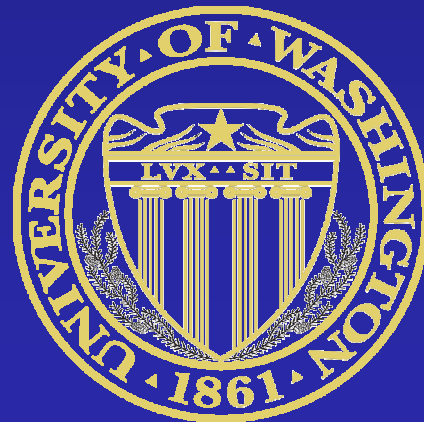


The Role of the Equilibrative Nucleoside Transporter 1 (Ent1) in Ribavirin Disposition in Mice

Christopher J. Endres

University of Washington
Department of Pharmaceutics

GPEN 2006 - Strategies in Drug Delivery: Intestines to
Intracellular Organelles



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Overview

- Nucleoside drugs such as:
 - Ribavirin
 - Gemcitabine
 - Fialuridineare substrates of the nucleoside transport systems
- These transporters may therefore contribute to the absorption, disposition, efficacy or toxicity of these drugs
 - e.g. Ribavirin: hemolytic anemia
 - Fialuridine: hepatotoxicity
- To better guide the future development of nucleoside drugs, the contribution of the nucleoside transporters to absorption, disposition, efficacy or toxicity must be characterized

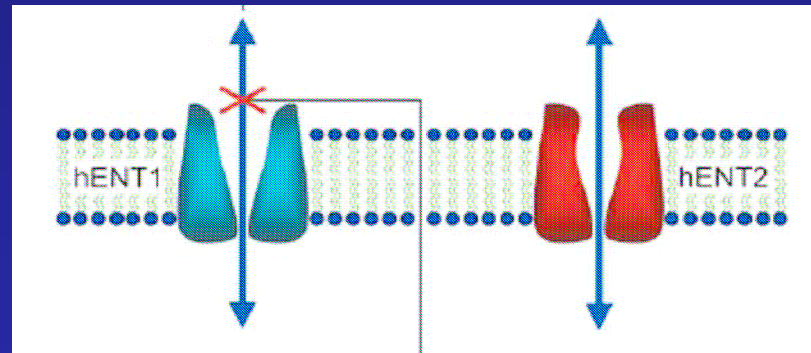
Outline

- Background
 - Nucleoside Transporters
 - Ribavirin
- Research Design, Methods and Preliminary Results
 - *Ex vivo* Erythrocyte Transport of Ribavirin
 - Pharmacokinetics and Tissue Distribution
- Conclusions

Nucleoside Transporters

- Endogenous role in nucleoside salvage for nucleotide biosynthesis and autocrine and paracrine cellular signaling
- Two major families:

Equilibrative nucleoside transporters (ENTs)

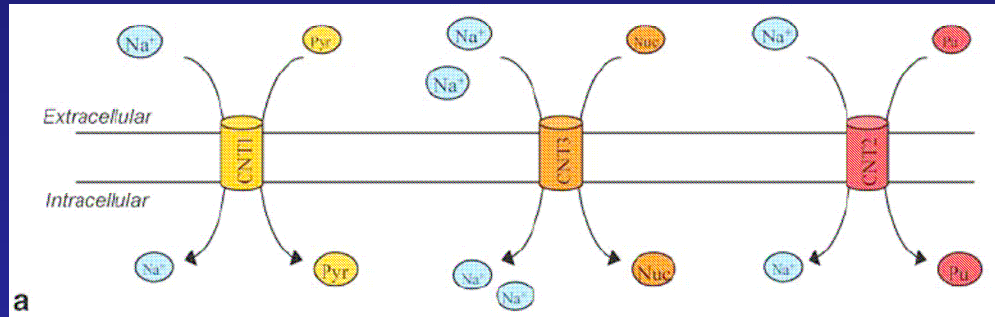


Na⁺-independent
Low affinity/high capacity
Three members: ENT1, ENT2, ENT3

Nucleoside Transporters

- Two major families:

Concentrative nucleoside transporters (CNTs)



Na⁺-dependent
High affinity/low capacity
Three members: CNT1, CNT2, CNT3

Nucleoside Transporters Function

Substrate	CNT						ENT			
	1		2		3		1		2	
	R	H	R	H	M	H	R	H	R	H
<i>Naturally occurring compounds</i>										
Adenosine	26		6	8		15		50		140
Uridine	37	45	21	80		22	150	480	300	270
Cytidine		34				15		680		5210
Thymidine	14	16				21		240		620
Inosine			28	5		53		200		50
Guanosine						43		140		2700

Substrate	CNT						ENT			
	1		2		3		1		2	
	R	H	R	H	M	H	R	H	R	H
<i>Antiviral agents</i>										
Zidovudine	500									
Zalcitabine	500							23000		
Didanosine			46	19				7400		2300
Floxidine	50							50		320
Lamivudine										
Ribavirin								1150		
Acyclovir										
Gancyclovir										
Stavudine										

Substrate	CNT						ENT			
	1		2		3		1		2	
	R	H	R	H	M	H	R	H	R	H
<i>Antineoplastic agents and metabolites</i>										
Cytarabine	1880							1500		1200
Gemcitabine		24						160		740
Cladribine			13	371				71		
Fluorouridine								50		220
5-Fluorouracil										
Capecitabine										
SdFU								18		340
Fludarabine										
Vidarabine										
Trox										
Zebularine										

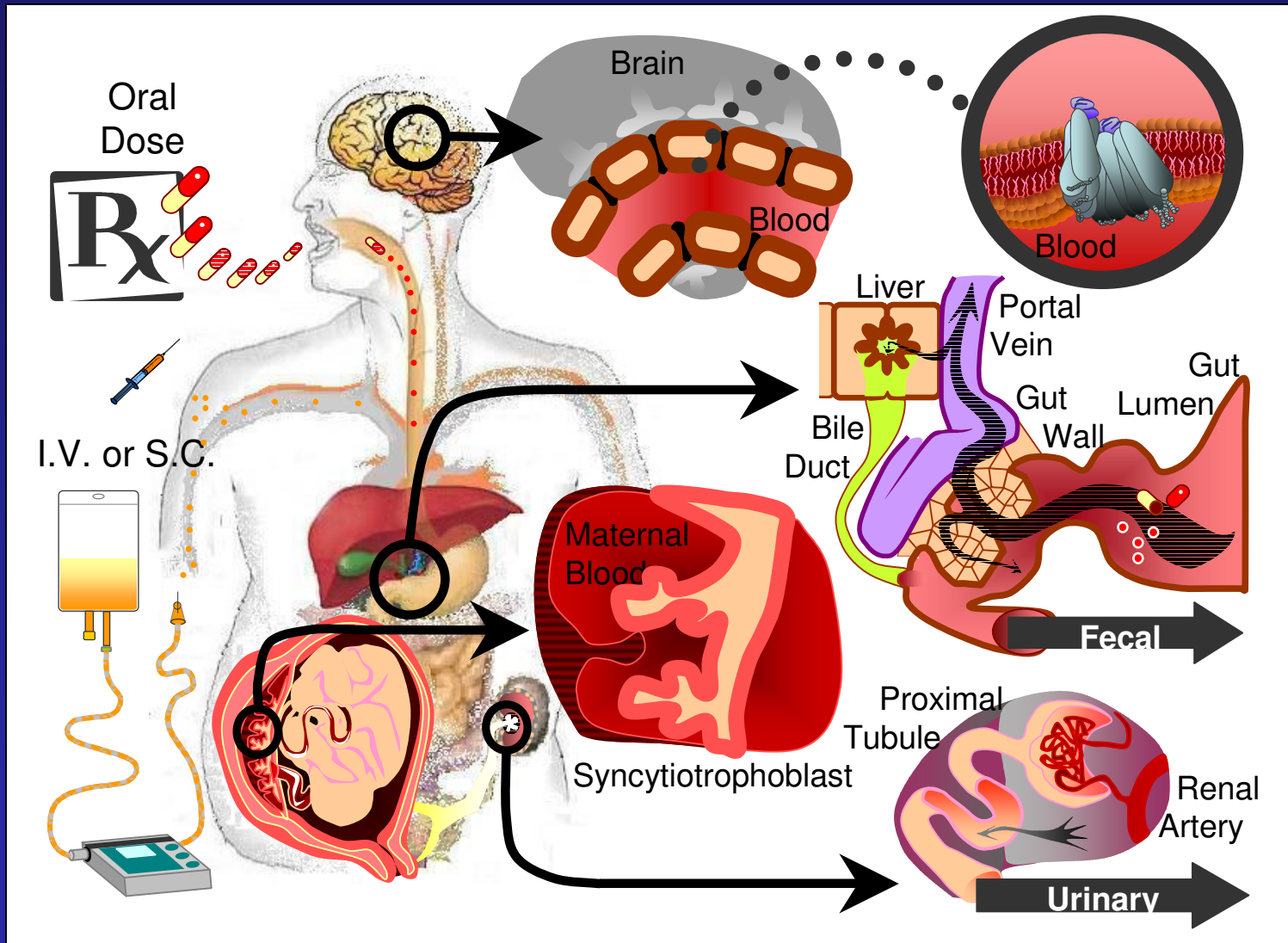
Values (in μM):
 K_m (black)
 IC_{50} (black/ital)
 K_i (blue)
 EC_{50} (green)

Yellow: Known Substrate
 Red: Known Inhibitor
 Blue: Neither
 White: Unknown

(Ribavirin IC_{50} of $[^3\text{H}]$ -inosine uptake in hENT1 expressed in yeast)

Drug Transporters

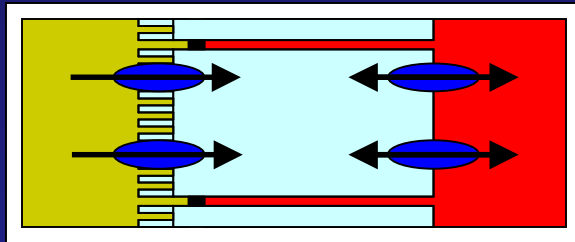
Role of Tissue Distribution on Drug Disposition



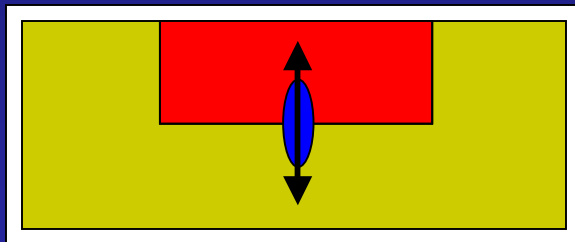
Nucleoside Transporters

Tissue Distribution

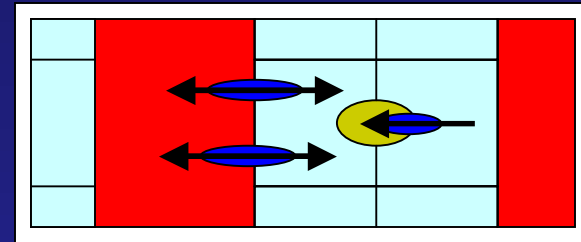
Polarized Epithelial Cells
(e.g. Intestine, Kidney)



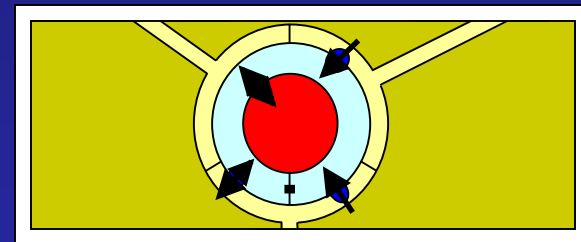
Erythrocytes



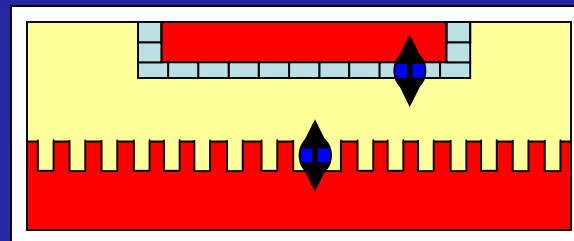
Hepatocytes



Blood Brain Barrier

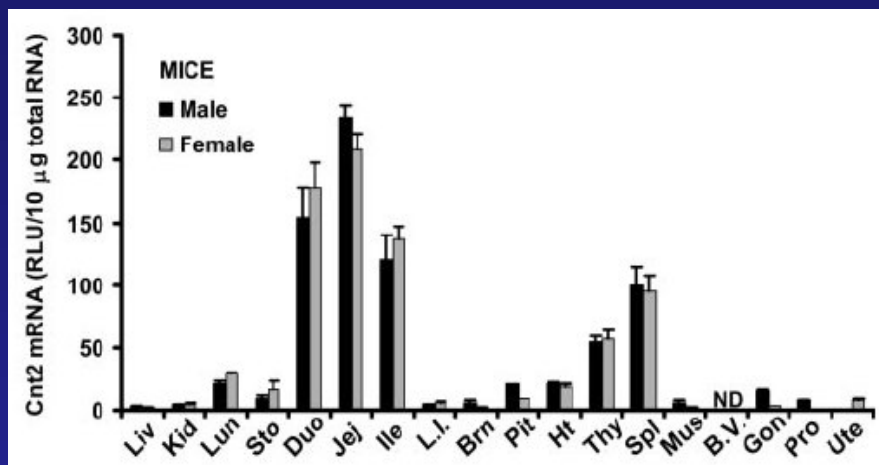


Placental Syncytiotrophoblasts

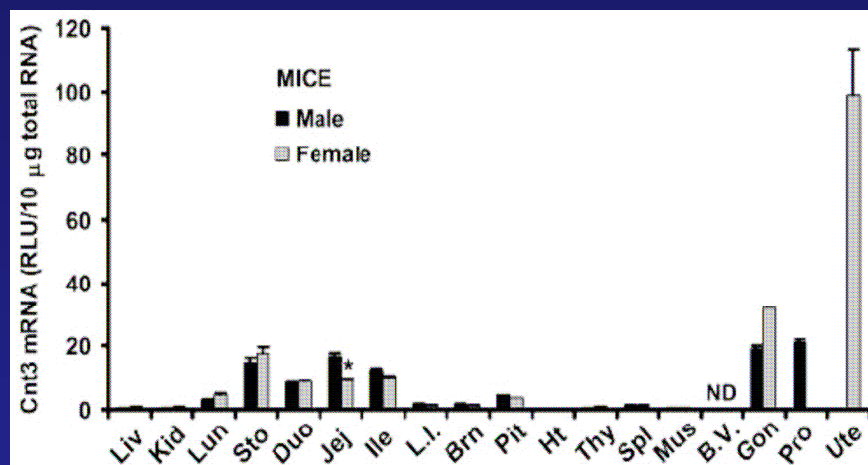


Nucleoside Transporters Mouse Tissue Distribution

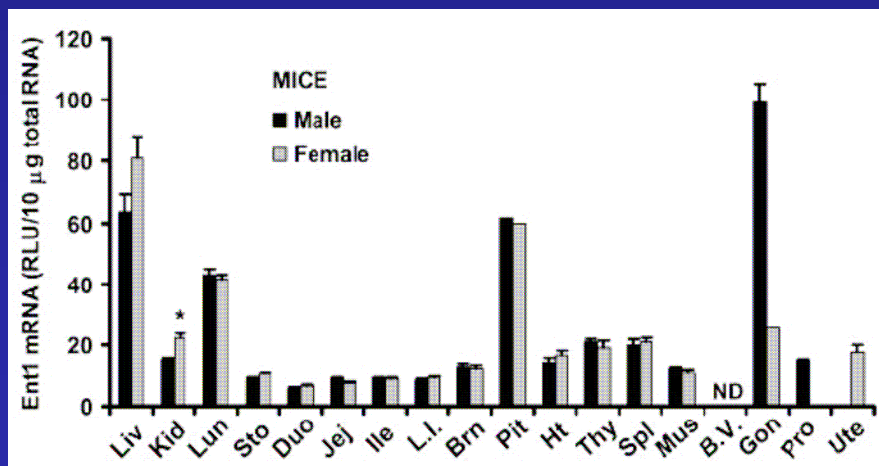
Cnt2



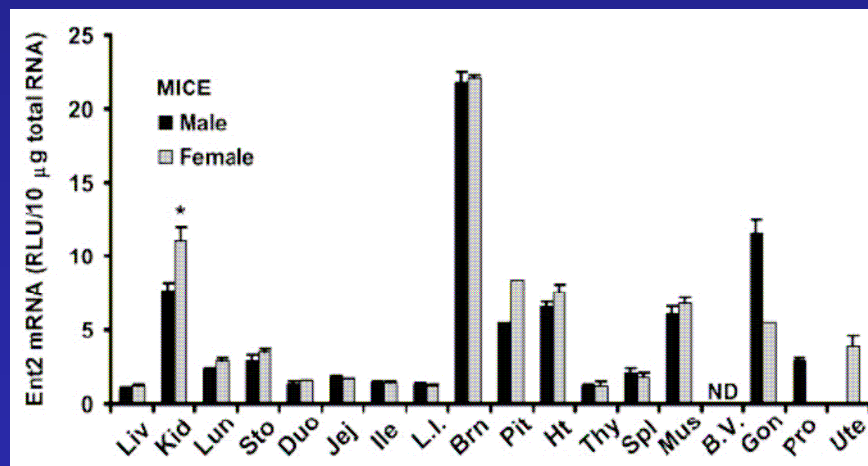
Cnt3



Ent1



Ent2



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Ribavirin

Introduction

- Ribavirin is used as first line treatment of compensated chronic hepatitis C virus (HCV) infection and is co-administered with interferon- α at doses of 800 to 1200 mg/kg/day
- The dose-limiting toxicity of ribavirin is hemolytic anemia, occurring in 10-13% of patients
- Ribavirin is contraindicated in pregnancy because of teratogenicity observed in animals and in case reports

Ribavirin

Pharmacokinetics

- The C_{\max} after a single oral dose of ribavirin was: 9.9 μM .
- After discontinuation of an intravenous infusion to steady-state, the plasma concentration time profile exhibited tri-exponential pharmacokinetics with half-lives of:
 - α : 0.2 - 0.9 hours
 - β : 1.6 - 2.0 hours
 - γ : 35.5 – 60 hours
- After discontinuation of oral dosing to steady-state, the terminal elimination half-life was:
 - λ_z : 298 hoursand ribavirin steady-state concentrations exhibited approximately 6-fold accumulation (compared to after a single oral dose).

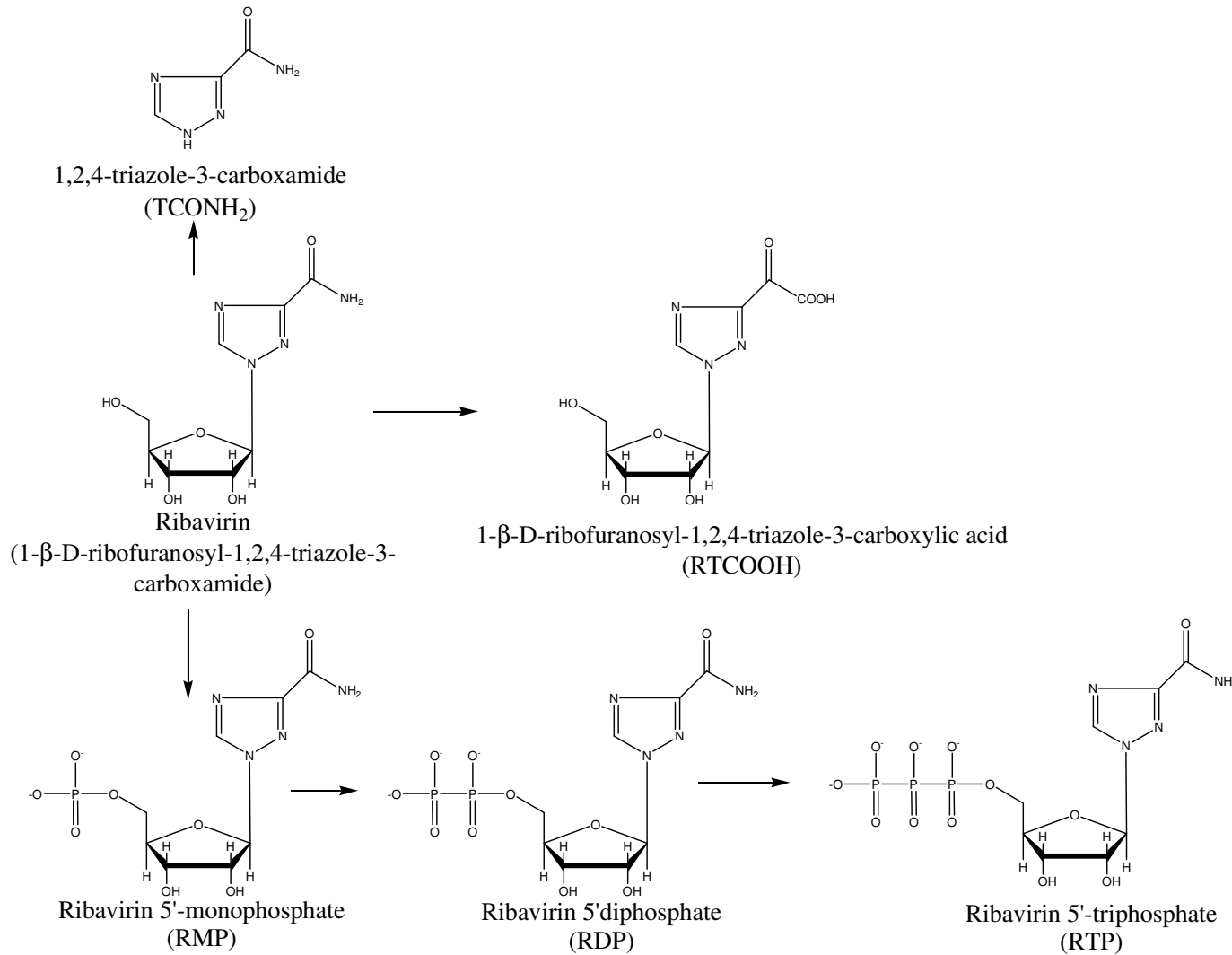
Ribavirin

Pharmacokinetics

- The systemic clearance of ribavirin is 280-400 mL/min
- The renal clearance of ribavirin is 99 mL/min
- Ribavirin is completely unbound ($0 \pm 7\%$), and is not saturated between 1 and 50 μM
- The V_{ss} of ribavirin after oral dosing is approximately 650 to 1100 L
- The oral bioavailability of ribavirin is 33 to 64%

Ribavirin

Metabolism and Distribution

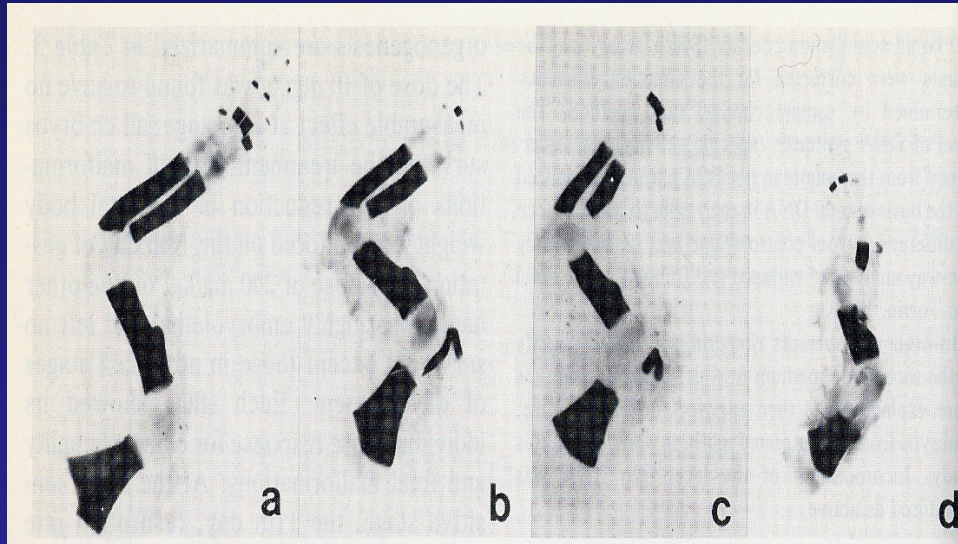


Ribavirin

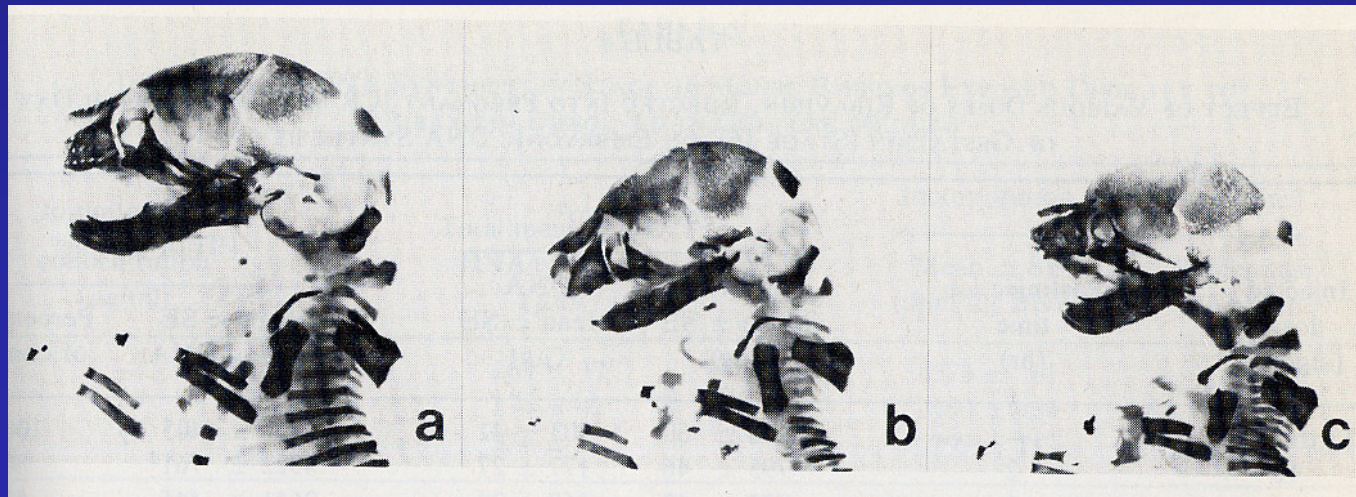
Metabolism and Distribution

- In *M. mulatta* (Rhesus macaques) 8 hours after receiving [¹⁴C]-ribavirin by intramuscular and intravenous administration, the recovery of total radioactivity in the major organs of distribution was (% of total body radioactivity):
 - 37.1 % in skeletal muscle
 - 14.1 % in erythrocytes
 - 8.1 % in the liver
- minor amounts were recovered in:
 - 1.0 % kidneys
 - 0.5 % brain

Ribavirin Fetal Toxicity



Single Dose (mg/kg)	% Fetuses Resorbed	% Surviving Fetuses Malformed
0	0	0
10	0	0
25	0	0
50	42	41
100	44	77
200	100	NA

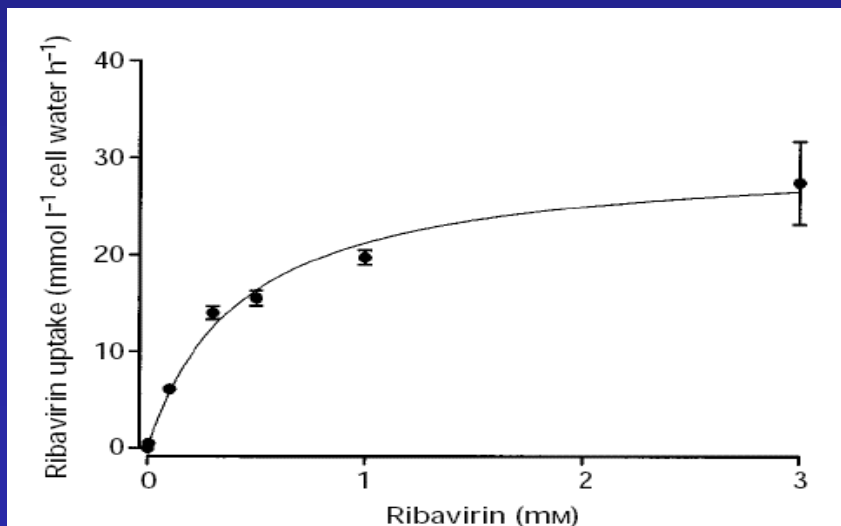
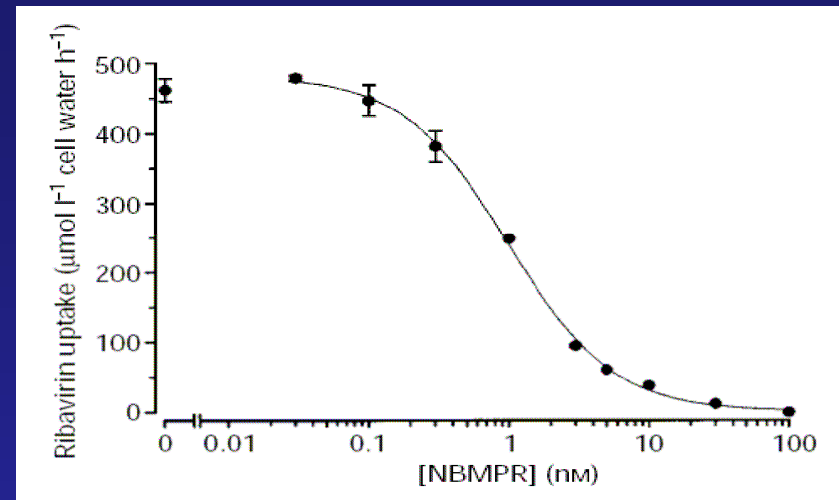
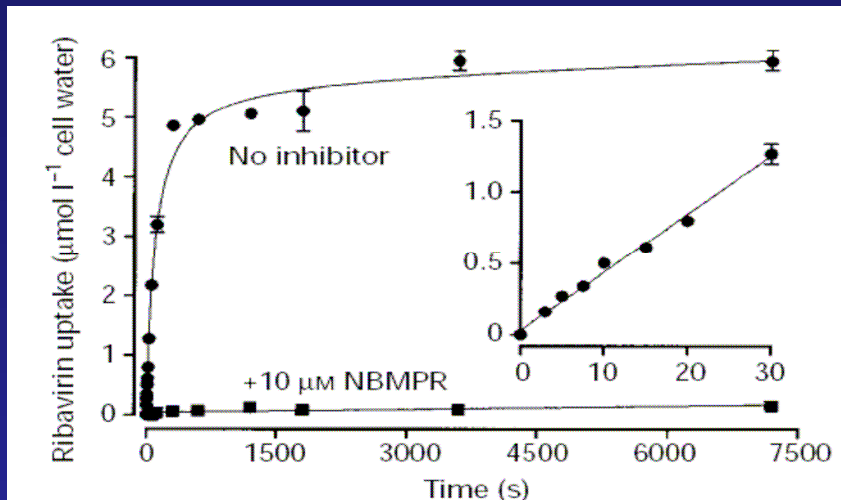


Christopher J. Endres

16 Kochhar, D.M., et al. *Tox. Appl. Pharmacol.* (1980) **52**: 99-112
Kochhar, D.M. *Pediat. Infect. Dis. J.* (1990) **9**: S88-S90

Nucleoside Transporters

Ribavirin Transport: hENT1



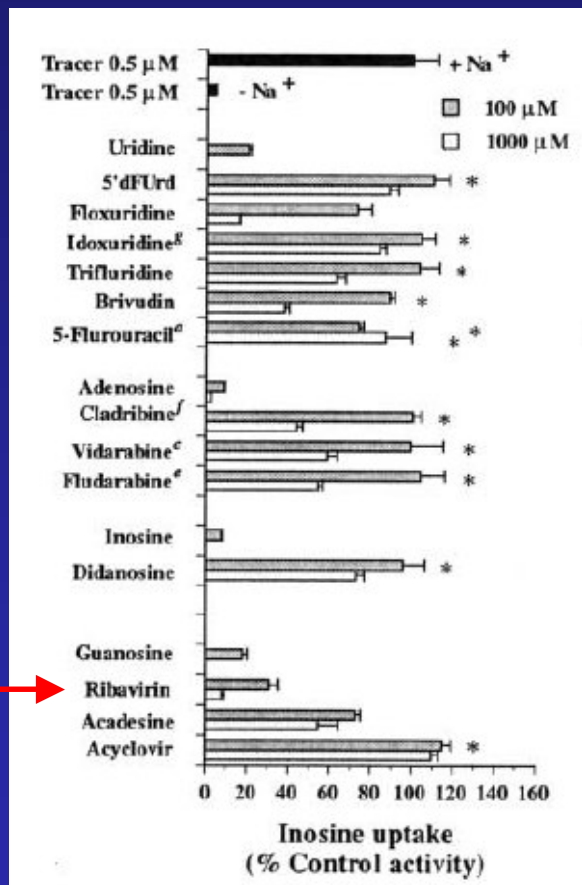
Ribavirin
 V_{max} : 30 ± 1.6 mmol/hr/L cell water
 K_m : 420 ± 67 μM

NBMPR
 IC_{50} : 0.99 ± 0.05 nM

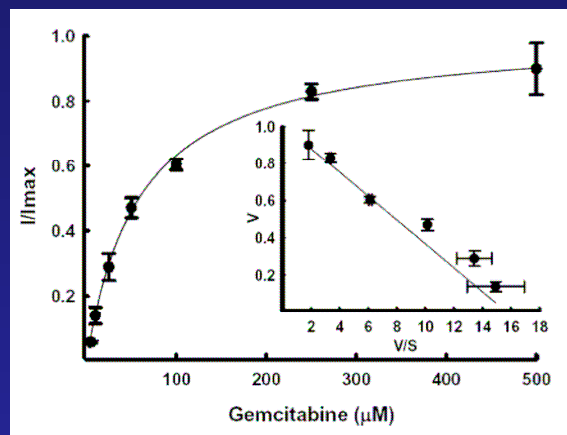
Nucleoside Transporters

Ribavirin Transport: hCNTs

hCNT2 Mediated [³H]-inosine uptake in human BBMVs



Ribavirin stimulated inward current in *X. laevis* oocytes expressing hCNT3



Ribavirin
 V_{max} : 638 ± 58.1 nA
 K_m : 59.7 ± 17.5 μ M

Inhibition of [³H]-uridine transport, and ribavirin stimulated inward current in *X. laevis* oocytes expressing hCNT1

Conc. (μ M)	% Inhibition	I_{RBV}/I_{URI}
1000	51 ± 7	0.06 ± 0.01

Hypothesis

“The mouse equilibrative nucleoside transporter 1 (mEnt1) significantly contributes to the absorption, tissue distribution, elimination and toxicity of ribavirin.”

Specific Aims

1. To characterize the *ex vivo* transport of [³H]-ribavirin in erythrocytes from mEnt1(+ / +) and mEnt1(- / -) mice.
2. To characterize the pharmacokinetics and tissue distribution of ribavirin in mEnt1(+ / +) and mEnt1(- / -) mice.

Outline

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Ex vivo Erythrocyte Transport of Ribavirin

Introduction

- The goal of this aim is to characterize the transport kinetics of ribavirin in erythrocytes from mEnt1(+/-) and mEnt1(-/-) mice.
- The:
 - time-course
 - activity
 - and kinetics

were examined.

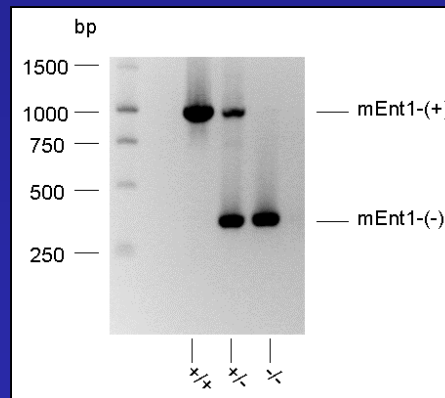
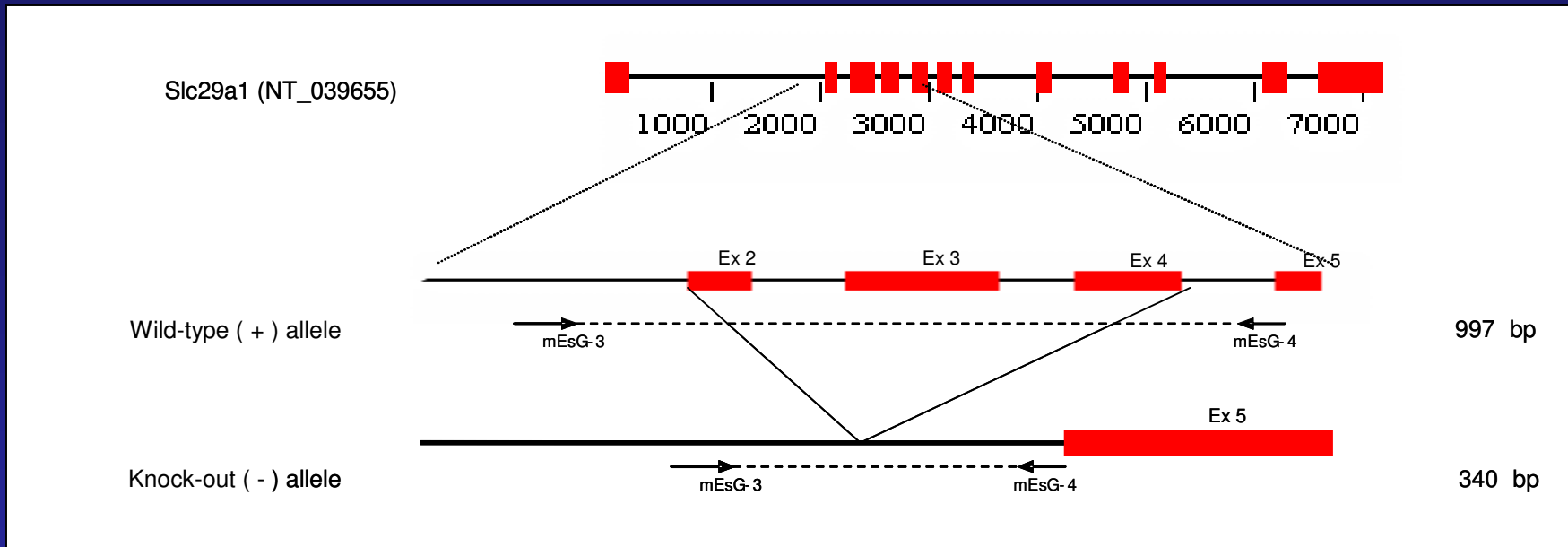
Ex vivo Erythrocyte Transport of Ribavirin

Experimental Design

- Mouse erythrocytes were washed in transport buffer and used within 2 hours of collection
- Transport was initiated by adding 0.77 μM [^3H]-ribavirin to erythrocytes in transport buffer (20% hematocrit), and stopped by dilution the suspensions 5-fold in transport buffer containing 10 mM NBMPR. The erythrocytes were immediately pelleted into an oil-layer (“oil-stop”), to limit further diffusional uptake
- Transport rates were calculated and normalized to total total protein amount
- Kinetic parameters (V_{max} and K_m) were determined using a “tracer-displacement” analysis

Ex vivo Erythrocyte Transport of Ribavirin

PCR Genotyping Assay of mEnt1 in Mice

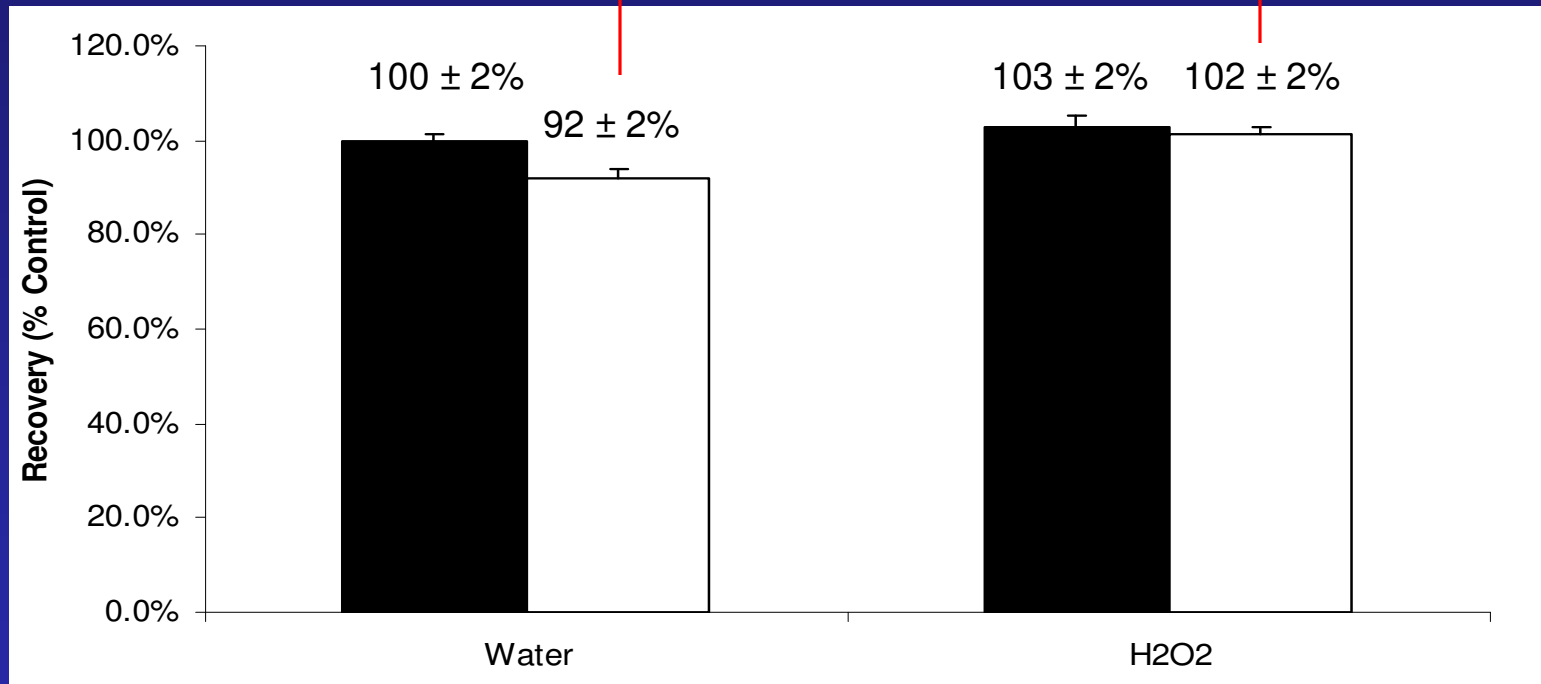


Ex vivo Erythrocyte Transport of Ribavirin

Spike Recovery from Erythrocytes

Color quenching by erythrocytes reduced recovery of erythrocyte spikes

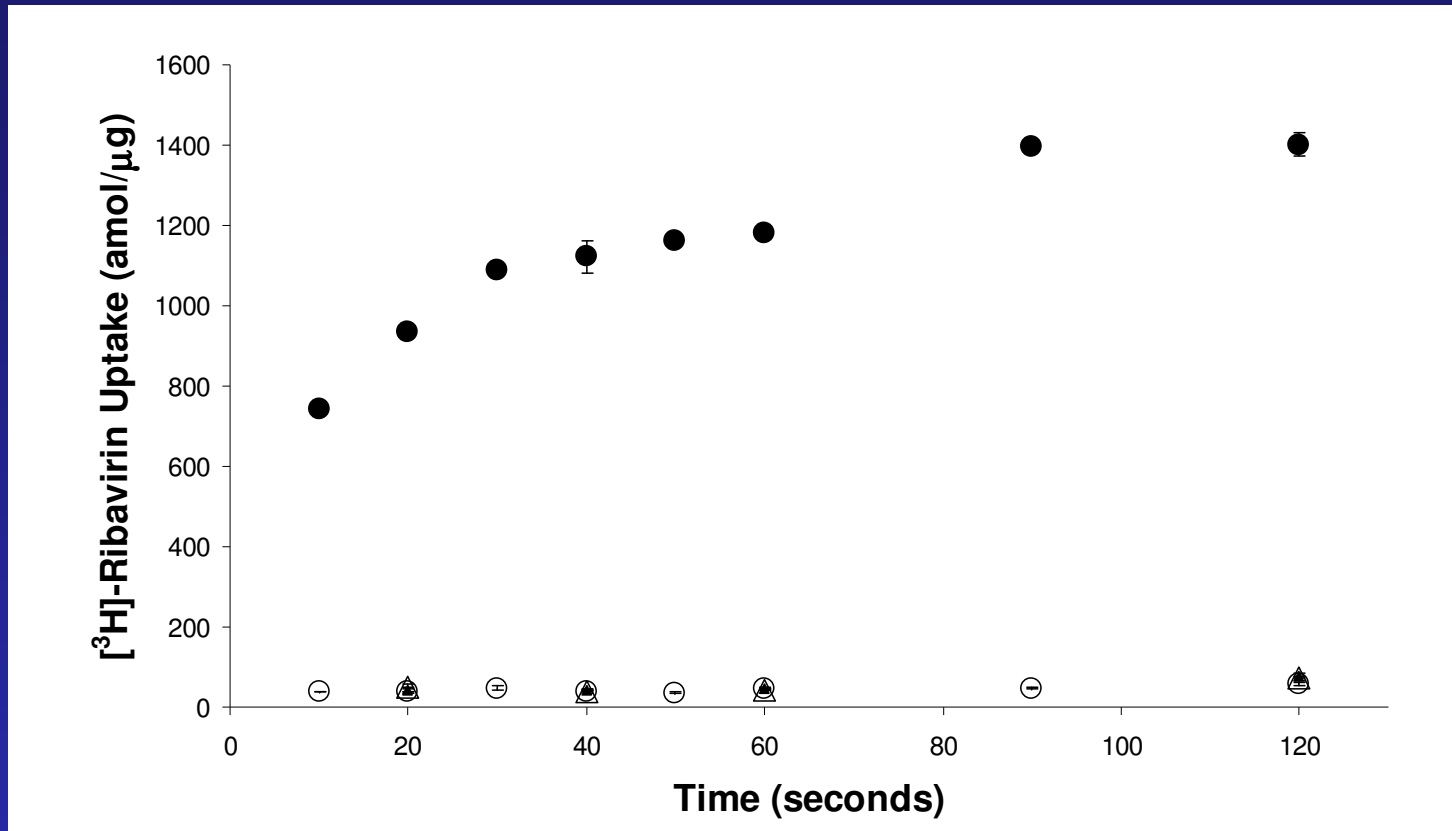
Decolorization with 30% H₂O₂ restores reduced recovery of erythrocyte spikes



Solid Bars: Water Spike
Open Bars: Erythrocyte Spike

Ex vivo Erythrocyte Transport of Ribavirin

Time Course of Erythrocyte Ribavirin Uptake



Filled: Vehicle

Open: 10 μM NBMPR

mEnt1(+/+): circles

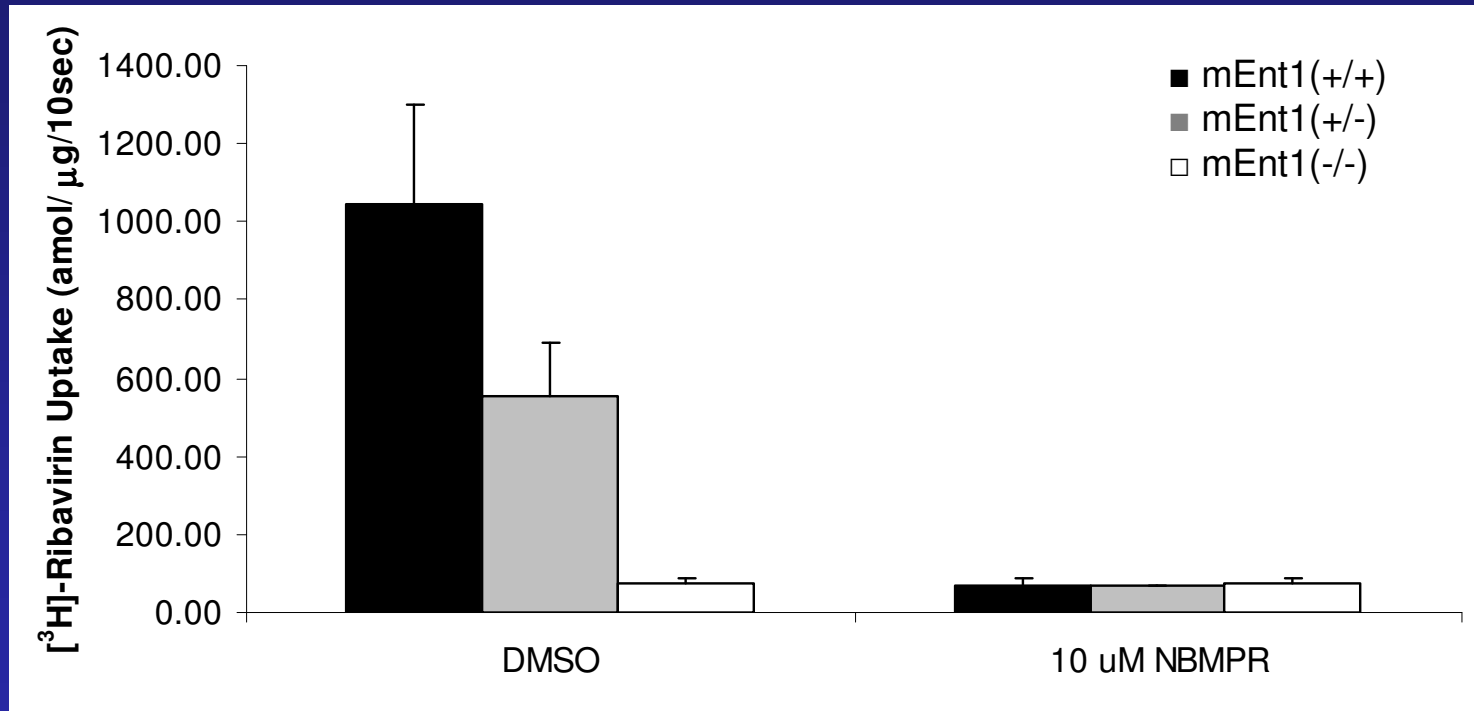
mEnt1(-/-): triangles

Values represent mean ± S.D. (n=3)

Ex vivo Erythrocyte Transport of Ribavirin

Ribavirin Transport Activity in Erythrocytes

[³H]-Ribavirin Uptake After 10 Seconds of Transport

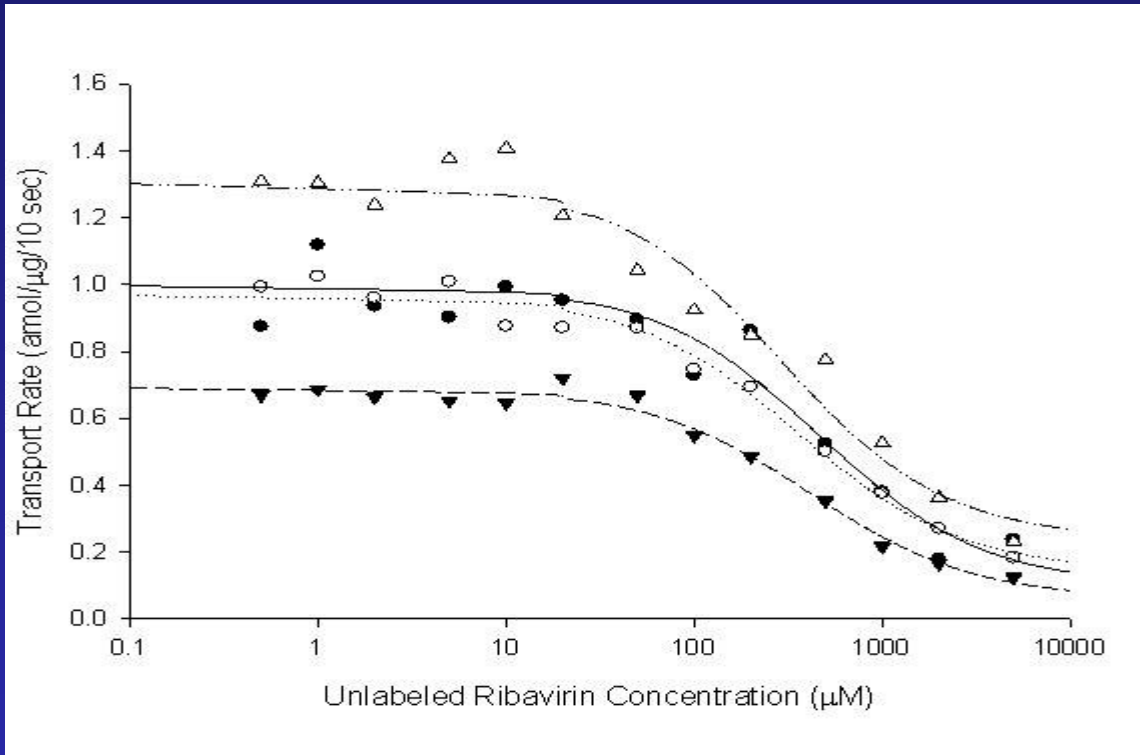


Values represent mean ± S.D. (n=3)

Ex vivo Erythrocyte Transport of Ribavirin

Ribavirin Transport Kinetics in Erythrocytes

Tracer Displacement Kinetics Analysis



V_{max}
 $417 \pm 86.7 \text{ amol}/\mu\text{g}/10 \text{ sec}$

K_m
 $382 \pm 75.1 \mu\text{M}$

Mean \pm S.D. (n=4)

Values represent mean \pm S.D. (n=3)

Ex vivo Erythrocyte Transport of Ribavirin

Conclusions

- The time-course of [³H]-ribavirin uptake in mEnt1(+/-) erythrocytes rapidly reaches equilibrium within 60 seconds, and was completely inhibited by 10 μM NBMPR.
- There is no NBMPR inhibitable [³H]-ribavirin transport in erythrocytes from mEnt1(-/-) mice.
- The transport activity of the mEnt1(+/-) erythrocytes was approximately half that of the mEnt1(+/+) erythrocytes, suggesting a gene-dose effect on activity.
- The K_m of [³H]-ribavirin transport in mEnt1(+/-) erythrocytes was similar to that observed in humans (420 μM).

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Pharmacokinetics and Tissue Distribution

Introduction

- The goal of this aim is to characterize the pharmacokinetics and tissue distribution of ribavirin in mEnt1(+/-) and mEnt1(-/-) mice.
- We will estimate pharmacokinetic parameters after both oral and intravenous administration of [³H]-ribavirin
- We will examine the tissue distribution of [³H]-ribavirin after intravenous administration in tissues where mEnt1 expression may be important in drug distribution:
 - i.e. liver, intestine, kidney, brain, skeletal muscle, heart and pancreas

Pharmacokinetics and Tissue Distribution

Experimental Design

- [³H]-Ribavirin was administered orally (10 μg/g) or intravenously (3 μg/g) to mEnt1(+ / +) and mEnt1(- / -) mice.
- Plasma, and erythrocyte samples were obtained by retro-orbital bleeding 15, 30, 60, 120, 240, 480, 720 and 1440 minutes after administration
- Ribavirin concentrations were determined by:
 - Direct counting for total radioactivity
 - HPLC / Fraction collection to determine percent ribavirin composition of the total radioactivity (t.b.d)

Pharmacokinetics and Tissue Distribution

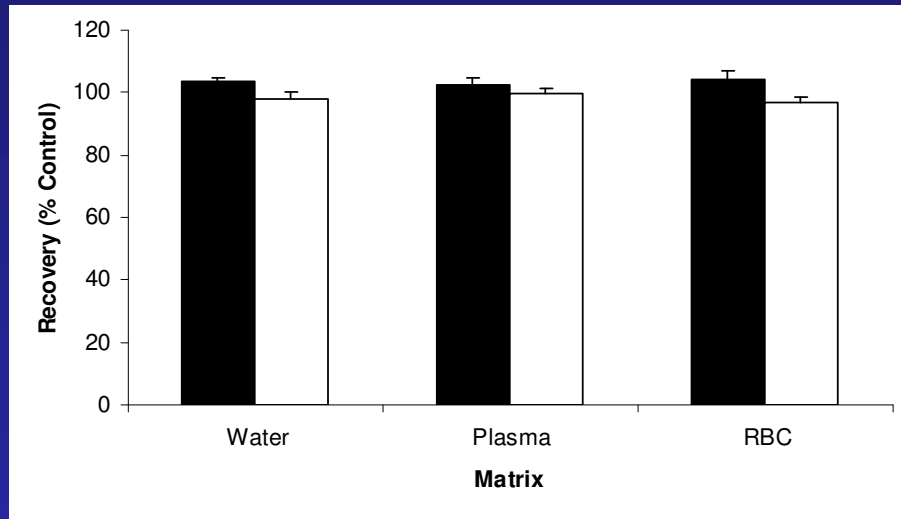
Experimental Design

- Tissues were collected at necropsy and concentrations were determined using a similar methodology (direct counting/HPLC fraction collection).
- [^{14}C]-Sucrose (tracer dose, $0.05 \mu\text{Ci/g}$) was administered to correct tissue ribavirin concentrations for ribavirin present in the tissue vascular volume.

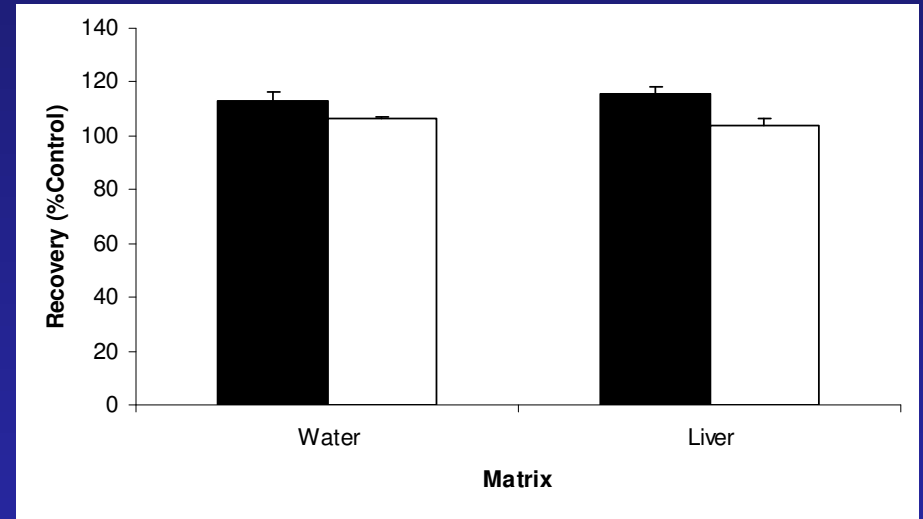
Pharmacokinetics and Tissue Distribution

Direct Counting Fluid and Tissue Spike Recovery

Fluid Spike Recovery



Tissue Spike Recovery



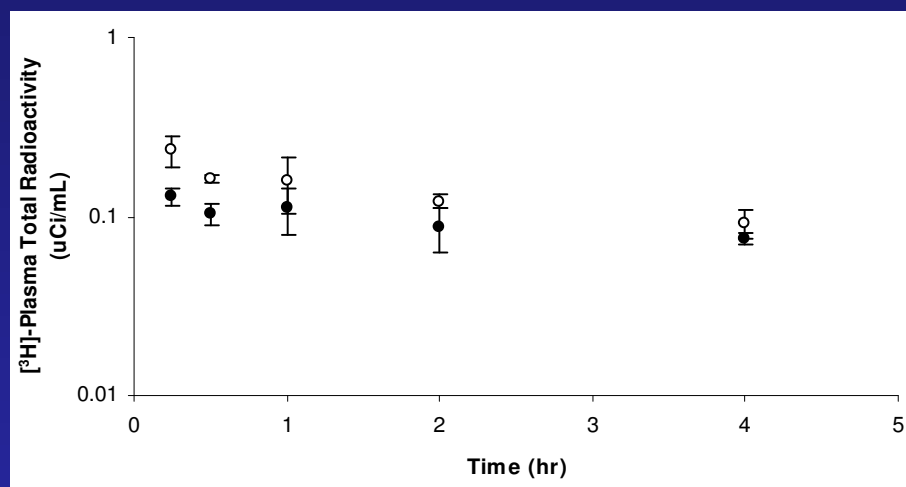
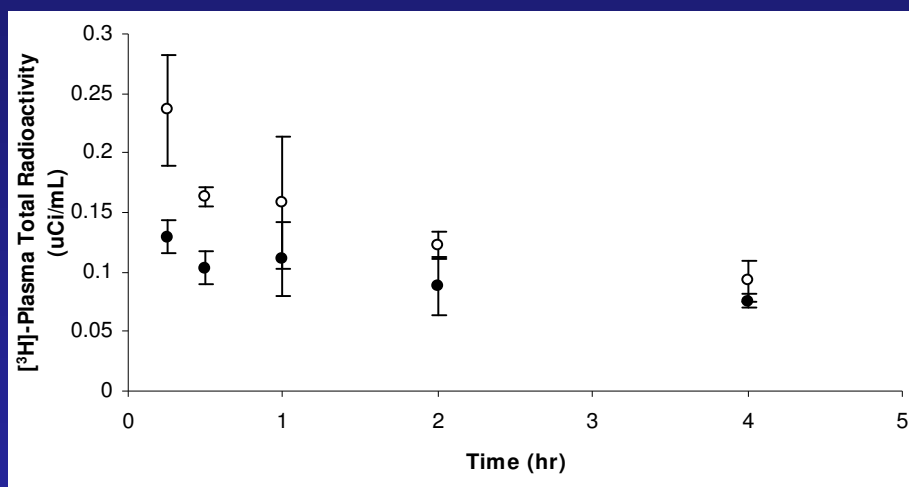
Solid bars: [³H]-Ribavirin
Open bars: [¹⁴C]-Sucrose

Values represent mean ± S.D. percent recovery
n=3

Pharmacokinetics and Tissue Distribution

Plasma Pharmacokinetics: [³H]-Total Radioactivity

Intravenous [³H]-Rivavirin Dose (3 μg/g; 0.4 μCi/g)



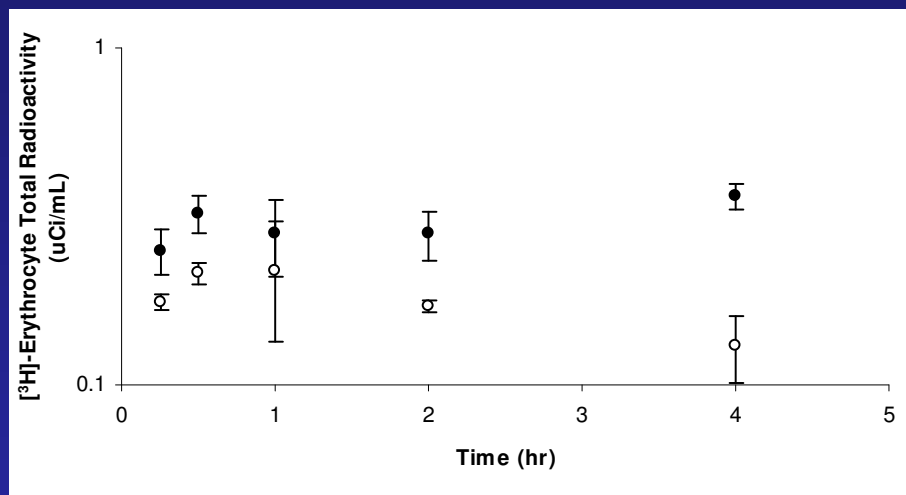
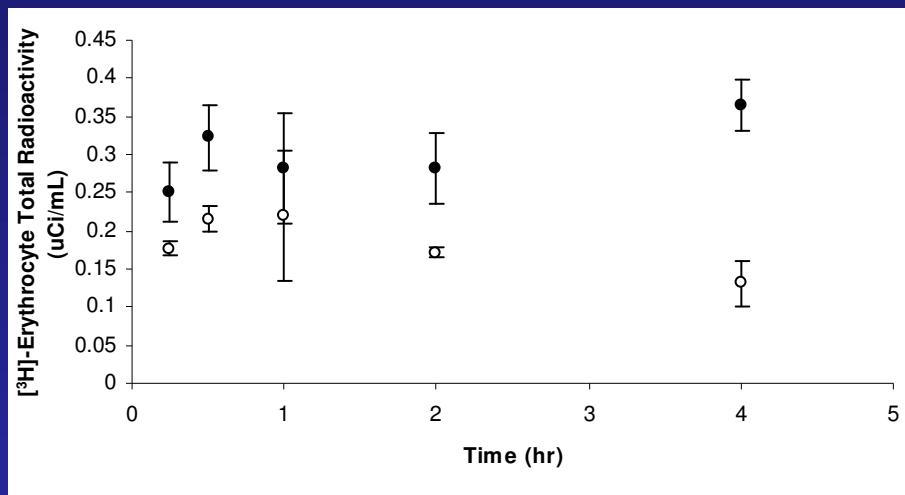
Filled Circles: mEnt1(+/+)
Open Circles: mEnt1(-/-)

Values represent mean ± S.D. percent recovery
n=2-3

Pharmacokinetics and Tissue Distribution

Erythrocyte Pharmacokinetics: ^3H -Total Radioactivity

Intravenous [^3H]-Rivavirin Dose ($3 \mu\text{g/g}$; $0.4 \mu\text{Ci/g}$)



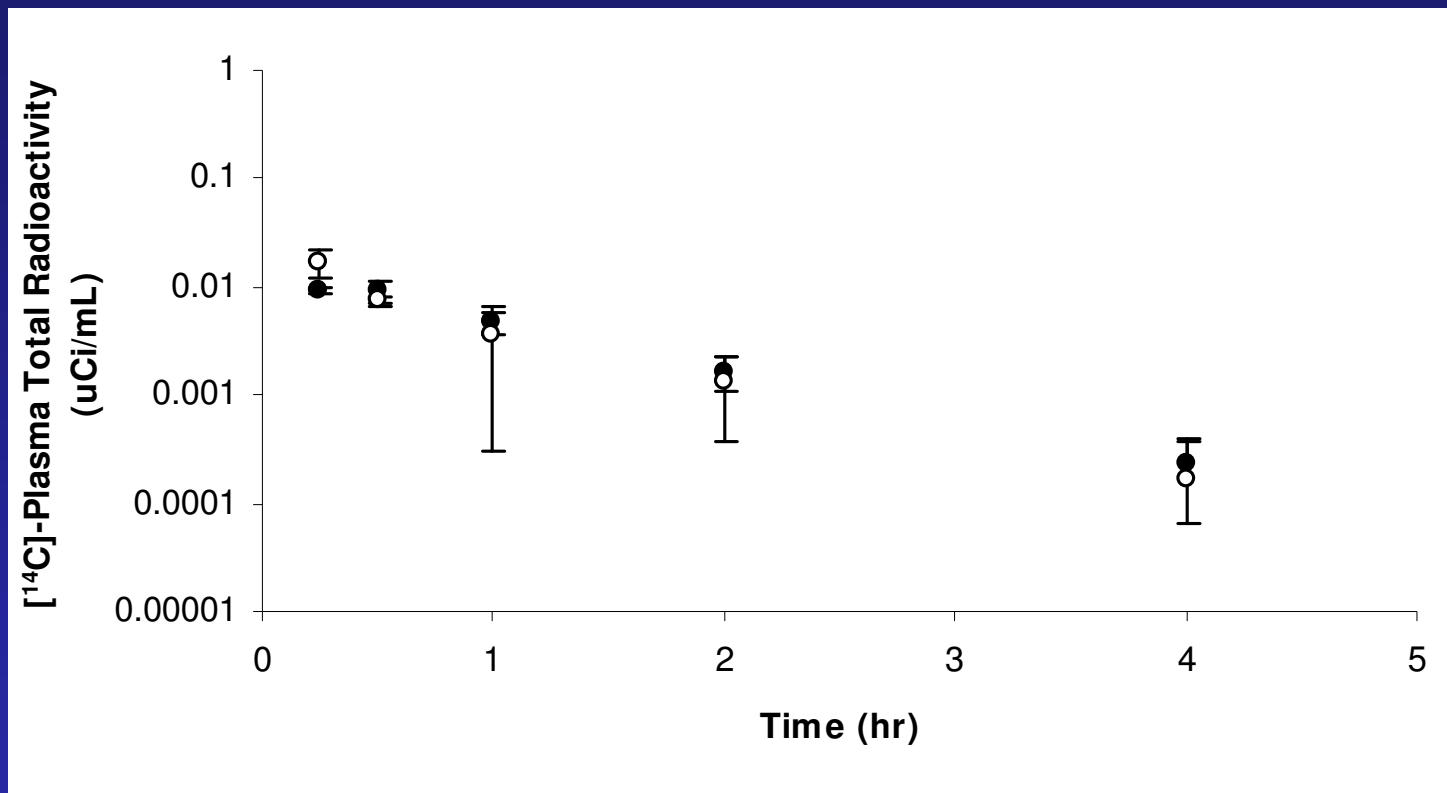
Filled Circles: mEnt1(+/+)
Open Circles: mEnt1(-/-)

Values represent mean \pm S.D. percent recovery
n=2-3

Pharmacokinetics and Tissue Distribution

Plasma Pharmacokinetics:¹⁴C-Total Radioactivity

Intravenous [¹⁴C]-Sucrose Dose (0.05 μCi/g)



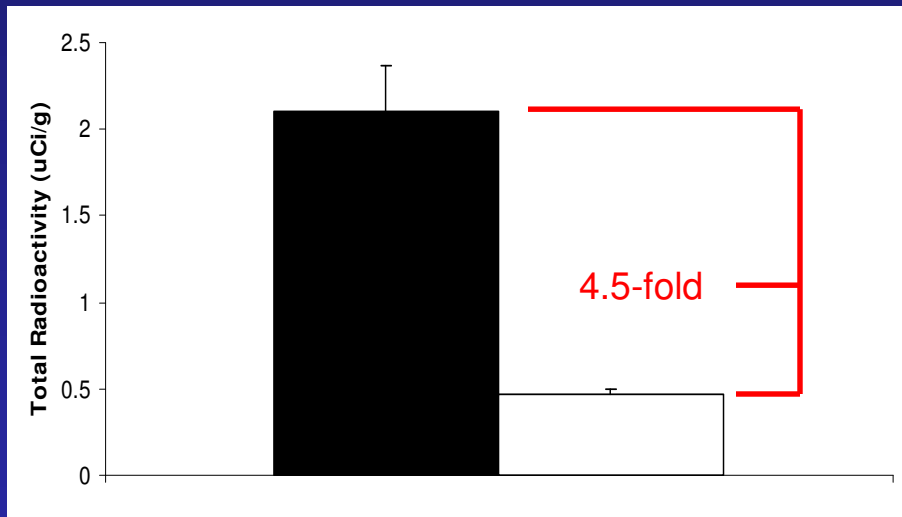
Values represent mean ± S.D. percent recovery, n=2-3

Pharmacokinetics and Tissue Distribution

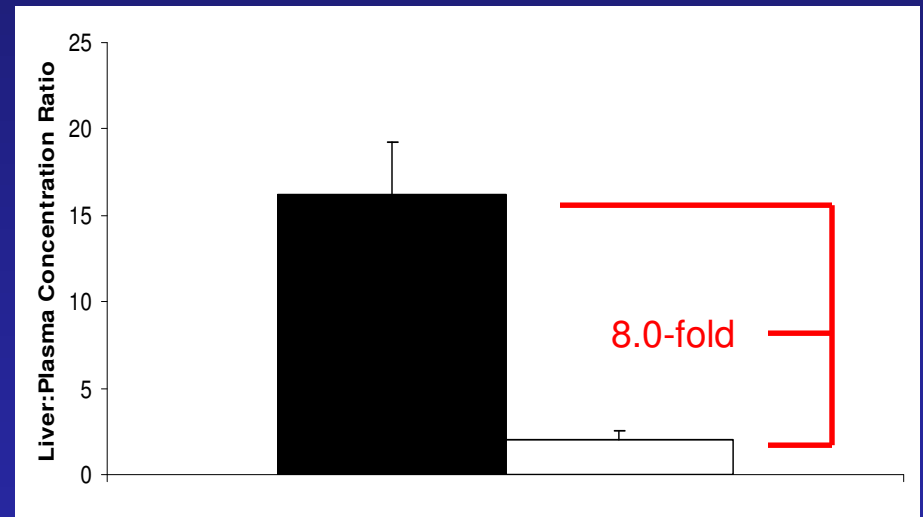
Liver Distribution: [³H]-Total Radioactivity

Liver [³H]-Total Radioactivity Concentration (μCi/g tissue) 15 Minutes After 3 μg/g (0.4 μCi/g) [³H]-Ribavirin Retro-Orbital Injection

Total Radioactivity



Liver : Plasma Ratio



Solid Bars: mEnt1(+/+)
Open Bars: mEnt1(-/-)

Data correct for vascular contribution of [³H]-total radioactivity
Values represent mean ± S.D. percent recovery
n=3

Pharmacokinetics and Tissue Distribution

Conclusions

- There is an 8-fold decrease in hepatic distribution in the mEnt1(-/-) mice
 - This is consistent with mEnt1 expression on the hepatic sinusoidal membrane,
 - and mEnt1 mediated hepatic uptake of ribavirin

Pharmacokinetics and Tissue Distribution

Conclusions

- The plasma [³H]-total radioactivity concentrations were greater in the mEnt1(-/-) mice than the mEnt1(+/-) mice consistent with decreased distribution to the peripheral compartment(s) in the mEnt1(-/-) mice.
- The erythrocyte [³H]-total radioactivity concentrations were greater in the mEnt1(+/-) mice than the mEnt1(-/-) mice, consistent with reduced uptake in the mEnt1(-/-) mice. Surprisingly, the magnitude of this difference was much smaller than that observed *in vitro*.

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General Conclusions

- mEnt1 contributes to erythrocyte uptake of ribavirin *ex vivo*.
- mEnt1 significantly contributes to the hepatic and erythrocyte distribution of ribavirin *in vivo*
- Because of this, the ENT1 may also play an important role in other tissue distribution, elimination and toxicity of ribavirin and other nucleoside drugs.

Future Directions

- Validate HPLC assay for [^3H]-ribavirin quantitation from fluids and tissue
- Complete I.V. ribavirin pharmacokinetics and tissue distribution
- Complete oral ribavirin pharmacokinetics

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GPEN 2006

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Joanne Wang, Ph.D.

Unadkat Lab

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Brian Kirby

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plank@u.washington.edu