HOST-BASED MECHANSIMS OF RIBAVIRIN RESISTANCE: IMPLICATIONS IN TREATMENT RESPONSE OF HEPATITIS C VIRUS INFECTION

APPROVED BY SUPERVISORY COMMITTEE

Julie Pfeiffer, Ph.D.
Supervising Professor

Pinghui Feng, Ph.D.
Chair, Examining Committee

Nitin Karandikar, M.D., Ph.D.
Committee Member

Tawanda Gumbo, M.D.
Committee Member

DEDICATION

This work is dedicated to those who have irrevocably touched my life and for whom I am eternally grateful, Ms. Linda Banks and Alexander (Sasha) Parfenov. Although I was not her own child, Linda took me in and taught me how to live again. Where there was once anger, she placed love, compassion and understanding. When I was alone, she gave me a family to call my own. Linda helped guide me to where I am today and taught me how to hope when I didn't know how. As for my partner Sasha, words cannot describe my love and gratitude. He has been my partner, helping me navigate through this world, tirelessly standing by my side despite numerous obstacles, holding me up when I felt too weak to stand, and believing in me when my own faith subsided. I dedicate this, and all future work that I may be blessed to perform, to both of them.

Acknowledgements

I would like to thank my sister Priscilla for all of her support and encouragement throughout the years. Despite the fact that our family was reduced to only the two of us, and conditions required us to be separated for a time, we managed to prevail. I would also like to thank my "surrogate" sisters, Monique and Tiffany Banks for whom I am eternally grateful for being allowed to be part of their lives and received as family. They provided a continual source of support and love.

I would also like to express my appreciation to former and current lab members (Chris Etheredge, Andrea Erickson, Karen Lancaster, Sharon Kuss and Gavin Best) for all of their help and support provided throughout the years. I am especially indebted to my committee members, Dr. Pinghui Feng, Dr. Tawanda Gumbo and Dr. Nitin Karandikar for tirelessly providing exceptional advice and guidance.

Last but not least, I would thank to express my gratitude to my mentor Dr. Julie Pfeiffer. Graduate life can be tumultuous, to say the least, and she was always there to provide guidance for whatever purpose I may have required, whether the issue was scientific, work-related, career-related, or any other topic. For that and everything else, I am extremely grateful.

HOST-BASED MECHANSIMS OF RIBAVIRIN RESISTANCE: IMPLICATIONS IN TREATMENT RESPONSE OF HEPATATIS C VIRUS INFECTION

by

KRISTIE DAWN IBARRA

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2010

HOST-BASED MECHANSIMS OF RIBAVIRIN RESISTANCE: IMPLICATIONS IN TREATMENT RESPONSE OF HEPATATIS C VIRUS INFECTION

Kristie Dawn Ibarra

The University of Texas Southwestern Medical Center at Dallas, 2010

Supervising Professor: Julie Pfeiffer, Ph.D.

Many individuals infected with hepatitis C virus (HCV) fail to respond to therapy, resulting in the development of chronic infection and increased risk for fibrosis, cirrhosis, and hepatocellular carcinoma. The current standard of care consists of pegylated interferon and ribavirin (RBV), a nucleoside analog. While RBV improves treatment outcome, and will likely be an important component of therapy with next-generation viral inhibitors, RBV's mechanism is controversial. Most of RBV's proposed mechanisms require RBV import into cells. Therefore, we examined whether host-based RBV

resistance develops through reduced cellular uptake, analogous to chemotherapy resistance in some cancers. We examined the effect of host-based RBV resistance on a model RNA virus, poliovirus, HCV replication in cultured hepatoma liver cells, and whether RBV resistance develops in HCV patients. When liver cells permissive for poliovirus or HCV replication were exposed to RBV, resistance developed through reduced activity of the ENT1 nucleoside transporter, and antiviral efficacy was reduced. Importantly, RBV uptake significantly declined in HCV peripheral blood mononuclear cells (PBMCs) following four weeks of therapy. Furthermore, maintenance of RBV uptake correlated with rapid treatment response. Our results uncovered a novel form of antiviral drug resistance, suggesting that host-based RBV resistance develops in HCV patients undergoing therapy and that maintenance of RBV uptake may contribute to rapid viral clearance.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Ibarra, K.D., Jain, M.K., Pfeiffer, J.K. 2010. Host-based ribavirin resistance influences hepatitis C virus treatment response. J Virol (Submitted)

Ibarra, K. D. and J. K. Pfeiffer (2009). "Reduced ribavirin antiviral efficacy via nucleoside transporter-mediated drug resistance." J Virol 83(9): 4538-4547.

TABLE OF FIGURES

LIST OF ABBREVIATIONS

4E-BP	4E-binding protein
ADA	Adenosine deaminase
ALT	Alanine aminotransferase
AML	Acute myeloid leukemia
AMPK	AMP-activated protein kinase
Ara-C	Cytosine arabinoside
ASM	American Society for Microbiology
CDC	Centers for disease control and prevention
CNT2	Concentrative nucleoside transporter 2
CNT3	Concentrative nucleoside transporter 3
CPM	Counts per minute
DMEM	Dulbecco's modified Eagle medium
eIF2	Eukaryotic translation initiation factor 2
eIF4E	Eukaryotic translation initiation factor 4E
ENT1	Equilibrative nucleoside transporter 1
ENT2	Equilibrative nucleoside transporter 2
ETR	End-of-treatment response
EVR	Early virological response
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFU	Focal forming units
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GTP	Guanosine triphosphate
Guo	Guanosine
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HRP	Horseradish peroxidase conjugated
HSC	Hepatitic stellate cell
HIV	Human immunodeficiency virus
IFN	Interferon
IFN-α	Interferon alpha
IMP	Inosine monophosphate
IMPDH	Inosine monophosphate dehydrogenase
IRF3	Interferon-regulatory factor 3
IRF7	Interferon-regulatory factor 7
IRF9	Interferon-regulatory factor 9
ISDR	Interferon-α sensitivity-determining region
ISG	Interferon-stimulated genes
IU	International units
M	Mock
m^7G cap	7-methyl Guanosine cap

MOI	Multiplicity of infection
NANBH	Non-A, non-B hepatitis
NBS1	Nijmegen breakage syndrome 1
NBMPR	Nitrobenzylmercaptopurine riboside
Non-RR	Non-Rapid response
NR	Non-response (or Non-responder)
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Peg-IFN	Pegylated interferon
PFA	Paraformaldehyde
PFU	Plaque forming units
PKR	Double-stranded RNA-activated protein kinase
PRPP	Phosphoribosyl pyrophosphate
RBV	Ribavirin
RBV^R	Ribavirin resistant
RBV^S	Ribavirin sensitive
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic acid inducible gene
RMP	Ribavirin monophosphate
RR	Rapid response (or rapid responder)
RSV	Respiratory syncytial virus
RT	Reverse transcriptase
RTP	Ribavirin triphosphate
RVR	Rapid virological response
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
SOC	Standard of care
STAT-C	Specifically targeted antiviral therapy
SVR	Sustained virological response
TGF-β	Transforming growth factor-β

CHAPTER ONE
Introduction

HEPATITIS C VIRUS

Discovery of Hepatitis C Virus

Hepatitis C virus (HCV) infection was initially believed to be transfusion-associated [1]. During the mid 1970s available diagnostics determined that 90% of transfusion-associated hepatitis patients were surprisingly type A and B negative, coining the name "non- A, non-B hepatitis" (NANBH) [2, 3] . Subsequent studies of community acquired NANBH by the CDC, in conjunction with national surveillance data, demonstrated NANBH transmission via routes other than blood transfusion. Further research provided evidence for a blood-borne agent which was readily transmissible to chimpanzees and believed to be a small enveloped virus [2]. The causative agent was not discovered until 1989 when Choo *et al.* successfully isolated a cDNA clone from a patient diagnosed with NANBH and designated the virus as hepatitis C virus [4].

Epidemiology

HCV is a bloodborne pathogen primarily transmitted through exposure to infected blood. Isolation and identification of HCV led to the development of serological assays and the implementation of sensitive blood screening methods. By 1992 the risk for transfusion-associated HCV decreased to 0.001% per unit transfected [3]. With the safety of the blood supply secured, vertical transmission became the leading cause of infection among children. Of pediatric HCV infections, 65% can be attributed to mother-

to-child transmission [5]. While the risk for infant transmission from HCV mono-infected mothers ranges from 4 – 6%, the presence of HIV co-infection greatly increases the likelihood of transmission to 15 – 22%. Within the adult population, sexual transmission of HCV occurs infrequently. However, high-risk behaviors such as multiple sexual partners and practices that increase the likelihood of blood exposure can increase the risk of transmission [5, 6]. Intravenous drug use now accounts for the majority of new infections in the U.S. Finally, occupational health workers represent an additional at risk group due to the inherent nature of their work which requires direct patient contact and the potential for needlestick injuries. Consequently, numerous studies have cited sharps related injuries as the principal means of exposure [7]. In response, the Needlestick Safety and Prevention Act was signed in to law in 2000. This resulted in OSHA provisions to the Bloodborne Pathogens Standard and required employers to implement changes to improve workplace safety.

Biology

HCV is an enveloped, positive-sense, single-stranded RNA virus classified under the *Flaviviradae* family. The genome is approximately 9.6 kb in length and encodes one large polyprotein which is processed into ten structural and non-structural proteins by host and viral proteases [8]. Replication, as with other RNA viruses, is error prone. The viral RNA-dependent RNA polymerase (RdRp) lacks proof-reading capacity. This feature, combined with high virion production rates of 10^{10} - 10^{12} daily, produces a highly versatile, dynamic and complex population of viruses referred to as a quasispecies

[6, 9]. There are currently six major genotypes, each sharing approximately 70% sequence homology and further divided into subtypes (i.e. 1a, 1b, 2a and so forth). Within the U.S. and Europe genotype 1 is the predominant form [10].

Pathogenesis

Worldwide approximately 170 million people are infected with HCV, with roughly 3 – 4 million new infections occurring each year [11]. Within the U.S. an estimated 3.2 million suffer from chronic infection [12]. As a result of complications from disease progression, HCV has become the leading cause of liver transplants in the U.S. and an increasing cause of cancer related death.

HCV is a hepatotropic virus and replicates primarily within hepatocytes. Due to the noncytopathic nature of HCV, each hepatocyte may give rise to vast quantities of virions over its lifetime. The unique regenerative ability of the liver provides a continual reservoir for replication. Although infection is noncytopathic, it does result in chronic liver inflammation (hepatitis) and induction of immune mediated liver damage [11].

Given the largely asymptomatic nature of acute infection, tracking disease progression in newly infected individuals can be difficult. However, several studies have provided some insight. Following transmission roughly 20% of individuals acutely resolve the infection (**Fig. 1**) [11]. In studies conducted with transfusion patients, approximately 70 – 80% of acute infections were asymptomatic [13]. Interestingly, there is evidence to suggest that those who experience symptoms during the acute phase have a greater chance for spontaneous HCV clearance [13, 14]. As a result, some have recommended an 8 - 12 week waiting period, should spontaneous recovery occur, to avoid potentially

unnecessary treatment. Symptoms can include jaundice, malaise, weakness, nausea, anorexia, abdominal pain or elevated serum alanine aminotransferase levels (ALT) [12, 13]. Unfortunately, clearance of HCV does not confer immunity and failure to acutely resolve the infection results in the development of chronicity [15]. The transition from acute to chronic infection is not definitive and diagnosis depends on several factors, including clinical presentation. Seroconversion within a six month time is generally an accepted method of diagnosis [14]. However, physicians rarely know when their patient's infection began, other than cases such as needlestick injuries or clinical surveillance trials. Individuals who test positive for HCV RNA via reverse-transcriptase PCR anyalsis (RT-PCR), or real time RT-PCR analysis, and negative for anti-HCV antibodies likely represent those who were recently infected [6]. Humoral immune responses are typically delayed and detection of HCV specific antibodies may take between 8 – 12 weeks.

For the 80% of genotype 1 patients who are unable to acutely resolve their infections, disease progression results in liver damage. Repeated cycles of low level damage triggers inflammatory and fibrogenic repair processes, which can eventually lead to the development of fibrosis and cirrhosis. Following the establishment of cirrhosis, hepatocellular carcinoma (HCC) develops in approximately 1 – 4 % of patients annually (**Fig. 1**). The mortality rate of HCC is extremely high, with a survival rate of less than 11% over five years. Regrettably, treatment options are limited. The most effective include surgical resection or liver transplantation [11, 16, 17].

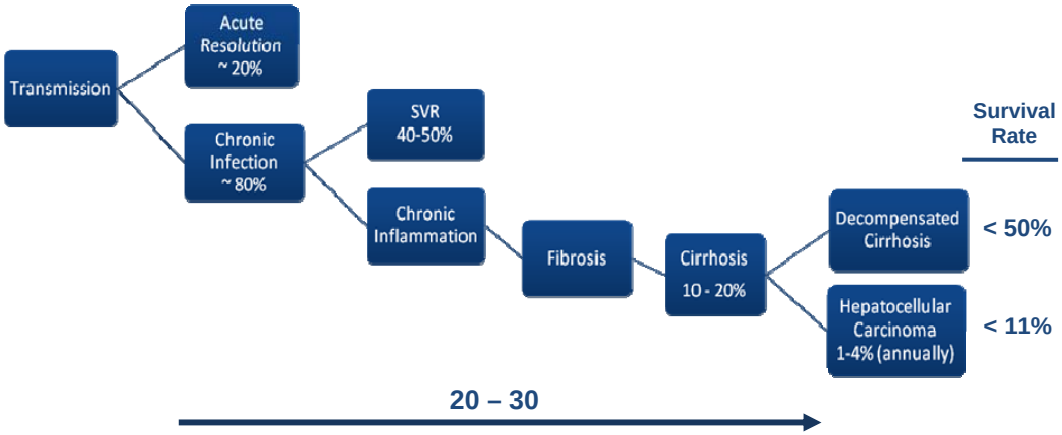


Figure 1. Disease progression in HCV infection.
Following transmission roughly 80% of genotype 1 infections result in chronic infection. Current combination Peg-IFN therapy provides a sustained virological response (SVR) in 40 – 50% of patients. Over the period of 20 – 30 years approximately 10 – 20% of chronically infected patients will develop cirrhosis. Once the disease has progressed to the point of cirrhosis, hepatocellular carcinoma occurs at annual rate of 1 – 4% and has a survival rate of less than 11% after 5 years. The development of decompensated cirrhosis is equally detrimental, with 5 year survival rates of less than 50%.

TREATMENT

Current Treatment and Classifications

Since 2001 the current standard of care (SOC) for genotype 1 infection has consisted of a combination of pegylated interferon (Peg-IFN) and ribavirin (RBV) [18]. Treatment duration can range from 24 – 48 weeks, depending on viral genotype. Infections of genotype 2 and 3 are typically more responsive to therapy (70 – 80% effective) and may require only 24 weeks. Genotype 1 infections, which are more difficult to treat, require a full 48 weeks and produce response rates of only 40 – 50%.

Patient treatment response is based on viral RNA levels, and the ultimate goal is to achieve a sustained virological response (SVR; defined as undetectable HCV RNA for at least 24 weeks post-therapy) [6]. On-treatment monitoring of HCV RNA generally occurs at weeks 4, 12, 24, 48 and 72 (**Fig. 2**). Patients who attain a rapid virological response (RVR; undetectable HCV RNA by week 4) are more likely to achieve SVR [19, 20]. Lack of an early virological response (EVR; ≥ 2 log decline in HCV RNA by week 12) is generally an indicator of those who will not respond to therapy [6]. Additional classifications include breakthrough, relapse and non-response (NR). Patients who transiently achieve undetectable HCV RNA while on therapy are classified under breakthrough. Relapsers remain HCV negative throughout the duration of treatment, but then rebound at some point after therapy. Relapses are typically seen within the first 12 weeks following end-of-treatment response (ETR; HCV RNA negative 24 - 48 weeks post-therapy). Although rare, relapse can occur after prolonged periods, such as 24 weeks

beyond ETR. Finally, those classified as NR fail to exhibit a minimum of a 2 log decline in viral RNA, or the complete clearance of RNA after 24 weeks of therapy.

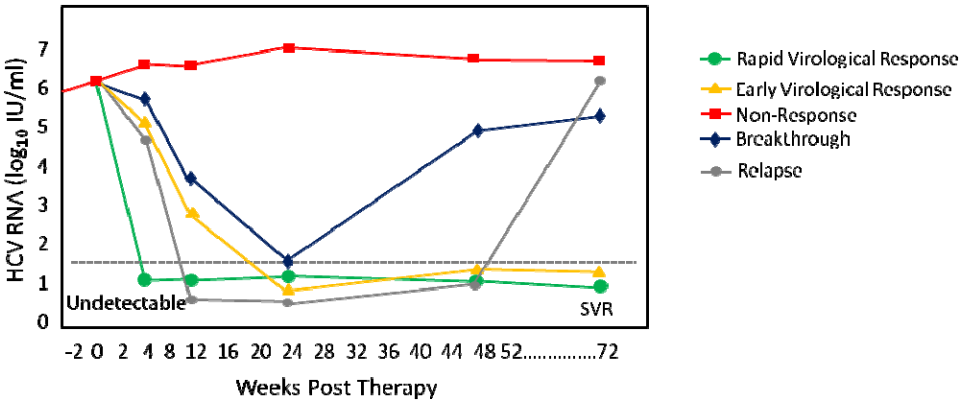


Figure 2. Patient virological response to Peg-IFN/RBV therapy.
Treatment response is based on viral RNA levels (IU/ml). Rapid virological response (RVR) is defined by the absence of detectable HCV RNA 4 weeks post-treatment. Early virological response (EVR) is defined as a decrease of 2 logs or greater by week 12. Non-responders are unable to obtain undetectable RNA levels by week 24, or fail to exhibit decreases of at least 2 logs or greater by week 12. Transiently undetectable levels of HCV RNA during treatment is considered breakthrough; whereas patients who remain HCV RNA negative while on treatment but become HCV RNA positive at some point after are considered relapsers.

Viral and Host Factors

Several viral factors are associated with treatment response. As mentioned previously, genotype 1 infections respond less favorably to therapy. Baseline viral load is an additional predictor of SVR. Patients with lower baseline viral RNA (< 60,000 – 80,000 IU/ml) typically fare better than those with high baseline levels [6, 21]. Some studies have additionally suggested that a correlation exists within a small segment of the carboxy-terminal of the NS5A protein of HCV, the IFN-α sensitivity-determining region (ISDR), and SVR. However, the results of those studies are inconclusive. For example,

some have reported an increased number of ISDR mutations in patients who attained SVR, while others found few, if any, mutations within the same region [22].

In regards to host factors, gender, age, race, specific alleles of the *IL-28B* gene, liver disease, obesity, co-morbidities such as HIV-coinfection, and baseline expression of IFN-stimulated genes have all been found to impact treatment response. Positive predictors of SVR include female gender, younger age, and the absence of fibrosis or cirrhosis [6]. African-Americans have historically responded poorly to therapy and recent studies indicate allelic variation within the *IL-28B* gene may be responsible. Initially reported by Ge *et al.,* a polymorphism near the *IL-28B* gene strongly correlated with SVR across all populations examined [23]. In addition, the C/C allele occurred at a substantially greater frequency in those of European ancestry and associated with SVR. Although African-Americans had an overall low C allele frequency, those who were genotype C/C also experienced significantly higher rates of SVR, as opposed to African-Americans who were either C/T or T/T. This suggests that individual genetics, rather than race, is a better predictor of treatment response. Two additional genome-wide association studies found similar associations for *IL-28B* regions within Japanese and Australian populations [24, 25]. Obesity and HIV co-infection are also negatively linked with disease prognosis. HIV/HCV infected individuals have lower rates of spontaneous clearance, increased disease progression, a twofold greater chance of developing cirrhosis, greater occurrences of RBV-induced anemia and lower overall SVR rates [6, 26]. Lastly, baseline levels of interferon-stimulated genes (ISGs) may additionally affect patient response to therapy. Several groups have shown that various ISGs are upregulated in non-responders (NRs) [27-29]. Researchers speculate that increased ISG expression

prior to therapy may represent an already maximally induced immune system. In accordance, gene expression profiling of ISGs in patients receiving treatment show similar expression levels in both NRs and rapid responders (RR, or those who achieve RVR). However, normalization of ISG expression to pre-treatment levels produced a greater fold change in ISGs for RRs, as opposed to non-RRs. This may explain why heightened ISG expression prior to therapy may not be advantageous; as there is little room for improvement once IFN is given.

New and Experimental Treatment Options

Despite extensive efforts to alter the current drug regimen, the SOC has remained unchanged for nearly ten years. Both RBV and IFN are known to have side effects and RBV in particular is known to induce hemolytic anemia [6]. Consequently, substantial attempts have been made to replace RBV with a prodrug that produces fewer side effects and specifically targets the liver. Unfortunately, these attempts have been unsuccessful. Phase III trials with the RBV prodrug teribavirin, formerly known as viramidine, produced significantly fewer cases of RBV-induced anemia and significantly lower SVR rates [30]. Several specifically targeted antiviral therapy (STAT-C) candidates have demonstrated promising results and may be used in combination with IFN and RBV based therapies. For example, telaprevir, an HCV NS3/4A protease inhibitor, in combination with IFN/RBV therapy resulted in increased SVR rates in PROVE 2 and 3 trials when compared to standard IFN/RBV therapy or IFN/telaprevir combination therapy [31]. Triple combination treatment with boceprevir, another protease inhibitor, also demonstrated increased efficacy over standard IFN/RBV combination [32].

Unfortunately, resistant HCV variants were identified in patients for both telaprevir and boceprevir, suggesting that targeting the virus directly may result in increased selection of resistant mutants [33]. Whether this becomes problematic in patients receiving triple combination therapy remains to be determined.

RIBAVIRIN

History

Synthesized in 1972, RBV was initially found to have broad antiviral activity against several DNA and RNA viruses [34, 35]. Its clinical history began in 1986 when the FDA officially approved RBV for use in the treatment of pediatric respiratory syncytial virus (RSV) infections [36]. RBV was also investigated as a potential therapeutic for other viral infections, including Lassa fever, influenza, herpes and measles. Surprisingly, investigations into RBV's use for HCV infections did not occur until the 1990s when a pilot study by Reichard *et al.* reported reductions in serum ALT levels following RBV treatment of HCV patients [37]. Research into RBV's use for hepatitis patients continued throughout the 1990s and eventually, in 1998, it was approved by the FDA for use in combination treatment with IFN-α [38].

Mechanism of Action

Despite significant effort to understand the mechanism for which RBV acts against HCV, the precise answer remains elusive. Several theories have been proposed and include: host enzyme inosine monophosphate dehydrogenase (IMPDH) inhibition, viral RdRp inhibition, mutagenesis, immunomodulation, and enhanced ISG induction [39, 40]. Following entry RBV is phosphorylated by various host kinases. Ribavirin monophosphate (RMP) is an excellent mimic of inosine monophosphate (IMP), the natural substrate of IMPDH. IMPDH is responsible for host *de novo* guanosine triphosphate (GTP) synthesis [41]. Binding of RBV to the substrate pocket of IMPDH

results in enzyme inhibition, and a subsequent reduction in host GTP pools. Therefore, viral replication can be inhibited due to a lack of available nucleotides. Some have additionally proposed that incorporation of ribavirin triphosphate (RTP) may directly inhibit viral replication [42-44]. Incorporation of RTP by the viral RdRp may block chain elongation, or result in the formation of stalled elongation complexes, which results in replication termination [39, 43]. Others have suggested that RBV acts a RNA virus mutagen. RTP incorporation into the viral genome can increase mutation rates [42, 44-46]. Since RNA viruses have high error frequencies, increased mutation rates can induce viral error catastrophe [47]. RBV exposure induced an increased number of transition mutations (C → U or G → A) within the poliovirus genome, used as a model RNA virus. Moreover, increased rates of transition mutations have been found within HCV patients [46, 48]. It has been further proposed that RBV acts indirectly against HCV by shifting the immune response from a humoral T_H2 response to a cell mediated T_H1 response [49, 50]. The most recently proposed anti-HCV mechanism of action, by Feld *et al.*, suggests that RBV enhances ISG induction when used in combination with IFN [28]. The authors examined hepatic gene expression in HCV patients prior to and during therapy. The addition of RBV to IFN enhanced expression of the IFNα receptor, IFN-regulatory factor 7 (IRF7) and IFN-regulatory factor 9 (IRF9). Therefore, RBV may act by increasing cell responsiveness to IFN and enhancing endogenous ISG production. Additionally, RBV treatment down-regulated hepatic stellate cell (HSC) activation, which may reduce hepatic fibrogenesis and increase apoptosis. In a recently published study, RBV therapy improved the kinetics of early responses to IFN therapy, but this result was only seen in patients who had an "adequate, but not overwhelming, response to interferon" [51].

Therapeutic Role

Prior to the addition of RBV, IFN-α monotherapy typically produced poor response rates ranging from 16 – 20% in HCV genotype 1 patients [21]. Following inclusion of RBV, SVR rates were doubled to roughly 38% [52]. Further advances were made with the advent of pegylated interferon (Peg-IFN) which increased the half-life of interferon through covalent attachment of a polyethyleneglycol molecule [21].

Exactly how RBV synergistically works with IFN remains to be determined. RBV alone does not impact serum HCV RNA levels to a large degree, but does reduce serum ALT levels [37, 53-55]. Several groups have demonstrated a correlation between higher RBV serum levels and successful treatment outcome [56-61]. In accordance, receiving less than 80% of the suggested RBV dosage resulted in a 20% drop in SVR [20]. Historically, RBV has had a more prominent role in the prevention of breakthrough and relapse, thus increasing the overall SVR rate [52, 62, 63]. More recent studies have suggested synergistic activity through augmentation of IFN responses [28, 51]. As RBV has been theorized, and observed in some

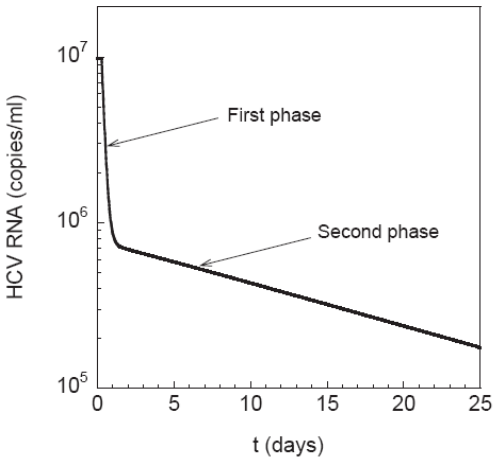


Figure 3. Typical two-phase profile of plasma HCV RNA decline in patients following the onset of interferon therapy.

Reprinted with kind permission from Springer Science + Business Media: Dixit NM, Perelson AS (2006). *Cell Mol Life Sci* **63:** 832-842.

clinical studies, to have a more pronounced effect on the second phase of viral decline (**Fig. 3**), increasing IFN responsiveness would appear to be consistent with the kinetics of viral decline [39, 51, 64].

NUCLEOSIDE TRANSPORTERS

Cellular and Therapeutic Roles

Nucleoside transporters are involved in the cellular import of natural and synthetic nucleosides and mediate uptake of the guanosine analog RBV [39]. ENT1 is the primary RBV transporter, however, CNT2, CNT3 and ENT2 may also transport RBV in various cell types [65-68]. Nucleoside transporters are divided into two categories, the equilibrative nucleoside transporters ENT1 – 4, which mediate facilitated bidirectional diffusion of nucleosides, and the concentrative nucleoside transporters CNT1 – 3 which are capable of transport of against a concentration gradient [69]. Each differs in their tissue distribution and substrate preference. ENT1 is ubiquitously expressed and known to have broad substrate selectively, transporting both purine and pyrimidine nucleosides. Likewise, CNT3 also exhibits broad substrate selectivity, although its tissue distribution is limited. In spite of the large volume of research, much remains to be elucidated in regards to regulation. Expression can vary depending on host deoxynucleotide levels, cell cycle, differentiation state, nutrient availability and under hypoxic conditions [69, 70]. Due to their ability to transport synthetic nucleosides, nucleoside transporters hold considerable clinical significance.

POLIOVIRUS AND HCV RESISTANCE STUDIES

Prior Work

Previous efforts to understand host-based mechanisms of RBV resistance originated from studies using the model RNA virus poliovirus, known to be sensitive to high concentrations of RBV [45]. In work published by Pfeiffer and Kirkegaard in 2003 a poliovirus mutant was generated which exhibited RBV resistance [71]. Analysis of the mutant determined that resistance had arisen from a single mutation, G64S, within the coding region of the viral RdRp. The resulting effect was an increase in replication fidelity, which the authors hypothesized contributed to reduced nucleotide analog incorporation and resistance. In an effort to extend these studies, several RBV resistant (RBV^R) HCV replicon-containing cell lines were generated via passage in increasing concentrations of RBV [72]. At the time research was greatly limited due to the absence of an infectious cell culture system and relied upon HCV subgenomic replicon systems which allowed for HCV replication, but failed to produce infectious particles [73]. RBV^R replicons were tested to determine whether resistance was replicon or cell derived (**Fig. 4**). Whereas some low level resistance was found to be replicon derived, the majority was cell derived [72]. Analysis of RBV^R cells which had been cured of the replicon by treatment with IFN-α suggested resistance had occurred through reduced RBV import. These results laid the foundation for the current focus of this project, which attempts to understand host-based mechanisms of RBV resistance in relation to HCV treatment response.

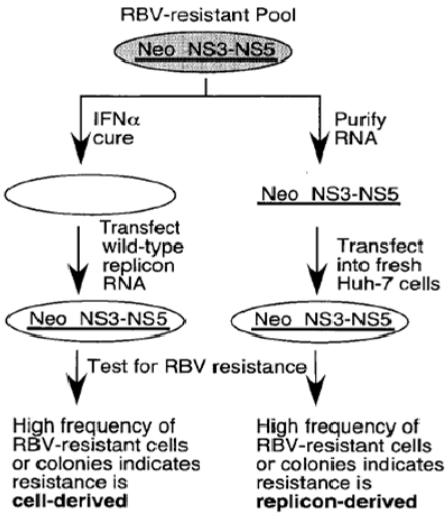


Figure 4. Flow chart for mapping RBV resistance to cell line or replicon.

The left branch depicts RBV-resistant cell lines cured of replicon RNA by passage in IFN-α in the absence of G418. Cured cells were then transfected with wild-type replicon RNA, and the resulting G418-resistant cells were pooled and tested for resistance to RBV. The right branch depicts total RNA harvested from RBV-resistant cell lines and transfected into fresh Huh-7 cells. After selection in G418, individual colonies were picked, expanded, and tested for resistance to RBV.

CHAPTER TWO
Methodology

Cell Lines

All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) or calf serum, and 100 U of penicillin-streptomycin/ml. The Cured 4 cell line has been previously described [72]. Briefly, RBV^R replicon-containing Huh-7 cells were generated by repeated passage in RBV, and the cells were cured of the replicon by interferon treatment. Huh 7.5 (human hepatoma) cells have also been previously described and were kindly provided by C. Rice [74]. Similar to the Cured 4 cell line, Huh 7.5 cells were also made from replicon-containing Huh-7 (human hepatoma) cells which had been cured of the replicon through high dose IFN treatment. The T55I mutation within the retinoic acid inducible gene (RIG-I) of Huh 7.5 cells results in a loss of signaling to interferon-regulatory factor 3 (IRF3) thereby allowing increased viral permissivity [75]. Additional cell lines include: 293 (human embryonic kidney), HeLa (human cervical carcinoma), HepG2 (human liver, a kind gift from M. Gale), PH5CH8 (human primary liver cells, a kind gift from M. Gale), MCF7 (human breast adenocarcinoma).

Generation of RBV^R Cell Lines and RBV Resistance Assay

RBV^R cell lines were generated as previously described [76]. Briefly, cells were initially passaged in media containing 100 μM RBV for approximately four weeks. The concentration of RBV was subsequently increased in two-week intervals until a concentration of 400 μM was achieved. Following approximately 2-4 weeks in media

containing 400 μM RBV, the level of RBV resistance was determined as previously described [76], by splitting cells every 1-3 days for 10 days and staining with crystal violet to visualize cellular viability.

Huh 7.5 Viability Assays

Viability and resistance was measured using a crystal violet assay previously described [72] in conjunction with viable cell counts following three-day exposure to increasing concentrations of RBV. For the crystal violet assay, cells were passaged at the indicated drug concentrations, split every 1-3 days for 7-10 days, and then visualized via crystal violet staining. Similar methods were employed for crystal violet viability assays using RBV and Guanosine (Guo) and included equimolar concentrations (0, 100, or 400 μM) of RBV and Guo, or Guo alone. For three-day viability assays, subconfluent cells were treated in duplicate with increasing concentrations of RBV, or RBV and Guo. Viable cell numbers were determined on day three using trypan blue exclusion and normalized as a percent of the 0 μM plates.

Virus

Mahoney serotype 1 poliovirus was generated as previously described from a single plaque following transfection of the viral cDNA clone [77]. High titer stocks were also generated as previously described [78]. HCV stocks were generated using infectious JFH-1 (genotype 2a) [79] from a synthetic clone kindly provided by M. Gale (to be described elsewhere). Supernatants from serially infected Huh 7.5 cells were concentrated using Centricon Plus-70 filters (Milipore) according to the manufacturer's

recommendation. Briefly, membranes were pre-rinsed with phosphate-buffered saline (PBS; Fisher) to remove trace glycerol. The viral supernatants were then spun at up to 3,500 x g for approximately 20 – 25 minutes, flow through discarded, inverted and given a shorter spin at no more than 1,000 x g for 2 – 5 minutes. This process was repeated several times until all of the supernatant had been collected, after which aliquots were made and stored at -80˚C until needed.

Poliovirus Infections

All plaque assays were performed at 37°C. For the Cured 4, Huh-7, HeLa, HepG2 and PH5CH8 cells, virus was added at 70-100 plaque forming units (PFU)/monolayer in 60 mm tissue culture plates. The MCF7 cells were infected with 10-fold more virus (700 pfu/plate) due to decreased permissivity. Following a 30-minute incubation period, virus was aspirated and an agar overlay, with or without 400 μM RBV (Sigma), was added. After 48 hours incubation, the cells were stained with crystal violet to visualize plaques.

HCV (JFH-1) Infections

In figure 13a and 13b experiments, cells were pretreated for two hours with 0 μM RBV, 15 μM RBV, 50 μM RBV, 100 μM RBV, 100 μM Guo, or 100 μM RBV and 100 μM Guo, infected at an multiplicity of infection (MOI) of 0.05, and given fresh drug-containing media after infection. After 72 hours, viral titers from cell supernatants were determined using a focus forming assay, described below [80]. For serial passage infections in figure 13c and 13d, cells were infected at an MOI of 0.1 for three days,

expanded, and on day six, the supernatant was harvested for titering and to begin a new infection in freshly plated cells (passage 2) and so forth. Infections continued through completion of six passages, representing 36 days of viral culture.

Focus Forming Assay

Huh 7.5 cells were seeded on 48-well plates 24 hours in advance to allow for subconfluent conditions the day of infection. On the following day serial dilutions were prepared from supernatants which were previously collected from viral infections. The cells were rinsed with PBS and incubated at 37˚C with 100 µL undiluted, or serially diluted, viral sample for approximately one hour. Each condition was performed in triplicate. Following incubation, each well was rinsed twice with PBS to remove any non-bound virus and given fresh, complete, DMEM and allowed to proceed for 48 hours. At the end of the infection period each well was rinsed twice with PBS and fixed with 4% paraformaldehyde (PFA). The plates were then either permeabilized with 0.2% Triton-X or stored at 4˚C until the rest of the assay could be completed. Following permeabilization blocking was conducted with 10% FBS for at least 10 minutes. Primary probing was performed using human antiserum specific to HCV and capable of detecting HCV genotypes 1 and 2, kindly provide by M. Gale [81]. Secondary binding was conducted using donkey anti-human horseradish peroxidase conjugated (HRP) IgG (Jackson ImmunoResearch). Infected cells were then visualized using the Vector VIP peroxidase substrate kit (Vector Laboratories) according to the manufacturer's recommendations and quantified by visual microscopy counts. Focal forming units

(FFU)/mL were determined with the following formula FFU x dilution factor x (1000 µl / 100 µl innoculum) = FFU/mL.

Uptake Assays

Cells were plated the day prior to the assay on 35 mm tissue culture plates to generate roughly 50-80% confluency on the day of the experiment. For the [^{3}H]-RBV uptake assays, cells were treated with 100 µl media containing 5 µM RBV, 1% of which was [^{3}H]-labeled to serve as a tracer ([^{3}H]-RBV, Moravek, Brea, Calif). Following a 30 minute incubation period at 37°C, the cells were washed twice with ice-cold PBS to terminate uptake. The cells were then trypsinized and collected via centrifugation. Cell pellets were resuspended in 100 µl cold lysis buffer [10 mM Tris at pH 8, 10 mM NaCl, 1.5 mM MgCl2 and 0.1 % NP40]. Cell debris was removed by centrifugation and 40 µl of the supernatant was quantified by scintillation counting. Cell number was determined by trypan blue counting trypsinized cells from an additional plate. Uptake values were determined by multiplying the counts per minute (CPM)/cell by 2.5 to account for the amount of lysate counted in the scintillator. The hypoxanthine uptake assays were performed in the same manner, except that the incubation period was reduced to five minutes to minimize the amount of [^{3}H]-hypoxanthine (Moravek, Brea, Calif) metabolized and exported out of the cell.

Inhibitor Assays

For uptake assays involving the inhibitor nitrobenzylmercaptopurine riboside (NBMPR, Sigma), cells were pretreated at the appropriate concentration for 15 minutes.

For those involving phloridzin (Sigma), 15 minutes of pretreatment was not required and therefore the pretreatment incubation period was shortened to five minutes. Following inhibitor pretreatment, uptake assays were preformed as described above, with the exception that the incubation period was reduced to five minutes.

Transfections

Transfections were performed using 2 μg of plasmid DNA [pcDNA 3.1, pENT1, pCNT1, pCNT2, pCNT3 (kindly provided by K. Giacomini, UCSF) or pLacZ], and Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Briefly, cells were prepared 24 hours in advance to allow for subconfluency the day of transfection. The following day solutions of lipofectamine and DMEM was prepared and combined with a prepared solution containing DMEM and 2 μg of the appropriate plasmid DNA. The lipofectamine/DNA mixture was then allowed to sit at room temperature for 20 minutes. Following the 20 minute incubation the cells were washed with DMEM and then incubated with the previously prepared liopfectamine/DNA solutions at 37˚C. Then the transfection media was removed and replaced with fresh, complete, DMEM. Twenty-four hours later the uptake assay was performed as described above. pLacZ-transfected cells were stained as previously described [82] with X-gal at 24 hours post-transfection to monitor transfection efficiency, which ranged from 40-90%, based on the number of blue cells observed under the microscope.

Real-time RT-PCR

Total RNA was isolated from RBV^R and RBV^S Huh-7 and Huh 7.5 cells as previously described [83]. Briefly, 1 x 10^7 cells were collected and resuspended in cell lysis buffer, and placed on ice for five minutes. Following centrifugation, sodium dodecyl sulfate (SDS) buffer was added and samples were vortexed, followed by incubating the samples with proteinase K. Nucleic acids were phenol/chloroform/isoamyl alcohol extracted, treated with a DNase solution, and subjected to an additional phenol/chloroform/isoamyl alcohol extraction. The relative levels of ENT1, CNT3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH - as an internal control) RNA were assessed using one-step RT PCR and the comparative (ΔΔCt) method [84]. Reactions were performed in an ABI 7500 sequence detection system (Applied Biosystems) and analyzed using ABI Sequence Detection 1.3 software. Reaction mixtures were plated in triplicate, each in a total of 25 µl, and consisted of 50 ng of whole cell RNA from RBV^R and RBV^S Huh-7 cells, SYBR GREEN PCR master mix (Applied Biosystems), 1.25 µM primer, RNasin (40 U/µl) and SuperScript II reverse transcriptase (200 U/µl). Reactions were performed according to the manufacturer's suggestions (Applied Biosystems). Cycling conditions were as follows: 1 cycle of 30 minutes at 42°C and 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The ENT1 and GAPDH primers have been previously reported [85, 86]. The CNT3 primer was designed using Primer3 v. 0.4.0 [87]. The forward and reverse CNT3 primer sequences are ACCTGATGGCCAAATACGAAC and GAGCTCCAGATCACCCACTTC, respectively. Each primer was validated according to standard protocol [84] and the products were additionally verified by agarose gel electrophoresis. Analysis and methods

used to determine fold change ($2^{-\Delta\Delta Ct}$) using the comparative ($\Delta\Delta$Ct) method have been previously described [84].

Immunoblot Analysis

Approximately 5 x 10^5 Huh-7 and RBV^R Huh-7 or Huh 7.5 cells were collected via trypsinization and centriGuotion, followed by three washes with ice-cold PBS. Cell pellets were resuspended in a lysis buffer (10 mM Tris, 150 mM NaCl, 0.02% NaN_3, 1% Na-deoxycholate, 1% Triton-X 100, 0.1% SDS) containing 10 µl/mL each of protease inhibitor cocktail (Sigma) and phosphatase inhibitor (Calbiochem). Lysates were centrifuged and supernatants were removed and transferred to fresh tubes for protein concentration determination using the Bio-rad protein assay. Then, 20 µg of protein was separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Following primary (rabbit polyclonal Ig ENT1, Abgent) and secondary (donkey anti-rabbit IgG-HRP, Jackson Immunoresearch for Huh-7 or donkey anti-rabbit IgG-HRP, Jackson Immunoresearch for Huh 7.5 cells) antibody probing, blots were developed using ECL Plus chemiluminescence reagent (Amersham) according to the manufacturer's recommendations. For analysis of GAPDH, blots were probed with goat anti-human GAPDH (V-18, Santa Cruz) diluted in 5% bovine serum albumin (BSA, Fisher) in PBS-T/0.15 M NaCl, and donkey anti-goat IgG-HRP (Santa Cruz).

PKR Analysis

Subconfluent cells were treated with media (mock), 100 μM of RBV, or 100 U/ml of human IFN-α (PBL Interferon Source) for 24 hours. After incubation the cells were collected and prepared for immunoblot analysis as described above.

Subjects and PBMC processing

Blood from eight healthy donors and 16 genotype 1 HCV-infected patients was collected and processed for peripheral blood mononuclear cell (PBMC) isolation by Ficoll centrifugation. Isolated PBMCs were stored in a standard freezing mix containing 15% FBS and stored in liquid nitrogen following preliminary freezing at -80˚C in an isopropanol bath. All patients received 800-1200 mg/day RBV (per body weight) in combination with pegylated-IFN-alpha 2a (Pegasys® ; F. Hoffman-La Roche;Nutley, NJ) at a dose of 180 mcg subcutaneously for 48 weeks. Blood was collected prior to the onset of treatment and at 14 and 28 days post-treatment. Serum HCV RNA levels on day 28 were used to determine rapid treatment response. Rapid response was defined as having undetectable HCV-RNA in branch DNA analysis (bDNA; < 615 RNA Units IU/mL) or PCR analysis (< 50 IU/mL). Signed consent was obtained from all HCV-infected patients and healthy donors, and all research was pre-approved by the UT Southwestern IRB. HCV patients were included if they were receiving HCV treatment and were willing to provide consent (UT Southwestern IRB protocols #102005-009 to M. Jain, and #082007-080 to J. Pfeiffer). Further patient information is available in table 2. For healthy donors, the only exclusion criteria were based on general health and willingness to participate and provide consent (UT Southwestern IRB protocol #082006-081 to J. Pfeiffer).

RBV Uptake in PBMCs

The [^{3}H]-RBV uptake assay described above was modified for use in PBMCs. Frozen PBMCs from healthy donors were quickly thawed, resuspended in RPMI media (Thermo Scientific) and incubated for a 6-hour recovery period at 37˚C prior to the assay. Viable cell numbers were determined post-recovery using trypan blue exclusion. To determine the linear range of the assay PBMCs were resuspended in DMEM, in duplicate, at various concentrations (Total PBMC/mL medium). Each was then treated with 5 μM RBV (1% of which was [^{3}H]-labeled) for 15 minutes, and CPM in lysates were quantified in a scintillation counter. The amount of RBV uptake linearly correlated with the number of cells used in the assay in the range of 1 x 10^5 to 1.2 x 10^6 cells and is shown in appendix B (GraphPad Prism 5.03, linear regression analysis). The control sample consisted of healthy donor PBMCs frozen into aliquots to allow normalization for experiments performed on different days. Normalized values were obtained by calculating the average CPM/cell of each sample to a percent of the average CPM/cell of the control sample (HCV PBMC Avg. CPM/cell divided by Healthy Donor Control PBMC Avg. CPM/cell). The control CPM/cell was set to 100%. The absolute CPM/cell for the control sample ranged from 2.50 x 10^{-4} to 9.97 x 10^{-4} (Avg., 5.34 x 10^{-4}, SD, 1.67 x 10^{-4}).

Statistical Analysis

Statistical significance for studies presented in chapter four was determined using independent two-sample Student's *t* test. Statistical significance was assigned to p values less than 0.05. In the following chapter for host-based RBV resistance in HCV patients,

statistical significance was determined as follows: for RBV^R and RBV^S Huh 7.5 experiments, excluding real time RT-PCR analysis, significance was determined using independent two-sample Student's *t* test. In assays using PBMCs, the Student's *t* test was two-tailed and either paired (for comparison within a donor) or unpaired (for comparison between donors).

CHAPTER THREE
Reduced Ribavirin Antiviral Efficacy via Nucleoside Transporter-Mediated Drug Resistance

INTRODUCTION

Approximately 170 million people are infected with hepatitis C virus (HCV), with the majority developing chronic infection [88]. With no vaccine currently available, the only approved treatment consists of a combination of interferon-α. (IFN-α) and ribavirin (RBV), a Guanosine nucleoside analog. IFN-α monotherapy has limited success, with only 16-20% of genotype 1 infected patients achieving a sustained virological response (SVR). However, the addition of RBV doubled response rates to 35-40%. Current treatment regimens including pegylated interferon and RBV achieve SVR rates of 54-56% in genotype 1 infected patients, while SVR rates of 70-80% are achieved in genotype 2/3 infected patients. Patient response is divided into three categories: SVR, end of treatment response (ETR) and relapse, and non-response (NR). Little is known about factors that influence treatment response, although various host and viral factors have been implicated. For instance, genotype 1 infections are more difficult to treat than other genotypes. Additionally, male gender, African-American race, advanced age, fibrosis, obesity, HIV co-infection, and low RBV serum concentrations have been negatively correlated with treatment success [21, 56-60].

Although RBV clearly plays a role in HCV treatment response, the antiviral mechanism remains controversial. There are many proposed mechanisms of action for RBV [39, 42-44]. First, RBV directly inhibits the viral RNA-dependent RNA polymerase through incorporation of RBV triphosphate (RTP). Incorporation of RTP inhibits chain

elongation and cause termination. Second, RBV inhibits the activity of inosine monophosphate dehydrogenase (IMPDH) [41, 89, 90], the host enzyme responsible for de novo synthesis of GTP. The monophosphorylated form of RBV, RMP, binds to the substrate pocket of IMPDH, thereby inhibiting the enzyme and reducing host nucleotide pools, which are required for viral replication. Third, RBV is a viral mutagen [9, 45, 46]. For poliovirus, incorporation of RTP into the viral RNA causes transition mutations [45]. Fourth, RBV shifts the immune response to a beneficial T_H1 cell mediated response [49, 50].Fifth, RBV enhances ISG induction when used in combination with IFN [28].

Factors that influence treatment response are not completely understood. Unlike HIV, there have been no clear HCV drug resistance mutations identified which can account for treatment failure [22, 91-95]. Therefore, HCV treatment response may be influenced more by host factors than by viral factors. Our previous work sought to determine whether RBV-resistant (RBV^R) HCV replicon-containing cells could be generated [76]. Whereas some low-level resistance occurred through mutations in the replicon, the majority of resistance occurred through changes in the cell line. These RBV^R cells demonstrated a RBV uptake defect.

RBV is imported into cells through host nucleoside transporters, which are divided into two categories, equilibrative and concentrative [66, 69]. The equilibrative nucleoside transporters include ENT1, ENT2, ENT3 and ENT4, which are carrier proteins that mediate facilitated bi-directional diffusion of nucleosides across the cell membrane. The concentrative transporters CNT1, CNT2 and CNT3 are able to transport nucleosides against a concentration gradient by coupling transport to the inwardly directed sodium gradient. The transporters differ in tissue distribution and preference of

substrate. ENT1 is generally considered ubiquitous and has broad substrate selectivity. Although ENT2 and CNT3 are also broadly selective, ENT2 is predominantly expressed in skeletal muscle, while CNT3 is largely found in the mammary gland, pancreas, bone marrow, and trachea [69, 96, 97]. In addition to importing natural nucleosides, both the concentrative and equilibrative transporters are capable of importing synthetic nucleosides [65, 98-102]. Therefore, nucleoside transporters are important clinically, since many nucleoside analogs are currently in use for the treatment of cancer and viral infections. Although the anti-HCV mechanism of RBV is controversial, at least four of the five proposed mechanisms require RBV import into the cell. We have therefore focused our efforts toward examining RBV uptake and the effect of uptake on antiviral efficacy.

In this study we sought to understand RBV uptake and cell-based mechanisms of RBV resistance. We were able to generate RBV^R cells in all cell lines tested, and we demonstrated that resistance is likely achieved by reduced ENT1-mediated uptake. We found that, in the presence of RBV, RBV^R cells facilitate increased growth of poliovirus, a model RNA virus with known sensitivity to RBV, compared to RBV-sensitive (RBV^S) cells. Additionally, by inhibiting equilibrative transport, the RBV^R phenotype was mimicked in RBV^S cells, for both RBV uptake and poliovirus growth. Our results suggest that the level of RBV uptake may affect HCV treatment response, either through natural equilibrative transport variance in patients or through acquired resistance.

RESULTS

RBV Uptake and Poliovirus Growth in Different Cell Lines

To assess whether the amount of RBV uptake could impact viral replication, we measured RBV uptake in a variety of cell lines and measured the growth of a model RNA virus, poliovirus. Various cell lines were tested, including several liver cell lines (Huh-7, HepG2, and PH5CH8) and a Huh-7-based cell line previously shown to have reduced RBV uptake (Cured 4)[76] . As expected, the RBV^R Cured 4 cells took up significantly less RBV than all cell lines tested (**Fig. 5A**, $p \leq 0.005$). Although the amount of RBV uptake varied between the remaining cell lines, Huh-7 cells took up significantly less than HeLa, HepG2, PH5CH8, and MCF7 cells ($p \leq 0.01$). We then determined whether differences in RBV uptake correlated with poliovirus growth efficiency in the presence of RBV. We chose poliovirus as a model RNA virus because RBV's antiviral mechanism for poliovirus, error catastrophe, has been established [45]. Since the mutagenic effects of RBV are cumulative and require several rounds of viral replication, an infection carried out over several days allows differences in viral growth to be observed [72] [71] [46] [45]. Therefore, we performed plaque assays in each of the cell lines in the absence or presence of 400 μM RBV. Although the addition of RBV greatly inhibited viral growth in the majority of cell lines (**Fig. 5 B**), those with lower RBV uptake levels generally displayed enhanced viral growth, as measured by plaque size. The addition of RBV to the Cured 4 cell line had little effect on viral plaque size. Likewise, although a reduction in viral growth was observed in Huh-7 cells upon addition of RBV, the antiviral efficacy of the drug was reduced compared with cells having higher levels of

uptake. These results suggest the possibility that low drug uptake facilitated enhanced viral growth in the presence of RBV.

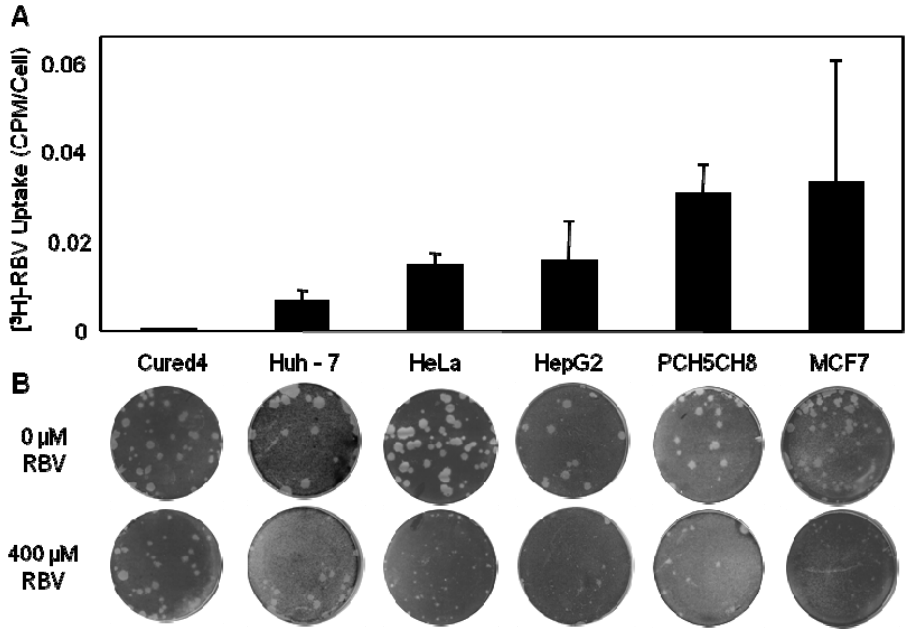


Figure 5. RBV uptake and poliovirus growth in several cell lines.
RBV uptake and poliovirus growth in several cell lines. (A) [3H]-RBV uptake assay. Cells were incubated in media containing 5 µM [3H]-labeled RBV at 37˚C. After 30 minutes, cells were chilled, washed, harvested, and the level of [3H]-RBV uptake was determined by scintillation counting. The data were normalized to account for cell number, and are reported as counts per minute (CPM) per cell. The average values and SD from at least three independent experiments performed in duplicate are shown. The difference between Cured 4 values and all other values was statistically significant ($p \leq 0.005$, Student's t test). Huh-7 cells took up significantly less than HeLa, HepG2, PH5CH8, and MCF7 cells ($p \leq 0.01$). (B) Poliovirus plaque assays. Cells were infected with poliovirus and agar overlays, containing 0 µM or 400 µM RBV, were added. Following 48 hours the overlays were removed and cells were stained with crystal violet to visualize plaques.

Generation and Characterization of New RBV^R Cell Lines

The RBV^R Cured 4 cell line generated previously had initially contained the HCV replicon, which was cured by interferon treatment [72]. Consequently, it is possible that cellular resistance could have arisen due to virally induced changes in the cells. For this reason, we generated new RBV^R versions of three cell lines, Huh-7, 293, and HeLa. RBV is toxic to a variety of cell lines at high μM concentrations [72, 103]. Therefore, we generated RBV^R cell lines by passage in increasing doses of RBV as previously described (**Fig. 6A**) [72]. Once the cells were able to withstand high concentrations of RBV, they were tested for resistance alongside the parental RBV^S cell lines. Both the RBV^S and RBV^R cells were passaged for 10 days in media containing 0 μM, 100 μM, or 400 μM RBV. As shown in Figure 2B, each of the RBV^R cell lines survived treatment with either high or low dose RBV, whereas their RBV^S counterparts could not. Importantly, all of the RBV^R cells demonstrated significantly reduced RBV uptake (**Fig. 6C**, $p < 0.01$).

Our previous results suggested that reduced RBV uptake promotes increased viral growth [72]. Therefore, we performed poliovirus plaque assays to determine whether the new RBV^R cell lines demonstrate increased permissivity for viral growth in the presence of RBV. Whereas RBV treatment severely limited viral growth in RBV^S HeLa cells, it had little effect on viral growth in RBV^R HeLa cells (**Fig. 6D**). Poliovirus grown in RBV^R Huh-7 cells also displayed increased plaque size in the presence of RBV, although the viral growth difference between RBV^S and RBV^R Huh-7 cells was less than the viral growth difference observed in HeLa cells. The minimal increase in plaque size in the RBV^R Huh-7 cells in the presence of RBV could be due to the reduced uptake in

these cells (**Fig. 5**), or other host factors that enhance viral growth. Overall, these results indicate that a small reduction in RBV uptake (~50% reduction in HeLa cells, **Fig. 6C**) can have a major impact on poliovirus growth (**Fig. 6D**, HeLa cells).

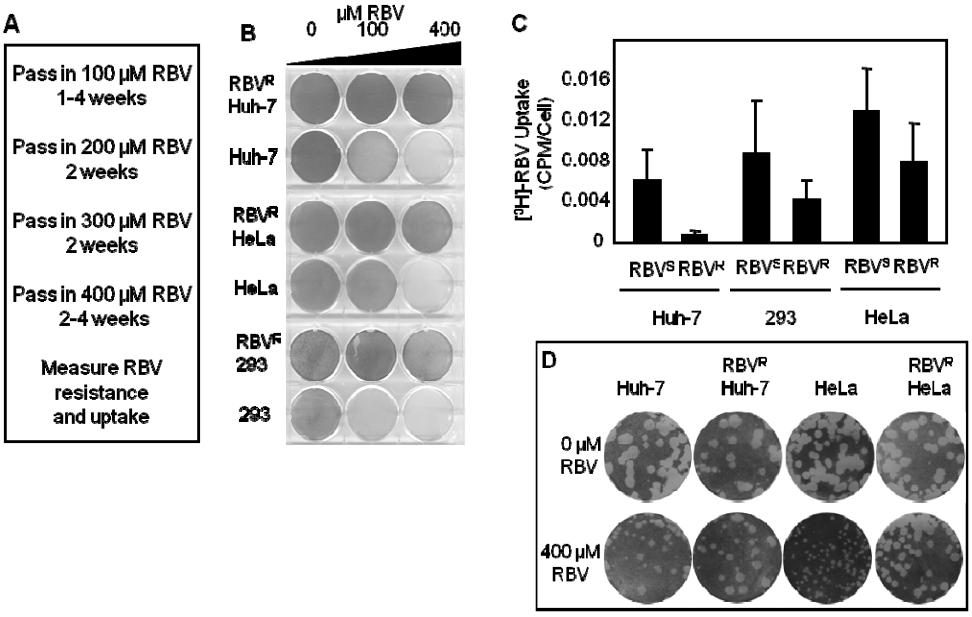


Figure 6. Generation of RBV^R cell lines and the effect of RBV resistance on poliovirus growth.

(A) Generation of RBV^R cell lines. Huh-7, HeLa, and 293 cells were passed for 1-4 weeks in media containing 100 µM RBV. The dosage of RBV was increased by 100 µM every two weeks until a concentration of 400 µM was achieved. Following 2-4 weeks in 400 µM RBV, the cells were assessed for RBV resistance and RBV uptake. (B) RBV resistance assay. The RBV^R and RBV^S parental cells were passed for 10 days in media containing 0 µM, 100 µM or 400 µM RBV. The cells were then stained with crystal violet to visualize cellular viability. (C) [^{3}H]-RBV uptake. Cells were incubated in media containing [^{3}H]-RBV for 30 min. at 37˚C and the level of [^{3}H]-RBV uptake determined by scintillation counting of cell lysates. The average values and SD from 6 independent experiments are shown. The difference in uptake between RBV^S and RBV^R cell lines was statistically significant ($p < 0.01$, Student's *t* test). (D) Plaque assays for RBV^R and RBV^S parental cells. Cells were infected with poliovirus and agar overlays, containing 0 µM or 400 µM RBV, were added. Following 48 hours the overlays were removed and cells were stained with crystal violet to visualize plaques.

The Role of Nucleoside Transporters in RBV Uptake and Resistance

RBV is transported into cells via nucleoside transporters. ENT1 is thought to be the primary RBV transporter [66], although ENT2 and CNT2 may also transport RBV in human liver [104]. However, most previous studies measured RBV uptake in erythrocytes, and it is well known that nucleoside transporter expression varies in different tissues. To determine which transporters are capable of importing RBV into liver cell lines, we transfected Huh-7 cells with several nucleoside transporter expression plasmids to determine which could increase RBV uptake. As shown in **Figure 7A,** transfection of ENT1 and CNT3 expression plasmids increased RBV uptake. We next determined whether over-expression of ENT1 or CNT3 could rescue the RBV uptake defect in RBV^R cells (**Fig. 7B**). Transfection of ENT1 or CNT3 expression plasmids increased the level of RBV uptake in all RBV^S cell lines tested, as well as in the majority of RBV^R cell lines. Although the level of uptake increased ~3 fold in RBV^R Huh-7 cells, nucleoside transporter over-expression was not able to fully restore the uptake defect (**Fig. 7C**). While we cannot definitively rule out a role for ENT2, CNT1, and CNT2 in RBV transport based on this experiment, in later inhibition experiments, we confirm the role of ENT1 in RBV uptake (**Fig. 8**).

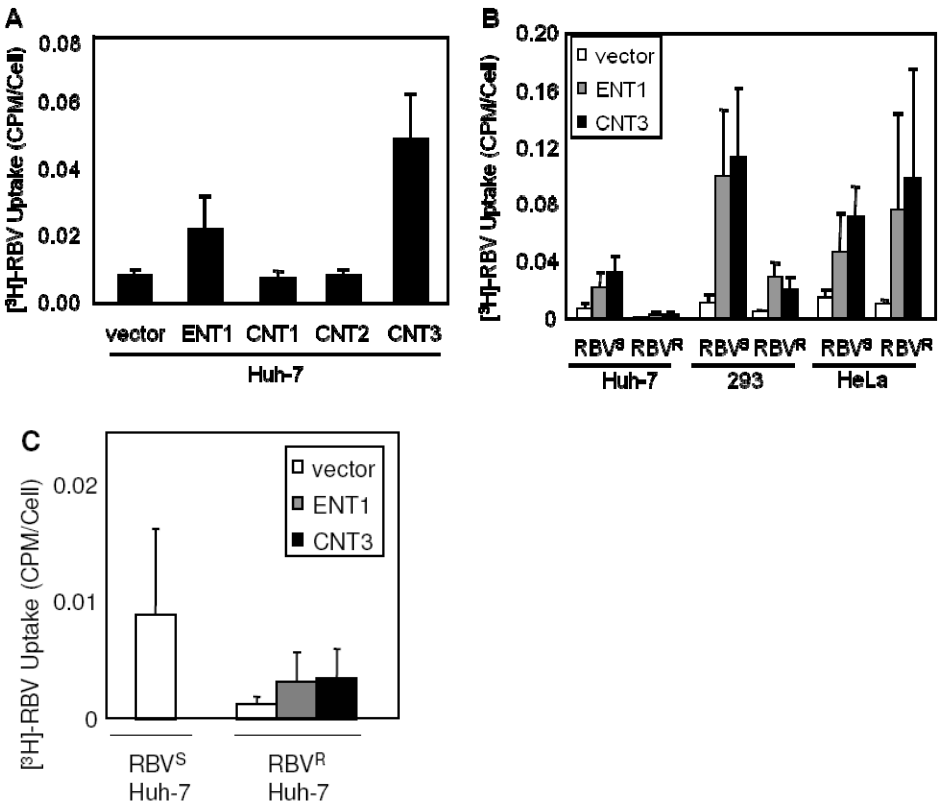


Figure 7. The effect of nucleoside transporter over-expression on RBV uptake.
(A) RBV^S Huh-7 cells were transfected with nucleoside transporter expression plasmids or a vector control plasmid, and the [^{3}H]-RBV uptake assay was performed at 24 hours post-transfection. The average values and SD from three independent experiments are shown. The difference in uptake between vector and ENT1 or CNT3 transfected cells was statistically significant for all cell lines ($p \leq 0.03$, Student's *t* test). (B) RBV^R and RBV^S Huh-7, 293 and HeLa cells were transfected with the vector control, ENT1, or CNT3 transporter expression plasmids, and the [^{3}H]-RBV uptake assay was performed at 24 hours post-transfection. The average values and SD from at least three independent experiments are shown. The difference in uptake between vector and ENT1 or CNT3 transfected cells was statistically significant for all cell lines ($p \leq 0.03$, Student's *t* test). (C) Huh-7 uptake data from panel B graphed on an appropriate scale to highlight uptake differences. The average values and SD from at least three independent experiments are shown.

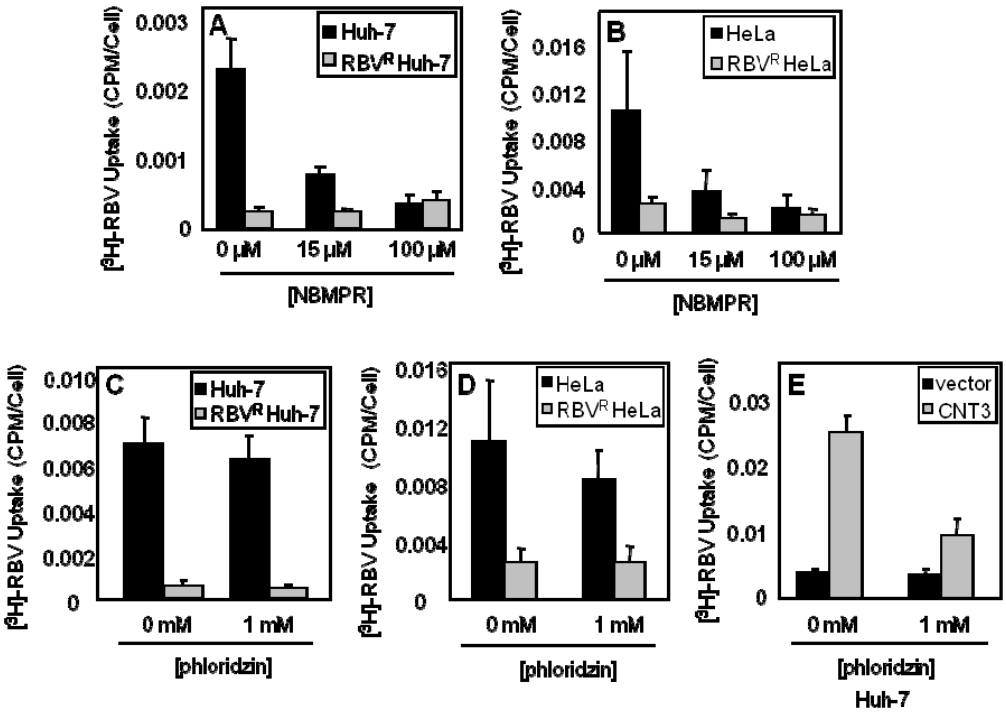


Figure 8. Inhibition of endogenous RBV uptake in Huh-7 and HeLa cells.
(A,B) [^{3}H]-RBV uptake in the presence of NBMPR. Huh-7 cells (A) or HeLa cells (B) were pretreated for 15 min. with 15 µM NBMPR to inhibit ENT1 transport, 100 µM NBMPR to inhibit ENT1 and ENT2 transport, or media lacking NBMPR. Following inhibitor treatment, the level of [^{3}H]-RBV uptake was determined over a 5 min. incubation period. The difference in uptake between NBMPR-treated and untreated cells was statistically significant for both Huh-7 and HeLa cells ($p < 0.04$, Student's *t* test). (C, D) [^{3}H]-RBV uptake in the presence of phloridzin. Huh-7 cells (C) or HeLa cells (D) were pretreated for 5 min. with 1 mM phloridzin, to inhibit concentrative nucleoside transport, or media lacking phloridzin. Following inhibitor treatment, the level of [^{3}H]-RBV uptake was determined over a 5 min. incubation period. The average values and SD from two experiments performed in duplicate are shown. The difference in uptake between phloridzin-treated and untreated cells was not statistically significant ($p > 0.3$, Student's *t* test). (E) To ensure that phloridzin inhibits concentrative transport, Huh-7 cells were transfected with the vector control or CNT3 transporter expression plasmid, and the [^{3}H]-RBV uptake assay was performed at 24 hours post-transfection in the presence or absence of 1 mM phloridzin. The average values and SD from two independent experiments, performed in duplicate, are shown. The difference in uptake between phloridzin-treated and untreated CNT3-transfected cells was statistically significant ($p = 0.001$, Student's *t* test).

Reprinted with permission from ASM: Ibarra KD, Pfeiffer JK (2009). *Journal of virology* **83:** 4538-4547.

The Effect of Nucleoside Transporter Inhibition on RBV Uptake in RBV^R and RBV^S Cells

If the RBV^R cells have some modification that alters nucleoside transporter expression or activity, then the RBV^R phenotype should be mimicked by inhibition of endogenous transporters in RBV^S cells. Therefore, we performed RBV uptake assays in RBV^R and RBV^S Huh-7 cells and HeLa cells in the presence of nucleoside transport inhibitors. Nitrobenzylmercaptopurine riboside (NBMPR) is an inosine analog commonly used as an equilibrative nucleoside transport inhibitor. ENT1 and ENT2-mediated transport can be differentiated based on the level of NBMPR sensitivity. ENT1 is NBMPR-sensitive and is inhibited within the nanomolar range, while ENT2 is insensitive to nanomolar concentrations of NBMPR, with inhibition requiring concentrations within the high micromolar range [69, 105]. The RBV uptake assay was performed in RBV^R and RBV^S Huh-7 or HeLa cells in the presence or absence of NBMPR. For both Huh-7 and HeLa cells, NBMPR treatment reduced RBV uptake in RBV^S cells by 3-fold, to an amount comparable to the uptake of RBV^R cells (**Fig. 8A, 8B**). Since treatment with 15 μM NBMPR was not sufficient to completely inhibit ENT1 uptake, we repeated the experiment using a higher dose, 100 μM NBMPR, which would completely block ENT1-mediated transport and likely ENT2-mediated transport as well. As anticipated, treatment of RBV^S Huh-7 or HeLa cells with 100 μM NBMPR reduced RBV uptake to match the low uptake of RBV^R cells (**Fig. 8A, 8B**). Although ENT3 or ENT4 are equilibrative transporters, they are not likely to be relevant for RBV uptake because ENT3 is localized in intracellular compartments, and ENT4 is a monoamine/organic cation transporter [70].

Because over-expression of CNT3 could also restore RBV uptake in RBV^R cells, we performed the inhibition assay using the drug phloridzin, which is a broad concentrative transport inhibitor [106-108]. We observed no significant difference in the level of uptake in either the RBV^R or RBV^S Huh-7 or HeLa cells in the presence of phloridzin (**Fig. 8C, 8D**). In order to confirm the drug concentration used was sufficient to inhibit concentrative transport, Huh-7 cells were transfected with CNT3, and then subjected to the RBV uptake assay in the presence of phloridzin. We observed a dramatic decrease in the level of RBV uptake in the CNT3 transfected cells (**Fig. 8E**), indicating that phloridzin was indeed capable of inhibiting concentrative transport. Taken together, these results suggest that endogenous uptake of RBV in Huh-7 and HeLa cells is primarily equilibrative, with the majority of uptake mediated by ENT1. Despite the fact that ENT1 overexpression could not completely restore RBV uptake in RBV^R Huh-7 cells to the amount of uptake observed in RBV^S Huh-7 cells, the robust NBMPR inhibition of RBV uptake in RBV^S Huh-7 cells (**Fig. 8A**) indicates that ENT1 is the likely RBV transporter in these cells. Additionally, our results indicate that for Huh-7 and HeLa cells, uptake is not mediated via concentrative transporters, since decreased uptake was not observed upon inhibition of concentrative transport.

Quantification of ENT1 Expression in RBV^S and RBV^R Cells

In some cases of acquired resistance to nucleoside analog drugs, a decrease in the level of nucleoside transporter RNA has been reported [109-111]. Therefore, we compared ENT1 and CNT3 RNA levels in RBV^R and RBV^S Huh-7 cells using real-time RT-PCR. CNT3 RNA was undetectable in Huh-7 cells by real-time RT-PCR analysis

(data not shown). We found a slight variation in ENT1 RNA levels between RBV^R and RBV^S Huh-7 cells, with a 0.2-fold reduction in ENT1 RNA in the RBV^R cell line (**Fig. 9A**). Although this small reduction in ENT1 RNA levels was statistically significant ($p < 0.01$), ENT1 protein levels were equivalent in RBV^S and RBV^R cells (**Fig. 9B**). Therefore, ENT1 RNA and protein levels do not correlate with RBV uptake or resistance, suggesting that altered ENT1 localization or activity may contribute to resistance. These results highlight the importance of assessing the activity of nucleoside transporters, rather than RNA or protein levels.

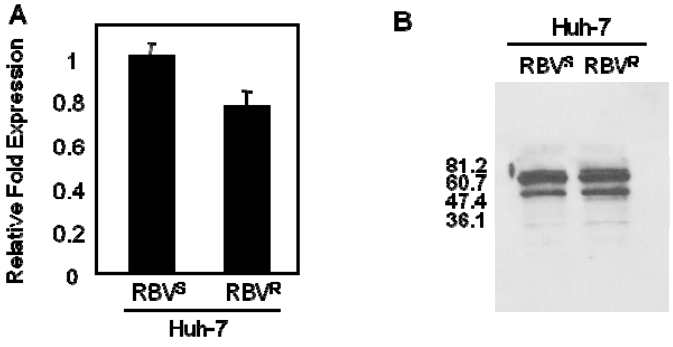


Figure 9. Nucleoside transporter RNA and protein levels in RBV^R and RBV^S cells.
(A) Total RNA was isolated from RBV^R and RBV^S Huh-7 cells and the relative levels of ENT1, CNT3, and GAPDH RNA were assessed with one-step real time RT-PCR using the comparative method (internal control not shown). CNT3 RNA was not detectable (data not shown). The average values and SD from three experiments performed in triplicate are shown. The difference in ENT1 RNA levels in RBV^R and RBV^S cells was statistically significant ($p < 0.01$, Student's *t* test). (B) Immunoblot analysis of ENT1 protein levels in RBV^R and RBV^S Huh-7 cells. Lysates were prepared and equivalent amounts of total protein were loaded. A representative immunoblot, probed with anti-human ENT1 antibody, is shown. The two bands may represent unglycosylated ENT1 (~45 kD) and glycosylated ENT1 (~55 kD).

The Effect of Nucleoside Transporter Activity on RBV-mediated Viral Growth Inhibition

Our results indicated that the level of RBV uptake affects poliovirus growth (**Fig. 5 and 6D**), and inhibition of equilibrative transport reduced RBV uptake in RBV^S cells (**Fig. 8A, 4B**). Thus, we sought to determine whether the antiviral efficacy of RBV could be reduced in RBV^S cells through equilibrative transport inhibition. Poliovirus plaque assays were performed in the absence or presence of RBV, and in the absence or presence of NBMPR. As anticipated, the inhibition of equilibrative transport facilitated robust viral growth, despite the presence of RBV (**Fig. 10**). We also performed the poliovirus plaque assays with the concentrative inhibitor phloridzin. We observed no difference in viral plaque size in the presence of phloridzin and RBV (data not shown). These results, in conjunction with our other data, indicate that endogenous ENT1 is responsible for RBV uptake in Huh-7 and HeLa cells, and that ENT1-mediated RBV uptake facilitates RBV's

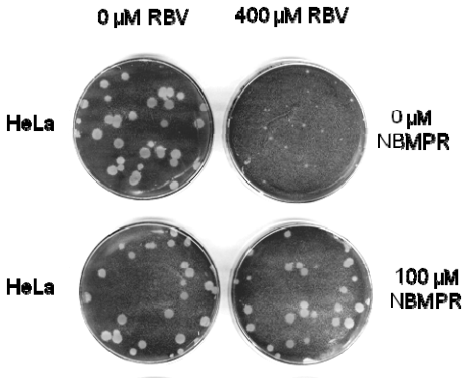


Figure 10. Effect of equilibrative nucleoside transporter inhibition on RBV-mediated viral growth inhibition.
HeLa cells were infected with poliovirus and an agar overlay containing 0 µM or 400 µM RBV and 0 µM or 100 µM NBMPR was added. After 48 hours the overlay was removed and cells were stained with crystal violet to visualize plaques.

antiviral effect in these cells.

Nucleoside Uptake Compensation Mechanism in RBV^R Huh-7 Cells

Whereas ENT1 clearly plays a pivotal function in the uptake of RBV in Huh-7 and HeLa cells, we could not dismiss the possibility that ENT2 contributed to RBV uptake (**Fig. 8A, 8B**). The simplest method of differentiating ENT1- and ENT2-mediated uptake, other than NBMPR sensitivity, is through nucleobase transport. While ENT2 has the capacity to transport both nucleosides and nucleobases, ENT1 cannot transport nucleobases [112-115]. Therefore, we monitored the uptake of [^{3}H]-hypoxanthine, a nucleobase. In contrast to the low level of RBV uptake in RBV^R Huh-7 cells, the level of hypoxanthine uptake in RBV^R Huh-7 cells was increased by almost three-fold compared with RBV^S Huh-7 cells (**Fig. 11 A,** $p < 0.01$). Although not initially expected, these data provide evidence for a possible cellular compensation mechanism for reduced ENT1-mediated uptake. A deficiency in a broad transporter such as ENT1 would be expected to confer a cellular growth defect. Perhaps to compensate for the reduction in available nucleosides imported by ENT1, the activity of ENT2, another broad nucleoside transporter, increased. Interestingly, the level of hypoxanthine uptake was equivalent in RBV^R and RBV^S HeLa cells (**Fig. 11 B**), suggesting that the ENT2-mediated compensation mechanism may be unique to Huh-7 cells.

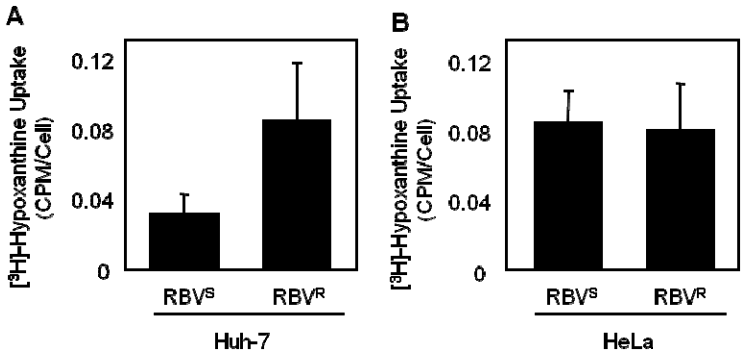


Figure 11. Hypoxanthine uptake in RBV^S and RBV^R cells.
(A) RBV^R and RBV^S Huh-7 cells or (B) RBV^R and RBV^S HeLa cells were incubated in media containing 5 μM [^{3}H]-labeled hypoxanthine for 5 min. at 37˚C and the level of [^{3}H]-hypoxanthine uptake was determined by scintillation counting. The average values and SD from at least two experiments performed in duplicate are shown. The difference in hypoxanthine uptake between RBV^R and RBV^S cells was statistically significant for Huh-7 cells ($p < 0.01$, Student's *t* test), but not for HeLa cells ($p = 0.68$).

DISCUSSION

Only limited success has been achieved in treating those infected with hepatitis C virus. Whereas much effort has been targeted towards understanding the mechanism of interferon and potential interferon resistance, relatively little effort has been directed towards understanding RBV. RBV has been shown to play an important role in treatment response [21] and higher RBV serum concentrations correlate with improved treatment outcome [57, 58]. However, we still do not understand how RBV works synergistically with interferon to improve SVR rates, or whether resistance to RBV develops in patients.

Our goal in this study was to gain a better understanding of potential RBV resistance mechanisms using a cell culture system. We began by examining the levels of RBV uptake among several cell lines and found it to vary. We next ascertained whether RBV uptake level influenced antiviral efficacy by performing poliovirus plaque assays. It is known that poliovirus is sensitive to RBV at high concentrations, such as 400 μM. Additionally, the antiviral mechanism of RBV, increasing the error rate to the point of error catastrophe [45], is well established for poliovirus. We observed an inverse relationship, where decreased RBV uptake facilitated increased viral growth in the presence of RBV (**Fig. 5B**). Importantly, a high dose of RBV was used in this study, and is required to observe effects on poliovirus growth [72] [71] [46] [45]. Although the concentration of RBV in plasma is significantly lower (~10 μM), RBV may accumulate to higher concentrations in the liver [116].

Since the focus our work was to understand potential host-mediated RBV resistance, we generated new RBV^R cell lines using cells that had not previously

contained the HCV replicon [72]. Resistance was achieved in all three cell lines and the RBV^R phenotype was stable, with cells maintaining their resistance despite months of culture in media lacking RBV (data not shown). When tested for RBV uptake, all RBV^R cell lines demonstrated significantly reduced uptake compared to their RBV^S counterparts (**Fig. 6C**). Upon performing poliovirus plaque assays in these new RBV^R cell lines, we found that RBV resistance also facilitated increased poliovirus growth in the presence of RBV (**Fig. 6D**). These results indicate that a reduction in RBV uptake can promote increased poliovirus growth.

As RBV is imported by nucleoside transporters, we assessed the contribution of several nucleoside transporters to RBV resistance in our cell lines. After surveying several transporters, over-expression of ENT1 and CNT3 increased RBV uptake. Although ENT1 is widely accepted as the primary RBV transporter, there have been additional reports suggesting other transporters can import RBV [65, 67, 68] [104]; indeed, our findings confirm CNT3-mediated RBV uptake. Additionally, we determined whether over-expression of ENT1 or CNT3 could restore the uptake defect in RBV^R cells. We found that in the majority of RBV^R cells, over-expression of ENT1 or CNT3 could restore RBV uptake (**Fig. 7B).** While transfection of ENT1 and CNT3 significantly increased uptake in RBV^S Huh-7, 293, and HeLa cells, it could completely restore the uptake defect in only RBV^R 293 and HeLa cells. The fact that uptake could not be completely restored in RBV^R Huh-7 cells (**Fig. 7C**) suggests other factors could interfere with RBV uptake in RBV^R Huh-7 cells, perhaps by limiting nucleoside transporter activity or localization. However, RBV uptake could be completely restored in ENT1 or CNT3-transfected RBV^R Huh-7.5 cells, a liver cell line derived from Huh-7 cells (data

not shown, to be described elsewhere). These results indicate that multiple pathways can lead to RBV resistance, even in transformed cells of the same lineage, such as Huh-7 and Huh-7.5. We hypothesize that ENT1 protein is mislocalized in RBV^R Huh-7 cells. There is evidence that localization of ENT1 and other transporters can change from the plasma membrane to intracellular membrane compartments in response to various stimuli, including adaptation to two-dimensional tissue culture [104] (see below). Similarly, expression and activity of transporters can change in response to transformation [117]. Therefore, although our results with Huh-7 cells may or may not apply to liver cells in vivo, they provide a foundation for future studies on the role of nucleoside transporters in host-based RBV resistance and antiviral efficacy.

Whereas ENT1 is ubiquitously expressed, expression of CNT3 is normally restricted to a few tissues, with low expression reported in the liver [118, 119]. The most common method of determining endogenous transport activity is through uptake assays performed in the presence of specific inhibitors. Experiments performed using NBMPR (an ENT1 inhibitor) and phloridzin (a broad concentrative nucleoside transport inhibitor) indicated RBV uptake occurs primarily through ENT1 (**Fig. 8A, 8B**). While CNT3 was capable of increasing RBV uptake into the cells upon transfection (**Fig. 7B**), CNT3 expression was undetectable in non-transfected Huh-7 cells as measured by real time RT-PCR (data not shown). These data suggest that ENT1 is the RBV transporter in the Huh-7 hepatoma cell line.

Host-based resistance to treatment with nucleoside analogs is not uncommon in cancer patients and numerous studies linking nucleoside analog drug resistance to decreased nucleoside transporter expression have been reported [109-111]. Accordingly,

we quantified ENT1 RNA levels in the RBV^R and RBV^S Huh-7 cells, and found only slightly reduced ENT1 expression in RBV^R cells (**Fig. 9**). Immunoblot analysis indicated that ENT1 protein levels are equivalent in RBV^R and RBV^S cells (**Fig. 9B**), providing support for the idea that ENT1 activity or protein localization is altered in RBV^R cells. Perhaps some modification occurred that altered the activity of ENT1, or altered the plasma membrane localization of ENT1, thus conferring the RBV^R phenotype. While much remains to be understood regarding regulation, localization, and activity of nucleoside transporters, there is precedent for localization of transporters in internal cell compartments [104, 120-123]. Altered ENT1 localization would account for the similar ENT1 RNA and protein levels found in RBV^R and RBV^S cells. It is also possible that RBV resistance is conferred by altered RBV processing pathways, such as reduced phosphorylation or increased degradation. There have been several cases in which resistance correlates with either a decrease in the level of activating kinases required for nucleoside phosphorylation, or an increase in the level of 5' nucleotidases capable of dephosphorylating nucleoside analogs [110, 123, 124]. However, the fact that uptake of RBV was reduced in RBV^R cell lines, that no difference was found in the export of RBV (data not shown), and that inhibition of ENT activity mimicked the RBV^R cell phenotype, suggests that RBV resistance is due to altered ENT1 activity, and not phosphorylation, degradation, or efflux effects.

After establishing that the level of RBV uptake affects viral growth and that endogenous ENT1 was primarily responsible for uptake, the next step was to demonstrate the antiviral impact of altered uptake. Our results illustrated two key points. First, by blocking equilibrative transport, uptake in the RBV^S cells mirrored that in the RBV^R

cells. Second, inhibition of RBV uptake reduced antiviral efficacy, with poliovirus growth in the RBV^S cells mirroring that in the RBV^R cells in the presence of nucleoside transport inhibitor (**Fig. 10**). Overall, our results indicate a role for ENT1-mediated RBV transport in the uptake of RBV, and suggest that a reduction in ENT1 activity may play a role in the acquisition of resistance.

The idea that host-based nucleoside uptake defects can affect antiviral efficacy may have clinical relevance. Similar to cancer patients undergoing chemotherapy, chronically infected hepatitis C patients undergo harsh, toxic, long-term treatments. The standard of care for treating HCV infection includes a combination of pegylated IFN-α and RBV for 24 to 48 weeks. Treatment with interferon alone can have side effects, but RBV is also a toxic drug capable of inducing hemolytic anemia. As with long-term exposure to cytotoxic nucleoside analogs in chemotherapy, it is plausible that host-based drug resistance could occur in response to RBV treatment. Constant exposure to RBV toxicity could trigger reduced transporter activity. Liver cells, like many others, are capable of de novo nucleoside synthesis. Additionally, the liver is considered the main site of nucleoside synthesis, providing a reservoir of nucleosides for other cells in the body which lack *de novo* synthesis [69]. It is likely that any disadvantage conferred by down regulation of a broad transporter such as ENT1 would be outweighed by the advantage of hepatocyte survival.

Our work also suggests that, for RBV^R Huh-7 cells, a compensation mechanism upregulates the activity of an alternate transporter, ENT2 (**Fig. 11A).** Furthermore, these results provide additional evidence, indirectly, that ENT1 is indeed the primary RBV nucleoside transporter in Huh-7 cells. We surmise that upregulation of an alternate

transporter offsets the disadvantage caused by downregulation of ENT1 activity. Therefore, we suggest that ENT1 downregulation is primarily responsible for RBV resistance, and that Huh-7 cells compensate for reduced nucleoside uptake by upregulation of the ENT2 transporter. RBV^R HeLa cells did not exhibit this ENT2-mediated compensation mechanism (**Fig. 11B**). Interestingly, RBV^R HeLa cells grow significantly slower than RBV^R Huh-7 cells (data not shown).

It is possible that nucleoside transporter activity could aid in the prediction of treatment success. Perhaps elevated RBV uptake correlates with overall treatment success. Higher RBV serum concentrations have been associated with SVR [57, 58]. SVR rates of up to 90% have been achieved by using extremely high doses of RBV [59], albeit with increased toxicity. We are currently measuring RBV uptake in HCV patient samples and determining the effect of RBV uptake level on HCV treatment response and HCV replication. If RBV uptake can affect treatment response, either through natural transport differences in the population, or through acquired resistance, then tailored treatment regimens may enhance treatment response.

CHAPTER FOUR
Host-based Ribavirin Resistance Influences Hepatitis C Virus Treatment Response

INTRODUCTION

Current treatment for HCV infection is a combination of pegylated interferon-α (IFN) and ribavirin (RBV), a guanosine nucleoside analog. Despite decades of ongoing basic and clinical research, sustained virological response (SVR) is achieved in only half of patients infected with genotype 1[22]. IFN clearly plays an important role in HCV treatment response. Although somewhat unappreciated in the past, RBV is quickly becoming an indispensible component of HCV treatment. The addition of RBV to IFN therapy doubled response rates, illustrating the importance of RBV in HCV treatment [52]. Efforts to replace RBV with taribavirin, a RBV prodrug formerly known as viramidine, have been unsuccessful [30]. Although HCV protease and polymerase inhibitors are promising new antivirals, RBV will continue to be a critical part of combination therapy because eliminating RBV from telaprevir-IFN therapy significantly reduced response rates [31, 125].

Patient treatment response is based on viral RNA levels, and the ultimate goal is to achieve a sustained virological response (SVR; defined as undetectable HCV RNA for at least 24 weeks post-therapy) [6]. Patients with a rapid virological response (RVR; undetectable HCV RNA by week 4) are more likely to achieve SVR [19, 20]. Lack of an early virological response (EVR; ≥ 2 log decline in HCV RNA by week 12) is generally an indicator of those who will not respond to therapy [6]. Additionally, several viral and host factors are associated with a positive treatment response, such as low baseline viral

loads, female gender, lack of co-morbidities such as HIV-coinfection, low initial IFN stimulated gene (ISG) expression, and specific alleles of the *IL-28B* gene [6, 23, 51].

While RBV plays a crucial role in treatment, its antiviral mechanism against HCV is unclear, with five proposed mechanisms. First, RBV is phosphorylated by host kinases, and RBV monophosphate can reduce host GTP pools through inhibition of IMPDH, which is responsible for *de novo* GTP synthesis [41]. Therefore, viral replication can be inhibited due to a lack of available nucleotides. Second, RBV triphosphate (RTP) may directly inhibit the viral RNA-dependent RNA polymerase [39, 43]. Third, RTP can be incorporated into the viral genome, which can increase mutation rates [42, 44-46]. Fourth, it has been suggested that RBV acts indirectly against HCV by shifting the immune response from a humoral T_H2 response to a cell mediated T_H1 response [49, 50]. Fifth, RBV enhances ISG expression [28]. Nearly all proposed mechanisms require import of RBV into the cell for antiviral activity. Therefore, we have focused on understanding host-based mechanisms of RBV uptake and resistance.

Nucleoside transporters are involved in the cellular import of natural and synthetic nucleosides and mediate RBV uptake [39]. Numerous studies have linked nucleoside analog chemotherapy drug resistance to decreased nucleoside transporter expression [111]. ENT1 is the primary RBV transporter, however CNT2, CNT3 and ENT2 may also transport RBV in various cell types [65-68]. Our previous work determined that ENT1 was the primary RBV transporter in Huh 7 liver cells. RBV-resistant (RBV^R) Huh 7 cells were generated by passage in increasing concentrations of RBV, and resistance was mediated by reduced RBV transport through ENT1 [126]. As a result of decreased RBV import, RBV^R Huh 7 cells supported robust poliovirus

replication in the presence of RBV compared to RBV-sensitive (RBV^S) Huh 7 cells, suggesting that the level of RBV import impacts antiviral efficacy.

In this study, we examined whether reduced cellular import of RBV affects HCV replication *in vitro* and treatment failure in HCV patients. Our results suggest that RBV resistance promotes robust HCV replication in the presence of RBV. Moreover, PBMCs obtained from patients exhibited declines in RBV uptake as therapy progressed, suggesting that patients undergoing combination IFN/RBV therapy may develop resistance to RBV. Importantly, rapid treatment response correlated with maintenance of RBV uptake, suggesting that maintaining basal RBV uptake levels may improve treatment efficacy.

RESULTS

Generation of RBV^R Huh 7.5 cells with reduced RBV uptake

We previously demonstrated that host-based RBV resistance can develop and reduce RBV antiviral efficacy in a poliovirus model [126]; however, the effect on HCV was unknown. Therefore, we generated RBV-resistant (RBV^R) human hepatoma cells (Huh 7.5), permissive for HCV replication [74], by passage in increasing concentrations of RBV. Viability assays confirmed resistance in the RBV^R cells when exposed to concentrations of RBV that were toxic to the RBV-sensitive (RBV^S) parental cells [**Fig. 12A**]. The RBV^R cells also exhibited reduced uptake of radiolabeled RBV (^{3}H-RBV), suggesting that reduced RBV uptake enhances cellular survival in the presence of RBV [**Fig. 12B**].

To dissect the mechanism of RBV resistance, nucleoside transporter over-expression and inhibition studies were conducted. We previously demonstrated that the nucleoside transporter ENT1 mediated RBV transport and resistance in Huh-7 liver cells [126]. Although the Huh 7.5 cells used in this study were derived from Huh-7 cells, resistance to nucleoside analogs can develop through various pathways [102]; therefore, the mechanism of resistance was analyzed further. Over-expression of ENT1, or the concentrative nucleoside transporter CNT3, restored RBV uptake in RBV^R cells [**Fig. 12C**]. To examine the activity of endogenous transporters, inhibition assays were performed. The addition of nitrobenzylmercaptopurine riboside (NBMPR), an inosine analog known to specifically inhibit ENT1 at low concentrations [69], decreased RBV uptake in RBV^S cells to levels observed in RBV^R cells [**Fig. 12D**].

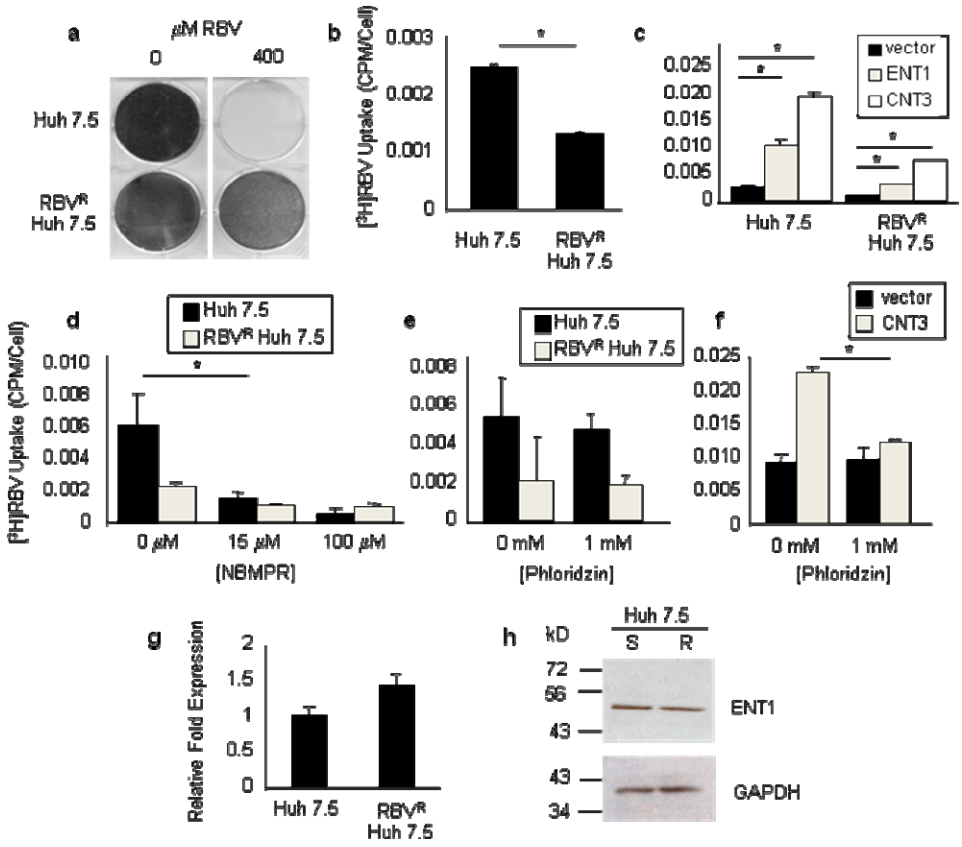


Figure 12. Characterization of RBV^R Huh 7.5 cells.
(**a**) Crystal violet viability assay. RBV^R and RBV^S Huh 7.5 cells were passaged for 10 days in medium containing 0 μM or 400 μM RBV and stained with crystal violet for visualization. (**b**) RBV uptake assay. Cells were incubated with [^{3}H]-labeled RBV and uptake was quantified by scintillation counting. The average from two experiments is shown as counts per minute (CPM) per cell (P = 0.001). (**c**) RBV uptake following nucleoside transporter transfection. Cells were transfected with plasmids expressing ENT1, CNT3, or vector. The RBV uptake assay was performed 24 hours post-transfection (hpt). One representative experiment, out of three, is shown. Asterisks denote $P \leq 0.01$. Inhibition of endogenous RBV uptake (**d – e**). (**d**) Cells were pretreated with the ENT inhibitor NBMPR followed by the RBV uptake assay (P = 0.04) (**e**) Cells were pretreated with phloridzin followed the RBV uptake assay. (**f**) Phloridzin control. Huh 7.5 cells were transfected with the CNT3 expression plasmid or vector and at 24 hpt a RBV uptake assay was performed in the presence of phloridzin (P = 0.01) (**g**) Real-time RT-PCR analysis of ENT1 RNA levels. The average relative fold expression from three experiments is shown. (**h**) ENT1 immunoblot analysis. Shown is one of two representative experiments, with GAPDH as a loading control. S, RBV^S Huh 7.5 cells; R, RBV^R Huh 7.5 cells. Error bars for all panels (**b – g**) represent standard deviation (SD), with significance obtained using unpaired, two-tailed, Student's t-tests.

No significant reduction in RBV uptake was observed with a higher concentration of NBMPR (100 μM), which inhibits both ENT1 and ENT2. Likewise, inhibition of concentrative transport through phloridzin treatment, an inhibitor of sodium-dependent nucleoside transport [106, 107], did not reduce RBV uptake in cells, unless they expressed CNT3 via transfection [**Fig. 12E – F].** This was expected, because our prior work determined that endogenous CNT3 RNA was undetectable via real-time RT-PCR analysis, but CNT3 was capable of transport if exogenously expressed through transfection [126]. Analysis of ENT1 protein and RNA levels revealed no significant difference between resistant and sensitive cells [**Fig. 12G – H**], suggesting that reduced ENT1 activity and/or transporter mislocalization may contribute to RBV resistance, rather than reduced ENT1 levels. Collectively, these results indicate that RBV resistance in Huh 7.5 cells occurs through reduced ENT1-mediated RBV uptake.

HCV replication in RBV^S and RBV^S Huh 7.5 cells

With the development of RBV^R Huh 7.5 cells, we were in a unique position to examine the effects of RBV on HCV replication, potential mechanisms of RBV action, and the consequences of RBV resistance. HCV infections were performed in RBV^R and RBV^S Huh 7.5 cells in the presence or absence of 100 μM RBV. RBV treatment induced a dramatic viral titer reduction in RBV^S cells [**Fig. 13A**]. RBV^R cells displayed no titer reduction in the presence of RBV, despite having only a two-fold RBV uptake defect; suggesting a modest reduction in RBV uptake can have a major impact on viral replication. Reduced cell health from RBV toxicity may have reduced viral replication in RBV^S cells. Studies have shown that guanosine supplementation can counter RBV-

induced toxicity by restoring host GTP pools [90]. Although IMPDH inhibition cannot completely account for RBV's antiviral activity [40], the addition of guanosine can reduce cellular toxicity through restoration of nucleotide pool balance [40, 90]. Therefore, infections were performed in the RBV^S cells in the presence of equimolar concentrations of RBV and guanosine. Supplementation with guanosine restored viral titers to levels observed in the absence of RBV [**Fig. 13A**]. Since 100 μM RBV likely exceeds most physiological concentrations, we wondered whether viral titers would be reduced at lower RBV concentrations. Infections were conducted using 15 μM RBV, to mimic achievable plasma concentrations in patients, as well as 50 μM RBV [**Fig. 13B**]. No titer reductions were observed at either concentration, indicating that RBV toxicity may have inhibited HCV growth at high RBV concentrations.

We considered the possibility that a 72-hour infection period was insufficient for RBV antiviral effects, especially if RBV-induced mutagenesis plays a role. RBV-induced mutagenesis requires multiple cycles of viral replication for mutations to accumulate and lead to observable antiviral effects [45, 46, 64]. To examine RBV's effect on HCV following extended exposure, HCV was serially passaged in RBV^S and RBV^R cells in the absence or presence of 15 or 50 μM RBV. Each six-day passage was followed by supernatant harvest and infection of naïve cells, with titer analysis after each passage. No significant RBV-mediated titer reduction was observed in either RBV^S or RBV^R cells [**Fig. 13C – D**]. This suggests that, at high concentrations, RBV may act indirectly against HCV, potentially through toxicity-mediated mechanisms.

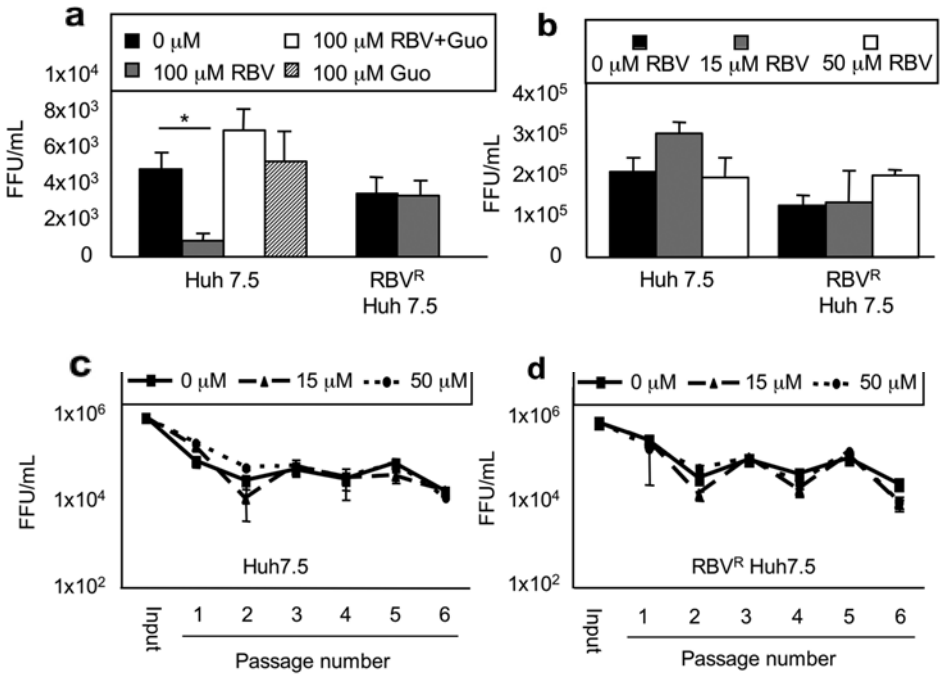


Figure 13. The impact of RBV resistance on HCV replication *in vitro*.
(**a**) Three day HCV infections in the absence or presence of high concentrations of RBV and guanosine. Cells were treated with 0 or 100 μM RBV and/or guanosine (Guo), and infected with HCV for three days. Titers were determined in triplicate at the end of each infection and results are shown in focus forming units per mL (FFU/mL) with SD. One of three representative experiments is shown (P = 0.002; unpaired, two-tailed, Student's t-test). (**b**) Three day HCV infections in the absence or presence of low RBV concentrations. Infections were performed as described in (a), except that 0, 15, or 50 μM RBV was used. Serial infections performed in the presence of 0, 15, or 50 μM RBV in Huh7.5 cells (**c**) or RBV^R Huh7.5 cells (**d**). To begin passage one (P1), cells were infected at a MOI of 0.1. After cell expansion at day 3, on day 6, the supernatant from each plate was used for viral titer analysis, and to initiate a new infection (P2). Serial infections continued for six passages, and the experiment was performed twice. The titers results from one representative experiment, performed in triplicate, is shown with SD.

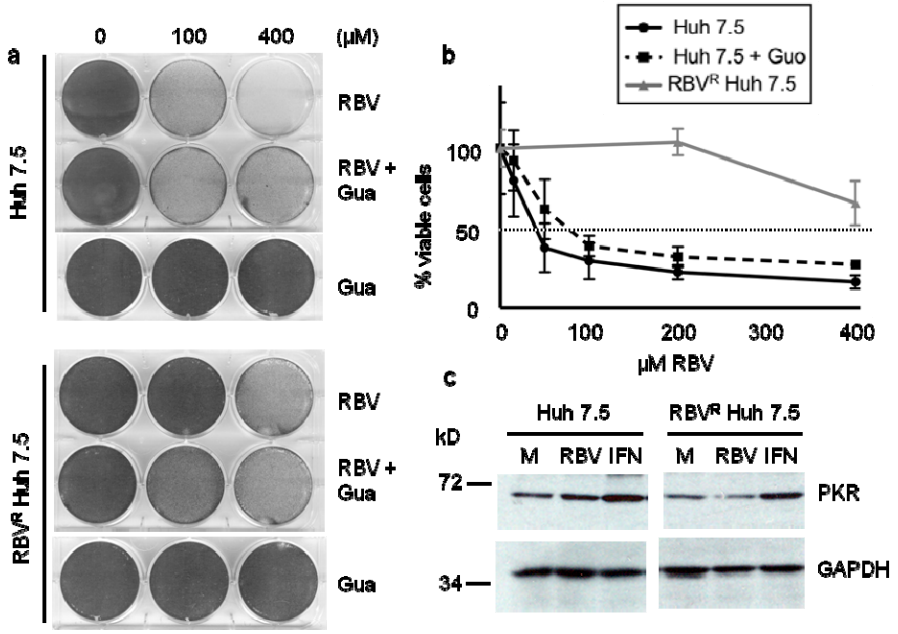


Figure 14. Cellular viability and PKR expression studies.
(**a**) Cells were passaged with the indicated concentrations of RBV and Guo for seven days. On day seven, viable cells were visualized using crystal violet staining. Shown is one of two representative experiments. (**b**) Huh 7.5 cells were passaged in various concentrations of RBV, or RBV and Guo at equimolar concentrations, for three days. On day three, viable cell counts were obtained using trypan blue exclusion and normalized to a percent of the no drug control (0 μM). Shown is the combined average from two experiments (for RBV^S cells) or one of three representative experiments (for RBV^R cells). Error bars indicate SD. (**c**) Cells were treated with 100 μM RBV, 100 U/mL interferon-α (IFN), or media alone (mock, M) for 24 hours, and cell lysates were collected and used for immunoblot analysis. One representative immunoblot, out of three, probed with anti-human PKR and GAPDH as an internal control is shown.

RBV toxicity in RBV^S and RBV^R Huh 7.5 cells

To further investigate the effects of RBV on cell survival and toxicity, cellular viability assays were performed in the presence of RBV and guanosine. Cells were exposed to RBV for seven days, or for three days to mimic infection conditions used in Figure 13 experiments, and cellular viability was determined. Guanosine enhanced cellular viability in RBV^S cells at high concentrations of RBV (400 μM) [**Fig. 14A**].

Additionally, RBV induced a 50% decline in RBV^S cell numbers at an estimated concentration of 70-80 μM in the presence of guanosine, and 40-50 μM in the absence of guanosine [**Fig. 14B**]. RBV^R cells never reached 50% population loss, despite treatment with 800 μM RBV [**Fig. 14B** and data not shown]. The combination of RBV's known cytostatic effect [127] in conjunction with toxicity likely resulted in decreased cell survival in RBV^S cells. Nevertheless, toxicity could not be completely overcome with guanosine treatment, suggesting that mechanisms other than IMPDH-induced alterations in nucleotide pools contribute to RBV toxicity.

One potential source of toxicity is RBV-mediated induction of double-stranded RNA-activated protein kinase (PKR). The ability of PKR to inhibit cellular proliferation and induce apoptosis is well characterized [128, 129]. RBV has been shown to induce PKR expression, PKR activation, and apoptosis which is thought to contribute to its antiviral activity against HCV [130, 131]. To determine the effect of reduced RBV uptake on PKR expression, we analyzed PKR levels in RBV^S and RBV^R cells following 100 μM RBV treatment for 24 hours [**Fig. 14C**]. Upon RBV treatment, PKR levels increased in RBV^S cells, but not in RBV^R cells. Because PKR can induce toxicity [129], these results suggest that RBV-mediated PKR induction may contribute to RBV's toxicity and HCV replication inhibition.

RBV uptake in PBMCs from healthy donors and HCV patients

Our cell culture results suggest that RBV resistance develops in many cultured cell lines [126] and impacts antiviral efficacy; however the development of host-based RBV resistance has yet to be described *in vivo*. Therefore, we determined whether host-

based resistance to RBV develops using PBMCs from healthy donors and HCV-infected patients undergoing combination IFN/RBV therapy. We began by examining whether *ex vivo* exposure to a physiologically relevant concentration of RBV can reduce RBV uptake in PBMCs from healthy donors. PBMCs were cultured in the absence or presence of 10 μM RBV for seven days and RBV uptake was quantified. Short-term, low-concentration RBV exposure significantly reduced RBV uptake in PBMCs from healthy donors [**Fig. 15A - C**]. RBV uptake declined in PBMCs from every donor examined [**Fig. 15C**]. Given that HCV patients receive 24 – 48 weeks of IFN/RBV treatment, we hypothesized that RBV uptake declines over time. We quantified RBV uptake in PBMCs isolated from HCV genotype 1-infected patients before therapy (day 0) and after 14 and 28 days of pegylated IFN/RBV therapy. PBMC RBV uptake was significantly reduced at days 14 and 28 compared with day 0 [**Fig. 15D**]. To ascertain whether host-based resistance could play a role in treatment response, we compared RBV uptake in patients with rapid response (RR; viral clearance by day 28) to RBV uptake in patients with slow-response or non-response (non-RR). We chose to examine rapid treatment response since it is highly predictive of SVR [20]. Interestingly, RRs maintained significantly higher levels of RBV uptake compared with non-RRs [**Fig. 15E – F and Table 2**]. These results suggest that RBV resistance develops *in vivo* and that maintenance of basal uptake levels correlates with rapid clearance of HCV.

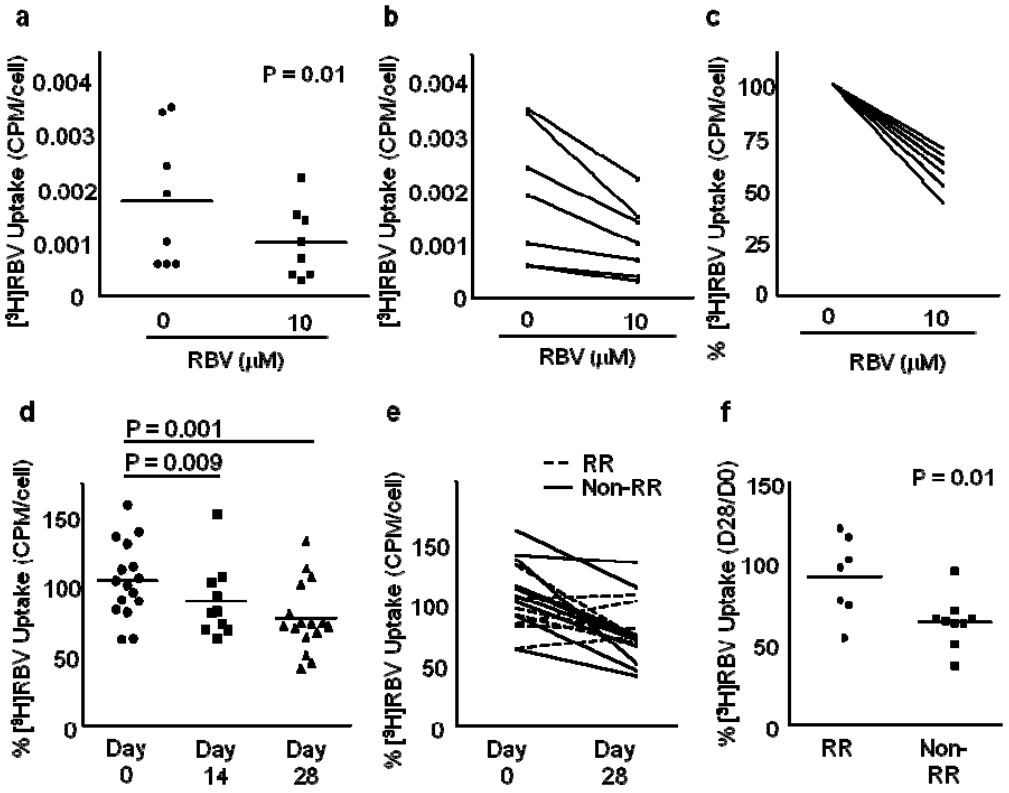


Figure 15. RBV uptake in PBMCs from healthy donors (a – c), and HCV patients (d - f). (**a**) PBMCs from healthy donors were cultured in 0 μM or 10 μM RBV for 7 days, followed by the RBV uptake assay. Shown is the average CPM/cell (n = 8, P = 0.01, paired, two-tailed, Student's t-test) (**b**) Values from (a) graphed to visualize pre- and post-treatment values. (**c**) Data from (a), normalized to 0 μM RBV values, to highlight trends following *ex vivo* treatment with 10 μM RBV, with 0 μM values set to 100%. (**d**) RBV uptake was quantified in HCV patient PBMCs isolated prior to the onset of treatment (Day 0), and 14 or 28 days post-treatment. To account for assay variability on different days, the average CPM/cell has been normalized to RBV uptake values from an aliquoted healthy donor PBMC control sample assayed in parallel (CPM/cell for patient sample divided by CPM/cell for the control)(n = 16 for day 0 vs. day 28, P = 0.001; n = 10 for day 0 vs. day 14, P = 0.009 using paired, two-tailed Student's t-tests). (**e**) RBV uptake before and after 28 days of treatment for each HCV patient, with treatment response indicated (rapid responder = RR, non-rapid responder = Non-RR; n =16) (**f**) Day 28 RBV uptake values from (e) categorized by treatment response (n = 7 and 9 for RRs and Non-RRs, respectively; P=0.01 in unpaired, two-tailed, Student's t-test). Horizontal lines within each group represent the mean.

DISCUSSION

In conclusion, our results uncovered a novel form of host-based antiviral drug resistance as a potential predictor of HCV treatment response. The data suggest that RBV toxicity drives the development of host-based resistance through reduced cellular uptake. Recent studies have demonstrated that RBV activates PKR and other interferon stimulated genes [28, 132], which are likely to contribute to RBV's antiviral effects. Notably, our results also suggest that patients undergoing combination pegylated IFN/RBV therapy develop resistance to RBV *in vivo*, with significantly reduced cellular uptake over time. Despite the fact that HCV replicates in liver cells, a correlation between RBV uptake in PBMCs and treatment response was observed, suggesting that either liver cells also experience RBV uptake declines, or, that altered PBMC function due to reduced RBV uptake impacted innate or adaptive antiviral responses in the liver. Overall, PBMC RBV uptake could be used as a diagnostic tool to predict treatment response and aid dosing regimens.

CHAPTER FIVE
Summary

CONCLUSIONS AND RECOMMENDATIONS

Although the concept of host-based resistance to nucleoside analog therapy is well documented in cancer studies, it is generally not considered in antiviral therapy resistance. Nucleoside and deoxynucleoside analogs are routinely used in chemotherapy, and their cytotoxicity can result in severe side effects and the development of resistance [102, 133]. Nearly all proposed mechanisms of action for RBV require RBV import. Therefore, there is a need to understand factors that impact cellular RBV import and the development of host-based RBV resistance.

Chemotherapy nucleoside analog resistance can occur through numerous mechanisms and is often associated with proteins or enzymes involved in the import, activation or degradation of the nucleoside analog in question [102, 133]. Following entry, nucleoside analogs are phosphorylated by host kinases, eventually resulting in formation of the active triphosphate derivative. Catabolism of the triphosphate derivative, or derivatives thereof, occurs via various deaminases or 5'-nucleotidases. Alterations within this pathway can result in the development of resistance. For example, cytosine arabinoside (Ara-C; cytarabine), which is used in the treatment of acute myeloid leukemia (AML), is imported primarily through ENT1 [102, 134]. Studies using Ara-C resistant cells have linked resistance to decreased ENT1 expression and protein levels, and increased adenosine deaminase (ADA) expression [109, 135]. Similar results were

obtained with gemcitabine therapy in pancreatic cancer patients, where ENT1 expression correlated with clinical outcome [136].

In an effort to determine whether reduced cellular import of RBV can contribute to HCV treatment failure we began by modeling the development of RBV resistance using a cell culture system. RBV^R cells were generated by serial passage in increasing concentrations of RBV over several weeks. Analysis of both liver cell lines examined, Huh 7 and Huh 7.5, suggests that RBV import occurs primarily through ENT1 and that resistance is ENT1-mediated.

The specific mechanism by which ENT1 is altered remains to be elucidated. RBV^S and RBV^R cells exhibit similar ENT1 RNA and protein levels (**Fig. 9A – B** and **12G – H**), suggesting that changes in transport activity and/or membrane localization may contribute to RBV resistance. ENT1 has been found in association with the mitochondria in ENT1-expressing MDCK cells [137]. Likewise, analysis of prolymphocyte cell populations from chronic lymphocytic leukemia patients determined that CNT3 was mislocalized to cytoplasm [86]. Internal localization would reduce transporter activity, while maintaining protein and RNA levels. Preliminary attempts to identify the extent of surface ENT1 expression within RBV^R and RBV^S cells using immunofluorescence or [^{3}H]-NBMPR binding techniques have not been successful (data not shown). Alternate techniques, such as plasma membrane isolation in conjunction with western analysis of ENT1, may provide an additional means to determine whether mislocalization of ENT1 within RBV^R cells has occurred. Alternatively, post-translational modifications of ENT1 protein or mutations in the ENT1 sequence may have occurred, reducing ENT1 transporter activity. Due to the integral role nucleoside

transporters play in human fitness, and consequently strong negative selection, non-synonymous mutations were believed to occur rarely [138, 139]. Recent evidence suggests this may not be the case. A polymorphism within a transmembrane domain of human CNT3 (C602R) resulted in altered sodium-binding capacity and specificity for several nucleoside-derived drugs [140, 141]. Although the mutation was identified using tissue derived from a patient with renal carcinoma, the allelic frequency occurred in 1% of the Spanish population. Similarly, genetic analysis of CNT2 polymorphisms within ethnically diverse populations located a variant with altered specificity for inosine and uridine [142]. During the preparation of this dissertation, Morello, J. *et al.* published several SNPs within the ENT1 gene (*SLC29A1*) [143]. One of the SNPs (rs760370A→G; located within a non-coding region) correlated with rapid treatment response in HCV patients, further supporting the role of ENT1-mediated RBV uptake in HCV treatment response. Mutations in *SLC29A1* could alter splicing which could affect ENT1 protein stability, localization, and activity. Splice variants have been identified that inactivate ENT1, or alter RBV uptake [144, 145]. Although no differences were found in ENT1 RNA or protein levels in the RBV^S and RBV^R cells examined here, subtle changes such as mutations or altered splicing could have occurred in RBV^R cells which may have been masked in our ENT1 protein and RNA analysis. Sequencing ENT1 from both RBV^R and RBV^S cells may determine whether either occurred. Nevertheless, the restoration of RBV uptake in RBV^R cells following ENT1 over-expression, and the recapitulation of RBV^R uptake levels in RBV^S cells during inhibition of endogenous ENT1 transport, indicates that RBV uptake is primarily ENT1-mediated. Alterations in ENT1 transport activity, and/or localization, may contribute to the development of resistance in RBV^R cells.

The effect of RBV import on poliovirus and HCV replication was also investigated. In both cases, reduced RBV uptake resulted in increased viral growth when a high concentration of RBV was used (100 – 400 μM). It has been established that RBV works against poliovirus through mutagenesis and that high concentrations (400 μM and above) are required to observe this effect [46]. The mechanism of action for RBV against HCV on the other hand, has yet to be determined. Although RBV plasma concentrations can range from 10 – 15 μM in patients, the RTP derivative can accumulate to greater than 100 μM intracellularly after only a few hours of exposure to physiological concentrations [39]. Moreover, pharmacokinetic analysis of HCV patients suggests that concentrations within hepatocytes may reach as high as 250 μM [45]. RBV may exert its antiviral effects indirectly as a result of reduced cell health since viral replication was restored in the presence of guanosine and HCV replication was not inhibited at low concentrations of RBV (15 and 50 μM; **Fig. 13**). If RBV were to primarily act through mutagenesis, viral titers would decline in serial passage infections, even at low concentrations of RBV, as has been observed with poliovirus [45, 46]. HCV titers were not reduced in RBV^S or RBV^R cells following a total of 36 days of viral culture in the presence of 15 or 50 μM RBV. Overall, our results indicate that, at high concentrations, RBV may inhibit HCV replication indirectly, potentially through cellular toxicity.

RBV-induced toxicity may arise through a variety of mechanisms, including IMPDH inhibition, PKR expression and activation, eukaryotic translation initiation factor 4E (eIF4E) inhibition, and others which have yet to be elucidated. Clinically, RBV can induce hemolytic anemia from the accumulation of RTP within erythrocytes [146]. However, the ability of RBV to interfere with cellular processes is not limited to

erythrocytes. RBV is a known cytostatic agent, at least partially through IMPDH inhibition and reduced GTP levels [41, 90]. Indeed, when we examined cellular viability after RBV exposure, the addition of Guanosine partially restored viability in RBV^S cells (**Fig. 14**). The fact that viability was not completely restored suggests RBV-induced toxicity occurs through additional mechanisms and is not solely dependent on IMPDH inhibition. As such, the induction of PKR expression or activation may provide an additional means by which RBV may affect cell health. RBV treatment increased PKR levels in RBV^S cells in the absence of viral infection or IFN treatment (**Fig. 12**). This is consistent with results from Liu *et al.*, where RBV treatment increased the levels of phosphorylated PKR and phosphorylated eIF2α in primary human hepatocytes [130]. RBV may also inhibit the activities of eIF4E [147-150]. eIF4E is a 7-methyl Guanosine cap (m^7G cap) binding protein that recruits mRNA to the ribosome and plays a crucial role in the regulation of translation [151]. It also functions in the export of specific mRNAs to the cytoplasm. RBV was found to bind eIF4E near the m^7G cap-binding site, which resulted in reduced translation of eIF4E sensitive transcripts and inhibition of eIF4E mediated mRNA translocation [147, 148]. As a result, RBV gained interest as a potential anti-cancer agent and has had some success in phase II clinical trials in AML patients [150]. eIF4E inhibition would explain the discordant results seen with Guanosine supplementation (**Fig. 13 and 14**). Contrary to cellular growth requirements, HCV replication is cap-independent. Therefore, restoration of host nucleotide pools would be expected to have an increased effect on viral replication, as opposed to cellular proliferation. Guanosine supplementation would oppose RBV-mediated GTP pool depletion, but it would not rescue eIF4E mediated inhibition. It is likely that the

combination of both IMPDH inhibition and eIF4E inhibition contribute to RBV's cytostatic and cytotoxic abilities (discussed further in **Epilogue** and **Fig. 16**). One means to test this would be to determine the effect eIF4E overexpression on RBV^S cells in the presence of RBV and Guo. RBV may induce low-level toxicity or stress responses which limit viral replication

Finally, our results indicate that host-based resistance to RBV may occur in HCV patients undergoing combination IFN/RBV therapy. In all cases examined, RBV exposure reduced RBV uptake in PBMCs from healthy donors (**Fig. 15A – C**). Declines in RBV uptake occurred after only 7 days of culture with a low, clinically relevant plasma concentration of RBV (10 μM). RBV uptake was also reduced in PBMCs from patients undergoing combination IFN/RBV therapy (**Fig. 15D – F**), suggesting the development of resistance is a general phenomenon and not limited to cell culture. Perhaps most interesting, we found that PBMCs from patients who responded rapidly to treatment (RRs) had statistically higher RBV uptake at day 28, compared with PBMCs from patients who were slow or non-responders. These results suggest that maintenance of basal RBV uptake levels may improve treatment response. While HCV replicates in hepatocytes, we used PBMCs for this study due to their high viability and ease of sample acquisition at multiple time points. In PBMCs, RBV uptake was reduced following *ex vivo* and *in vivo* exposure to RBV. Because treatment response correlated with RBV uptake in PBMCs, this suggests that either RBV uptake is similarly affected in liver cells, or, PBMC function is altered by RBV treatment, impacting innate or adaptive antiviral responses in the liver. Further research aimed at understanding the precise mechanisms

by which RBV can affect cellular processes is warranted and may improve our understanding of RBV's antiviral activity against HCV.

In conclusion, our results uncovered a novel form of host-based antiviral drug resistance that correlates with HCV treatment response. The data suggest that RBV toxicity drives the development of host-based resistance through reduced cellular uptake. Notably, our results also suggest that patients undergoing combination IFN/RBV therapy develop resistance *in vivo*, with significantly reduced cellular RBV uptake over time. RBV uptake in PBMCs could be used as a diagnostic tool to predict treatment response. The knowledge that host-based RBV resistance may impact treatment outcome suggests that the development of drugs specifically targeted to counter the development of resistance may improve HCV treatment response.

EPILOGUE

Despite the abundance of information which supports the significance of RBV within IFN-based treatment, we still do not understand why or how RBV synergistically improves IFN therapy within HCV patients. This is likely due to the manner by which RBV exerts its antiviral mechanism of action against HCV. It's possible that RBV does not act through a singular mechanism, or the combination of several discordant mechanisms. Evidence suggests HCV replication is enhanced in proliferating cells [152]. Furthermore, HCV proteins can induce cellular proliferation, exhibit transformation potential in tissue culture, and in some cases are proposed to be oncogenic. Perhaps the accumulation of cytostatic or cytotoxic interactions induced by RBV within the cell could cumulatively result in the production of an antiproliferative state, which can be in effect antiviral. If so, the ability of RBV to work synergistically with IFN could be seen as an indirect effect of RBV's cytostatic and cytotoxic abilities.

One could imagine such a scenario and map out where and how RBV would improve antiviral efficacy over IFN alone, accounting for many effects that have been attributed to RBV by considering toxicity-mediated events alone. In an effort to increase my own understanding of the results published from other groups, in addition to the apparent discordant results of guanosine supplementation within my own experiments (viral replication in the presence of RBV and Guo as opposed to cellular viability in the presence of both) I devised a model which incorporates the findings of several groups across the fields of cell biology, cancer and viral research. I propose that the synergistic activity of RBV primarily originates from its ability to act as a cytostatic and cytotoxic

agent, and that RBV augments the IFN response in part by restoring inhibition of pathways which were previously manipulated by HCV. It should be noted that all of the mechanisms discussed (**Fig. 16**) require RBV import.

To begin, it is well established that RBV can inhibit IMPDH, thereby depleting host GTP pools as mentioned previously [41]; however, full consideration of this concept has additional repercussions. GTP depletion can impact viral replication, due to a lack of available GTP nucleotides and increased incorporation of RTP by the viral RdRp, and host cellular processes. Depletion of host GTP pools leads to increased IMP and adenine nucleotide pools, which negatively feedback to inhibit phosphoribosyl pyrophosphate (PRPP) synthetase thereby reducing formation of PRPP, a common precursor in the synthesis of both purines and pyrimdines [153, 154]. This can eventually result in ATR and p53 activation, both capable of resulting in cell cycle arrest or apoptosis (**Fig. 16**) [155, 156].

RBV additionally acts to inhibit proliferation by binding eIF4E [147, 148]. Inhibition of eIF4E inhibits cap-dependent translation, most notably for those transcripts which are eIF4E sensitive [157]. It also prevents the selective eIF4E-mediated export of specific mRNA transcripts from the nucleus, many of which are known for their oncogenic potential. One of those transcripts includes Nijmegen breakage syndrome 1 (NBS1). eIF4E can upregulate NBS1, an upstream activator of the Akt pathway. The Akt pathway is known to stimulate cellular proliferation, cellular survival and inhibit apoptosis [158]. HCV directly and indirectly activates this pathway by stimulating the expression of transforming growth factor-β (TGF-β), demonstrating its significance [17, 159]. Activation of the Akt pathway additionally results in mTor signaling and

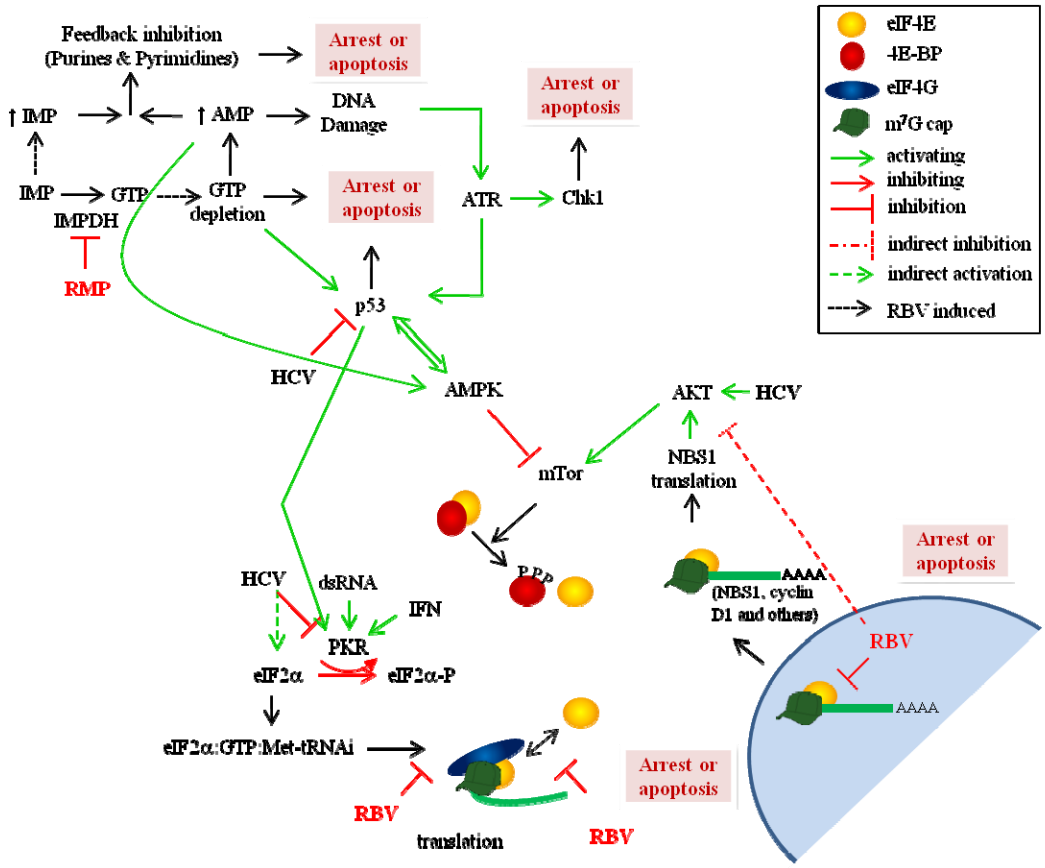


Figure 16. Ribavirin, multiple pathways to an antiviral state.
Model illustrating the effects of IMPDH and eIF4E inhibition on cellular proliferation. Some of the HCV-mediated effects previously described in the literature have also been mapped to illustrate over-lapping pathways between RBV and HCV, and IMPDH/eIF4E inhibition. All interactions shown have been previously described from separate fields of study. The results of those studies, in combination with those of RBV, have been mapped to provide an overall picture of RBV-induced pathways which ultimately result in the induction of cell cycle arrest or apoptosis.

phosphorylation of 4E-binding protein (4E-BP), which results in the release of another eIF4E-mediated inhibition mechanism (**Fig. 16**) [160]. This provides an additional means by which HCV can promote cellular proliferation, and thus viral growth. By inhibiting eIF4E, RBV indirectly acts to oppose Akt activation, thereby reducing cellular proliferation and viral replication

PKR, a well described IFN-induced gene, functions to halt cellular proliferation by phosphorylating eukaryotic translation initiation factor 2 (eIF2α) and inhibiting protein synthesis [129]. HCV is known to counter this blockade by directly interacting with and inhibiting PKR [129]. Nevertheless, restoration of unphosphorylated eIF2α levels will not affect limiting host GTP levels, or replication blocks of essential host proteins through eIF4E RBV-mediated inhibition. Interestingly, PKR activation also results in decreased cyclin D1 levels, which are known to stimulate proliferation and survival [157]. Cyclin D1 happens to be one of the selectively regulated transcripts of eIF4E which may contribute to RBV's ability to counter cellular proliferation.

Finally, the effects of IMPDH and eIF4E inhibition can be linked through p53 (**Fig. 16**). Recent evidence suggests that p53 can induce the expression of PKR, independent of viral infection or type I IFN [161]. Furthermore, p53 is a known activator of AMP-activated protein kinase (AMPK), a known inhibitor of mTor signalling [162]. The NS5A protein of HCV has been shown to directly bind and suppress p53 function, once more illustrating the importance of cell proliferation blockades. By inducing host GTP depletion, RBV can indirectly lead to p53 activation which results in downstream activation of PKR and AMPK. This provides additional mechanisms by which RBV may restore cell proliferation blockades, synergistically improving the outcome over IFN alone.

In summary, by considering all of the repercussions of RBV-mediated toxicity, one can begin to see a picture where RBV, through different mechanisms, consistently results in cell cycle arrest or apoptosis. By initiating these events, multiple cell cycle

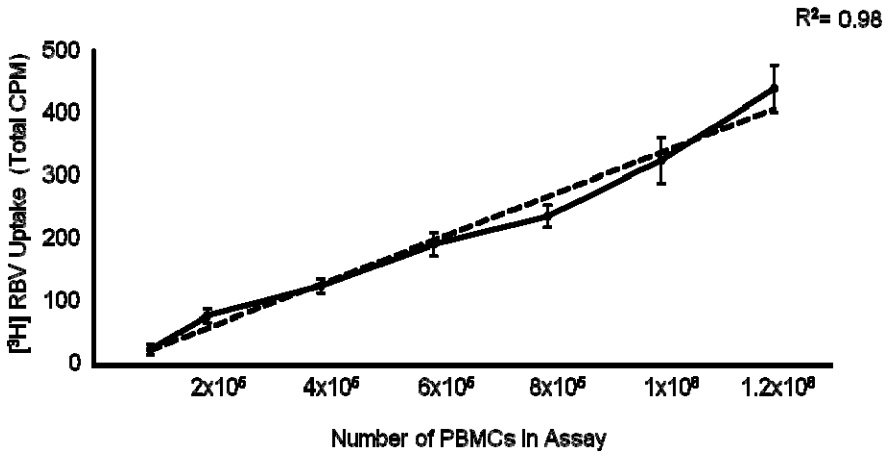
blockades provide additional measures to inhibit HCV replication and growth. RBV also functions to indirectly promote apoptosis, amplify the effects of IFN, and restore IFN-induced blockades which were previously antagonized by HCV. Given the extensive pathways and pleiotropic effects involved, it's no wonder a consensus on RBV's anti-HCV mechanism of action has not be reached thus far.

APPENDIX A
CELL LINE DATA

Cell Line Data	
Cell Line	**Description**
293	Human embryonic kidney
Cured 4	Replicon containing Huh 7 cells that were previously cured of the replicon through IFN treatment
HepG2	Human liver
HeLa	Human cervial carcinoma
Huh-7	Human hepatoma
Huh 7.5	Derivative Huh 7 cell containing a mutation within RIG-I which results in a loss of signalling to IRF3. Previously cured of the replicon through IFN treatment
MCF7	Human breast adenocarcinoma
PH5CH8	Human primary liver

Table 1. List of cell lines used.

APPENDIX B
LINEAR RANGE OF [^{3}H]-RBV UPTAKE IN PBMCS



Appendix B. Linear range of [^{3}H]-RBV uptake in PBMCs. PBMCs from a healthy donor were resuspended at various concentrations (PBMC total/ml) and treated with [^{3}H]-labeled RBV for 15 minutes. RBV uptake was quantified and is shown as the average total CPM for each concentration. Linear regression analysis is represented by a dashed line, $R^2 = 0.98$. Error bars represent SD.

APPENDIX C
PATIENT DATA

Patient ID	HCV Genotype	Response	Gender	Race	Age	HIV	Weight (kg)	RBV (mg)	RBV Dose (mg/kg)	% RBV Uptake (D28/D0)
70	1a	RR	Male	White	35	+	81.8	1200	14.67	121.98
65	1a	RR	Male	White	49	+	76.1	1200	15.77	116.67
41	1a	RR	Male	White	38	+	70.1	800*	11.41	102.86
64	1a	RR	Male	White	39	+	80.8	1200	14.85	97.91
69	1a	Non-RR	Male	White	38	+	93.9	1200	12.78	95.53
12	1a	RR	Male	White	49	+	79.7	1000	12.55	77.66
63	1a	RR	Male	Black	47	+	67.2	1000	14.88	74.52
68	1b	Non-RR	Male	White	32	+	69	1000	14.49	71.10
51	1b	Non-RR	Male	Black	43	+	118.7	1200	10.11	66.36
7	1a	Non-RR	Female	White	36	+	85.1	1200	14.10	66.12
66	1b	Non-RR	Female	Black	56	+	94	1200	12.77	65.02
45	1a	Non-RR	Female	Black	59	+	74.6	1000	13.40	63.73
53	1a	Non-RR	Male	White	37	-	70.5	1000	14.18	63.72
61	1a	RR	Male	White	44	+	69.2	1000	14.45	54.82
58	1a	Non-RR	Female	Black	45	+	87.6	1200	13.70	50.62
49	1b	Non-RR	Female	Black	54	+	79.2	1200	15.15	37.23

Table 2. Patient data. RR = rapid response. Non-RR = non-rapid response. The % RBV Uptake (D28/D0) is the amount of RBV uptake in PBMCs isolated at day 28 post-therapy relative to the amount of RBV uptake prior to therapy on day 0 (see Fig. 15f). The patient marked with an asterisk was given a lower RBV dose due to inclusion in a separate study comparing the efficacy of 800 mg RBV to 1000 -1200 mg.

BIBLIOGRAPY

1. Main, J., *Hepatitis C: clinical aspects.* J Infect, 1995. **30**(2): p. 103-6.

2. Houghton, M., *The long and winding road leading to the identification of the hepatitis C virus.* J Hepatol, 2009. **51**(5): p. 939-48.

3. *Recommendations for prevention and control of hepatitis C virus (HCV) infection and HCV-related chronic disease. Centers for Disease Control and Prevention.* MMWR Recomm Rep, 1998. **47**(RR-19): p. 1-39.

4. Choo, Q.L., et al., *Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome.* Science, 1989. **244**(4902): p. 359-62.

5. Hsu, E.K. and K.F. Murray, *Hepatitis B and C in children.* Nat Clin Pract Gastroenterol Hepatol, 2008. **5**(6): p. 311-20.

6. Ghany, M.G., et al., *Diagnosis, management, and treatment of hepatitis C: an update.* Hepatology, 2009. **49**(4): p. 1335-74.

7. MacCannell, T., et al., *Occupational exposure of health care personnel to hepatitis B and hepatitis C: prevention and surveillance strategies.* Clin Liver Dis, 2010. **14**(1): p. 23-36, vii.

8. Lloyd, A.R., et al., *Host and viral factors in the immunopathogenesis of primary hepatitis C virus infection.* Immunol Cell Biol, 2007. **85**(1): p. 24-32.

9. Vignuzzi, M., J.K. Stone, and R. Andino, *Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications.* Virus Res, 2005. **107**(2): p. 173-81.

10. Asselah, T., et al., *Hepatitis C: viral and host factors associated with non-response to pegylated interferon plus ribavirin.* Liver Int, 2010.

11. Castello, G., et al., *HCV-related hepatocellular carcinoma: From chronic inflammation to cancer.* Clin Immunol, 2010. **134**(3): p. 237-50.

12. Daniels, D., S. Grytdal, and A. Wasley, *Surveillance for acute viral hepatitis - United States, 2007.* MMWR Surveill Summ, 2009. **58**(3): p. 1-27.

13. Chen, S.L. and T.R. Morgan, *The natural history of hepatitis C virus (HCV) infection.* Int J Med Sci, 2006. **3**(2): p. 47-52.

14. Maheshwari, A. and P.J. Thuluvath, *Management of acute hepatitis C.* Clin Liver Dis, 2010. **14**(1): p. 169-76; x.

15. Pawlotsky, J.M., *Pathophysiology of hepatitis C virus infection and related liver disease.* Trends Microbiol, 2004. **12**(2): p. 96-102.

16. El-Serag, H.B., *Hepatocellular carcinoma and hepatitis C in the United States.* Hepatology, 2002. **36**(5 Suppl 1): p. S74-83.

17. Tsai, W.L. and R.T. Chung, *Viral hepatocarcinogenesis.* Oncogene, 2010. **29**(16): p. 2309-24.

18. Bacon, B.R. and J.G. McHutchison, *Into the light: strategies for battling hepatitis C.* Am J Manag Care, 2007. **13 Suppl 12**: p. S319-26.

19. Poordad, F., K.R. Reddy, and P. Martin, *Rapid virologic response: a new milestone in the management of chronic hepatitis C.* Clin Infect Dis, 2008. **46**(1): p. 78-84.

20. Ferenci, P., et al., *Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a (40 KD)/ribavirin.* J Hepatol, 2005. **43**(3): p. 425-33.

21. Feld, J.J. and J.H. Hoofnagle, *Mechanism of action of interferon and ribavirin in treatment of hepatitis C.* Nature, 2005. **436**(7053): p. 967-72.

22. Wohnsland, A., W.P. Hofmann, and C. Sarrazin, *Viral determinants of resistance to treatment in patients with hepatitis C.* Clin Microbiol Rev, 2007. **20**(1): p. 23-38.

23. Ge, D., et al., *Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance.* Nature, 2009. **461**(7262): p. 399-401.

24. Suppiah, V., et al., *IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy.* Nat Genet, 2009. **41**(10): p. 1100-4.

25. Tanaka, Y., et al., *Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C.* Nat Genet, 2009. **41**(10): p. 1105-9.

26. Jain, M.K. and C. Zoellner, *Role of ribavirin in HCV treatment response: now and in the future.* Expert Opin Pharmacother, 2010. **11**(4): p. 673-83.

27. Chen, L., et al., *Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection.* Gastroenterology, 2005. **128**(5): p. 1437-44.

28. Feld, J.J., et al., *Hepatic gene expression during treatment with peginterferon and ribavirin: Identifying molecular pathways for treatment response.* Hepatology, 2007. **46**(5): p. 1548-63.

29. Asselah, T., et al., *Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C.* Gut, 2008. **57**(4): p. 516-24.

30. Benhamou, Y., et al., *A phase III study of the safety and efficacy of viramidine versus ribavirin in treatment-naive patients with chronic hepatitis C: ViSER1 results.* Hepatology, 2009. **50**(3): p. 717-26.

31. Shiffman, M.L., *What future for ribavirin?* Liver Int, 2009. **29 Suppl 1**: p. 68-73.

32. Pereira, A.A. and I.M. Jacobson, *New and experimental therapies for HCV.* Nat Rev Gastroenterol Hepatol, 2009. **6**(7): p. 403-11.

33. Sarrazin, C. and S. Zeuzem, *Resistance to direct antiviral agents in patients with hepatitis C virus infection.* Gastroenterology, 2010. **138**(2): p. 447-62.

34. Fernandez, H., G. Banks, and R. Smith, *Ribavirin: a clinical overview.* Eur J Epidemiol, 1986. **2**(1): p. 1-14.

35. Witkowski, J.T., et al., *Design, synthesis, and broad spectrum antiviral activity of 1- -D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides.* J Med Chem, 1972. **15**(11): p. 1150-4.

36. Patterson, J.L. and R. Fernandez-Larsson, *Molecular mechanisms of action of ribavirin.* Rev Infect Dis, 1990. **12**(6): p. 1139-46.

37. Reichard, O., et al., *Ribavirin treatment for chronic hepatitis C.* Lancet, 1991. **337**(8749): p. 1058-61.

38. James, J.S., *Ribavirin approved for hepatitis C combination treatment.* AIDS Treat News, 1998(No 297): p. 7.

39. Dixit, N.M. and A.S. Perelson, *The metabolism, pharmacokinetics and mechanisms of antiviral activity of ribavirin against hepatitis C virus.* Cell Mol Life Sci, 2006. **63**(7-8): p. 832-42.

40. Loustaud-Ratti, V., et al., *Ribavirin in chronic hepatitis C: past and future.* Expert Rev Anti Infect Ther, 2009. **7**(3): p. 249-53.

41. Sintchak, M.D. and E. Nimmesgern, *The structure of inosine 5'-monophosphate dehydrogenase and the design of novel inhibitors.* Immunopharmacology, 2000. **47**(2-3): p. 163-84.

42. Bougie, I. and M. Bisaillon, *Initial binding of the broad spectrum antiviral nucleoside ribavirin to the hepatitis C virus RNA polymerase.* J Biol Chem, 2003. **278**(52): p. 52471-8.

43. Maag, D., et al., *Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin.* J Biol Chem, 2001. **276**(49): p. 46094-8.

44. Vo, N.V., K.C. Young, and M.M. Lai, *Mutagenic and inhibitory effects of ribavirin on hepatitis C virus RNA polymerase.* Biochemistry, 2003. **42**(35): p. 10462-71.

45. Crotty, S., et al., *The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen.* Nat Med, 2000. **6**(12): p. 1375-9.

46. Crotty, S., C.E. Cameron, and R. Andino, *RNA virus error catastrophe: direct molecular test by using ribavirin.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6895-900.

47. Domingo, E. and J.J. Holland, *RNA virus mutations and fitness for survival.* Annu Rev Microbiol, 1997. **51**: p. 151-78.

48. Cuevas, J.M., et al., *Effect of ribavirin on the mutation rate and spectrum of hepatitis C virus in vivo.* J Virol, 2009. **83**(11): p. 5760-4.

49. Peavy, D.L., C.N. Powers, and V. Knight, *Inhibition of murine plaque-forming cell responses in vivo by ribavirin.* J Immunol, 1981. **126**(3): p. 861-4.

50. Tam, R.C., et al., *Ribavirin polarizes human T cell responses towards a Type 1 cytokine profile.* J Hepatol, 1999. **30**(3): p. 376-82.

51. Feld, J.J., et al., *Ribavirin Improves Early Responses To Peginterferon Through Improved Interferon Signaling.* Gastroenterology, 2010.

52. McHutchison, J.G., et al., *Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group.* N Engl J Med, 1998. **339**(21): p. 1485-92.

53. Reichard, O., R. Schvarcz, and O. Weiland, *Therapy of hepatitis C: alpha interferon and ribavirin.* Hepatology, 1997. **26**(3 Suppl 1): p. 108S-111S.

54. Khakoo, S., et al., *Ribavirin and interferon alfa-2b in chronic hepatitis C: assessment of possible pharmacokinetic and pharmacodynamic interactions.* Br J Clin Pharmacol, 1998. **46**(6): p. 563-70.

55. Pawlotsky, J.M., et al., *Antiviral action of ribavirin in chronic hepatitis C.* Gastroenterology, 2004. **126**(3): p. 703-14.

56. Jen, J.F., et al., *Population pharmacokinetic and pharmacodynamic analysis of ribavirin in patients with chronic hepatitis C.* Ther Drug Monit, 2000. **22**(5): p. 555-65.

57. Jen, J., et al., *Ribavirin dosing in chronic hepatitis C: application of population pharmacokinetic-pharmacodynamic models.* Clin Pharmacol Ther, 2002. **72**(4): p. 349-61.

58. Larrat, S., et al., *Ribavirin quantification in combination treatment of chronic hepatitis C.* Antimicrob Agents Chemother, 2003. **47**(1): p. 124-9.

59. Lindahl, K., et al., *High-dose ribavirin in combination with standard dose peginterferon for treatment of patients with chronic hepatitis C.* Hepatology, 2005. **41**(2): p. 275-9.

60. Bain, V.G., et al., *Clinical trial: exposure to ribavirin predicts EVR and SVR in patients with HCV genotype 1 infection treated with peginterferon alpha-2a plus ribavirin.* Aliment Pharmacol Ther, 2008. **28**(1): p. 43-50.

61. Loustaud-Ratti, V., et al., *Ribavirin exposure after the first dose is predictive of sustained virological response in chronic hepatitis C.* Hepatology, 2008. **47**(5): p. 1453-61.

62. Manns, M.P., et al., *Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial.* Lancet, 2001. **358**(9286): p. 958-65.

63. Bronowicki, J.P., et al., *Effect of ribavirin in genotype 1 patients with hepatitis C responding to pegylated interferon alfa-2a plus ribavirin.* Gastroenterology, 2006. **131**(4): p. 1040-8.

64. Dixit, N.M., et al., *Modelling how ribavirin improves interferon response rates in hepatitis C virus infection.* Nature, 2004. **432**(7019): p. 922-4.

65. Errasti-Murugarren, E., M. Pastor-Anglada, and F.J. Casado, *Role of CNT3 in the transepithelial flux of nucleosides and nucleoside-derived drugs.* J Physiol, 2007. **582**(Pt 3): p. 1249-60.

66. Jarvis, S.M., J.A. Thorn, and P. Glue, *Ribavirin uptake by human erythrocytes and the involvement of nitrobenzylthioinosine-sensitive (es)-nucleoside transporters.* Br J Pharmacol, 1998. **123**(8): p. 1587-92.

67. Patil, S.D., et al., *Intestinal absorption of ribavirin is preferentially mediated by the Na+-nucleoside purine (N1) transporter.* Pharm Res, 1998. **15**(6): p. 950-2.

68. Yamamoto, T., et al., *Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters.* Eur J Pharmacol, 2007. **557**(1): p. 1-8.

69. Kong, W., K. Engel, and J. Wang, *Mammalian nucleoside transporters.* Curr Drug Metab, 2004. **5**(1): p. 63-84.

70. Young, J.D., et al., *Human equilibrative nucleoside transporter (ENT) family of nucleoside and nucleobase transporter proteins.* Xenobiotica, 2008. **38**(7-8): p. 995-1021.

71. Pfeiffer, J.K. and K. Kirkegaard, *A single mutation in poliovirus RNA-dependent RNA polymerase confers resistance to mutagenic nucleotide analogs via increased fidelity.* Proc Natl Acad Sci U S A, 2003. **100**(12): p. 7289-94.

72. Pfeiffer, J.K. and K. Kirkegaard, *Ribavirin resistance in hepatitis C virus replicon-containing cell lines conferred by changes in the cell line or mutations in the replicon RNA.* J Virol, 2005. **79**(4): p. 2346-55.

73. Lohmann, V., et al., *Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line.* Science, 1999. **285**(5424): p. 110-3.

74. Blight, K.J., J.A. McKeating, and C.M. Rice, *Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication.* J Virol, 2002. **76**(24): p. 13001-14.

75. Sumpter, R., Jr., et al., *Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I.* J Virol, 2005. **79**(5): p. 2689-99.

76. Pfeiffer, J.K. and K. Kirkegaard, *Increased fidelity reduces poliovirus fitness and virulence under selective pressure in mice.* PLoS Pathog, 2005. **1**(2): p. e11.

77. Racaniello, V.R. and D. Baltimore, *Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome.* Proc Natl Acad Sci U S A, 1981. **78**(8): p. 4887-91.

78. Kirkegaard, K. and D. Baltimore, *The mechanism of RNA recombination in poliovirus.* Cell, 1986. **47**(3): p. 433-43.

79. Wakita, T., et al., *Production of infectious hepatitis C virus in tissue culture from a cloned viral genome.* Nat Med, 2005. **11**(7): p. 791-6.

80. Erickson, A.K., S. Seiwert, and M. Gale, Jr., *Antiviral potency analysis and functional comparison of consensus interferon, interferon-alpha2a and pegylated interferon-alpha2b against hepatitis C virus infection.* Antivir Ther, 2008. **13**(7): p. 851-62.

81. Loo, Y.M., et al., *Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection.* Proc Natl Acad Sci U S A, 2006. **103**(15): p. 6001-6.

82. Pfeiffer, J.K., et al., *Altering the intracellular environment increases the frequency of tandem repeat deletion during Moloney murine leukemia virus reverse transcription.* J Virol, 1999. **73**(10): p. 8441-7.

83. Ribaudo, R., Gilman, M., Kingston, R.E, Chomczynski, P., Sacchi, N. , *Preparation and Analysis of RNA*, in *Current Protocols in Immunology*, J.E. Coligan, Bierer, B.E., Margulies, D.H., Shevach, E.M., Strober, W., Brown, P., Donovan, J.C. , Editor. 2007, John Wiley and Sons, Inc.

84. Bookout, A.L., Cummins,C.L. , Kramer, M.F., Pesola, J.M, and Mangelsdorf, D.J., *High-Throughput Real-Time Quantitative Reverse Transcription PCR*, in *Current Protocols in Molecular Biology*. 2006 John Wiley & Sons, Inc.

85. Maes, B., et al., *The NPM-ALK and the ATIC-ALK fusion genes can be detected in non-neoplastic cells.* Am J Pathol, 2001. **158**(6): p. 2185-93.

86. Mackey, J.R., et al., *Immunohistochemical variation of human equilibrative nucleoside transporter 1 protein in primary breast cancers.* Clin Cancer Res, 2002. **8**(1): p. 110-6.

87. Rozen, S.a.S., H.J, *Primer3 on the WWW for general users and for biologist programmers*, in *Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology*. 2000, Humana Press, Totowa, NJ, pp 365-386

88. *Hepatitis C--global prevalence (update).* Wkly Epidemiol Rec, 2000. **75**(3): p. 18-9.

89. Muller, W.E., et al., *Virazole (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; a cytostatic agent.* Biochem Pharmacol, 1977. **26**(11): p. 1071-5.

90. Lowe, J.K., L. Brox, and J.F. Henderson, *Consequences of inhibition of Guonine nucleotide synthesis by mycophenolic acid and virazole.* Cancer Res, 1977. **37**(3): p. 736-43.

91. Bacheler, L., et al., *Genotypic correlates of phenotypic resistance to efavirenz in virus isolates from patients failing nonnucleoside reverse transcriptase inhibitor therapy.* J Virol, 2001. **75**(11): p. 4999-5008.

92. Enomoto, N., et al., *Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region.* J Clin Invest, 1995. **96**(1): p. 224-30.

93. Hofmann, W.P., et al., *Mutations within the CD81-binding sites and hypervariable region 2 of the envelope 2 protein: correlation with treatment response in hepatitis C virus-infected patients.* J Infect Dis, 2003. **187**(6): p. 982-7.

94. Johnson, V.A., et al., *Update of the drug resistance mutations in HIV-1: 2007.* Top HIV Med, 2007. **15**(4): p. 119-25.

95. Sarrazin, C., et al., *Clinical significance of in vitro replication-enhancing mutations of the hepatitis C virus (HCV) replicon in patients with chronic HCV infection.* J Infect Dis, 2005. **192**(10): p. 1710-9.

96. Damaraju, V.L., et al., *Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy.* Oncogene, 2003. **22**(47): p. 7524-36.

97. Pennycooke, M., et al., *Differential expression of human nucleoside transporters in normal and tumor tissue.* Biochem Biophys Res Commun, 2001. **280**(3): p. 951-9.

98. King, K.M., et al., *A comparison of the transportability, and its role in cytotoxicity, of clofarabine, cladribine, and fludarabine by recombinant human nucleoside transporters produced in three model expression systems.* Mol Pharmacol, 2006. **69**(1): p. 346-53.

99. Lang, T.T., J.D. Young, and C.E. Cass, *Interactions of nucleoside analogs, caffeine, and nicotine with human concentrative nucleoside transporters 1 and 2 stably produced in a transport-defective human cell line.* Mol Pharmacol, 2004. **65**(4): p. 925-33.

100. Molina-Arcas, M., et al., *Fludarabine uptake mechanisms in B-cell chronic lymphocytic leukemia.* Blood, 2003. **101**(6): p. 2328-34.

101. Ritzel, M.W., et al., *Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib).* J Biol Chem, 2001. **276**(4): p. 2914-27.

102. Zhang, J., et al., *The role of nucleoside transporters in cancer chemotherapy with nucleoside drugs.* Cancer Metastasis Rev, 2007. **26**(1): p. 85-110.

103. Zhou, S., et al., *The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA.* Virology, 2003. **310**(2): p. 333-42.

104. Govindarajan, R., et al., *Expression and hepatobiliary transport characteristics of the concentrative and equilibrative nucleoside transporters in sandwich-cultured human hepatocytes.* Am J Physiol Gastrointest Liver Physiol, 2008. **295**(3): p. G570-80.

105. Yao, S.Y., et al., *Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues.* J Biol Chem, 1997. **272**(45): p. 28423-30.

106. Toan, S.V., et al., *Genomic organization and functional characterization of the human concentrative nucleoside transporter-3 isoform (hCNT3) expressed in mammalian cells.* Pflugers Arch, 2003. **447**(2): p. 195-204.

107. Griffith, D.A. and S.M. Jarvis, *Nucleoside and nucleobase transport systems of mammalian cells.* Biochim Biophys Acta, 1996. **1286**(3): p. 153-81.

108. Ngo, L.Y., S.D. Patil, and J.D. Unadkat, *Ontogenic and longitudinal activity of Na(+)-nucleoside transporters in the human intestine.* Am J Physiol Gastrointest Liver Physiol, 2001. **280**(3): p. G475-81.

109. Takagaki, K., et al., *Gene-expression profiling reveals down-regulation of equilibrative nucleoside transporter 1 (ENT1) in Ara-C-resistant CCRF-CEM-derived cells.* J Biochem (Tokyo), 2004. **136**(5): p. 733-40.

110. Galmarini, C.M., et al., *Potential mechanisms of resistance to cytarabine in AML patients.* Leuk Res, 2002. **26**(7): p. 621-9.

111. Fotoohi, A.K., et al., *Involvement of the concentrative nucleoside transporter 3 and equilibrative nucleoside transporter 2 in the resistance of T-lymphoblastic cell lines to thiopurines.* Biochem Biophys Res Commun, 2006. **343**(1): p. 208-15.

112. Yao, S.Y., et al., *Functional and molecular characterization of nucleobase transport by recombinant human and rat equilibrative nucleoside transporters 1*

and 2. Chimeric constructs reveal a role for the ENT2 helix 5-6 region in nucleobase translocation. J Biol Chem, 2002. **277**(28): p. 24938-48.

113. Isakovic, A.J., et al., *Transport of [14C]hypoxanthine by sheep choroid plexus epithelium as a monolayer in primary culture: Na+-dependent and Na+-independent uptake by the apical membrane and rapid intracellular metabolic conversion to nucleotides.* Neurosci Lett, 2008. **431**(2): p. 135-40.

114. Robillard, K.R., D.B. Bone, and J.R. Hammond, *Hypoxanthine uptake and release by equilibrative nucleoside transporter 2 (ENT2) of rat microvascular endothelial cells.* Microvasc Res, 2007.

115. Ward, J.L., et al., *Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for Guanosine and cytidine but a high affinity for inosine.* J Biol Chem, 2000. **275**(12): p. 8375-81.

116. Glue, P., *The clinical pharmacology of ribavirin.* Semin Liver Dis, 1999. **19 Suppl 1**: p. 17-24.

117. Dragan, Y., et al., *Selective loss of nucleoside carrier expression in rat hepatocarcinomas.* Hepatology, 2000. **32**(2): p. 239-46.

118. Baldwin, S.A., et al., *The equilibrative nucleoside transporter family, SLC29.* Pflugers Arch, 2004. **447**(5): p. 735-43.

119. Gray, J.H., R.P. Owen, and K.M. Giacomini, *The concentrative nucleoside transporter family, SLC28.* Pflugers Arch, 2004. **447**(5): p. 728-34.

120. Mani, R.S., et al., *Demonstration of equilibrative nucleoside transporters (hENT1 and hENT2) in nuclear envelopes of cultured human choriocarcinoma (BeWo) cells by functional reconstitution in proteoliposomes.* J Biol Chem, 1998. **273**(46): p. 30818-25.

121. Pisoni, R.L. and J.G. Thoene, *Detection and characterization of a nucleoside transport system in human fibroblast lysosomes.* J Biol Chem, 1989. **264**(9): p. 4850-6.

122. Lai, Y., C.M. Tse, and J.D. Unadkat, *Mitochondrial expression of the human equilibrative nucleoside transporter 1 (hENT1) results in enhanced mitochondrial toxicity of antiviral drugs.* J Biol Chem, 2004. **279**(6): p. 4490-7.

123. Mackey, J.R., et al., *Quantitative analysis of nucleoside transporter and metabolism gene expression in chronic lymphocytic leukemia (CLL): identification of fludarabine-sensitive and -insensitive populations.* Blood, 2005. **105**(2): p. 767-74.

124. Galmarini, C.M., et al., *Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the deoxycytidine kinase gene.* BMC Pharmacol, 2004. **4**: p. 8.

125. Hezode, C., et al., *Telaprevir and peginterferon with or without ribavirin for chronic HCV infection.* N Engl J Med, 2009. **360**(18): p. 1839-50.

126. Ibarra, K.D. and J.K. Pfeiffer, *Reduced ribavirin antiviral efficacy via nucleoside transporter-mediated drug resistance.* J Virol, 2009. **83**(9): p. 4538-47.

127. Graci, J.D. and C.E. Cameron, *Mechanisms of action of ribavirin against distinct viruses.* Rev Med Virol, 2006. **16**(1): p. 37-48.

128. Garcia, M.A., et al., *Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action.* Microbiol Mol Biol Rev, 2006. **70**(4): p. 1032-60.

129. Garcia, M.A., E.F. Meurs, and M. Esteban, *The dsRNA protein kinase PKR: virus and cell control.* Biochimie, 2007. **89**(6-7): p. 799-811.

130. Liu, W.L., et al., *Ribavirin up-regulates the activity of double-stranded RNA-activated protein kinase and enhances the action of interferon-alpha against hepatitis C virus.* J Infect Dis, 2007. **196**(3): p. 425-34.

131. Schlosser, S.F., et al., *Ribavirin and alpha interferon enhance death receptor-mediated apoptosis and caspase activation in human hepatoma cells.* Antimicrob Agents Chemother, 2003. **47**(6): p. 1912-21.

132. Feld, J.J., et al., *Ribavirin Improves Early Responses To Peginterferon Through Improved Interferon Signaling.* Gastroenterology.

133. Jordheim, L.P. and C. Dumontet, *Review of recent studies on resistance to cytotoxic deoxynucleoside analogues.* Biochim Biophys Acta, 2007. **1776**(2): p. 138-59.

134. Wiley, J.S., et al., *Cytosine arabinoside influx and nucleoside transport sites in acute leukemia.* J Clin Invest, 1982. **69**(2): p. 479-89.

135. Sarkar, M., et al., *Cytosine arabinoside affects multiple cellular factors and induces drug resistance in human lymphoid cells.* Biochem Pharmacol, 2005. **70**(3): p. 426-32.

136. Giovannetti, E., et al., *Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine.* Cancer Res, 2006. **66**(7): p. 3928-35.

137. Lai, Y., A.H. Bakken, and J.D. Unadkat, *Simultaneous expression of hCNT1-CFP and hENT1-YFP in Madin-Darby canine kidney cells. Localization and vectorial transport studies.* J Biol Chem, 2002. **277**(40): p. 37711-7.

138. Badagnani, I., et al., *Functional analysis of genetic variants in the human concentrative nucleoside transporter 3 (CNT3; SLC28A3).* Pharmacogenomics J, 2005. **5**(3): p. 157-65.

139. Damaraju, S., et al., *Identification and functional characterization of variants in human concentrative nucleoside transporter 3, hCNT3 (SLC28A3), arising from single nucleotide polymorphisms in coding regions of the hCNT3 gene.* Pharmacogenet Genomics, 2005. **15**(3): p. 173-82.

140. Errasti-Murugarren, E., et al., *Functional characterization of a nucleoside-derived drug transporter variant (hCNT3C602R) showing altered sodium-binding capacity.* Mol Pharmacol, 2008. **73**(2): p. 379-86.

141. Errasti-Murugarren, E., et al., *The human concentrative nucleoside transporter-3 C602R variant shows impaired sorting to lipid rafts and altered specificity for nucleoside-derived drugs.* Mol Pharmacol, 2010. **78**(2): p. 157-65.

142. Owen, R.P., et al., *Genetic analysis and functional characterization of polymorphisms in the human concentrative nucleoside transporter, CNT2.* Pharmacogenet Genomics, 2005. **15**(2): p. 83-90.

143. Morello, J., et al., *Influence of a Single Nucleotide Polymorphism at the Main Ribavirin Transporter Gene on the Rapid Virological Response to Pegylated Interferon-Ribavirin Therapy in Patients with Chronic Hepatitis C Virus Infection.* J Infect Dis, 2010.

144. Cai, J., et al., *Two distinct molecular mechanisms underlying cytarabine resistance in human leukemic cells.* Cancer Res, 2008. **68**(7): p. 2349-57.

145. Fukuchi, Y., et al., *Characterization of ribavirin uptake systems in human hepatocytes.* J Hepatol, 2010. **52**(4): p. 486-92.

146. Gish, R.G., *Treating HCV with ribavirin analogues and ribavirin-like molecules.* J Antimicrob Chemother, 2006. **57**(1): p. 8-13.

147. Kentsis, A., et al., *Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical mimicry of the 7-methyl Guanosine mRNA cap.* Proc Natl Acad Sci U S A, 2004. **101**(52): p. 18105-10.

148. Kentsis, A., et al., *Further evidence that ribavirin interacts with eIF4E.* RNA, 2005. **11**(12): p. 1762-6.

149. Tan, K., et al., *Ribavirin targets eIF4E dependent Akt survival signaling.* Biochem Biophys Res Commun, 2008. **375**(3): p. 341-5.

150. Assouline, S., et al., *Molecular targeting of the oncogene eIF4E in acute myeloid leukemia (AML): a proof-of-principle clinical trial with ribavirin.* Blood, 2009. **114**(2): p. 257-60.

151. Rhoads, R.E., *eIF4E: new family members, new binding partners, new roles.* J Biol Chem, 2009. **284**(25): p. 16711-5.

152. McGivern, D.R. and S.M. Lemon, *Tumor suppressors, chromosomal instability, and hepatitis C virus-associated liver cancer.* Annu Rev Pathol, 2009. **4**: p. 399-415.

153. Hedstrom, L., *IMP dehydrogenase: structure, mechanism, and inhibition.* Chem Rev, 2009. **109**(7): p. 2903-28.

154. Jeremy M. Berg, J.L.T., Lubert Stryer, *Biosynthesis of nucleotides*, in *Biochemistry*. 2006, Freeman, W. H. & Company: New York.

155. Hastak, K., et al., *DNA synthesis from unbalanced nucleotide pools causes limited DNA damage that triggers ATR-CHK1-dependent p53 activation.* Proc Natl Acad Sci U S A, 2008. **105**(17): p. 6314-9.

156. Raven, J.F. and A.E. Koromilas, *PERK and PKR: old kinases learn new tricks.* Cell Cycle, 2008. **7**(9): p. 1146-50.

157. Fischer, P.M., *Cap in hand: targeting eIF4E.* Cell Cycle, 2009. **8**(16): p. 2535-41.

158. Culjkovic, B., et al., *The eIF4E RNA regulon promotes the Akt signaling pathway.* J Cell Biol, 2008. **181**(1): p. 51-63.

159. Mannova, P. and L. Beretta, *Activation of the N-Ras-PI3K-Akt-mTOR pathway by hepatitis C virus: control of cell survival and viral replication.* J Virol, 2005. **79**(14): p. 8742-9.

160. Richter, J.D. and N. Sonenberg, *Regulation of cap-dependent translation by eIF4E inhibitory proteins.* Nature, 2005. **433**(7025): p. 477-80.

161. Yoon, C.H., et al., *PKR, a p53 target gene, plays a crucial role in the tumor-suppressor function of p53.* Proc Natl Acad Sci U S A, 2009. **106**(19): p. 7852-7.

162. Huang, J. and B.D. Manning, *The TSC1-TSC2 complex: a molecular switchboard controlling cell growth.* Biochem J, 2008. **412**(2): p. 179-90.