The Role of the Equilibrative Nucleoside Transporter 1 (ENT1) in Ribavirin Disposition in Mice

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

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Department of Pharmaceutics

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

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Overview

- Nucleoside drugs such as:

  Ribavirin
  Gemcitabine
  Fialuridine

  are 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

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Outline

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

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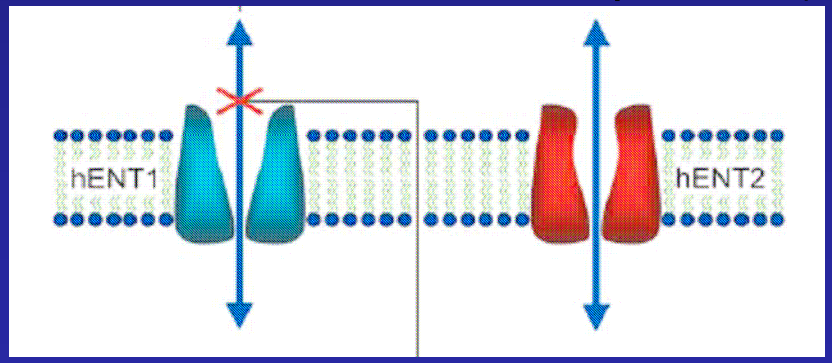
Nucleoside Transporters

- 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

Baldwin, S.A., *et al. Pflugers Arch. – Eur. J. Physiol.* (2004) **447**: 735-743

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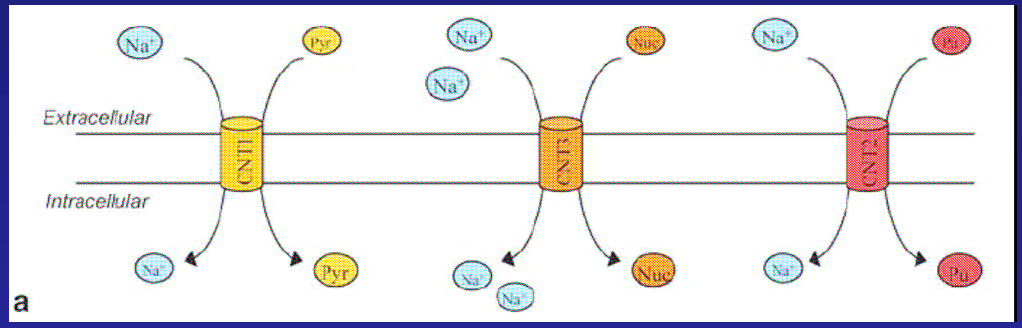
Nucleoside Transporters

- Two major families:

Concentrative nucleoside transporters (CNTs)



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

Gray, J.H., *et al. Pflugers Arch. – Eur. J. Physiol.* (2004) **447**: 728-734

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Nucleoside Transporters
Function

Substrate	CNT						ENT			
	1		2		3		1		2	
	R	H	R	H	M	H	R	H	R	H
Naturally occurring compounds										
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
Antiviral agents										
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
Antineoplastic agents and metabolites										
Cytarabine	*1880*							1500		1200
Gemcitabine		24						160		740
Cladribine			*13*	*371*				71		
Fluorouridine								50		220
5-Flurouracil										
Capecitabine										
5dFU								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}H]-inosine uptake in hENT1 expressed in yeast)

Mangrivite, L.M., *et al. Eu. J. Pharmacol.* (2003) **479**: 269-281

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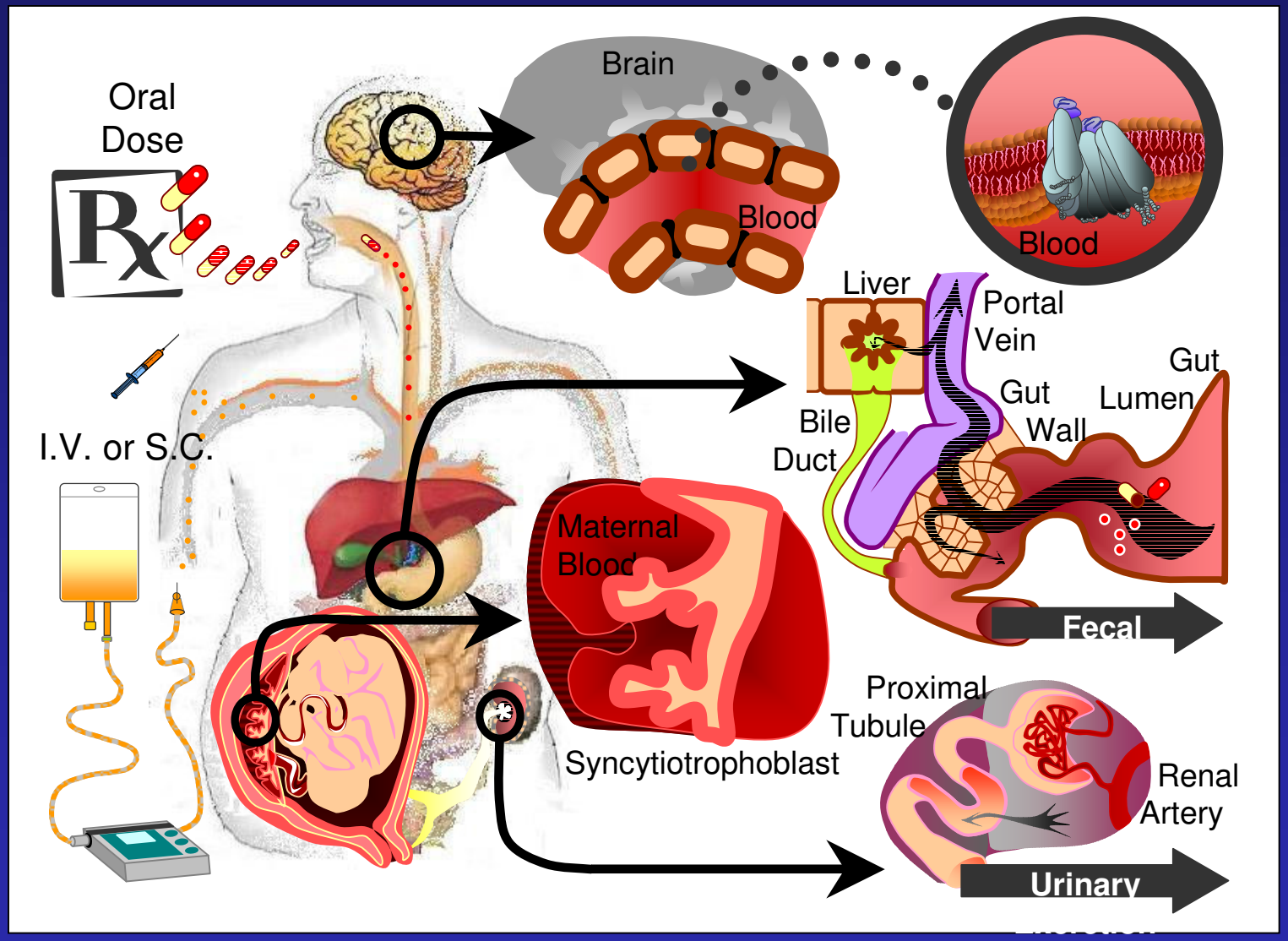
Drug Transporters

Role of Tissue Distribution on Drug Disposition



Endres, C.J., *et al. Eu. J. Pharm. Sci.* (2006) **27**: 501-517

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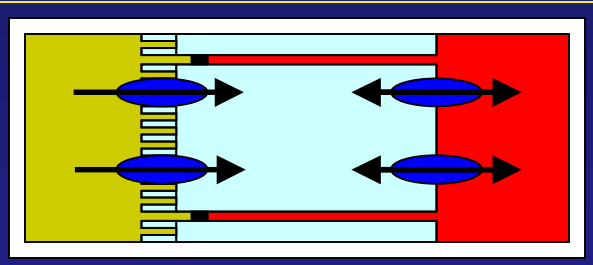
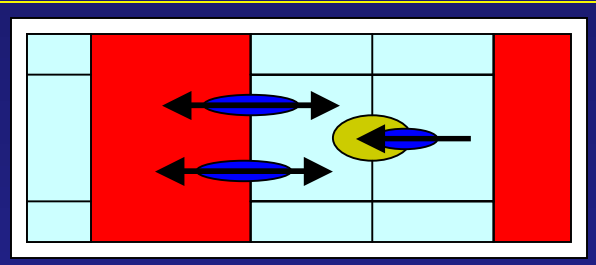
Nucleoside Transporters
Tissue Distribution

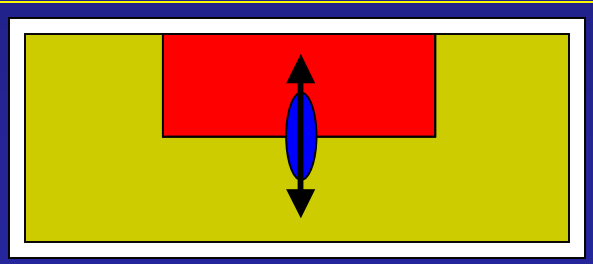
Polarized Epithelial Cells
(e.g. Intestine, Kidney)



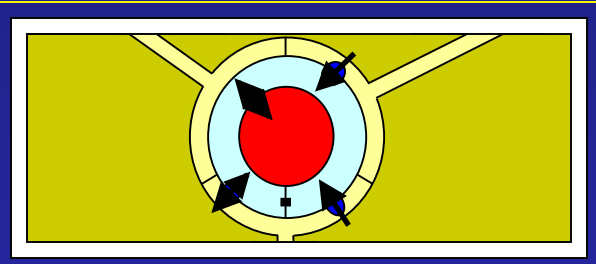
Hepatocytes



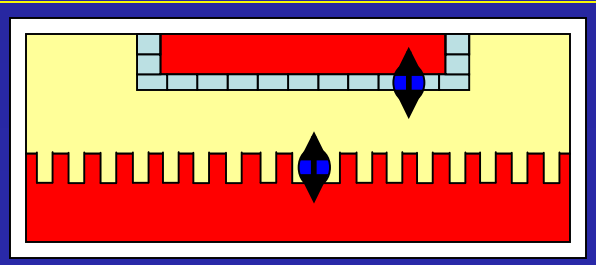
Erythrocytes



Blood Brain Barrier



Placental Syncytiotrophoblasts



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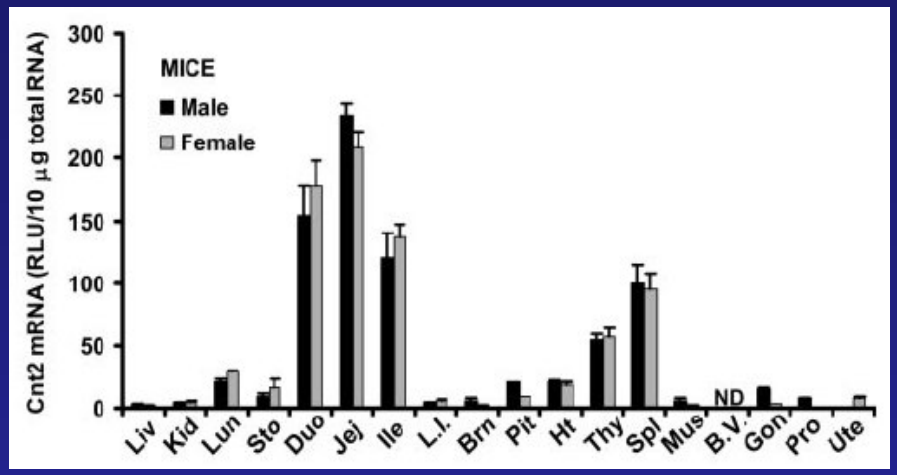
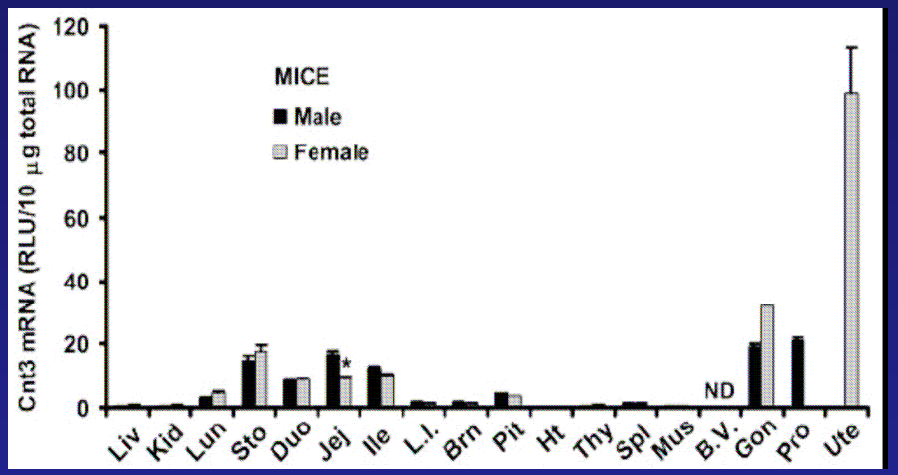
Nucleoside Transporters
Mouse Tissue Distribution

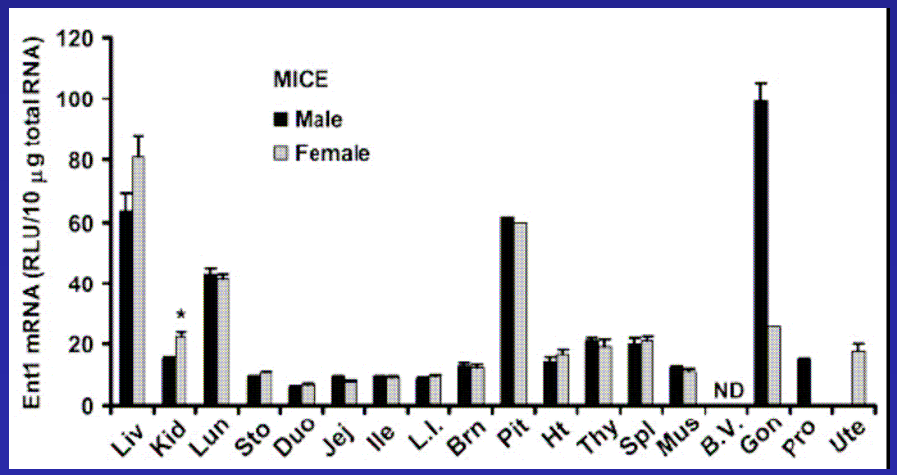
Cnt2



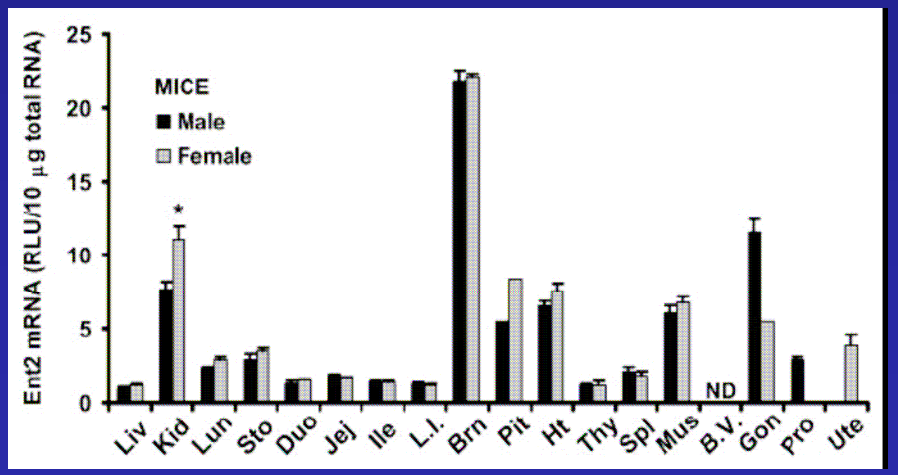
Cnt3



Ent1



Ent2



Lu, H., *et al. Drug Metab. Dispos.* (2004) **32**: 1455-1461

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Ribavirin
Introduction

- Ribavirin is used as first line treatment of compensated chronic hepatitis C virsus (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 an in case reports

FDA Revised Label. Rebetol® (2004)
FDA Revised Label. Copegus ® (2005)

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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 hours

  and ribavirin steady-state concentrations exhibited approximately 6-fold accumulation (compared to after a single oral dose).

Laskin *et al. Clin. Pharmacol. Ther.* (1987) **41**: 546-555
FDA Revised Label. Rebetol® (2004)
FDA Revised Label. Copegus ® (2005)

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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 ± 7%), and is not saturated between 1 and 50 μM
- The V_{ss} of ribavirin after oral dosing is approximatley 650 to 1100 L
- The oral bioavailability of ribavirin is 33 to 64%

Laskin *et al. Clin. Pharmacol. Ther.* (1987) **41**: 546-555
FDA Revised Label. Rebetol® (2004)
FDA Revised Label. Copegus ® (2005)

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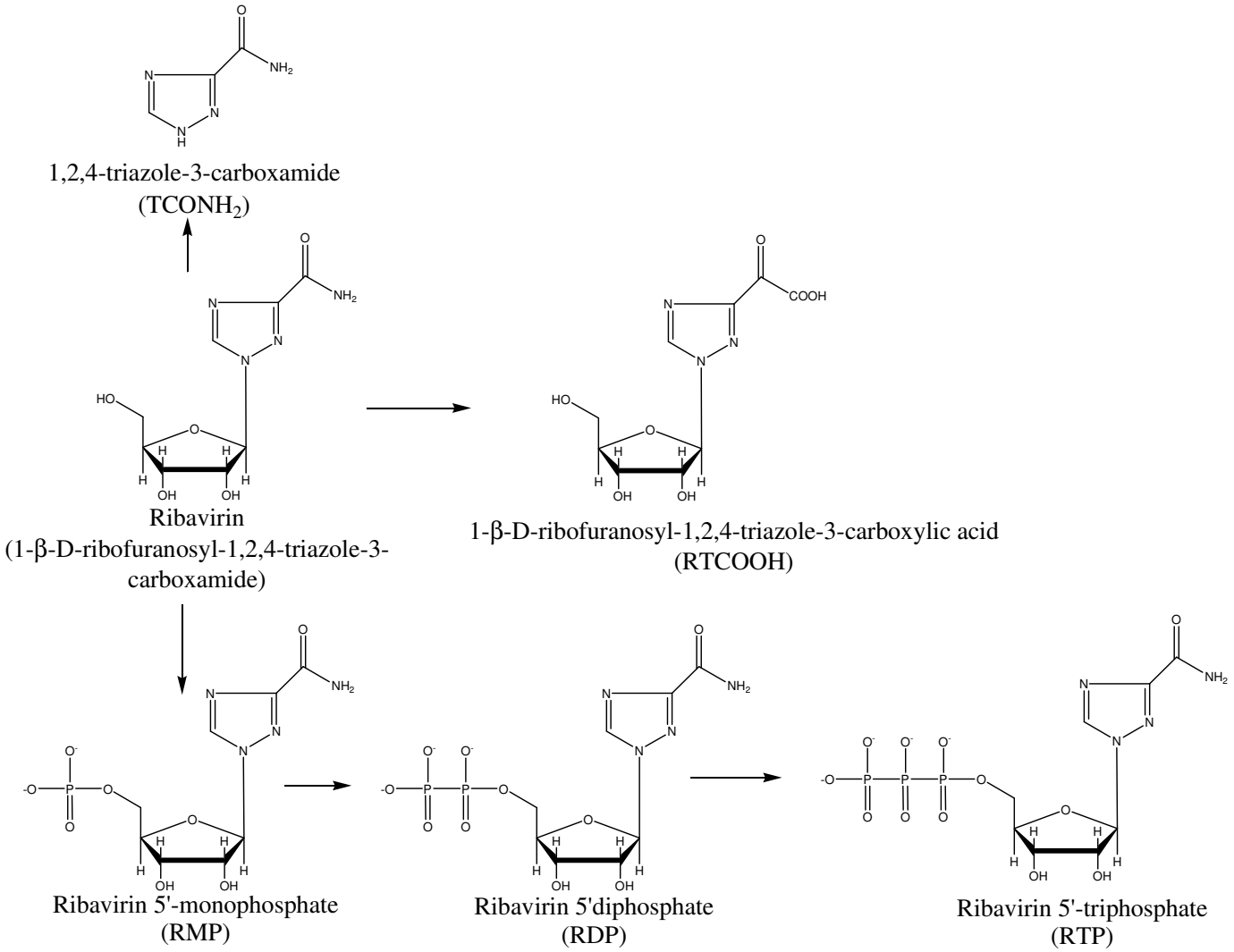
Ribavirin
Metabolism and Distribution



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Ribavirin
Metabolism and Distribution

- In *M. mulatta* (Rhesus macaques) 8 hours after receiving [^{14}C]-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

Ferrara, E.A. *et al. Antimicrob. Agents and Chemother.* (1981) **19**: 1042-1049

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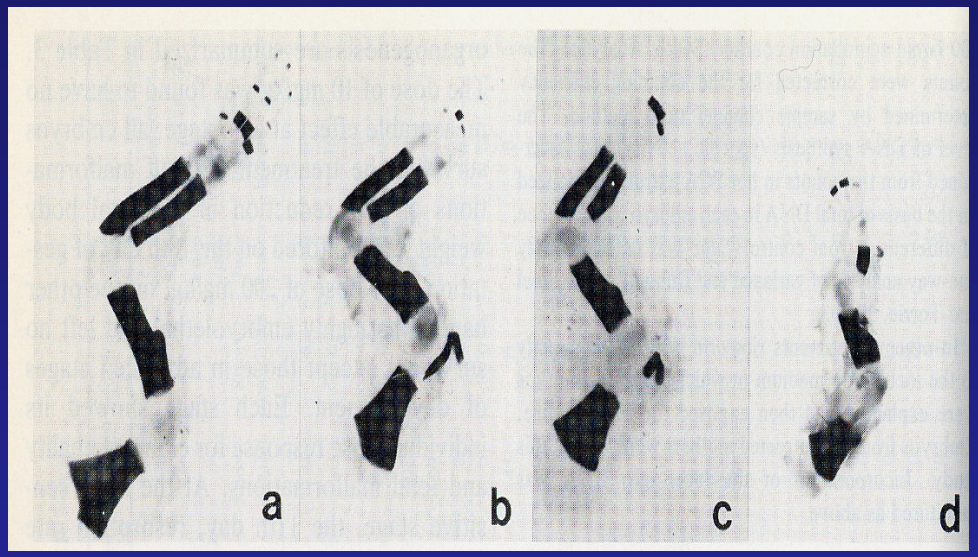
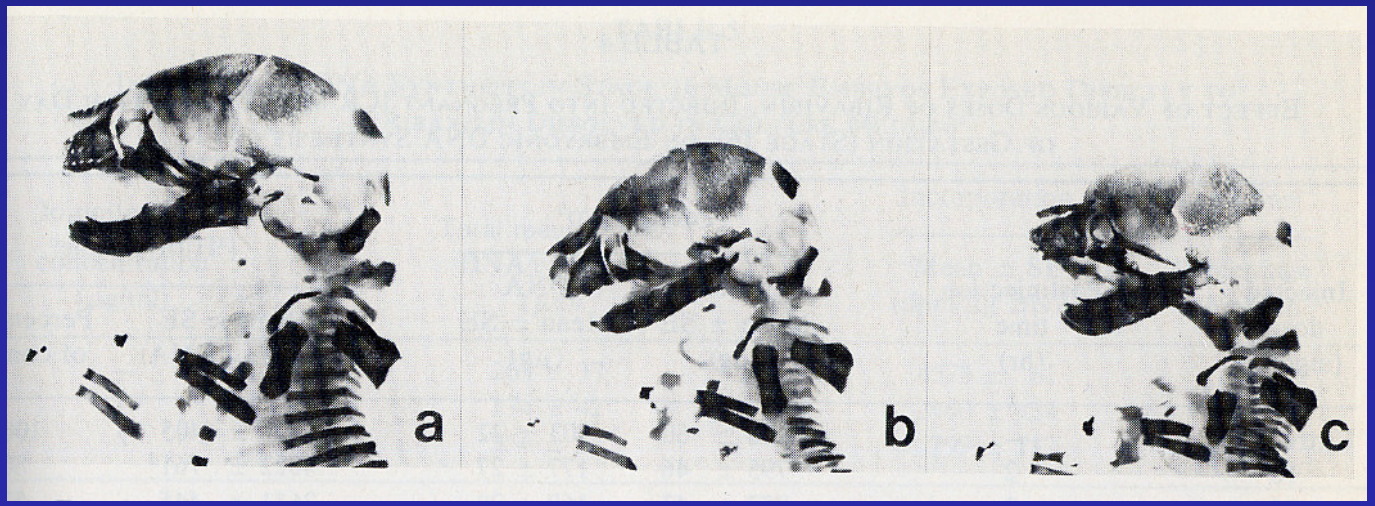
Ribavirin
Fetal Toxicity



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



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

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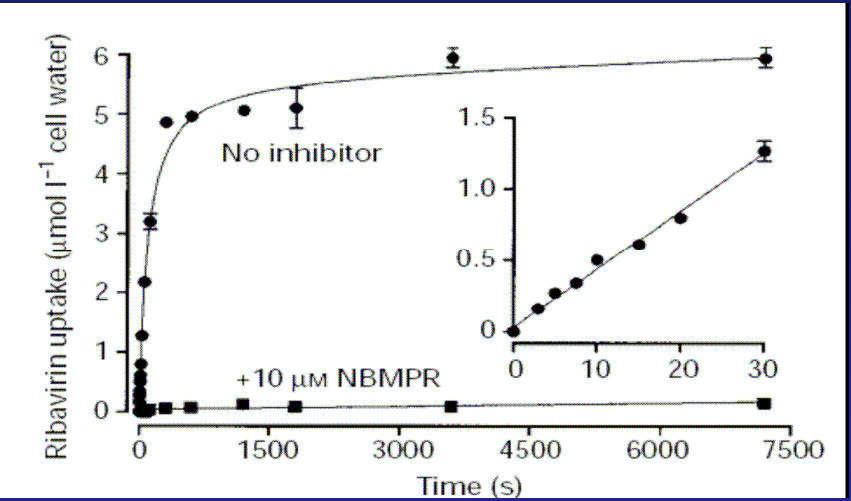
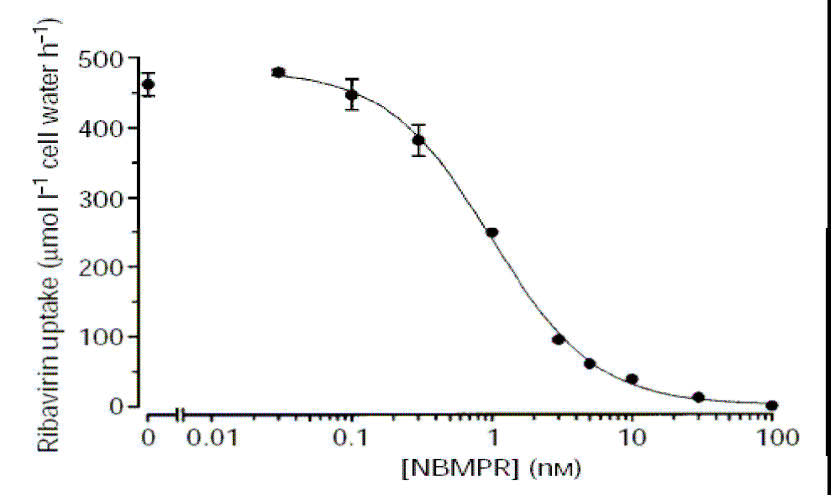
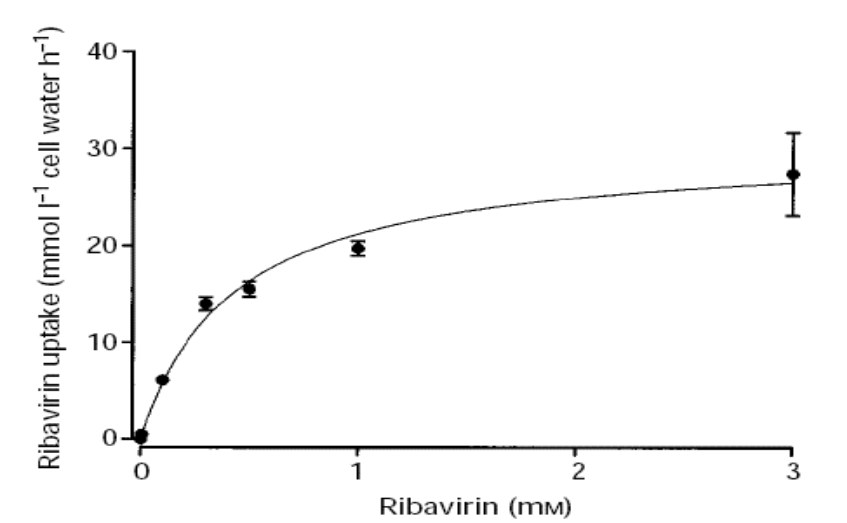
Nucleoside Transporters
Ribavirin Transport: hENT1







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

NBMPR
IC_{50}: 0.99 ± 0.05 nM

Jarvis, S.M. *et al. Br. J. Pharmacol.* (1998) **123**: 1587-1592

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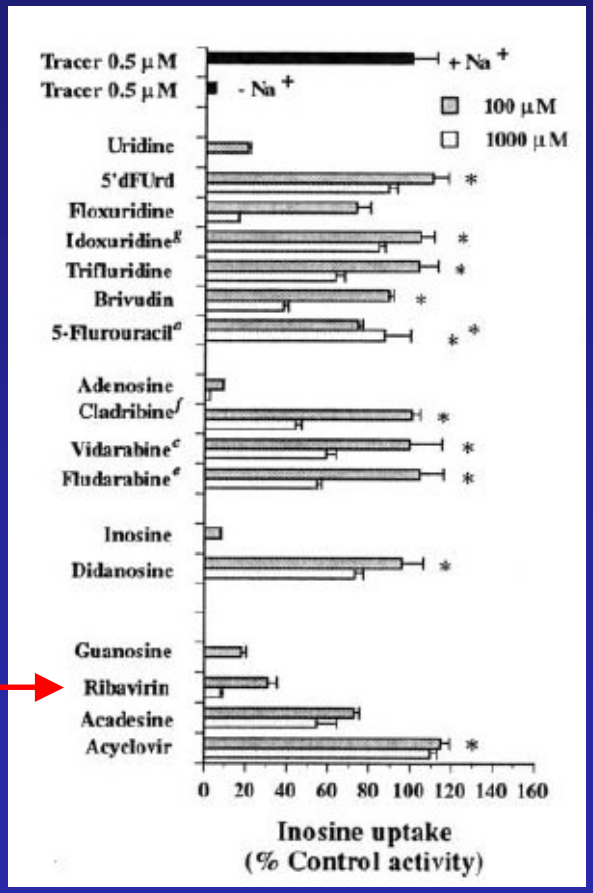
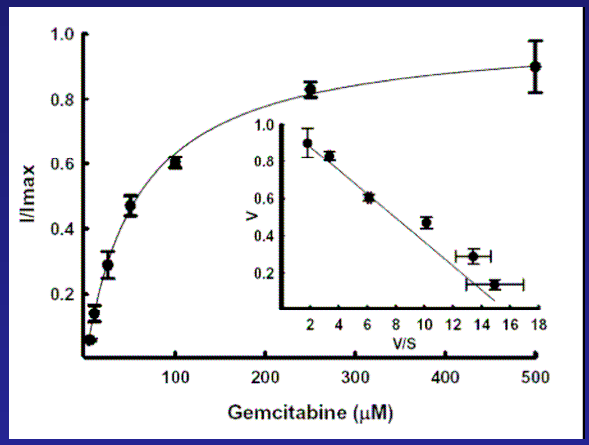
Nucleoside Transporters
Ribavirin Transport: hCNTs

hCNT2 Mediated [^{3}H]-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 µM

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

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

Christopher J. Endres

Dresser, M.J., *et al. Drug Metab. Disp.* (2000) **28**: 1135-1140
Hu, H., *et al. Mol. Pharmacol.* (2006) in press
Patil, S.D., *et al. Cancer Chemother. Pharmacol.* (2000) **46**: 394-402

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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 [^{3}H]-ribavirin in erythrocytes from mEnt1(+/+) and mEnt1(-/-) mice.

2. To characterize the pharmacokinetics and tissue distribution of ribavirin in mEnt1(+/+) and mEnt1(-/-) mice.

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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.

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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 [^{3}H]-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

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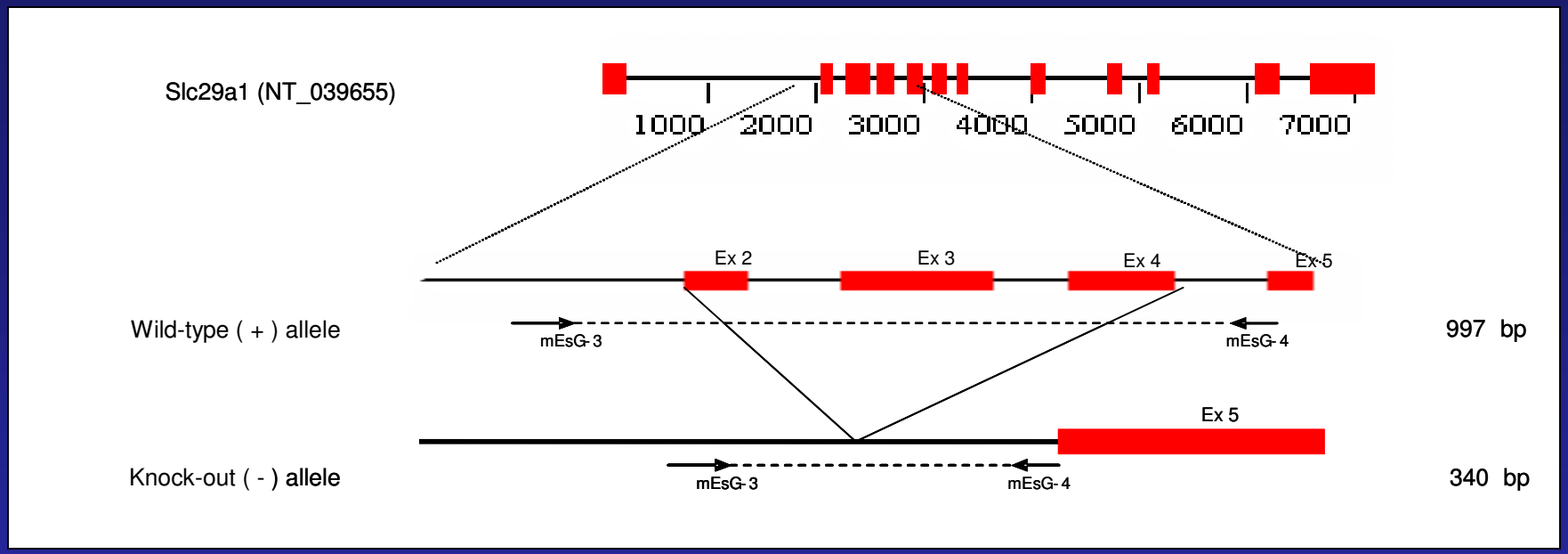
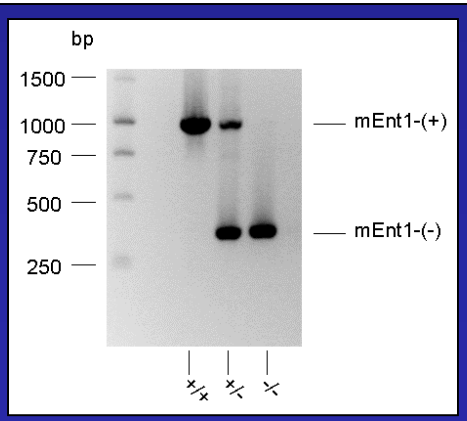
Ex vivo Erythrocyte Transport of Ribavirin

PCR Genotyping Assay of mEnt1 in Mice





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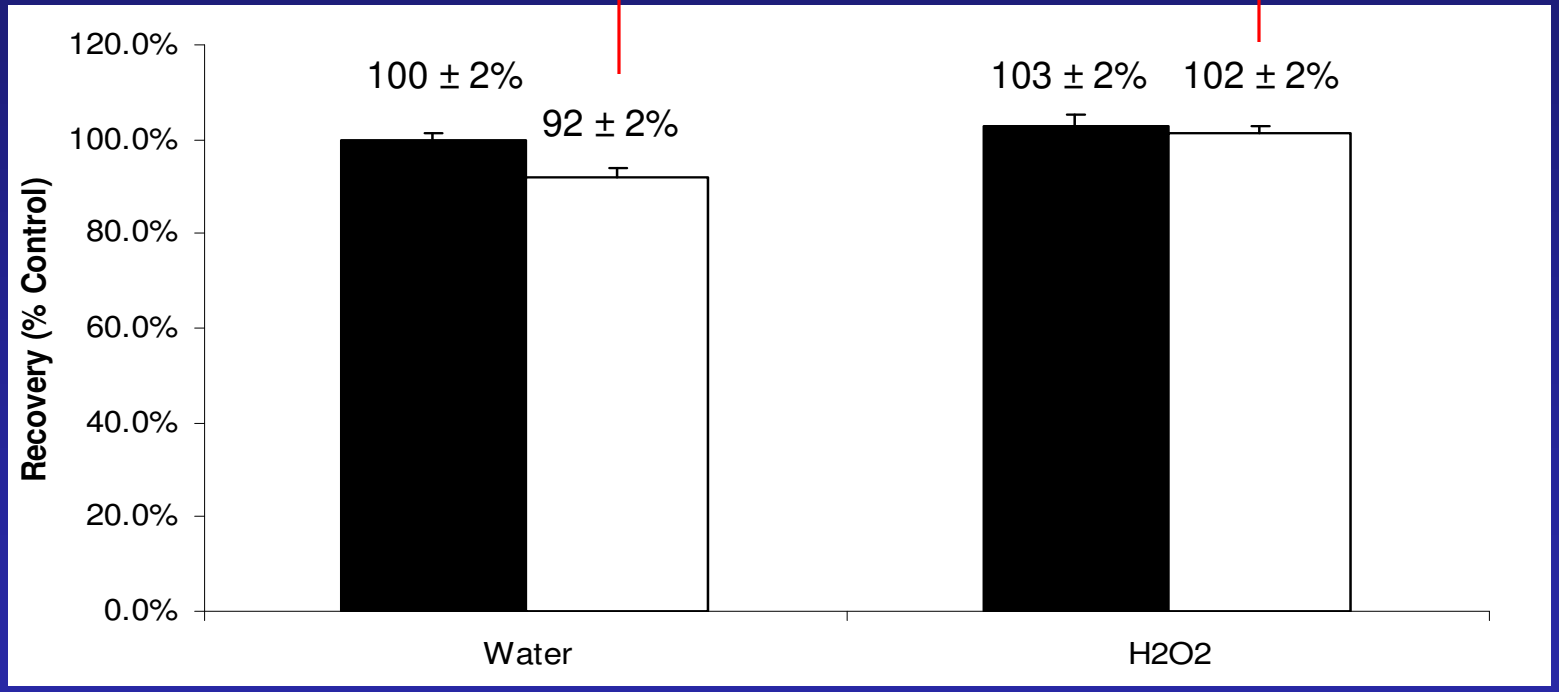
Ex vivo Erythrocyte Transport of Ribavirin

Spike Recovery from Erythrocytes

Color quenching by erythrocytes reduced recovery of erythrocyte spikes

Decolorization with 30% H_2O_2 restores reduced recovery of erythrocyte spikes



Solid Bars: Water Spike
Open Bars: Erythrocyte Spike

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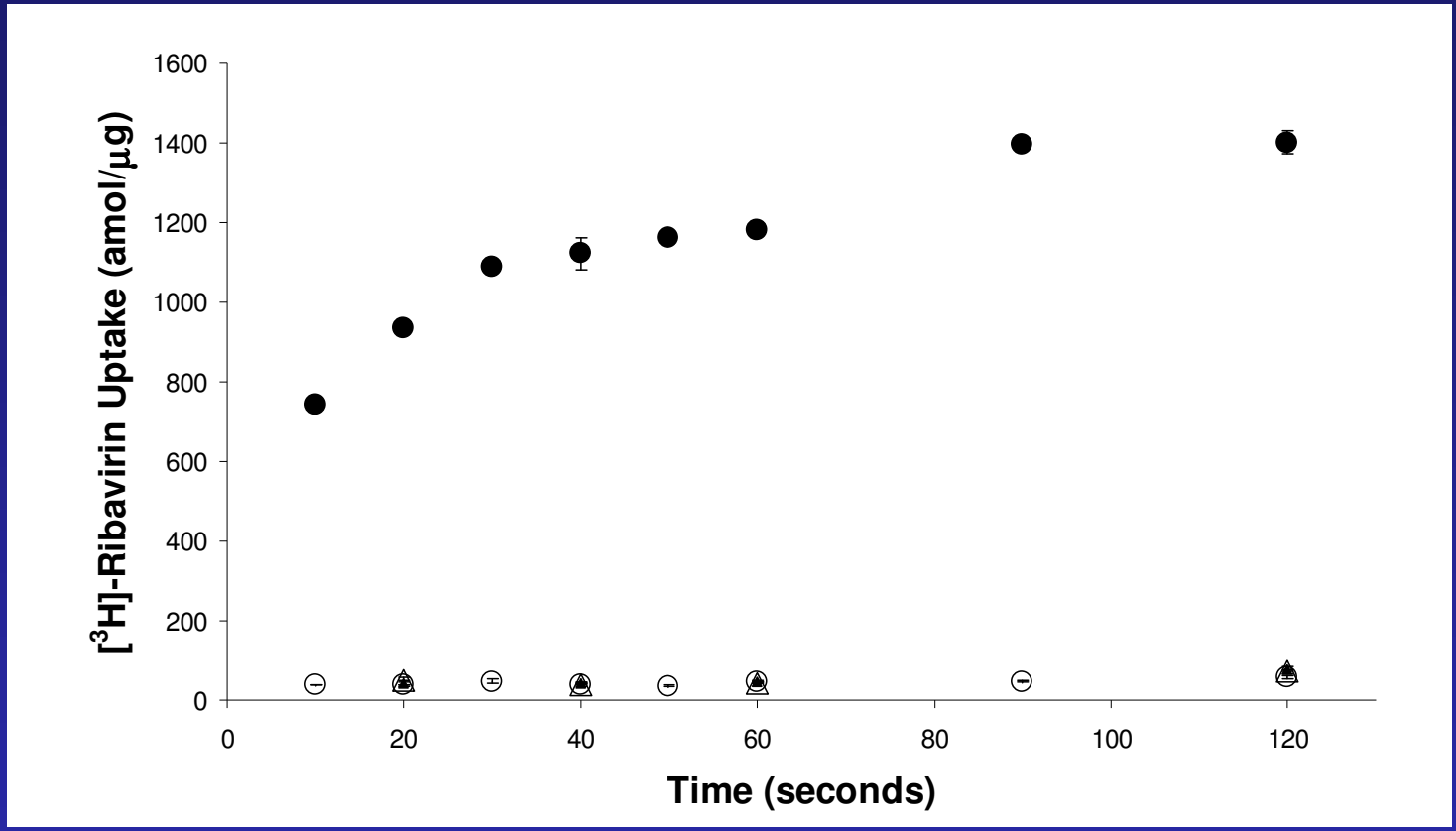
Ex vivo Erythrocyte Transport of Ribavirin

Time Course of Erythrocyte Ribavirin Uptake



Filled: Vehicle
Open: 10 µM NBMPR

mEnt1(+/+): circles
mEnt1(-/-): triangles
Values represent mean ± S.D. (n=3)

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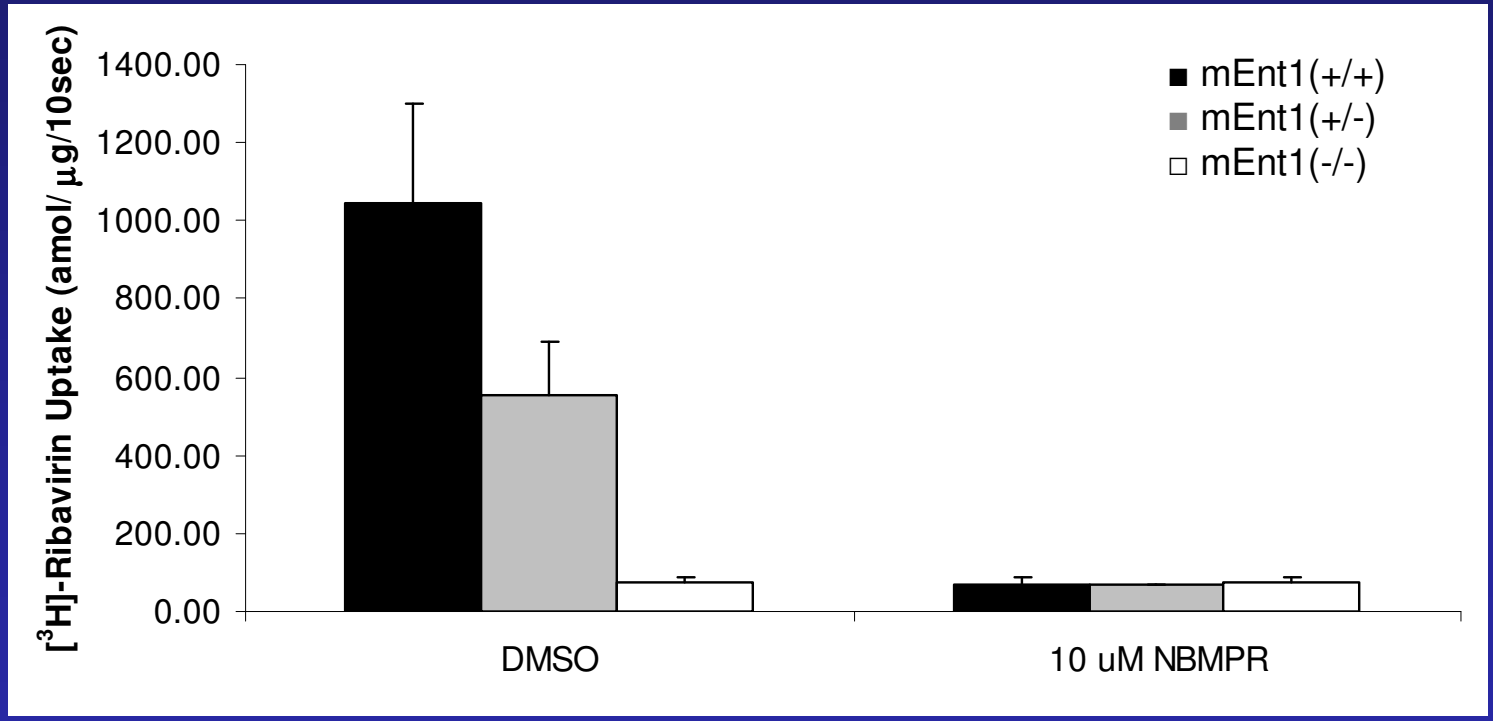
Ex vivo Erythrocyte Transport of Ribavirin

Ribavirin Transport Activity in Erythrocytes

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



Values represent mean ± S.D. (n=3)

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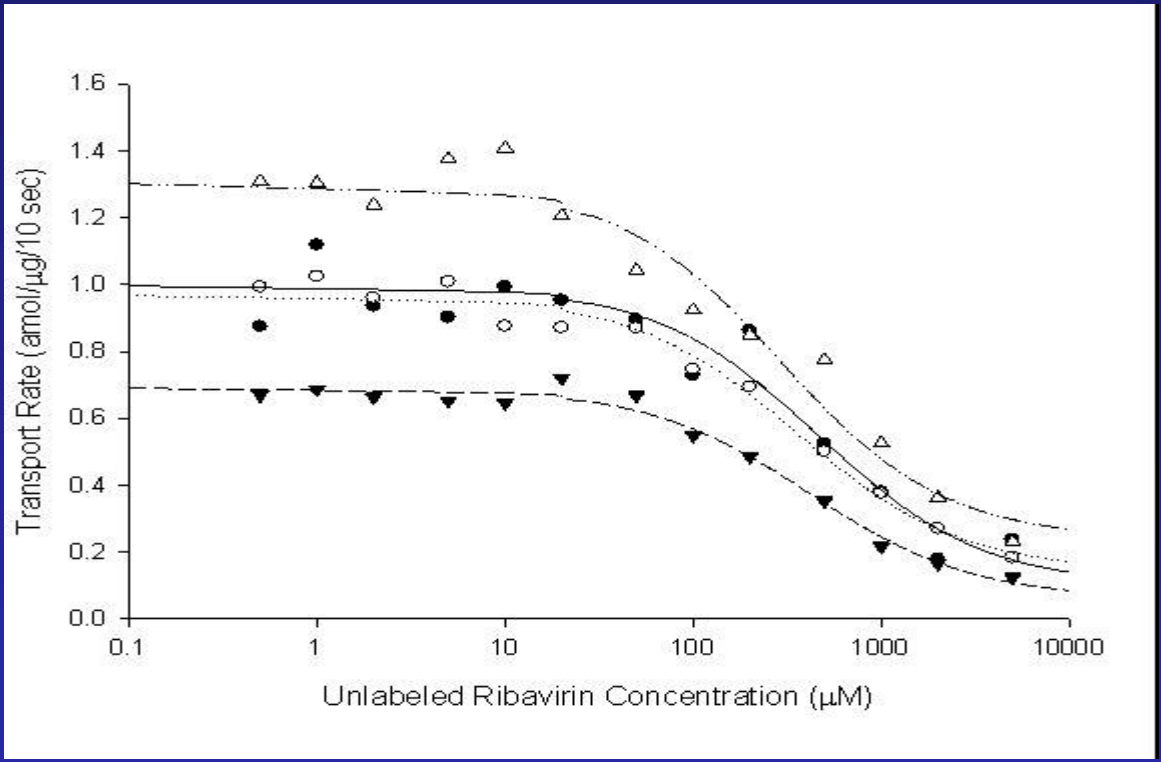
Ex vivo Erythrocyte Transport of Ribavirin

Ribavirin Transport Kinetics in Erythrocytes

Tracer Displacement Kinetics Analysis



V_{max}

417 ± 86.7 amol/µg/10 sec

K_m

382 ± 75.1 µM

Mean ± S.D. (n=4)

Values represent mean ± S.D. (n=3)

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Ex vivo Erythrocyte Transport of Ribavirin
Conclusions

- The time-course of [^{3}H]-ribavirin uptake in mEnt1(+/+) erythrocytes rapidly reaches equilibrium within 60 seconds, and was completely inhibited by 10 μM NBMPR.
- There is no NBMPR inhibitable [^{3}H]-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 [^{3}H]-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 [^{3}H]-ribavirin

- We will examine the tissue distribution of [^{3}H]-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

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Pharmacokinetics and Tissue Distribution
Experimental Design

- [^{3}H]-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)

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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 μCi/g) was administered to correct tissue ribavirin concentrations for ribavirin present in the tissue vascular volume.

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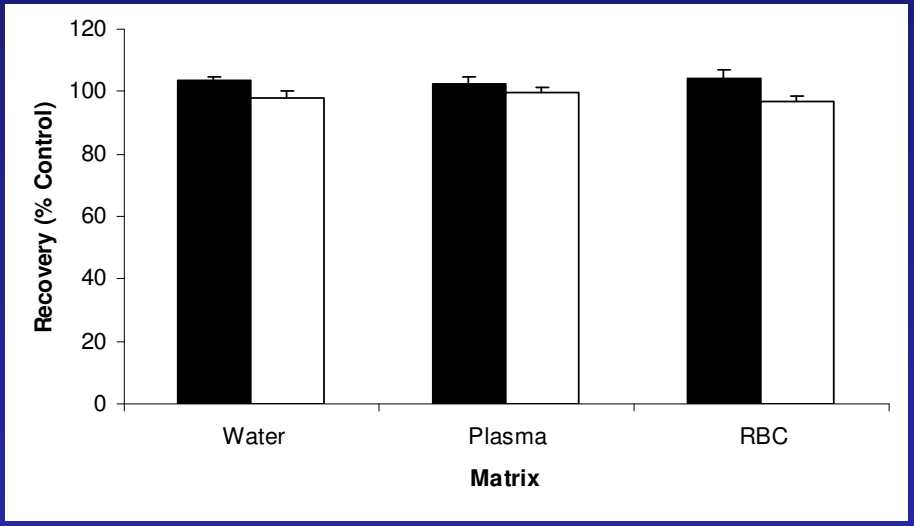
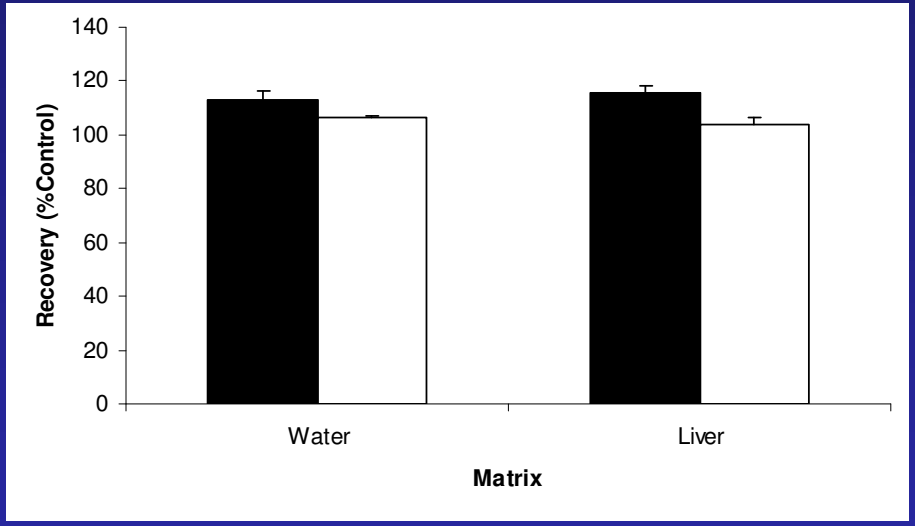
Pharmacokinetics and Tissue Distribution
Direct Counting Fluid and Tissue Spike Recovery

Fluid Spike Recovery



Tissue Spike Recovery



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

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

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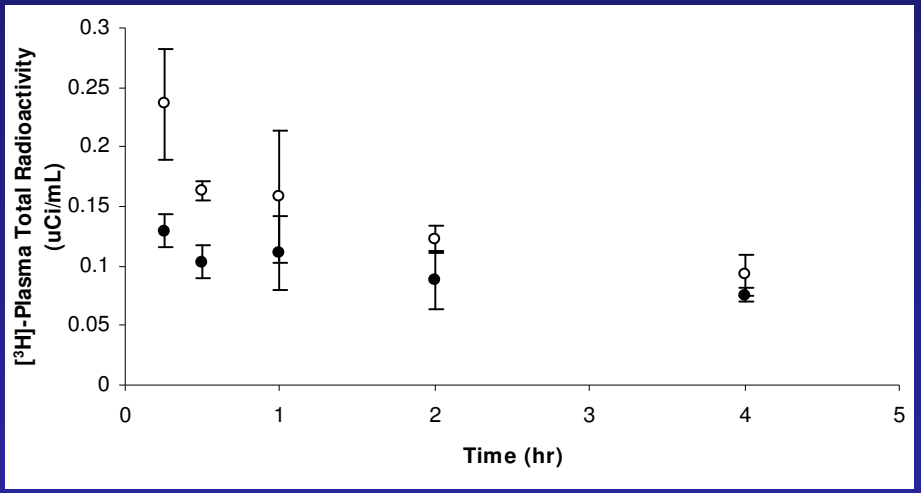
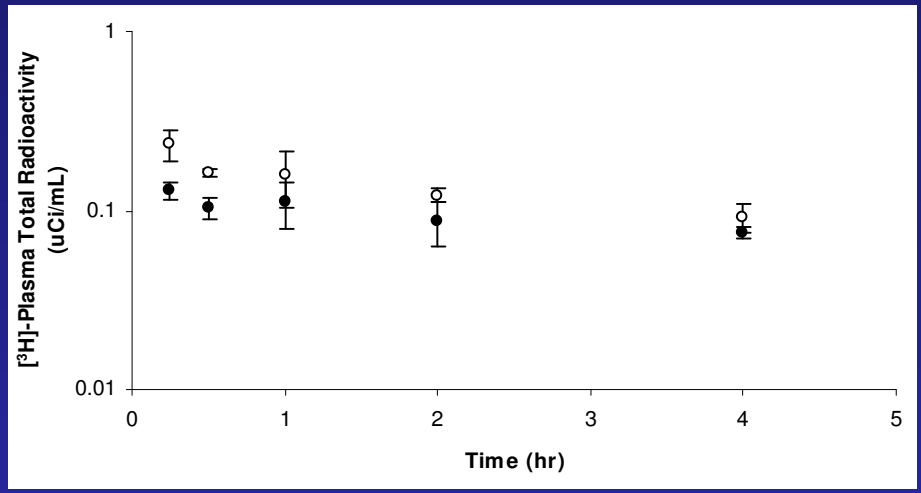
Pharmacokinetics and Tissue Distribution

Plasma Pharmacokinetics: [^{3}H]-Total Radioactivity

Intravenous [^{3}H]-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

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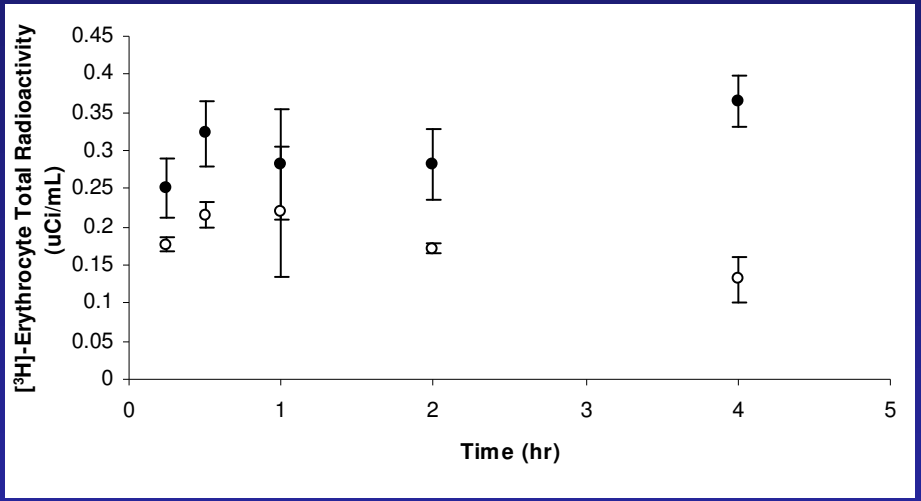
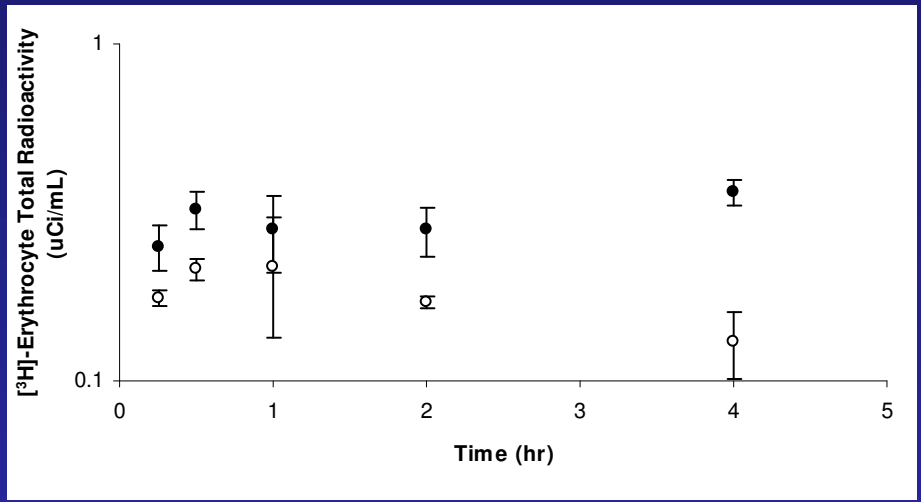
Pharmacokinetics and Tissue Distribution

Erythrocyte Pharmacokinetics: ^{3}H-Total Radioactivity

Intravenous [^{3}H]-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

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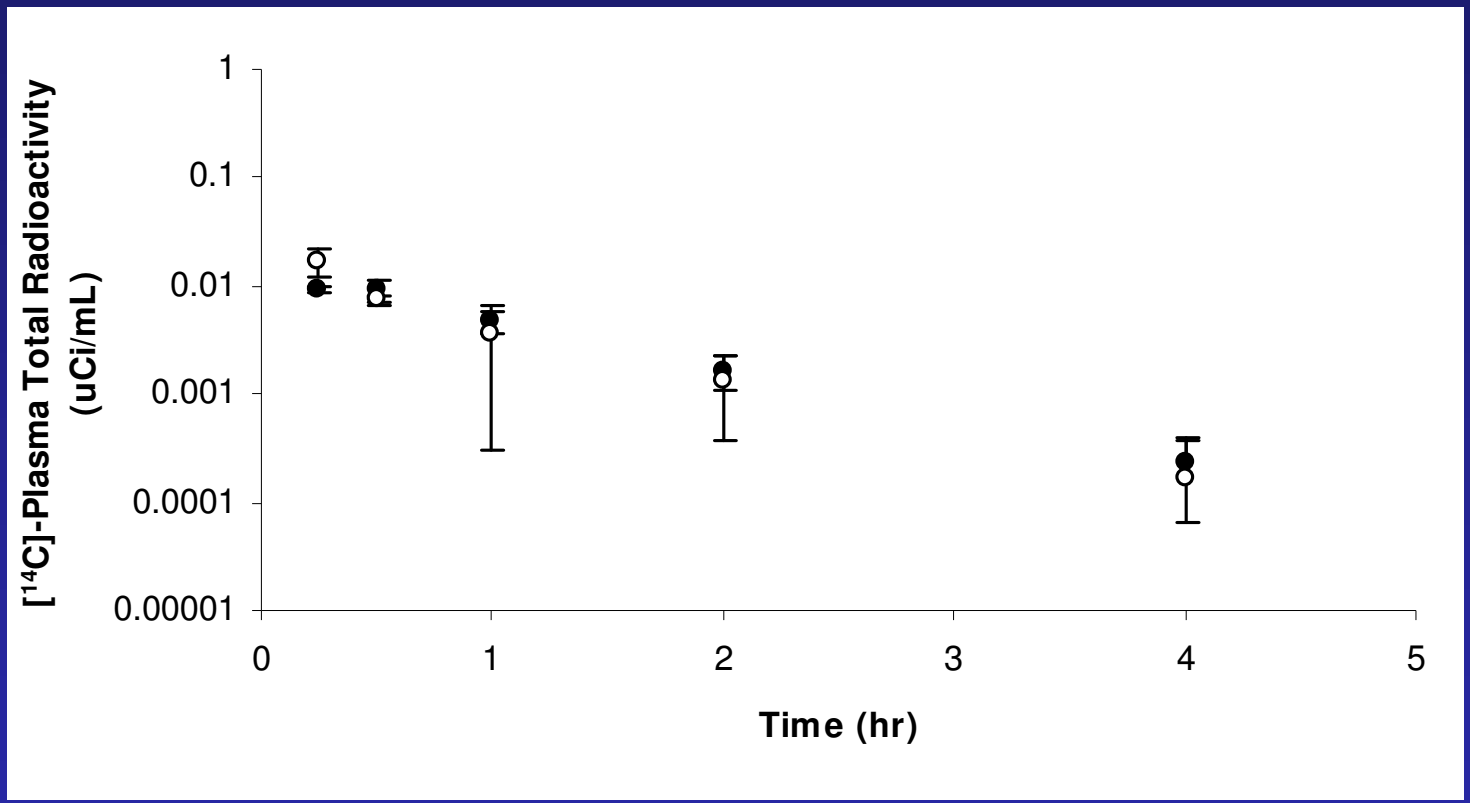
Pharmacokinetics and Tissue Distribution

Plasma Pharmacokinetics: ^{14}C-Total Radioactivity

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



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

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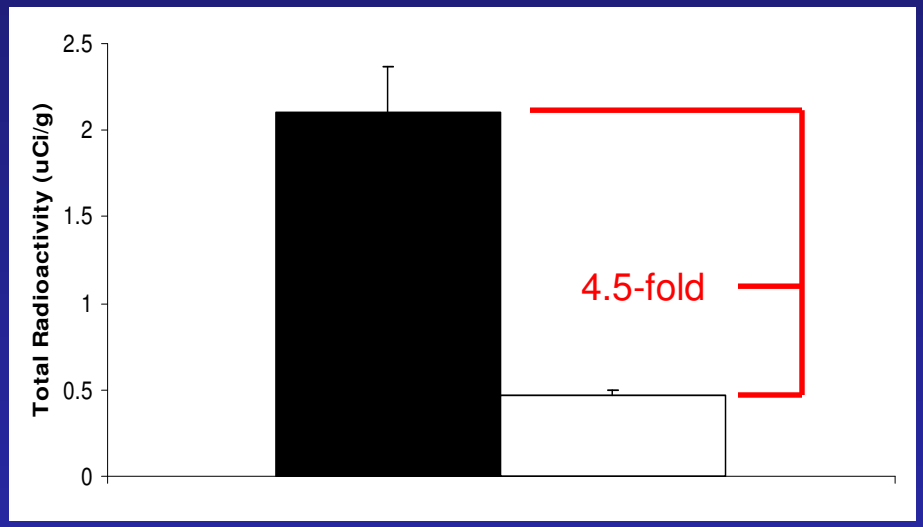
Pharmacokinetics and Tissue Distribution

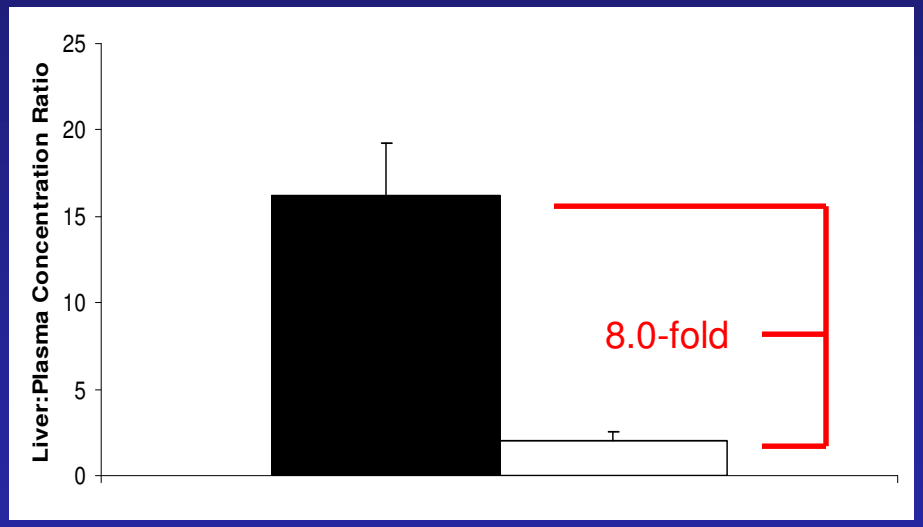
Liver Distribution: [^{3}H]-Total Radioactivity

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

Total Radioactivity



Liver : Plasma Ratio



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

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

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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

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Pharmacokinetics and Tissue Distribution
Conclusions

- The plasma [^{3}H]-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 [^{3}H]-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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Outline

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

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 [^{3}H]-ribavirin quantitation from fluids and tissue
- Complete I.V. ribavirin pharmacokinetics and tissue distribution
- Complete oral ribavirin pharmacokinetics

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