The Role of the Equilibrative Nucleoside Transporter 1 (ENT1) in Transport and Metabolism of Ribavirin by Human and Wild-Type or Ent1(–/–) Mouse Erythrocytes

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ABSTRACT

The polar nucleoside drug ribavirin is front-line treatment for chronic hepatitis C virus infection. The human equilibrative nucleoside transporter (ENT) 1 transports ribavirin into erythrocytes where it is phosphorylated. These phosphorylated metabolites accumulate in the erythrocytes and produce dose-limiting hemolytic anemia. Here, we examined the *in vitro* and *ex vivo* transport and metabolism of ribavirin by erythrocytes isolated from humans and Ent1-null mice. Ribavirin (2.4 μ M) uptake was significantly higher (1044 ± 255 amol/ μ g/10 s) into erythrocytes from Ent1(+/+) mice compared with that from Ent1(–/–) mice (76.48 ± 11.20 amol/ μ g/10 s). Our results showed a saturable (K_m of 382 ± 75.1 μ M) transport of [3 H]ribavirin into erythrocytes from Ent1(+/+) mice. We found that ribavirin concentration rapidly (within 60 s) reached equilibrium in erythrocytes using a time course of [3 H]ribavirin transport (2.5

μ M) and metabolism in mouse and human erythrocytes for 8 h. However, total radioactivity of ribavirin was predominantly attributed to the phosphorylated metabolites ribavirin monophosphate and ribavirin triphosphate. Our findings allow us to estimate ribavirin transport, diffusion, and metabolic clearance and to predict *in vivo* accumulation of ribavirin phosphates in erythrocytes of both mice and humans. Our modeling of ribavirin in erythrocytes on long-term administration of ribavirin suggests that the accumulation of ribavirin inside the cells is dependent on ENT1/Ent1 transport and the rates of intracellular phosphorylation and the degradation of the phosphorylated metabolites. We predict that Ent1(+/+) and Ent1(–/–) mice will serve as excellent models to investigate the contribution of Ent1 to the pharmacokinetics and toxicity of ribavirin *in vivo*.

The front-line treatment of compensated chronic hepatitis C virus infection is a combination therapy with the nucleoside drug ribavirin plus interferon- α (Food and Drug Administration, 2007a,b). Ribavirin acts as a prodrug and exerts its broad antiviral activity primarily through its active phosphorylated metabolite ribavirin 5'-triphosphate (RTP) (Martin and Jensen, 2008) and also possibly through the ribavirin 5'-monophosphate (RMP) (Parker, 2005). This metabolic activation occurs intracellularly by various kinases, including adenosine kinase (Russmann et al., 2006). The dose-limiting toxicity of ribavirin

is hemolytic anemia, which occurs in 10 to 13% of patients (Fried, 2002). This toxicity is believed to be because of significant intracellular accumulation of the phosphorylated metabolites of ribavirin. These metabolites accumulate in the erythrocyte because they are too polar to diffuse out of the cells and they are not catabolized in erythrocytes (Canonico et al., 1984). Unlike nucleated cells and serum, erythrocytes lack both 5'-nucleotidase and alkaline phosphatase (Willis et al., 1978; Page and Connor, 1990), which are capable of dephosphorylating RMP and RTP to ribavirin. Although the exact mechanism of the hemolytic anemia caused by ribavirin is unknown, recent studies have suggested that this may be a result of oxidative damage to the erythrocyte membrane resulting in erythrophagocytosis by the reticuloendothelial system (De Franceschi et al., 2000).

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ABBREVIATIONS: RTP, ribavirin triphosphate; RMP, ribavirin monophosphate; ENT, equilibrative nucleoside transporter; 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; NBMPR, S-(4-nitrobenzyl)-6-thioinosine; GFP, green fluorescent protein; MDCK, Madin-Darby canine kidney; dH₂O, deionized water; HPLC, high-performance liquid chromatography; AUC, area under the curve; CL, clearance; V, apparent volume of distribution; τ , dosing interval.

For ribavirin to produce its hematological toxicity, it must first be transported into erythrocytes. Because ribavirin is a polar molecule (log P from -2.03 to -2.54 ; National Institute of Allergy and Infectious Diseases National Institutes of Health Anti-HIV/OI Chemical Compound Database, Bethesda, MD), it diffuses poorly across cell membranes. In vitro studies have shown that the plasma membrane nucleoside transporter 1 (ENT1) transports ribavirin (Jarvis et al., 1998; Yamamoto et al., 2007). ENT1 is a member of the equilibrative nucleoside transporter family (ENT), which consists of four members (ENT1–4) (Kong et al., 2004). Of these four members, human erythrocytes express only ENT1 (Oliver and Paterson, 1971; Paterson and Oliver, 1971). The physiological role of nucleoside transporters is to mediate salvage of nucleoside and to regulate the autocrine and paracrine effects of adenosine (Kong et al., 2004). Extrapolating from in vitro studies, it is widely believed that, in vivo, ENT1 expression in erythrocytes is required for ribavirin to enter erythrocytes and exert its toxicity (Russmann et al., 2006). Despite this widely held belief, there is only indirect evidence to support this conclusion. For example, in several clinical studies, it has been suggested that ENT1 transport may be important in the toxicity of ribavirin, because erythrocyte ribavirin (and phosphorylated metabolite) concentrations are significantly correlated with decreases in hemoglobin (a marker of hemolytic anemia) (Homma et al., 2004; Inoue et al., 2006). However, in these two separate studies, the inverse correlation between ribavirin concentration and erythrocyte hemoglobin, although significant, was weak, with correlation coefficient (r) of 0.62 and 0.36 in each study, respectively. In addition, in these studies, there was substantial interindividual variation in the intracellular concentrations of the ribavirin (and its phosphorylated metabolites). Ribavirin undergoes intracellular metabolism (Fig. 1), including phosphorylation to form the active RMP and RTP metabolites, as well as a nonphosphorylated metabolism to form the primary [1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxylic acid (RTCOOH)] and 1,2,4-triazole-3-carboxamide (TCONH₂) and secondary (1,2,4-triazole-3-carboxylic acid;

TCOOH) nonphosphorylated inactive metabolites (Wu et al., 2003, 2005). Because of this, variability in the intracellular metabolism of ribavirin may also contribute to variability in its toxicity.

As part of a larger series of studies on the role of ENT1 in the disposition of ribavirin, we studied in detail the contribution of ENT1 to the ex vivo distribution of ribavirin and its metabolism to the active phosphorylated metabolites in erythrocytes of both mice and humans. We used both wild-type and Ent1(–/–) mice to determine whether entry of ribavirin into mouse erythrocytes requires ENT1. We compared our findings with studies of human erythrocytes in which we blocked ENT1 activity with the potent ENT1 inhibitor *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR). Finally, to gain insight into factors that contribute to the accumulation of ribavirin and its metabolites in erythrocytes, we analyzed the data using compartmental models and predicted the accumulation of the phosphorylated metabolites in erythrocytes on multiple dosing of ribavirin.

Materials and Methods

Retroviral Transduction of Ent1 GFP-LNCX2 into MDCK Cells. The cDNA of mouse Ent1 was cloned into the Kpn1 sites of the green fluorescent protein vector pGFP-C1 (Clontech, Mountain View, CA). For retroviral expression studies, Ent1-GFP was excised from pGFP-C1 plasmid at the NheI and KpnI sites and cloned into the NotI site of the pLNCX2 vector. Blunt end ligation was used to facilitate ligation. The Phoenix amphotropic retroviral packaging cell line was cultured according to the manufacturer's instructions (Orbigen, Inc., San Diego, CA). FuGENE 6 (Roche Applied Science, Mannheim, Germany) was used to transfect the Ent1-GFP LNCX2 vector into the Phoenix cells according to the manufacturer's instructions. In brief, approximately 5×10^4 cells were seeded in T-25 flasks containing 5 ml of Complete medium and incubated at 37°C. After 16 h, cells were transfected with the Ent1-GFP containing LNCX2 plasmid using 24 μ l of FuGENE 6 reagent with 8 μ g of DNA complex in 800 μ l of serum-free media. Concurrently, wild-type MDCK cells were grown at 37°C with 5% CO₂ in minimal essential medium

Fig. 1. In vivo metabolism of ribavirin. Ribavirin undergoes 5' phosphorylation to form RMP, RDP, and RTP; deribosylation to form TCONH₂; and amide hydrolysis to form RTCOOH. Both TCONH₂ and RTCOOH are sequentially metabolized to form TCOOH.

media supplemented with 1% penicillin/streptomycin. The media from the Phoenix cells containing virus were aspirated and filtered through a 0.2- μ m syringe filter. Polybrene (Sigma, St. Louis, MO; 5 mg/ml) was added to the filtered media containing virus and mixed gently before the solution was added to the MDCK cells. This application was repeated 24 and 48 h later for maximal viral exposure. MDCK cells infected with the Ent1-LNCX2 were selected with 400 μ g/ml Geneticin (G-418; Invitrogen, Carlsbad, CA) for 2 to 3 weeks and subsequently maintained in 200 μ g/ml G-418. The expression of Ent1-GFP in the MDCK cells was confirmed visually with an inverted IX70 fluorescence microscope (Olympus, Melville, NY).

Ribavirin Transport Kinetics in Ent1-Overexpressing Cells.

The kinetics of ribavirin transport was measured in MDCK cells stably overexpressing mouse Ent1 using a method similar to that described previously (Lai et al., 2005; Lee et al., 2006). The Ent1-MDCK cells were grown in monolayers in complete media to 85% confluence in 24-well polystyrene plates. At this time, the culture media were replaced with a 37°C sodium-free transport buffer (20 mM Tris-HCl, 3 mM K_2HPO_4 , 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2$, 5 mM glucose, and 130 mM *N*-methyl-D-glucamine, pH 7.4) to eliminate the contribution of sodium-dependent transport and was preincubated with (to inhibit Ent1 function) or without 10 μ M NBMPR. Transport was initiated by the timed addition of fresh sodium-free transport buffer containing 250 nM [3H]ribavirin plus unlabeled ribavirin ranging in concentration from 0.001 to 4.0 mM. After 15 min, uptake was terminated with the addition and subsequent washes with ice-cold 10 μ M NBMPR in sodium-free transport buffer. The cells were lysed with 1 N NaOH, neutralized with 1 N HCl, and 950 μ l of lysate was counted by scintillation counting.

Mouse Husbandry. All animal procedures were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee. We interbred F1 Ent1(+/-) animals (Choi et al., 2004) to produce Ent1(+/+), Ent1(+/-), and Ent1(-/-) animals in a hybrid (50% C57BL/6J, 50% SX1/SvJ) background. Animals were polymerase chain reaction genotyped using ear-clip biopsies obtained from 3-week-old pups. Total genomic DNA was isolated using the QIAamp DNA Mini kit (QIAGEN, Valencia, CA), and the wild-type and knockout alleles were amplified using polymerase chain reaction with forward (5'-GGT TCT GTC TCC CGT GTC AT-3') and reverse (5'-ATG CTG GGG AGT AGC AAA GA-3') primers.

Isolation of Mouse Erythrocytes. Ent1(+/+), Ent1(+/-), and Ent1(-/-) mice were anesthetized with pentobarbital and exsanguinated to collect blood samples in 1.5-ml microcentrifuge tubes containing ~20 U sodium heparin. The whole blood was briefly centrifuged. After aspiration of the plasma, the erythrocytes were gently washed three times in 20 volumes of transport buffer (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl, and 0.1 mM EDTA, pH 7.4). The erythrocytes were then taken up in 4 volumes of transport buffer and used within 2 h of collection.

Ribavirin Transport in Erythrocytes. Erythrocytes isolated as described above were used for the following transport studies using oil-stop methodology (Jarvis et al., 1980). Fifty microliters of erythrocyte suspensions were aliquoted into 1.5-ml microcentrifuge tubes, and transport was started by adding 16.6 μ l of transport buffer containing [3H]ribavirin (2.4 μ M; 0.42 μ Ci). Transport was stopped by transferring 50 μ l of the samples to a 1.5-ml microcentrifuge tube containing 1000 μ l of 10 μ M NBMPR layered on top of 200 μ l of dibutyl phthalate (oil). The samples were then immediately centrifuged at 5000g for 30 s. The transport buffer and oil were then aspirated, and the insides of the tube were dried with a cotton-tipped applicator. Each sample was vortexed briefly after adding 1 ml of deionized water (dH_2O) to each sample to hypotonically lyse the erythrocytes. Two hundred fifty microliters of the lysate was added in triplicate to 10-ml scintillation vials, and the samples were decolorized by adding 300 μ l of 30% hydrogen peroxide. After 20 min of shaking on a plate shaker, 10 ml of scintillation fluid was added, and the total radioactivity was measured by scintillation counting. Total protein in each sample was determined using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL). Cell

count (determined by using an Improved Neubauer hemocytometer; Hausser Scientific, Horsham, PA) was used to estimate intracellular ribavirin concentration.

Ribavirin Transport and Inhibition Kinetics in Mouse Erythrocytes. Ribavirin transport kinetic parameters were determined by measuring the 10-s transport rate of [3H]ribavirin (0.77 μ M) in the presence of increasing concentrations of unlabeled ribavirin (0–5 mM). The kinetics of inhibition of ribavirin transport by its metabolites was determined by measuring the 10-s transport rate of [3H]ribavirin (0.77 μ M) in the presence of increasing concentrations of the following ribavirin metabolites: RTCOOH, TCONH₂, and TCOOH (0–2 mM).

Isolation of Human Erythrocytes. Studies using human erythrocytes were approved by the University of Washington Institutional Review Board. Volunteers between 18 and 45 years, in good health and taking no medications were enrolled after providing informed written consent. Whole blood (30 ml) was drawn by venipuncture into blood collection tubes containing sodium heparin as a preservative. The whole human blood was processed and resuspended as described above for mouse blood.

Transport and Metabolism Time Course in Mouse and Human Erythrocytes. [3H]Ribavirin (2.5 μ M) transport into erythrocytes was measured at various time points (10 s–8 h), and total radioactivity was determined by scintillation counting. In addition, the intracellular composition of ribavirin and its metabolites (phosphorylated and nonphosphorylated) was determined at 1 and 15 min and at 1, 4, 6, and 8 h. For these samples, the erythrocyte pellet was immediately resuspended in 150 μ l of dH_2O and enzymatic activity was immediately quenched by the addition of 60 μ l of 6% perchloric acid, which was then neutralized by the addition of 20 μ l of 2 M K_2HPO_4 . The sample was then centrifuged at 20,000g for 10 min at 4°C and stored for analysis.

HPLC Analysis to Determine Ribavirin and Metabolite Composition. Fifty microliters of the supernatant from the metabolite samples (see above) was added to a 7-ml scintillation vial containing 100 μ l of dH_2O and counted using scintillation counting to determine total radioactivity and workup recovery. One hundred twenty microliters of the supernatant was analyzed by HPLC (Alliance 2695; Waters, Milford, MA) using a method that separates ribavirin from RTCOOH, TCONH₂, TCOOH (kindly provided by Valeant Pharmaceuticals, Aliso Viejo, CA) and RMP and RTP (Moravsek Biochemicals, Brea, CA). The HPLC method used an Atlantis dC18 column (3 μ ; 4.6 $\times$ 150 mm; Waters) eluted with a mobile phase that consisted of 100 mM potassium phosphate, pH 6.2, containing the ion-pairing reagent 0.1% *N,N*-dimethylhexylamine (A) and methanol (B) at a flow rate of 0.75 ml/min. The initial condition was 100% A. Between 4.5 and 6.0 min, the mobile phase composition linearly decreased from 100% A to 70% A and was held at 70% A until the end of the run at 22 min. After 22 min, the column was allowed to immediately return to 100% A and re-equilibrated for 5 min before the next injection. The UV absorbance was monitored at 207 nm, and fractions were collected every 20 s, beginning at the solvent front (approximately 1.5 min after injection) through the elution of the RTP peak. The radioactivity in each fraction was determined by scintillation counting and expressed as ribavirin and metabolite amounts and concentrations.

Data Analysis. Radiolabeled ribavirin transport rate was normalized to micrograms of protein (using the bicinchoninic acid assay and bovine serum albumin protein concentration standard curve) or cell count (hematocytometer). Intracellular ribavirin (or metabolite) concentrations were estimated by using an intracellular erythrocyte volume of 90 fl (μ^3) in humans (Bessman and Johnson, 1975) and 48 fl (Abraham et al., 1978) in mice.

Transport kinetic parameters V_{max} , K_m , and the diffusion constant K_{dif} were determined by nonlinear regression analysis (WinNonlin; Pharsight, Mountain View, CA) of the transport rates in the presence of increasing unlabeled substrate (1.3 μ M–4 mM) in the absence or presence of 10 μ M NBMPR. The following equations were simulta-

neously fit to the data (Malo and Berteloot, 1991; Chenu and Berteloot, 1993):

$$v_1^* = \frac{V_{\max} T}{K_m + S_{\text{cold}} + T} + K_{\text{dif}} T \quad (1)$$

$$v_2^* = K_{\text{dif}} T \quad (2)$$

where v_1^* is the velocity of transport of the labeled substrate in the absence of 10 μM NBMPR, v_2^* is the velocity of transport of the labeled substrate in the presence of 10 μM NBMPR, S_{cold} is the concentration of the unlabeled substrate, T is the concentration of the labeled substrate, $V_{\max}$ and K_m are the transport parameters, and K_{dif} is the diffusion rate constant. Data were analyzed from three independent experiments to determine the mean and S.D. of the parameter estimates.

A modified Hill equation

$$E = E_{\max} - (E_{\max} - E_0) \times \frac{I}{I + IC_{50}} \quad (3)$$

was fit to the metabolite inhibition data, where $E_{\max}$ is the transport activity in absence of the inhibitor, E_0 is the transport activity at infinite inhibitor concentration (representing the diffusional component and nonspecific binding of substrate), I is the inhibitor concentration, and IC_{50} is the inhibitor concentration at which transport activity is half the difference between $E_{\max}$ and E_0 .

Simulation of Ribavirin Disposition in Erythrocytes. Various disposition models for the transport and metabolism of ribavirin in erythrocytes were simulated or modeled using both WinNonlin and SAAM II (University of Washington, Seattle, WA). In the first model, we assumed that ribavirin was not metabolized in the erythrocytes. This simple model involved only a transport and diffusional clearance to mediate the distribution of ribavirin into and out of erythrocytes:

$$\frac{dC_{\text{RBV}}^{\text{ex}}}{dt} = -C_{\text{RBV}}^{\text{ex}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_1} + C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_1} \quad (4)$$

$$\frac{dC_{\text{RBV}}^{\text{in}}}{dt} = C_{\text{RBV}}^{\text{ex}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_2} - C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_2} \quad (5)$$

where $C_{\text{RBV}}^{\text{ex}}$ and $C_{\text{RBV}}^{\text{in}}$ are the extracellular and erythrocyte concentrations of ribavirin, respectively; CL_{dif} and CL_{ent} are the diffusional and Ent1-mediated transmembrane distributional clearances of ribavirin, respectively; and V_1 and V_2 are the extracellular and intracellular distributional volumes of ribavirin, respectively. In the second model, we assumed that ribavirin is metabolized intracellularly (via a single intracellular metabolic pathway), and there was no efflux or further metabolism of the metabolite. Equation 4 and the following differential equations were used to describe the intracellular disposition of both ribavirin and its metabolite:

$$\frac{dC_{\text{RBV}}^{\text{in}}}{dt} = C_{\text{RBV}}^{\text{ex}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_2} - C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}} + CL_{\text{met}})}{V_2} \quad (6)$$

$$\frac{dC_{\text{met}}^{\text{in}}}{dt} = C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{met}})}{V_2} \quad (7)$$

where $C_{\text{met}}^{\text{in}}$ is the intracellular concentration of the metabolite, and CL_{met} is the intracellular metabolic clearance of ribavirin.

Modeling of Ribavirin Disposition in Erythrocytes. The ex vivo transport and metabolism of ribavirin into human and mouse erythrocytes was modeled by fitting the intracellular ribavirin total radioactivity, and parent and metabolite (phosphorylated and nonphosphorylated) concentrations were determined at various time points between 10 s and 8 h. We assumed that ribavirin was metabolized via two pathways, a nonphosphorylation pathway ($CL_{\text{nonphosph}}$) and a phosphorylation (CL_{phosph}) pathway. In addition, because of the extended experimental duration (8 h), we accounted for the ex vivo time-dependent decrease in CL_{phosph} that was most probably because

of intracellular ATP depletion. Equation 4 and the following differential equations were fit to the data:

$$\frac{dC_{\text{RBV}}^{\text{in}}}{dt} = C_{\text{RBV}}^{\text{ex}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_2} - C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}} + CL_{\text{non-phosph}} + (CL_{\text{phosph}} \cdot e^{-K(\text{nor}-t)}))}{V_2} \quad (8)$$

$$\frac{dC_{\text{nonphosph}}^{\text{in}}}{dt} = C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{nonphosph}})}{V_2} \quad (9)$$

$$\frac{dC_{\text{phosph}}^{\text{in}}}{dt} = C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{phosph}} \cdot e^{-K(\text{nor}-t)})}{V_2} - C_{\text{phosph}}^{\text{in}} \cdot K_{\text{deg}} \quad (10)$$

where $C_{\text{nonphosph}}^{\text{in}}$ is the intracellular amount of nonphosphorylated metabolite (RTCOOH, TCONH₂, and TCOOH; pooled), $C_{\text{phosph}}^{\text{in}}$ is the intracellular amount of phosphorylated metabolite (RMP, ribavirin 5'-diphosphate, and RTP; pooled), $CL_{\text{nonphosph}}$ is the nonphosphorylated metabolite formation clearance, CL_{phosph} is the phosphorylated metabolite formation clearance, K_{deg} is the in vivo elimination rate constant of the phosphorylated metabolite, and K_{inact} is the ex vivo inactivation rate constant of CL_{phosph} . In modeling the ex vivo data, we fixed K_{deg} at 0, because the phosphorylated metabolites were shown to be stable in erythrocytes through 4 h (Page and Connor, 1990). The data obtained from human erythrocytes (absence and presence of 10 μM NBMPR) were simultaneously modeled using two sets of differential equations, one set with and one set without the CL_{ent} parameter. The data obtained from Ent1(+/-) and Ent1(-/-) mouse erythrocytes were randomly paired and also simultaneously modeled. In mice, parameter estimates of CL_{phosph} and $CL_{\text{nonphosph}}$ were allowed to be unique for each genotype.

Simulation of In Vivo Transport and Metabolism of Ribavirin. In vivo ribavirin transport and metabolism were simulated using eqs. 8 to 10 (without the inactivation of CL_{phosph} ; i.e., $K_{\text{inact}} = 0$) and the following one-compartment model describing the plasma disposition of ribavirin

$$\frac{dC_{\text{RBV}}^{\text{ex}}}{dt} = -C_{\text{RBV}}^{\text{ex}} \cdot \left(\frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_1} - K_{\text{sys}} \right) + C_{\text{RBV}}^{\text{in}} \cdot \frac{(CL_{\text{dif}} + CL_{\text{ent}})}{V_1} \quad (11)$$

where K_{sys} is the systemic elimination rate constant. Plasma ribavirin and erythrocyte phosphorylated metabolite concentrations (and AUC ratios) were simulated after single or multiple dosing of ribavirin to steady state [dosing interval (τ): humans, 12 h; mice, 12 h] using the parameters estimated ex vivo and various values of the in vivo systemic plasma elimination constant (K_{sys}) and in vivo phosphorylated metabolite degradation rate constants (K_{deg}). The extracellular volume of distribution of ribavirin was fixed at approximately 1500-fold the intracellular distribution volume (V_2) to minimize the erythrocyte distribution of ribavirin from modulating its plasma concentration. In humans, plasma ribavirin and intracellular phosphorylated metabolite concentration-time profiles were simulated at steady state after fixing K_{sys} at $0.000077 \text{ min}^{-1}$ ($t_{1/2} = 151 \text{ h}$) and K_{deg} at 0.00048 min^{-1} ($t_{1/2} = 1 \text{ day}$). K_{sys} was chosen based on the reported systemic half-life after multiple dosing of ribavirin to steady state (Lertora et al., 1991), and K_{deg} was chosen to produce a degree of accumulation of the phosphorylated metabolite that is observed in vivo (Lertora et al., 1991).

Results

Mouse Ent1 Transport Activity and Kinetics in MDCK Cells. The uptake of [³H]ribavirin increased linearly between 5 and 20 min in cells expressing mouse Ent1 and was substantially reduced in the presence of 10 μM NBMPR or in untransfected control cells (Fig. 2). Subsequent uptake

Fig. 2. Time course of [^3H]ribavirin uptake into MDCK cells expressing mouse Ent1. The time course of [^3H]ribavirin uptake (0.25 μM) by mouse Ent1-expressing MDCK cells was determined in the absence (●) or presence (■) of 10 μM NMBPR or in untransfected MDCK cells in the absence (○) of 10 μM NMBPR.

experiments were conducted over 15 min. In MDCK cells expressing mouse Ent1, the V_{max} of ribavirin transport was $15.6 \pm 1.9 \mu\text{mol/mg protein/15 min}$, the K_m was $321.1 \pm 49.8 \mu\text{M}$, and the K_{dif} was $0.016 \pm 0.002 \text{ l/mg protein/15 min}$.

Mouse Erythrocyte Transport Activity and Kinetics. The uptake of [^3H]ribavirin into erythrocytes from Ent1(+/-), Ent1(+/-), and Ent1(-/-) mice was linear through 30 s, and, unless otherwise noted, all subsequent experiments were conducted for 10 s. The uptake of [^3H]ribavirin into Ent1(+/-) and (-/-) erythrocytes was significantly reduced compared with that into Ent1(+/+) erythrocytes (Fig. 3). In the presence of 10 μM NMBPR, there was no significant difference in the uptake of [^3H]ribavirin into Ent1(+/-), Ent1(+/-), or Ent1(-/-) erythrocytes. The uptake into Ent1(-/-) erythrocytes in the absence of 10 μM NMBPR was significantly different from that into Ent1(+/+) and Ent1(+/-) erythrocytes. In addition, uptake into Ent1(+/+) erythrocytes treated with 10 μM NMBPR was not significantly different from uptake measured into Ent1(-/-) erythrocytes in the absence of NMBPR. The mean ($n = 4$) V_{max} and K_m of [^3H]riba-

virin transport by mouse Ent1(+/+) erythrocytes was $417 \pm 86.7 \text{ fmol}/\mu\text{g}/10 \text{ s}$ and $382 \pm 75.1 \mu\text{M}$, respectively.

Inhibition of Ribavirin Transport by Ribavirin Metabolites in Mouse Erythrocytes. The potency with which ribavirin metabolites RTCOOH, TCONH₂, and TCOOH inhibit the uptake of [^3H]ribavirin into erythrocytes isolated from Ent1(+/+) mice could not be determined because of their limited solubility at high concentrations. These three metabolites did not significantly inhibit Ent1-mediated [^3H]ribavirin transport at concentrations between 0.5 μM and 1 mM (data not shown).

Mouse and Human Erythrocyte Uptake and Metabolism Time Course. Erythrocytes from both humans and Ent1(+/+) mice exhibited no decrease in transport activity (as measured by 10-s uptake) up to 8 h after collection and isolation (data not shown). In addition, there was no significant depletion (<5%) of the extracellular ribavirin concentration through 8 h (data not shown).

The time course of [^3H]ribavirin uptake between 10 s and 8 h was measured in erythrocytes obtained from Ent1(+/+) mice.

Fig. 3. Ribavirin uptake into erythrocytes from Ent1(+/-), Ent1(+/-), and Ent1(-/-) Mice. Uptake of [^3H]ribavirin (2.4 μM) by Ent1(+/+) (■), Ent1(+/-) (▨), and Ent1(-/-) (□) mouse erythrocytes in the absence [vehicle, dimethyl sulfoxide (DMSO)] and presence of 10 μM NMBPR. The values represent mean $\pm$ S.D. of independent measurements with erythrocytes obtained from four mice. *, $P < 0.05$ compared with Ent1(+/+) mice; †, $P < 0.05$ compared with Ent1(+/-) mice.

and Ent1(−/−) mice or from those obtained from humans in the absence (dimethyl sulfoxide, vehicle) or presence of 10 μ M NBMPR. The uptake of total radioactivity rapidly reached equilibrium (within 60 s) in the Ent1(+/+) mouse erythrocyte and was substantially reduced in the Ent1(−/−) erythrocytes (Fig. 4). Similar results were observed in the human erythrocytes, whereby the uptake of total radioactivity rapidly reached equilibrium in the absence of NBMPR and was also substantially reduced in the presence of NBMPR (Fig. 4).

The uptake of 3 H total radioactivity seemed to increase linearly between 60 s and 15 min in both species when ENT1 was present. The simplest erythrocyte distributional model (eqs. 4 and 5; Fig. 5A) with no intracellular metabolism of ribavirin predicted that once equilibrium is achieved, there should be no change in intracellular total radioactivity. In contrast, simulation of ribavirin distribution into an erythrocyte that included intracellular metabolism (eqs. 4, 6, and 7; Fig. 5B) predicted that such metabolism would allow the concentration of total intracellular radioactivity to continually increase, even after ribavirin intracellular and extracellular concentrations reached equilibrium. This accumulation would plateau only if metabolism decreases to zero or if there was elimination of the metabolites from the erythrocytes. Indeed, the time course of intracellular total radioactivity concentration during [3 H]ribavirin uptake by both mouse and human erythrocytes between 5 min and 8 h (Fig. 6, circles) suggested that one of these mechanisms was operational. In the mouse Ent1(+/+) erythrocytes, the intracellular total radioactivity concentration continued to increase up to approximately 6 h and then remained unchanged between 6 and 8 h. In the mouse Ent1(−/−) erythrocytes, the increase of total radioactivity concentration was substantially reduced compared with the Ent1(+/+) erythrocytes and began to plateau at 6 h. The magnitude of the difference in intracellular total radioactivity concentration between the mouse Ent1(+/+) and Ent1(−/−) erythrocytes decreased from 26-fold after 5 min to 3.2-fold after 8 h.

Like the Ent1(+/+) erythrocytes, the intracellular total radioactivity concentration in human erythrocytes, in the absence of 10 μ M NBMPR, continued to increase to 6 h and then remained unchanged between 6 and 8 h (Fig. 6, triangles). In the presence of 10 μ M NBMPR, the intracellular total radioactivity in human erythrocytes increased through 6 h at a rate substantially slower than in the Ent1(−/−) mouse erythrocytes. The magnitude of the difference in intracellular total radioactivity in human erythrocytes in the absence and presence of 10 μ M NBMPR was 52-fold at 5 min and 18-fold at 8 h.

In both vehicle-treated human (Fig. 7A) and mouse Ent1(+/+) (Fig. 7B) erythrocytes, the intracellular ribavirin amount remained relatively constant over time, further suggesting that extracellular-intracellular ribavirin equilibrium was rapidly achieved when Ent1/ENT1 was present. In addition, in humans and in mice, in the absence of NBMPR, the nonphosphorylated and phosphorylated metabolites increased with time.

Ex Vivo Erythrocyte Disposition Modeling and Simulation. The total radioactivity (Figs. 4 and 6) and ribavirin and metabolite data (Fig. 7, A and B) were simultaneously fit to a disposition model of ribavirin in both mouse and human erythrocytes (eqs. 4 and 8–10; Fig. 7C), with K_{sys} fixed at 0 (i.e., no elimination of ribavirin from the buffer). In mice, parameters were estimated after pairing animals from each genotype (Table 1). The Ent1-mediated distributional clearance of ribavirin into erythrocytes (CL_{ent}) was 821-fold greater than the diffusional clearance (CL_{dif}). There were no significant difference in the parameter estimates for the formation clearance of the nonphosphorylated metabolites ($CL_{nonphosp}$) or phosphorylated metabolites (CL_{phosp}) between the Ent1(+/+) and Ent1(−/−) animals.

In humans, parameters were estimated from three subjects and the ENT1-mediated distribution clearance was approximately 11,500-fold that of the diffusional clearance of ribavirin. In humans, the ENT-mediated distribution was

Fig. 4. Time course (0–15 min) of ribavirin uptake into mouse and human erythrocytes as measured by total radioactivity concentration. Time course of [3 H]ribavirin (2.5 μ M) uptake into erythrocytes from Ent1(+/+) mice (●) or humans (▲) as measured by total radioactivity (micromolar ribavirin equivalents). The corresponding open symbols represent erythrocytes from Ent1(−/−) mice or those from humans treated with 10 μ M NBMPR. Values represent mean $\pm$ S.D. for three animals or subjects.

Fig. 5. Simulation and modeling of ribavirin disposition in erythrocytes. Simulation of the extracellular ribavirin (C_{RBV}^{ex} ; circles) and intracellular ribavirin (C_{RBV}^{in} ; squares) metabolite C_{met}^{in} ; triangles) or total radioactivity (diamonds; intracellular ribavirin plus intracellular metabolite) concentration-time profile for the ex vivo erythrocyte uptake of ribavirin in the absence (A) and presence (B) of intracellular metabolism. The filled symbols represent simulation with both Ent and diffusional components, whereas the open symbols represent simulation with only the diffusional component. CL_{ent} and CL_{dif} are the Ent and diffusional mediated clearances, respectively and CL_{met} is the metabolic clearance. CL_{ent} and CL_{dif} were set at 0.1 and 0.005, respectively, and CL_{met} was set at 0.01. For simplicity the extracellular and intracellular volume of distribution (V_1 and V_2 , respectively) were set equal to each other. A.U., arbitrary units.

Fig. 6. Time course (0–8 h) of ribavirin uptake into mouse and human erythrocytes as measured by total radioactivity concentration. Time course of [3H]ribavirin (2.5 μM) uptake into erythrocytes from Ent1(+/+) mice (●) or humans (▲) as measured by uptake of total radioactivity concentration (micromolar ribavirin equivalents). The corresponding open symbols represent erythrocytes from Ent1(-/-) mice or those from humans treated with 10 μM NBMPR. Values represent mean $\pm$ S.D. for three animals or subjects.

Fig. 7. Time course and disposition model for ribavirin and its metabolites in mouse and human erythrocytes. The time course (0–8 h) of the amount of ribavirin (circles), nonphosphorylated metabolite (squares), and phosphorylated metabolites (triangles) in erythrocytes from humans (A) or mice (B). The filled symbols represent erythrocytes from humans or

1.9-fold greater and significantly different, whereas the diffusional clearance (CL_{dif}) was approximately 7.3-fold lower and significantly different from that in Ent1(–/–) mice.

In Vivo Erythrocyte Disposition Simulation. Using the parameters estimated above, the steady-state and single dose accumulation ratio of erythrocyte phosphorylated metabolite to plasma ribavirin concentration was predicted for both humans and mice using various values of ribavirin systemic elimination (K_{sys}) and degradation rate of ribavirin phosphates (K_{deg}) (Tables 2 and 3). K_{inact} was set to 0, because the ATP-depletion phenomenon observed in the ex vivo system was not expected to be present in vivo. The erythrocyte to plasma accumulation ratio depended on CL_{ent} , CL_{phosp} , and K_{deg} , whereas the difference in the accumulation ratio between the presence and absence of ENT/Ent1 was relatively insensitive to these parameters. In humans, the predicted difference in the erythrocyte phosphorylated metabolite to plasma ribavirin $AUC_{0-\tau}$ ratio after a single dose and at steady state between the presence and absence of ENT1 was approximately 37-fold, whereas the corresponding predicted difference in this ratio for mice was approximately 14-fold.

The plasma ribavirin and erythrocyte-phosphorylated metabolite concentration-time profiles were simulated after multiple dosing in humans (Fig. 8). The dose was adjusted to produce plasma C_{ss} observed clinically. In humans, the predicted steady-state concentration of plasma ribavirin was 15.5 μ M (C_{max}) and 14.6 μ M (C_{min}), and the concentrations of phosphorylated metabolites in the erythrocytes were 569 μ M (C_{max}) and 567 μ M (C_{min}), respectively. In addition, in humans, the predicted erythrocyte phosphorylated metabolite to plasma ribavirin concentration ratio was 37-fold at plasma C_{max} and 39-fold at plasma C_{min} .

Discussion

Erythrocytes from humans exclusively express high amounts of ENT1 (Jarvis and Young, 1980; Jarvis et al., 1982). The transport of ribavirin by human ENT1 has been characterized previously in erythrocytes, showing a rapid equilibrium and complete inhibition by 10 nM NBMPR (Jarvis et al., 1998). This complete inhibition suggests that ENT1 but not ENT2 facilitates transport of ribavirin into the erythrocyte. In addition, ribavirin transported by human ENT1 is saturable, with a K_m value of 420 μ M (Jarvis et al., 1998). The K_m value of ribavirin transported in both mouse Ent1 expressed in MDCK cells and in erythrocytes was similar to that observed in humans. As in human erythrocytes, ribavirin was rapidly transported into mouse erythrocytes expressing Ent1, reaching equilibrium within 60 s. The transport activity of Ent1(+/-) erythrocytes was approximately half that of the Ent1(+/+) erythrocytes (Fig. 3), suggesting there is a gene-dose effect of Ent1 of [³H]ribavirin

Ent1(+/+) mice. The corresponding open symbols represent erythrocytes from humans treated with 10 μ M NBMPR or from Ent1(–/–) mice. Insets represent expanded scale. Values represent mean $\pm$ S.D. for three subjects. Compartmental model (C) 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 ($CL_{non-phosp}$) pathways. Extracellular ribavirin is eliminated by systemic elimination (K_{sys}), whereas intracellular phosphorylated metabolite is eliminated by degradation (K_{deg}).

TABLE 1

Estimates of the kinetic parameters of transport, diffusion, and metabolism of ribavirin using erythrocytes from Ent1(+/+) and Ent1(-/-) mice and humans

Intercompartmental clearance and volume parameters were estimated by simultaneously fitting the intracellular total radioactivity, ribavirin, pooled phosphorylated metabolite (RMP, RDP, and RTP), and nonphosphorylated metabolite (RTCOOH, TCONH₂, and TCOOH) amounts were determined in erythrocytes from Ent1(+/+) and Ent1(-/-) mice and humans. Values represent mean $\pm$ S.D. or parameter estimates from three independent animals (mice) or subjects (humans).

Parameter	Parameter Estimate (Mean $\pm$ S.D.)		
	Mouse		Human
	Ent1(+/+)	Ent1(-/-)	
CL _{ent} (μ l/min/10 ⁹ cells)	167.1 ^a $\pm$ 41.3	167.1 ^a $\pm$ 41.3	321.1 $\pm$ 76.8
CL _{diff} (μ l/min/10 ⁹ cells)	0.204 ^a $\pm$ 0.041	0.204 ^a $\pm$ 0.041	0.028 $\pm$ 0.004
CL _{phosp} (μ l/min/10 ⁹ cells)	2.89 ^a $\pm$ 0.11	6.05 $\pm$ 4.72	1.02 $\pm$ 0.24
CL _{nonphosp} (μ l/min/10 ⁹ cells)	0.046 $\pm$ 0.013	0.109 $\pm$ 0.067	0.024 $\pm$ 0.007
V ₂ (μ l/10 ⁹ cells)	42.7 ^a $\pm$ 6.7	42.7 ^a $\pm$ 6.7	55.9 $\pm$ 4.7
t1/2 _{inact} (min)	68.9 $\pm$ 12.0	68.9 $\pm$ 12.0	99.1 $\pm$ 29.7

^a $P < 0.05$ compared with humans.

TABLE 2

Predicted ratio of human erythrocyte ribavirin-phosphorylated metabolite to ribavirin plasma concentration in the absence or presence of ENT1

Concentration ratios were determined after simulation of multiple dosing of ribavirin to steady state (τ , 12 h) for various values of systemic (plasma) ribavirin and erythrocyte-phosphorylated metabolite half-lives (t1/2_{sys} and t1/2_{deg}, respectively) for erythrocytes in the presence (+) or absence (-) of ENT1. The clinically observed ribavirin t1/2 value after single and multiple doses is 27.1 and 151 h, respectively.

t1/2 _{sys}	t1/2 _{sys} / τ	Ratio	Steady-State or Single Dose AUC _{0-τ} Ratio ^a					
			t1/2 _{deg} 0.1 day		t1/2 _{deg} 1 day		t1/2 _{deg} 10 days	
			+	-	+	-	+	-
2.71 h	0.226	RBC:plasma +/-	3.4 (3.0)	0.09 (0.08)	35.8 (7.7)	1.0 (0.19)	340 (8.7)	9.1 (0.21)
				36.0 (37.3)		37.4 (40.5)		37.5 (41.0)
27.1 h	2.26	RBC:plasma +/-	3.8 (2.8)	0.10 (0.07)	37.7 (6.2)	1.0 (0.14)	355 (6.9)	9.4 (0.16)
				37.4 (40.4)		37.6 (42.5)		37.6 (42.8)
151 h	12.6	RBC:plasma +/-	3.8 (2.7)	0.10 (0.07)	37.8 (5.9)	1.0 (0.14)	338 (6.6)	8.8 (0.15)
				37.6 (40.8)		37.6 (42.7)		38.1 (43.1)

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

TABLE 3

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

Concentration ratios were determined after simulation of multiple dosing of ribavirin to steady state (τ , 12 h) for various values of systemic (plasma) ribavirin and erythrocyte-phosphorylated metabolite half-lives (t1/2_{sys} and t1/2_{deg}, respectively) for erythrocytes from Ent1(+/+) or Ent1(-/-) mice.

t1/2 _{sys}	t1/2 _{sys} / τ	Ratio	Steady-State or Single Dose AUC _{0-τ} Ratio ^a					
			t1/2 _{deg} 0.1 day		t1/2 _{deg} 1 day		t1/2 _{deg} 10 days	
			+/+	-/-	+/+	-/-	+/+	-/-
0.083 h	0.1	RBC:plasma +/+:-/-	8.9 (8.3)	0.63 (0.59)	101 (25.6)	7.1 (1.8)	971 (29.5)	67.7 (2.1)
				14.1 (14.1)		14.3 (14.4)		14.4 (14.4)
0.833 h	1	RBC:plasma +/+:-/-	13.5 (10.3)	0.94 (0.71)	137 (23.9)	9.5 (1.6)	1300 (26.7)	90.0 (1.8)
				14.4 (14.5)		14.4 (14.6)		14.4 (14.6)
8.33 h	10	RBC:plasma +/+:-/-	13.7 (9.8)	0.96 (0.67)	138 (21.7)	9.6 (1.5)	1300 (24.1)	87.1 (1.6)
				14.4 (14.5)		14.4 (14.7)		14.6 (14.7)

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

transport into the erythrocytes. These data confirm that Ent1 contributes to the rapid ex vivo uptake of ribavirin by mouse erythrocytes and that Ent1 probably contributes to ribavirin erythrocyte distribution in vivo.

Ribavirin undergoes extensive metabolism in humans. For this reason, we investigated whether the nonphosphorylated metabolites of ribavirin could inhibit mouse Ent1-mediated [³H]ribavirin transport. Because nucleotides (i.e., phosphorylated metabolites of nucleosides) have previously been shown not to be transported by ENT1 or to inhibit ENT1, it is unlikely that RMP, RDT, or RTP inhibit ENT1. Previous data on the structure-activity relationship of human ENT1 substrates suggested that RTCOOH may be either a sub-

strate or an inhibitor of Ent1/ENT1 (Chang et al., 2004). RTCOOH, TCONH₂, and TCOOH did not inhibit mouse Ent1 at concentrations up to 1 mM. Because these metabolites are most probably present in vivo at plasma concentrations substantially less than 1 mM, they are unlikely to behave as "inhibitory metabolites" with respect to Ent1 transport in the mouse.

Because ENT1/Ent1 is an equilibrative transporter, it may modulate the rate, but not the extent to which ribavirin distributes into the erythrocytes. This is illustrated by simulation of the simple distributional model (no intracellular metabolism) (Fig. 5A), where, in the absence or presence of ENT1/Ent1, the intracellular and extracellular concentra-

Fig. 8. Simulation of in vivo ribavirin plasma and phosphorylated metabolite erythrocyte concentrations. The plasma ribavirin (solid line) and erythrocyte phosphorylated metabolite (dashed line) concentration-time profile was simulated in humans dosed intravenously to steady state. K_{sys} was set at $0.000077 \text{ min}^{-1}$ ($t_{1/2}$ of 151 h) and K_{deg} was set at 0.00048 min^{-1} ($t_{1/2}$ of 1 day).

tions eventually reach equilibrium. In contrast, when there is intracellular metabolism and trapping of these metabolites (e.g., phosphorylated metabolites), the total intracellular ribavirin concentration (parent plus metabolites) increases with time even after the intracellular-extracellular ribavirin concentrations have reached equilibrium. This increase continues if extracellular drug concentration is not depleted. For this reason, these simulations predict that in the absence of Ent1, the non-Ent1 mediated distribution of ribavirin into the Ent1(−/−) erythrocytes (and subsequent metabolism there) over the long-term diminishes the initial difference in distribution of ribavirin between Ent1(+/+) and Ent1(−/−) erythrocytes. Our observations are in line with the above-mentioned predictions. The time course of [^3H] ribavirin transport by mouse and human erythrocytes between 10 s and 8 h revealed that despite apparently reaching equilibrium after 60 s of transport, ribavirin total radioactivity continued to accumulate in the erythrocytes up to 6 h in mice and humans. This is a result of a metabolic “sink” effect, whereby ribavirin is intracellularly phosphorylated and these phosphorylated metabolites are poorly cleared from the erythrocyte. In addition, with time, the magnitude of the difference in radioactivity content between the Ent1(+/+) and Ent1(−/−) erythrocytes decreased from ~26-fold at 5 min to ~3.2-fold at 8 h. Likewise, the difference in intracellular radioactivity content in human erythrocytes, in the presence and absence of 10 μM NBMRP, decreased from ~52-fold at 5 min to ~18-fold at 8 h.

We modeled the erythrocyte disposition data for both mice and humans to obtain parameter estimates for the various pathways that contribute to ribavirin disposition in erythrocytes. Our goal was to obtain values of these parameters “relative” to each other and to use them to predict the disposition of ribavirin and its metabolites in vivo. We examined various models to describe the disposition of ribavirin in erythrocytes, and the proposed model described the data best using standard diagnostic criteria (e.g., quality of fit, residual plots). A key feature of the ex vivo model was that it incorporated a time-dependent decrease in the formation of the intracellular phosphorylated metabolites. Without this feature, the intracellular

ribavirin phosphate concentration would continually increase without reaching a plateau. Incorporation of this feature in the model is reasonable, because the kinases that are responsible for the phosphorylation steps are ATP-dependent, and substantial ATP depletion has been observed in erythrocytes incubated ex vivo over similar time frames (Page and Connor, 1990; De Franceschi et al., 2000). In addition, during this 8-h time frame, this “steady state” is probably not a result of elimination of these phosphorylated metabolites, because direct measurement of the stability of RTP in human erythrocytes showed no degradation over 4 h (Page and Connor, 1990).

The parameter estimates obtained from modeling the erythrocyte data revealed that human erythrocytes have higher ENT1 activity (per 10^9 cells) than do mouse erythrocytes. In addition, the non-Ent1-mediated distribution of ribavirin into the erythrocytes, CL_{dif} , was approximately 7-fold greater in mice than in humans. It is possible that this difference may be because of a minor non-Ent1 transport pathway present in the mice that is not present (or active) in the human erythrocytes in the presence of 10 μM NBMPR. Such activity may be a result of very low Ent2 expression and activity in erythrocytes. We did not observe any significant difference in the 10-s transport activity of ribavirin in the Ent1(+/+) erythrocytes in the presence of 10 μM NBMPR and the Ent1(−/−) erythrocytes in the absence or presence of 10 μM NBMPR (Fig. 3). This further suggests that if Ent2 activity is present on the mouse erythrocytes, the activity is very low and undetectable at 10 s. In addition, Choi et al. (2004) also characterized the inhibition of [^3H] uridine transport by NBMPR in erythrocytes from Ent1(+/+) mice and detected no Ent2 type transport activity in erythrocytes from these animals. However, it is important to note that this low level of activity would not be detectable in shorter transport activity experiments and yet could be a significant contributor over longer time frames. We also observed minor differences in the magnitude of CL_{phosp} between the Ent1(+/+) and Ent1(−/−) erythrocytes. This difference may be because of small changes in the activity of the enzymes responsible for this pathway (adenosine kinase, and other kinases) because of differences in intracellular nucleoside pools that may compete for these metabolic pathways. It is not unreasonable

to speculate that these nucleoside pools may be lower in the *Ent1*($-/-$) mice resulting in increased rate of ribavirin phosphorylation.

Using the parameter estimates obtained from the *ex vivo* study, we simulated the effect of various values of systemic elimination (K_{sys}) and *in vivo* degradation of the phosphorylated metabolite (K_{deg}) on the accumulation of phosphorylated metabolites in both humans and mice after single and multiple dosing. In doing so, we assumed that there would be no ATP depletion *in vivo*; therefore, K_{inact} was set to zero. In addition, to keep the simulation simple, we assumed a simple one-compartment model for the systemic distribution and elimination of ribavirin and set the volume of this compartment large (~ 1500 -fold) compared with the erythrocyte distributional volume. As a result, the distribution of ribavirin into erythrocytes did not substantially affect its plasma concentrations. This allowed us to directly compare the differences in erythrocyte distribution in the presence and absence of ENT1/*Ent1*, because ribavirin plasma concentrations were unaffected. In humans, the reported terminal half-life of ribavirin is 27 and 151 h after single and multiple doses, respectively (Lertora et al., 1991), whereas in mice, after a single dose, we observed a half-life of ~ 4 to 16 h (unpublished observations). Therefore, for simulation of human data, we used K_{sys} values that correspond to plasma half-lives of 2.7, 27, and 151 h. For simulating mouse data, values of K_{sys} were chosen to correspond to plasma half-lives of 1, 10, and 100 h. For both humans and mice, a dosing interval of 12 h was chosen based on that used in the clinic and to reflect a practical dosing interval in a future multiple dosing/toxicity study in mice.

As expected, for a given $t_{1/2_{sys}}$ of ribavirin, as the half-life of degradation of the phosphorylated metabolites increased, both the single dose ($AUC_{0-\tau}^1$) and steady-state ($AUC_{0-\tau}^{ss}$) ratio of the erythrocyte-phosphorylated metabolite to plasma concentration of ribavirin increased. However, for a given $t_{1/2_{deg}}$ as $t_{1/2_{sys}}$ increased, the ratio of the phosphorylated metabolites of ribavirin to its plasma concentration did not change. This was because the concentrations of the phosphorylated metabolites and ribavirin increased in proportion to each other. Clinically, after multiple dosing of ribavirin in adults to steady state (400 mg b.i.d. for 10 weeks), erythrocyte concentrations of the phosphorylated metabolites of ribavirin accumulate 60-fold greater than the trough plasma ribavirin concentrations (C_{min}) (Lertora et al., 1991). In addition, in children after multiple dosing of ribavirin to steady state (6 and 10 mg/kg), the erythrocyte accumulation is 78- and 70-fold at C_{min} (Connor et al., 1993). When the $t_{1/2}$ of K_{deg} was set at 1 day, our simulations predicted a similar magnitude of accumulation (Fig. 8) of the phosphorylated metabolites in the erythrocyte. It is important to note that the accumulation of the phosphorylated metabolites depends on the relative activity of the formation pathway (CL_{phosp}) and the elimination pathway (K_{deg}) and the amount of intracellular ribavirin. This K_{deg} could represent several processes, either singly or in combination. For example, first, it could constitute the metabolism of the phosphorylated metabolites. Although Page and Connor (1990) showed no degradation of intracellular RTP over 4 h in human erythrocytes, a slow degradation of this metabolite (i.e., $t_{1/2}$ of 1 day) may not have been detectable in their *in vitro* system. Second, it could partially reflect more rapid degradation of the erythrocytes.

Healthy human erythrocytes have a fixed life span of approximately 120 days (Landaw, 1991), whereas in mice this life span is approximately 104 days (Abraham et al., 1978). Multiple dosing of ribavirin in monkeys has shown that the erythrocyte degradation was increased upon multiple dosing of ribavirin (Canonico et al., 1984). Third, it could represent the efflux of the phosphorylated metabolites by the transporters multidrug resistance protein 4 and multidrug resistance protein 5 that are expressed on erythrocytes and have been shown to transport cyclic phosphorylated nucleosides such as cAMP and cGMP (Köck et al., 2007). Fourth, it could represent the ribavirin phosphates inhibiting their own metabolism. For the purposes of the predictions made here, it is not crucial to tease out which of these mechanisms represent K_{deg} .

It is important to note that the magnitude of the accumulation of the intracellular phosphorylated metabolites observed in the *ex vivo* study is not representative of the accumulation observed after multiple dosing to steady state. This is because in the *ex vivo* study, the formation of these metabolites decreased with time, most probably as a result of the depletion of the intracellular ATP pool. We do not expect this ATP depletion to occur *in vivo*. This assumption is supported by the magnitude of accumulation of the intracellular phosphorylated metabolites observed *in vivo*. However, this accumulation does eventually reach a plateau at approximately 7 to 14 days after beginning ribavirin therapy (Lertora et al., 1991). As mentioned earlier, there are several possible mechanisms for the intracellular phosphorylated metabolite concentrations to plateau. *In vivo*, this accumulation would be further enhanced by multiple dosing, and attenuated by both the systemic elimination of ribavirin and degradation of the intracellular phosphorylated metabolites. In addition to these, it is clear from the above-mentioned simulations and modeling that *Ent1*/*ENT1* is also a major determinant of the extent of accumulation of ribavirin metabolites in erythrocytes on long-term administration of ribavirin. This is confirmed by our simulations where, on long-term administration of ribavirin, the predicted human erythrocyte-phosphorylated metabolite to plasma ribavirin concentration was ~ 30 -fold greater in the presence of ENT1 than the absence of ENT1 and ~ 15 -fold greater in *Ent1*($+/+$) mice versus *Ent1*($-/-$) mice.

Hematological toxicity of ribavirin is a dose-limiting factor in effective treatment of hepatitis C (Fried, 2002). Based on short-term transport data, ENT1 has been implicated as a major contributor to the hematological toxicity of ribavirin and its interindividual variability (Russmann et al., 2006). Our data show that, on long-term administration, ribavirin phosphates accumulate in *Ent1*($+/+$) mouse erythrocytes and that this accumulation is much greater in the presence of *Ent1* versus absence of *Ent1*. Our data also provide evidence that the rate of intracellular ribavirin phosphorylation and rate of elimination of the ribavirin phosphates may also contribute to this toxicity and its interindividual variability. Therefore, evaluating the interindividual variability in these processes may help explain and predict susceptibility to the hematological toxicity of ribavirin. Ribavirin does produce hematological toxicity in mice (Food and Drug Administration, 1998). Therefore, we predict that the toxicity of ribavirin will be significantly reduced in *Ent1*($-/-$) compared with *Ent1*($+/+$) mice, and we predict that the *Ent1*($-/-$) mouse

could serve as an excellent model to study the role of Ent1 in producing ribavirin toxicity.

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