0.014 ± 0.004</td>
</tr>
<tr>
<td>Ent1(-/-)</td>
<td>0.974 ± 0.072</td>
<td>0.039 ± 0.008</td>
<td>0.012 ± 0.005</td>
<td>0.015 ± 0.004</td>
</tr>
</tbody>
</table>

Values represent mean ± R.S.E. of parameter estimates.
### Table 3. Predicted and Observed Ratio of Ribavirin Phosphorylated Metabolite to Ribavirin Plasma Concentration in Mouse Ent1(+/+) and Ent1(-/-) Erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>CL\textsubscript{phosp} (\mu L/min/10^9 cells)</th>
<th>ex vivo\textsuperscript{b}</th>
<th>Predicted</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Steady-State or Single Dose AUC\textsubscript{0-\tau} Ratio\textsuperscript{a}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC\textsuperscript{c} :</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ent1(+/-)</td>
<td>(4.35)</td>
<td>8.1 (10.0)</td>
<td>3.2 (4.1)</td>
<td>1.9 (2.6)</td>
</tr>
<tr>
<td>Ent1(-/-)</td>
<td>(1.66)</td>
<td>0.3 (0.5)</td>
<td>0.3 (0.6)</td>
<td>0.4 (0.6)</td>
</tr>
<tr>
<td>Ent1(+/-) : Ent1(-/-)</td>
<td>(2.62)</td>
<td>27.2</td>
<td>9.7 (7.3)</td>
<td>5.2 (4.2)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Values represent steady-state erythrocyte phosphorylated metabolite (RBC) to plasma ribavirin AUC\textsubscript{0-\tau} ratio or Ent1(+/-) to Ent1(-/-) ratio. Values in parenthesis represent the same ratios after a single dose.

\textsuperscript{b}Values were 2.89 and 6.06 \mu L/10^9 cells/min for erythrocytes from Ent1(+/-) and Ent1(-/-) mice respectively (Chapter 2).

\textsuperscript{c}RBC represents both erythrocyte ribavirin and erythrocyte phosphorylated metabolite concentrations.
Table 4. Urinary Excretion (0 to 48 hr) After Intravenous Administration of Ribavirin to Ent1(+/+) and Ent1(-/-) Mice

<table>
<thead>
<tr>
<th>Ent1</th>
<th>Total-μCi (% Dose)</th>
<th>Urine Composition (% Total μCi Collected)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RBV</td>
</tr>
<tr>
<td>+/+</td>
<td>42.2 ±6.0</td>
<td>58.0 ±5.0</td>
</tr>
<tr>
<td></td>
<td>44.6 ±10.9</td>
<td>40.2 ±12.1</td>
</tr>
<tr>
<td>-/-</td>
<td>18.8 ±9.9</td>
<td>1.44 ±1.07</td>
</tr>
</tbody>
</table>

\(^a\)Unknown metabolite.

\(^*\)\(P\textless0.05\) when compared to Ent1(+/+). Values represent mean ±S.D.; \(n=3\). Values in parenthesis represent fold-Ent1(+/+). N.D.: Not detected.
Table 5. Summary of Ribavirin Pharmacokinetic Parameters (0 to 30 Minutes) After Oral Administration to Ent1(+/-) or Ent1(-/-) Mice

<table>
<thead>
<tr>
<th>Ent1</th>
<th>Dose (mg/kg)</th>
<th>Plasma AUC$_{0-30}$ min (μg min/ml)</th>
<th>Erythrocyte Blood</th>
<th>Plasma C$_{max}$/Dose (μg/ml)/(mg/kg)</th>
<th>Erythrocyte Blood</th>
<th>T$_{max}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>0.024</td>
<td>0.150 ±0.035</td>
<td>0.281 ±0.029</td>
<td>0.252 ±0.047</td>
<td>0.494 ±0.072</td>
<td>0.372 ±0.029</td>
</tr>
<tr>
<td></td>
<td>0.244</td>
<td>0.800 ±0.036</td>
<td>1.49 ±0.061</td>
<td>0.107† ±0.052</td>
<td>0.249† ±0.028</td>
<td>0.174† ±0.014</td>
</tr>
<tr>
<td></td>
<td>6.1</td>
<td>9.39 ±0.139</td>
<td>20.8 ±0.25</td>
<td>0.075† ±0.012</td>
<td>0.181† ±0.028</td>
<td>0.134† ±0.019</td>
</tr>
<tr>
<td>-/-</td>
<td>0.024</td>
<td>0.055* ±0.006</td>
<td>0.061* ±0.015</td>
<td>0.096* ±0.028</td>
<td>0.128* ±0.046</td>
<td>0.105* ±0.015</td>
</tr>
<tr>
<td></td>
<td>0.244</td>
<td>0.499* ±0.013</td>
<td>0.307* ±0.013</td>
<td>0.093* ±0.012</td>
<td>0.068* ±0.028</td>
<td>0.071* ±0.019</td>
</tr>
<tr>
<td></td>
<td>6.1</td>
<td>1.67 ±0.139</td>
<td>2.1 ±0.27</td>
<td>0.017 ±0.017</td>
<td>0.003 ±0.028</td>
<td>0.013 ±0.013</td>
</tr>
</tbody>
</table>

*P<0.05 when compared to 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 when compared to lowest dose at equivalent genotype. Values represent mean ±S.D.; n=3. Values in parenthesis represent fold-Ent1(+/-) at equivalent dose.
Table 6. Summary of Ribavirin Pharmacokinetic Parameters (0 to 12 Hours) After Oral Administration of the Drug to Ent1(+/+) or Ent1(-/-) Mice

<table>
<thead>
<tr>
<th>Ent1 Dose (mg/kg)</th>
<th>Plasma AUC$_{0-12\text{ hr}}$ (mg min/ml)</th>
<th>Erythrocyte AUC Ratio</th>
<th>Blood t$_{1/2\beta}$ (hr)</th>
<th>Ent1(+/+)</th>
<th>Ent1(-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/- 0.024</td>
<td>1.70 ±0.29</td>
<td>5.78 ±1.22</td>
<td>2.66 ±0.75</td>
<td>3.38 ±0.13</td>
<td>6.71 ±1.63</td>
</tr>
<tr>
<td>0.244</td>
<td>5.64 ±1.18</td>
<td>24.1 ±2.15</td>
<td>15.4 ±0.88</td>
<td>4.82 ±0.47</td>
<td>4.96 ±1.03</td>
</tr>
<tr>
<td>6.1</td>
<td>111.9 ±16.4</td>
<td>398.6 ±53.7</td>
<td>269.9 ±39.8</td>
<td>3.59 ±0.45</td>
<td>6.20 ±2.65</td>
</tr>
<tr>
<td>-/- 0.024</td>
<td>0.46* ±0.07</td>
<td>0.99* ±0.49</td>
<td>0.75* ±0.29</td>
<td>2.13 ±1.00</td>
<td>5.37 ±1.00</td>
</tr>
<tr>
<td>(3.70↓)</td>
<td>(5.86↓)</td>
<td>(3.52↓)</td>
<td>(1.58↓)</td>
<td>(1.25↓)</td>
<td>(1.22↓)</td>
</tr>
<tr>
<td>0.244</td>
<td>3.16* ±0.18</td>
<td>6.24* ±0.09</td>
<td>4.9* ±0.05</td>
<td>1.96* ±0.10</td>
<td>4.56 ±0.04</td>
</tr>
<tr>
<td>(1.78↓)</td>
<td>(3.87↓)</td>
<td>(3.13↓)</td>
<td>(2.46↓)</td>
<td>(1.09↓)</td>
<td>(1.22↓)</td>
</tr>
<tr>
<td>6.1</td>
<td>74.5* ±5.9</td>
<td>106.6* ±4.4</td>
<td>90.1* ±5.9</td>
<td>1.44* ±0.06</td>
<td>5.11 ±1.52</td>
</tr>
<tr>
<td>(1.50↓)</td>
<td>(3.74↓)</td>
<td>(3.00↓)</td>
<td>(2.50↓)</td>
<td>(1.21↓)</td>
<td>(1.37↑)</td>
</tr>
</tbody>
</table>

*P<0.05 when compared to Ent1(+/+) at equivalent dose. Values represent mean ±S.D.; n=3.

Values in parenthesis represent fold-Ent1(+/+) at equivalent dose.
Figure 2.

A.
Figure 2 cont.

B.
Figure 2 cont.

C.
Figure 3.
A.
Figure 3 cont.

B.
Figure 3 cont.

C.
Figure 4.
Figure 5.

A. 

B. 

C. 

Legend: 

* Significant difference from control group. 

† Significant difference from 0.024 mg/kg dose.