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

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

October 26, 2006
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
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

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Nucleoside Transporters

• Two major families:

Concentrative nucleoside transporters (CNTs)

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

Nucleoside Transporters

Function

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CNT 1</th>
<th>CNT 2</th>
<th>CNT 3</th>
<th>ENT 1</th>
<th>ENT 2</th>
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<th>CNT 2</th>
<th>CNT 3</th>
<th>ENT 1</th>
<th>ENT 2</th>
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<td>Floxidine</td>
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<td>Lamivudine</td>
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<td>Gancyclovir</td>
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<td>Stavudine</td>
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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 [3H]-inosine uptake in hENT1 expressed in yeast)

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Drug Transporters
Role of Tissue Distribution on Drug Disposition

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Nucleoside Transporters
Tissue Distribution

- Polarized Epithelial Cells (e.g. Intestine, Kidney)
- Erythrocytes
- Blood Brain Barrier
- Placental Syncytiotrophoblasts
- Hepatocytes

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Nucleoside Transporters
Mouse Tissue Distribution

Outline

• **Background**
  – Nucleoside Transporters
  – Ribavirin

• **Research Design, Methods and Preliminary Results**
  – *Ex vivo* Erythrocyte Transport of Ribavirin
  – Pharmacokinetics and Tissue Distribution

• **Conclusions**
Ribavirin

Introduction

- Ribavirin is used as first line treatment of compensated chronic hepatitis C virus (HCV) infection and is co-administered with interferon-\(\alpha\) 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
Ribavirin
Pharmacokinetics

- The $C_{\text{max}}$ 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:
  - $\alpha$: 0.2 - 0.9 hours
  - $\beta$: 1.6 - 2.0 hours
  - $\gamma$: 35.5 – 60 hours

- After discontinuation of oral dosing to steady-state, the terminal elimination half-life was:
  - $\lambda_z$: 298 hours
  and 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 ± 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

1,2,4-triazole-3-carboxamide (TCNH₂)

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide)

1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxylic acid (RTCOOH)

Ribavirin 5'-monophosphate (RMP)

Ribavirin 5'-diphosphate (RDP)

Ribavirin 5'-triphosphate (RTP)
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

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Ribavirin Fetal Toxicity

<table>
<thead>
<tr>
<th>Single Dose (mg/kg)</th>
<th>% Fetuses Resorbed</th>
<th>% Surviving Fetuses Malformed</th>
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<tr>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>10</td>
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<tr>
<td>25</td>
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<td>50</td>
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<tr>
<td>100</td>
<td>44</td>
<td>77</td>
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<tr>
<td>200</td>
<td>100</td>
<td>NA</td>
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</tbody>
</table>

Nucleoside Transporters
Ribavirin Transport: hENT1

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

NBMPR
$IC_{50}$: 0.99 ± 0.05 nM

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Nucleoside Transporters
Ribavirin Transport: hCNTs

Ribavirin stimulated inward current in X. laevis oocytes expressing hCNT3

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

<table>
<thead>
<tr>
<th>Conc. (µM)</th>
<th>% Inhibition</th>
<th>$I_{RBV}/I_{URI}$</th>
</tr>
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<tbody>
<tr>
<td>1000</td>
<td>51 ± 7</td>
<td>0.06 ± 0.01</td>
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</table>

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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 [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

• Background
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  – Ex vivo Erythrocyte Transport of Ribavirin
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• Conclusions
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

Slc29a1 (NT_039655)

Wild-type (+) allele

Knock-out (-) allele

997 bp

340 bp
Ex vivo Erythrocyte Transport of Ribavirin

Spike Recovery from Erythrocytes

Color quenching by erythrocytes reduced recovery of erythrocyte spikes

Decolorization with 30% H$_2$O$_2$ 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)

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Ex vivo Erythrocyte Transport of Ribavirin
Ribavirin Transport Activity in Erythrocytes

[\textsuperscript{3}H]-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_{\text{max}} = 417 \pm 86.7 \, \text{amol/\(\mu\text{g}/10 \, \text{sec}\) } \]
\[ K_m = 382 \pm 75.1 \, \mu\text{M} \]

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 $[^3\text{H}]-$ribavirin uptake in mEnt1(+/+) erythrocytes rapidly reaches equilibrium within 60 seconds, and was completely inhibited by 10 $\mu\text{M}$ NBMPR.

- There is no NBMPR inhibitable $[^3\text{H}]-$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 $[^3\text{H}]-$ribavirin transport in mEnt1(+/+) erythrocytes was similar to that observed in humans (420 $\mu\text{M}$).
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• Conclusions
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 $[^3\text{H}]-\text{ribavirin}$

• We will examine the tissue distribution of $[^3\text{H}]-\text{ribavirin}$ 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

- $[^{3}\text{H}]$-Ribavirin was administered orally (10 $\mu$g/g) or intravenously (3 $\mu$g/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}\text{C}\text{-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: $[^3\text{H}]-\text{Ribavirin}$
Open bars: $[^{14}\text{C}]-\text{Sucrose}$

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: $^3$H-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

Intravenous $[^{14}\text{C}]-\text{Sucrose Dose (0.05 } \mu\text{Ci/g)}$

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 ($\mu$Ci/g tissue) 15 Minutes After 3 $\mu$g/g (0.4 $\mu$Ci/g) $[^3]$H-Ribavirin Retro-Orbital Injection

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

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

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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
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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General Conclusions

- mEnt1 contributes to erythrocyte uptake of ribavirin \textit{ex vivo}.

- mEnt1 significantly contributes to the hepatic and erythrocyte distribution of ribavirin \textit{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 $[^3\text{H}]-$ribavirin quantitation from fluids and tissue

- Complete I.V. ribavirin pharmacokinetics and tissue distribution

- Complete oral ribavirin pharmacokinetics
Acknowlegments

GPEN 2006  
Shinji Yamashita, Ph.D.

**Doctoral Committee**  
Jashvant D. Unadkat, Ph.D.

Danny D. Shen, Ph.D.  
Kenneth E. Thummel, Ph.D.  
John T. Slattery, Ph.D.  
Joanne Wang, Ph.D.

**Unadkat Lab**  
Raj Govindarajan, Ph.D.  
Aaron Moss  
Peng Hsiao  
Brian Kirby  
Huixia Zhang

**Department of Pharmaceutics**  
Ed Kelley, Ph.D.

**Comparative Medicine**  
Rosita Morales  
Virginia Gunderson-Batterson, Ph.D.  
Ron Varnam  
Steve Marks  
Carol Ware, Ph.D.

**Ernest Gallo Research Center**  
Jackie Connolly  
Doo-Sup Choi, Ph.D.  
Robert Messing, Ph.D.

**Funding**  
NIH GM54447  
NIH GM07750

Christopher J. Endres
Thank You

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Christopher J. Endres