HOST FACTORS CONTROLLING VIRUS INFECTION: IMPLICATIONS FOR ANTIVIRALS AND VIROTHERAPY

by

Nirav Rajivkumar Shah

A dissertation submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Biology

Charlotte

2013

Approved by:

_____
Dr. Valery Grdzelishvili

_____
Dr. Pinku Mukherjee

_____
Dr. Dan Nelson

_____
Dr. Laura Schrum

_____
Dr. Mike Turner

ABSTRACT

NIRAV RAJIVKUMAR SHAH. Host factors controlling virus infection: implications for antivirals and virotherapy. (Under the direction of DR.VALERY GRDZELISHVILI)

Viruses are obligate intracellular parasites rely heavily on host components and pathways for their replication. Studying different cellular factors affecting viral infection can enable us to identify novel drug targets, improve current antiviral treatments and improve efficacy of virus based therapies. This dissertation examines two prototypic members of an order *Mononegavirales,* vesicular stomatitis virus (VSV) and Sendai virus (SeV) and is focused on: 1) resistance of some hosts to a broad spectrum antiviral drug ribavirin and 2) resistance of some pancreatic cancers to oncolytic virotherapy. Here, for the first time we examined whether certain cell types are naturally resistant to ribavirin even without prior drug exposure. Our results show striking differences between cell types in their response to ribavirin. Our data also suggest that this resistance was due to cellular factors rather than viral determinants and ribavirin may inhibit the same virus via different mechanisms in different cells depending on the ribavirin metabolism. Additionally, resistance of oncolytic VSV therapy in specific human pancreatic ductal adenocarcinoma (PDA) cells was investigated and this resistance was attributed to constitutive expression of the IFN-stimulated antiviral genes MxA and OAS. Decreasing the levels of MxA and OAS by inhibition of JAK/STAT signaling, improved VSV infection and oncolysis. Overall, our study demonstrated heterogeneity in the type I IFN signaling status of PDA cells and suggests MxA and OAS as potential biomarkers for PDA resistance to VSV and other oncolytic viruses (OVs) sensitive to type I IFN responses.

DEDICATION

To my parents, my family
and
my beautiful wife, Sapana.

ACKNOWLEDGEMENTS

My dissertation would not have been possible without the help of many individuals. First and foremost, I would like to thank my mentor Dr. Valery Grdzelishvili for providing the opportunity to work in his lab. His mentorship, insight, and knowledge have been an invaluable asset throughout my doctoral research. I would like to extend a special thanks to Dr. Dan Nelson for always being available to discuss and troubleshoot several issues. I would also like to thank the rest of the members of my dissertation committee, Dr. Pinku Mukherjee, Dr. Laura Schrum and Dr. Mike Turner for their guidance and criticism throughout the dissertation process. I am also very thankful to the National Institutes of Health for providing the funding for this research.

I appreciate the help I have received from all the members of the Grdzelishvili lab throughout my tenure especially, Megan Moerdyk-Schauwecker, Dr. Andrea Murphy, and Eric Hastie. I would like to thank the faculty and staff of the department of Biology at UNC Charlotte, especially, Dr. Topher Gee, my mentor as teaching assistant.

I would like to thank my parents and my brother for always believing in me and supporting me during some of the tough times. I am grateful for my parents for providing me every opportunity to attain the best education possible. I would also like to thank all my friends to support me during my career.

Lastly, I cannot accurately describe how grateful I am to my beautiful and ever smiling wife Sapana. She worked twice as hard to provide the opportunity for me to do what I love to do. I will be forever indebted to her for her kindness and support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ΔM51	deletion of methionine at position 51
^{3}H	*tritium*
aa	*amino acid*
ANOVA	*analysis of variance*
BSA	bovine serum albumin
CIU	cell infectious units
CRAds	conditionally replicative adenoviruses
d	days
DMEM	dulbecco's modified eagles medium
DMSO	dimethyl sulfoxide
DNA	*deoxyribonucleic acid*
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
F	fusion
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony stimulating factor
h	hours
h.p.i.	hours post infection
HBS	HEPES buffered saline

HPDE	human pancreatic duct epithelial cell line
HRP	horse radish peroxidase
HSV-1	herpes simplex virus type 1
hTERT	human telomerase reverse transcriptase
IFN	interferon
Ig	immunoglobulin
M	matrix protein
MDSCs	myeloid derived suppressor cells
MEM	modified eagle's medium
mg	milligrams
MHC	major histocompatibility complex
min	minutes
MOI	multiplicity of infection
mRNA	messenger RNA
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NIH	national institute of health
NNS	nonsegmented negative-strand
OD	optical density
ORF	open reading frame
OV	oncolytic virus
p.t.i.	post tumor injection
PanIN	pancreatic intraepithelial neoplasia
PBS	*phosphate buffered saline*

PCR	polymerase chain reaction
PDA	pancreatic ductal adenocarcinoma
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNase	*ribonuclease*
RSV	respiratory syncytial virus
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	*standard error of the mean*
SeV	Sendai virus
SFM	*serum free medium*
U	units
VSV	vesicular stomatitis virus
VVT7	vaccinia virus containing T7 RNA polymerase gene
wt	wild-type

CHAPTER 1: INTRODUCTION

1.1 Overview

Viruses are obligate intracellular parasites, rely heavily on host components and processes for their replication. Viruses are responsible for causing various human diseases. It has been estimated that different viral infections lead to ~3.5 million deaths annually worldwide (Krausslich and Bartenschlager 2009). RNA viruses are the most prevalent, and viruses such as Ebola and Marburg viruses (Paragas and Geisbert 2006), West Nile virus (Hayes and Gubler 2006), SARS (severe acute respiratory distress syndrome) coronavirus (Spicuzza, Spicuzza et al. 2007), Rift Valley fever virus (Flick and Bouloy 2005), Nipah and Hendra virus (Bossart and Broder 2006), hantaviruses (Sun, Chung et al. 2007), influenza virus (Severson, McDowell et al. 2008) can cause severe infectious and devastating diseases. In addition to the importance of viruses as pathogens, many viruses have been exploited as vaccine vectors, gene therapy vectors or anti-cancer agents. A detailed mechanistic understanding of viral replication cycle in different cell types is fundamental in developing reagents to prevent and combat viral diseases, and to exploit viruses in various health and technology applications.

The viruses consist of genetic material either as DNA or RNA. The genetic material of all viruses is protected by a viral protein coat, known as a capsid. Many viruses also possess a lipid bilayer (known as a viral envelope) that contains viral and host proteins. Based on the type of viral genetic material and its replication mechanism,

viruses are classified into seven groups according to a system designed by David Baltimore (Baltimore 1971). In the David Baltimore classification system of viruses, Group I and Group II consist of double-stranded (ds) DNA and single-stranded (ss) DNA viruses respectively. Their replication cycle follows the central dogma of molecular biology (DNA genome is transcribed into mRNA which is translated into proteins). Group III, IV, and V are dsRNA, ssRNA(+), ssRNA(-) viruses, respectively. The ssRNA(+) viruses have an RNA genome on mRNA polarity and structure, and it can be directly translated to proteins. However, genome of ssRNA(-) viruses and dsRNA must be transcribed first to synthesize translatable mRNAs before protein synthesis. Viruses of group VI are retroviruses, which first convert their RNA genome into DNA by reverse transcriptase. The viruses of group VII have dsDNA genome, but they utilize reverse transcriptase during their replication.

My dissertation research focuses on ssRNA(-) viruses, specifically those belonging to the non-segmented negative strand (NNS) RNA viruses. The ssRNA(-) include the families *Rhabdoviridae, Paramyxoviridae, Filoviridae, Bornaviridae*, *Orthomyxoviridae*, *Bunyaviridae* and *Arenaviridae*. First four families aggregate the order *Mononegavirales* (Table 1) and characterized by their non-segmented genome (Pringle 1999). Unlike members of the order *Mononegavirales*, viruses belonging to the *Orthomyxoviridae*, *Bunyaviridae* and *Arenaviridae* families have genome segmented consisting of more than one segment of negative strand RNA genomes (Pringle 1999).

Table 1. Classification of viruses of the order *Mononegavirales*

The NNS RNA viruses			
Order	Family Subfamily	Genus	Species
Mononegavirales	*Paramyxoviridae*	*Respirovirus*	*Sendai virus*
	[Paramyxovirinae]	*Morbillivirus*	*Measles virus*
		Rubulavirus	*Mumps virus*
		Henipavirus	*Hendra virus; Nipah virus*
	[Pneumovirinae]	*Pneumovirus*	*Human respiratory syncytial virus*
		Metapneumovirus	*Avian pneumovirus*
	Rhabdoviridae	*Vesiculovirus*	*Vesicular stomatitis Indiana virus*
		Lyssavirus	*Rabies virus*
		Ephemerovirus	*Bovine ephemeral fever virus*
		Cytorhabdovirus	*Lettuce necrotic yellows virus*
		Nucleorhabdovirus	*Potato yellow dwarf virus*
		Novirhabdovirus	*Infectious hematopoietic necrosis virus*
	Filoviridae	*"Marburg-like viruses"*	*Marburg virus*
		"Ebola-like viruses"	*Zaire Ebola virus*
	Bornaviridae	*Bornavirus*	*Borna disease virus*

Adapted from (Pringle 1999)

This dissertation focused on two prototypic members of the order *Mononegavirales*, vesicular stomatitis virus (VSV) and Sendai virus (SeV). This order includes many important plant, animal and human pathogens such as Ebola and Marburg viruses, Hendra, Nipah, mumps, measles, rabies and respiratory syncytial virus (RSV). A majority of current understanding of the molecular biology of *Mononegavirales* comes from widely studied prototypic models, VSV and SeV (Lamb and Parks 2007). VSV and SeV are both relatively weak human pathogens without any transforming properties; because both viruses replicate in cytoplasm and do not integrate their genomic material into host cellular DNA (Lawson, Stillman et al. 1995, Barber 2004). Moreover,

replication competent recombinant strains can be developed to accommodate relatively large gene inserts into their genome. Also, both VSV and SeV grow to high titers in vitro, which facilitate the purification with high yield. Both VSV and SeV are great models to study mechanisms of virus infection, antiviral drug resistance, vaccine vectors and oncolytic (anticancer) virus agents.

Replication of viruses in a host cell depends on the interaction of many viral and cellular ("host") factors. Studying specific host-viral interactions has important practical implications to develop effective antiviral treatments and effective viral vectors. This dissertation focuses on two different aspects of host-viral interactions: (1) Mechanisms of resistance of the antiviral drug ribavirin on VSV and SeV; (2) To identify potential biomarkers for pancreatic ductal adenocarcinoma (PDA) resistance to oncolytic VSV to improve anticancer treatment efficacy.

Although effective vaccines have been developed for many viral pathogens, it is more challenging to develop effective vaccines against many viruses due to high genome mutation frequency. Moreover, even though vaccines are effective as a prophylactic tool, they are generally not effective as a treatment post exposure. Although research from the past several decades resulted into the development of several antiviral drugs, to this day, only about 40 antiviral drugs have been developed and approved for different virus infections, a majority of them being against HIV infections (De Clercq 2004). An increasing number of drug resistant viral strains emphasize the urgent need to develop newer and more effective antiviral strategies to combat different viral infection (De Clercq 2004, Krausslich and Bartenschlager 2009). It is very difficult to develop antiviral drugs especially against viruses due to following reasons: (1) Viruses are heavily

dependent on host cellular pathways; (2) Viruses mutate rapidly compared to bacteria and develops rapid resistance to drug; (3) Most viruses have very few genes, hence few targets for the development of antiviral drugs. Furthermore, many patients do not respond to approved antiviral drugs treatments due to various host and viral factors such as insulin resistance, obesity, ethnicity, age, sex and viral genotypes (Asselah, Estrabaud et al. 2010) . Thus, it is increasingly important to identify factors that can allow us to predict the response antiviral therapies.

The goal of my first study was to determine the mechanism of resistance of certain cell types to the antiviral drug, ribavirin. This drug is the major antiviral treatment against hepatitis C virus (HCV) and some other clinically important human viruses. We found that specific cells can be naturally resistant to ribavirin treatment and specific cellular and not viral factors are responsible for this resistance. A broad spectrum antiviral drug, ribavirin in combination with IFN is a standard treatment for the patients with chronic HCV infection. However, this treatment is ineffective in ~45-50% of patients and these patients are called non-responders (Thomas, Feld et al. 2010). The mechanism of this non-responsiveness to ribavirin-IFN treatment is not completely understood. Virus-based resistance to ribavirin via generation of virus mutants has been extensively studied. However, recent reports suggest that cell based resistance to ribavirin can be developed upon repeated exposure to the drug via decreased uptake. But natural resistance of cells to ribavirin (without prior exposure) has not been investigated. Here, we investigated the role of specific cellular and not viral factors in natural ribavirin resistance against two NNS RNA viruses VSV and SeV in several cell types. Since it is difficult to generate drug resistance against host factors, studying host factors interacting

with viral factors during infection will be extremely critical to understand drug resistance and to develop novel drugs targeting host factors. (Lederman 1995; Tan, Ganji et al. 2007).

In the second part of my dissertation, I focus on a relatively recent anti-cancer approach utilizing viruses to specifically target and kill tumor cells without infecting healthy cells. Viruses used in such anti-cancer therapy are known as oncolytic viruses (OVs). Many studies have demonstrated that viruses can be used successfully as anticancer agents. However, great variability in the susceptibility to OV infection was observed against various malignancies (Paglino and van den Pol 2011; Murphy, Besmer et al. 2012). Thus, better understanding of the host-viral interactions can be beneficial to improve virus mediated therapy (Schwegmann and Brombacher 2008; Moerdyk-Schauwecker, Shah et al. 2012). Here we investigated the role of type I IFN signaling in oncolytic VSV resistance in PDA cells. The goal of my second study was to identify potential biomarkers associated with permissibility of human pancreatic cancer to oncolytic virotherapy using VSV and several other viruses. We were able to identify two potential biomarkers for oncolytic VSV infection resistance. Moreover, continuation of this project is presently underway which mainly focuses on NF-κB activation in cancer cells and resistance to OVs.

1.2 VSV and SeV as model RNA viruses

VSV is an enveloped virus with a helical nucleocapsid containing single-stranded, negative-sense RNA with an elongated bullet-like shape.VSV is the prototypic widely-studied member of the family *Rhabdoviridae*. A majority of knowledge about the replication of NNS RNA viruses has come from studying VSV (Lyles, 2007). VSV causes acute disease in livestock populations and is characterized by symptoms similar to foot-and-mouth disease virus including fever, vesicles in oral cavity and skin. Infection in humans by VSV is asymptomatic and laboratory-adapted strains are rarely pathogenic for humans (Letchworth, Rodriguez et al. 1999; Rodriguez 2002).

Similar to VSV, SeV (mouse parainfluenza virus type 1) is a prototype of the family *Paramyxoviridae* of the order *Mononegavirales*. This family includes some of the most ubiquitous disease causing viruses to humans and animals including measles, respiratory syncytial virus (RSV), parainfluenza viruses, mumps virus, Hendra virus, Nipah virus, human metapneumovirus, Newcastle disease virus, canine distemper virus and rinderpest virus (Lamb, Paterson et al. 2006). Similar to VSV, SeV also contains NNS RNA genomes with negative polarity and replicate within the cytoplasm (Lamb, Paterson et al. 2006).

The RNA genome of VSV is 11 to 12 kb, encapsidated by approximately 1,200 copies of VSV nucleoprotein (N) (Green, Zhang et al. 2006). The nucleocapsid is also associated with virus encoded phosphoprotein (P) and polymerase protein (L) with lesser extent (Green, Macpherson et al. 2000). L protein is mainly responsible for all of the enzymatic activities associated with the synthesis of both translatable and genetic RNA (Rahmeh, Schenk et al. 2010). Nucleocapsid is also associated with matrix (M) protein

which condenses the nucleocapsid into very tight nucleocapsid-matrix complex. VSV M also interacts with the lipid bilayer of virus envelop (Lenard and Vanderoef 1990; Swinteck and Lyles 2008). The VSV Glycoprotein (G) is responsible for enabling VSV to infect most of the mammalian cell types (Stanifer, Cureton et al. 2011). To date there has not been any specific cell surface receptor identified which is required by VSV G protein. Binding of VSV G to the cell surface occurs via negatively charged membrane lipids followed by actin and clathrin-dependent endocytosis (Cureton, Massol et al. 2010). After the particle has been internalized acidification of endosomal membrane facilitates fusion of viral envelop, allowing the release of viral ribonucleoprotein core into cytoplasm (Stanifer, Cureton et al. 2011).

SeV is spherical virion that range in average diameter from 150 to 200 nm. The virion consists of a nucleocapsid (also known as ribonucleoprotein or RNP) tightly packaged in a lipid envelope which is acquired from host cell membrane during budding. The envelope contains spike like projections composed of Hemaglutinin Neuraminidase (HN) and Fusion (F) transmembrane glycoproteins. The envelope is surrounded by a nonglycosylated matrix (M) protein from inside (Lamb, Mahy et al. 1976; Lamb and Parks 2007). SeV also has a P/V/C gene which can code for seven different polypeptides (Lamb and Parks 2007). However, V and C proteins are not required by SeV for replication (Fukuhara N, Huang C et al. 2002; Kato, Cortese-Grogan et al. 2004/7). SeV also has the large L polymerase having the catalytic role in viral RNA synthesis (Smallwood, Hovel et al. 2002/12/5; Lamb and Parks 2007) SeV HN is required for the virus adsorption to the cell surface molecule containing sialic acid. HN is also responsible for the enzymatic cleavage of sialic acid from both the surfaces of virions and infected

cells. Moreover, it has been shown that HN promotes viral membrane to cell membrane fusion activity (Lamb, Paterson et al. 2006). SeV F protein mediates viral penetration by fusion between the virions envelope and the host cell plasma membrane and this fusion event occurs at neutral pH (Lamb 1993). Fusion leads to the delivery of viral nucleocapsid into the cytoplasm and the fusion of infected cells with neighboring cells to form syncytia (multi-nucleated giant cell formation) (Lamb 1993; Lamb and Parks 2007).

Both VSV and SeV have served as excellent models to study fundamentals of virus pathogenesis, drug resistance and virotherapy. VSV can infect almost any vertebrate cells and have a short life cycle. Because of these advantages VSV has been used an excellent model virus to study virus entry, replication, and mechanisms of innate and adaptive immune response. The cell-free assays like *in vitro* transcription serve as a great tool to study virus transcription and replication (Horikami and Moyer 1995/8/20; Chen, Ogino et al. 2007).

Studying the host-viral interactions can be benefited greatly, if viruses harboring specific mutations in their genome can be engineered, such system is known as reverse genetics. The biggest challenge with NNS RNA viruses was a lack of such system to manipulate the RNA genome with negative polarity. In early 1990s the first reverse genetic system was established for NNS RNA viruses using VSV and SeV (Garcin, Pelet et al. 1995; Lawson, Stillman et al. 1995; Whelan, Ball et al. 1995). The reverse-genetics system is an extremely powerful tool to dissect the different aspects of NNS RNA genome replication and transcription (Whelan and Wertz 1999/1; Conzelmann 2004). VSV and SeV have also been used as vaccine vectors for several decades. Both VSV and

SeV have been shown to have promising results on animal models when used as vaccine vectors (Takimoto, Hurwitz et al. 2005; Mire, Miller et al. 2012).

Both VSV and SeV propagate rapidly in cell culture and infect various cell types from different sources, making them useful for the screening of different antiviral drugs and studying their mechanisms of action. Using reverse genetic approach recombinant VSV and SeV have been developed to facilitate the visualization of virus infection. These recombinant viruses replicate similarly to wt strains and utilize expression of fluorescent proteins to monitor infection. This approach facilitates the study of the mechanism of action of any antiviral drugs with these recombinant viruses in different cell types. Moreover, VSV can replicate poorly in non-transformed normal cells but replicates efficiently in transformed cell lines. This observation was exploited to use VSV as an anti cancer agent in the past decade (Stojdl, Lichty et al. 2000; Balachandran, Porosnicu et al. 2001; Giedlin, Cook et al. 2003; Obuchi M, Fernandez M et al. 2003; Barber 2004). Development of reverse genetic system have also provided a much better platform to synthesize highly specific, safe and effective recombinant oncolytic VSV for various type of cancers (Stojdl DF 2003; Barber 2004; Hastie and Grdzelishvili 2012).

CHAPTER 2: CELL TYPE MEDIATED RESISTANCE OF VESICULAR STOMATITIS VIRUS AND SENDAI VIRUS TO RIBAVIRIN

2.1 Background

Ribavirin: clinical importance.

Ribavirin (1-ß-D-ribofuranosyl-1,2,4-triazole-3-carboxamide): a nucleoside analog was first synthesized in 1972 (Sidwell, Huffman et al. 1972) and demonstrated its diverse antiviral activity against several RNA and DNA viruses (Crotty, Cameron et al. 2001; Parker 2005; Vignuzzi, Stone et al. 2005; Dixit and Perelson 2006). Ribavirin was originally approved in humans to treat respiratory syncytial virus infection in children. Later it was approved for the treatment of Lassa fever virus infection (Dixit and Perelson 2006). Most importantly to date ribavirin in combination with Interferon-α (IFN) is the most effective treatment available for HCV infection. ribavirin alone has a transient effect on HCV replication in patients (Thomas, Feld et al. 2010), however it dramatically improves the long term therapeutic response when treated in combination with IFN (Dixit and Perelson 2006; Thomas, Feld et al. 2010). It has been estimated that ~170 million people are living with HCV infection worldwide (Dixit and Perelson 2006). In ~70% of cases this infection becomes chronic and can lead to the development of cirrhosis, fibrosis and/or hepatocellular carcinoma (HCC) (Hoofnagle 2002; Dixit and Perelson 2006). Many patients infected with HCV have demonstrated a long lasting effect of ribavirin-IFN therapy. However, a growing number of individuals infected with hepatitis C virus (HCV) fail to respond to therapy. Although it was thought that development of

agents targeting each steps of HCV infection cycle would make ribavirin-IFN therapy antiquated, newer studies indicate otherwise (Thomas, Feld et al. 2010). Antiviral drugs which directly targets to the viral life cycle are likely to generate resistant strains. Ribavirin-IFN therapy is required to achieve optimal response. Moreover, recent clinical trials with HCV protease inhibitors have indicated that ribavirin therapy is required for the prevention of viral relapse (Shiffman 2009). Together these studies clearly suggest that studying the mechanisms of action of ribavirin and its resistance is a promising approach to improve the treatment options for HCV infection.

Structure and metabolism of ribavirin.

To understand the mechanism of action of any nucleoside analog, it is extremely important to study the metabolism at cellular and molecular levels (Parker 2005). Structurally ribavirin has no close resemblance to any natural nucleoside. However, the triazole ring is attached to the ribose sugar making ribavirin resembles to ribonucleoside and not the deoxyribonucleoside (Sidwell, Huffman et al. 1972). Once transported, ribavirin metabolizes in the cell by the enzymes involved in purine metabolism (Willis, Carson et al. 1978; Balzarini, Karlsson et al. 1993). First ribavirin gets phosphorylated by adenosine kinase to ribavirin mono-phosphate (RMP), which is successively phosphorylated to ribavirin-di (RDP) and ribavirin-tri phosphate (RTP) by nucleoside mono- and di-phosphate kinases respectively (Gallois-Montbrun, Chen et al. 2003). Mammalian adenosine kinase does not metabolize ribavirin as efficiently as adenosine. Since, RTP is the major metabolite inside the cell, it is evident that the adenosine kinase is the rate-limiting step for its synthesis (Zimmerman and Deeprose 1978; Smee and Matthews 1986; Balzarini, Karlsson et al. 1993). Pharmacologically relevant

concentration of RTP (10-100 μM) can be achieved in a couple of hours of ribavirin treatment in mammalian cells, which is equivalent to that of intracellular concentration of ATP and GTP in cells without any treatment (Zimmerman and Deeprose 1978; Jones 1980; Smee and Matthews 1986; Rankin, Eppes et al. 1989/1). Levels of RTP drop significantly if ribavirin is removed from the treated cells in cell culture except erythrocytes. (Smee and Matthews 1986; Page and Connor 1990). This rapid degradation of RTP can contribute towards the lack of persistent antiviral activity of ribavirin in cell culture (Kirsi, North et al. 1983). There have not been many studies done to evaluate the effect of virus infection on ribavirin metabolism. Infection of RSV had almost no effect on ribavirin metabolism (Smee and Matthews 1986). Deoxynucleotide of ribavirin have never been reported which suggests that RDP is not a substrate for ribonucleotide reductase. However, it is important to note that intracellular levels of deoxynucleotides are much lower than that of ribonucleotides, making their detection extremely difficult. None of the previous studies have found ribavirin incorporated in RNA of any ribavirin treated mammalian cell types (Zimmerman and Deeprose 1978). Also, ribavirin does not inhibit RNA polymerase I, RNA polymerase II activity (Eriksson, Helgstrand et al. 1977; Muller, Maidhof et al. 1977). These results indicated that mammalian RNA polymerases cannot use RTP as a substrate.

Mechanisms of action of ribavirin.

The better understanding of ribavirin treatment failures is complicated by an unclear mechanism of action of ribavirin, partly due to its pleiotropic nature (Martin and Jensen 2008; Shah, Sunderland et al. 2010). There are six distinct proposed mechanism of action of the antiviral activity of ribavirin. They can be subdivided into direct (if impacts

viral life cycle) or indirect mechanisms (Thomas, Feld et al. 2010). The direct mechanisms include (i) inhibition of viral RNA polymerase by phosphorylated ribavirin through physical interaction (Maag, Castro et al. 2001; Bougie and Bisaillon 2004/5/21). (ii) ribavirin acts as a mutagen by direct incorporation into viral genome, which results in inducing error catastrophe (Crotty, Cameron et al. 2001; Crotty, Cameron et al. 2002) (iii) ribavirin is also the substrate for viral guanylyl transferases, which leads to the inhibition of mRNA capping. The indirect mechanisms of ribavirin's action include (i) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH), which is an essential enzyme for the *de novo* synthesis of GTP. This results in the strong depletion of cellular GTP pools required by virus for efficient replication (Malinoski and Stollar 1980; Zhou, Liu et al. 2003). (ii) ribavirin has also been shown to modulate antiviral cellular responses such as the ability to induce a Th2 to Th1 shift in immune response to favor viral clearance (Tam, Pai et al. 1999). (iii) Recently, ribavirin has been shown to modulate the expression of interferon stimulated genes (ISGs) in cell culture system of RSV and in patients receiving ribavirin monotherapy before the start of standard treatment (Feld, Nanda et al. 2007). All the mechanisms of the antiviral activity of ribavirin are explained in detail below.

One of the first indirect mechanisms of action of ribavirin was through the inhibition of host enzyme IMPDH. Many studies have demonstrated that ribavirin treatment results in the inhibition of host enzyme IMPDH, which is required by cells for purine biosynthesis (Muller, Maidhof et al. 1977; Zimmerman and Deeprose 1978). The mechanism of IMPDH inhibition is also investigated. The phosphorylated form of ribavirin metabolite RMP is a competitive inhibitor of IMPDH. In cells IMPDH is

responsible for converting its natural substrate IMP into xanthosine mono phosphate in the *de novo* synthesis of GMP (Fig. 1). However, in the presence of RMP this enzyme activity is reduced which results into reduced levels of GTP pools (Streeter, Witkowski et al. 1973; Balzarini, Karlsson et al. 1993). This depletion in intracellular GTP pools reduces the supply of nucleotides for progeny viral RNA synthesis (Fig. 1). These results at least partially explained broad spectrum antiviral activity of ribavirin against many RNA and DNA viruses. Moreover, these results may also explain the toxicity of ribavirin observed in many human cells since ribavirin treatment modulates cellular nucleotide pools (Tam, Ramasamy et al. 2000). It became evident that ribavirin inhibits cellular IMPDH, however how much role this inhibition plays in the antiviral activity was not clear. This mechanism was supported by few independent studies with a drug-resistant strain of Sindbis virus, which replicated efficiently in cells with low intracellular GTP concentrations. This ribavirin resistant strain contained mutations in its viral guanylyl transferase gene, which increased its affinity for this enzyme (Malinoski and Stollar 1980; Scheidel and Stollar 1991; Leyssen, Balzarini et al. 2005).

With several viruses it has been demonstrated that antiviral activity of ribavirin can be at least partially reversed if cells are treated with guanosine (Streeter, Witkowski et al. 1973; Wray, Gilbert et al. 1985; Smee, Bray et al. 2001; Zhou, Liu et al. 2003). Guanosine treatment replenishes the GTP pool independent of IMPDH. Guanine nucleotide is synthesized by purine nucleoside phopshorylase, which gets converted to guanosine monophosphate by hypoxanthine transferase and does not require IMPDH. The reversal in the antiviral effect of ribavirin by guanosine treatment does not necessarily show that inhibition of IMPDH is responsible for the antiviral activity of

ribavirin. Guanosine treatment results in elevated levels of GTP to compete with RTP and prevent its antiviral actions on viral RNA polymerases (Parker 2005). To exclude the possibility of RNA polymerase as a target of ribavirin, activity of a known inhibitor of IMPDH against many viruses was evaluated. The orthopox viruses, Sindbis virus, dengue virus etc. are sensitive to the treatment of mycophenolic acid (MPA) (Malinoski and Stollar 1980; Smee, Bray et al. 2001; Marroqui, Estepa et al. 2008). MPA is a non-nucleoside inhibitor of IMPDH currently used in clinical settings as an immunosuppressive agent mainly for renal transplant (Kaplan 2006). Because the only mechanism of action known of MPA is through the inhibition of IMPDH, these results suggests that reduction in the GTP pool might be sufficient to achieve the antiviral effect against these viruses. However, MPA treatment did not show any activity against HCV (Lanford, Chavez et al. 2001; Zhou, Liu et al. 2003) indicating that suppression of GTP pools is not always sufficient to obtain antiviral activity at least against HCV. Also, several studies with RSV and vaccinia virus showed no reversal of antiviral activity of ribavirin when co-treated with guanosine (Robins, Revankar et al. 1985; Smee and Matthews 1986). Imbalance caused in nucleotide pool by the inhibition of IMPDH by ribavirin treatment could result in substitution of nucleotides by viral polymerases. This subsequently results in increased number of viral genome mutations, which have been reported in many cases.

Despite ribavirin have been shown to reduce cellular GTP pools, results from various studies suggest the involvement of other mechanisms of action of ribavirin. Ribavirin treatment results in higher accumulation of intracellular RTP. Intracellular concentrations of ATP and GTP are ~3 and 0.5 mM (Jones 1980). Intracellular

concentration of RTP >0.1 mM is high enough to compete with natural nucleotides. As mentioned earlier, the triazole ring structure of ribavirin does not closely resemble any purine and could interact with viral RNA polymerases as a GTP or ATP analog. This interaction is even more probable when GTP levels are significantly dropped. Under any circumstances if ribavirin is metabolized inside the cell it could modulate the activity of RNA polymerase in following ways: (1) RTP can physically inhibit viral polymerase by competing with natural nucleotides (2) RTP can cause chain termination by acting as alternative substrate (3) RTP can act as an alternative substrate and get incorporated into the viral genome, resulting into the formation of false copies. In both the first and second cases, RTP will immediately result in the inhibition of virus replication. However, in the third scenario viral genomes with many mistakes will be created and this will induce error catastrophe. Many *in vitro* studies have previously shown that RTP can inhibit viral RNA polymerases of various RNA viruses. Inhibition of influenza viral polymerase by RTP was competitive with the intracellular concentrations of ATP and GTP. (Eriksson, Helgstrand et al. 1977; Wray, Gilbert et al. 1985). Moreover, inhibition of viral RNA elongation required much higher concentration of RTP than incorporating RTP in viral RNA (Wray, Gilbert et al. 1985). This results suggest that RTP can inhibit viral RNA polymerase and cause chain termination but at much higher concentration (Fig 1). Studies with RNA polymerase of Reo virus and VSV have demonstrated that viral RNA polymerase can be inhibited by ribavirin nucleotides at lower concentrations than natural nucleotides (Toltzis, O'Connell et al. 1988/4; Rankin, Eppes et al. 1989/1). However, these studies did not evaluate the incorporation of ribavirin into viral genomic RNA and

inhibition of RNA synthesis does not imply that it occurred through competitive inhibition.

Recent publications have studied the interaction of RTP with RNA polymerases of polio virus, Hantan virus (HTNV) and HCV (Crotty, Maag et al. 2000; Maag, Castro et al. 2001; Bougie and Bisaillon 2003; Sun, Chung et al. 2007). RNA polymerase of polio virus successfully incorporated RTP into viral RNA and continued the elongation without terminating the chain. Also, RNA polymerase of polio virus recognized RMP in the template as AMP or GMP and subsequently added UMP or CMP into the progeny strand (Crotty, Maag et al. 2000; Crotty, Cameron et al. 2001). Studies with Hantaan virus also showed that RTP synthesis directly corresponds to the antiviral activity of ribavirin and it also increases the mutation frequency in viral genome (Severson, Schmaljohn et al. 2003; Sun, Chung et al. 2007). For HCV RNA polymerase RTP is a poor substrate when compared to natural nucleotides. Similar to polio viral RNA polymerase, HCV RNA polymerase also elongated the growing RNA strand after the incorporation of RMP and incorporated CMP or UMP in daughter strands (Maag, Castro et al. 2001; Vo, Young et al. 2003). However, significantly higher inhibition of HCV RNA elongation was also observed (Vo, Young et al. 2003). Moreover, at lower GTP levels RNA chain elongation was stalled by the HCV RNA polymerase and this was reversed by increasing the concentration of GTP (Vo, Young et al. 2003). Overall, these studies revealed that metabolites of ribavirin can both inhibit viral RNA synthesis and induce error catastrophe. Also, mechanisms of RTP activity can differ among different viral polymerases.

Another direct mechanism of action of ribavirin has been shown to negatively influence viral mRNA capping enzyme (Cameron and Castro 2001) (Fig. 1.). The 5'-phosphate of most eukaryotic mRNAs and many viral mRNAs possess a ^{m7}GpppN cap structure, which plays a central role in stability and protein synthesis of mRNAs (Furuichi, LaFiandra et al. 1977; Shatkin 1985/2; Bougie and Bisaillon 2004/5/21). This is a two step process, in the first step 5'-triphosphate of RNA is hydrolyzed by RNA triphosphatase to 5'-diphosphate end. In the second step RNA guanylyltransferase interacts with GTP to form intermediate GMP-enzyme complex. The GMP from this complex is then transferred to the 5'-diphosphate of RNA by the same enzyme to form GpppN (Shuman 1982). The guanosine is then methylated to form the ^{m7}GpppN cap by RNA methyl transferase. In a study with Sindbis virus genome suggested that resistance to ribavirin treatment is due to mutation in the coding region of RNA guanylyltransferase enzyme (Scheidel, Durbin et al. 1989/12; Scheidel and Stollar 1991). Similarly, ribavirin has been shown to inhibit the RNA cap synthesis of vaccinia virus (Goswami, Borek et al. 1979). More recently, a study provided evidence that RTP can be used as a substrate for vaccinia virus RNA capping enzyme and RMP-enzyme intermediate can be formed (Bougie and Bisaillon 2004/5/21). Further, in vitro viral mRNA transcript containing a 5'RpppN cap (where R is ribavirin and N is nucleotide) instead of 5'GpppN was synthesized (Bougie and Bisaillon 2004/5/21) (Fig. 1).

Ribavirin has also been shown to promote T-cell mediated immunity against viral infections (Fig. 1). Cytokine produced by $CD4^+$ and $CD8^+$ T cells can be categorized in two different phenotypes Th_1 and Th_2 (Mosmann and Sad 1996). The Th_1 ($CD4^+$) cells produce cytokines such as tumor necrosis factor-alpha (TNFα), interleukin (IL)-2 and

interferon gamma (IFNγ) especially to provide helper T cell driven cytotoxic T-cell response to viral infections. On the other hand Th_2 ($CD8^+$) cells produce IL-4, IL-5 and IL-10 which increases the synthesis of antibodies. This has been implicated with progression of specific viral diseases due to a shift in the cytokine profile from Th_1 to Th_2 in both $CD4^+$ and $CD8^+$ T cells (Mosmann and Sad 1996). Few studies have demonstrated that ribavirin treatment can shift the cytokine profile by shifting the balance from Th_2 to Th_1 in human T cells (Tam, Pai et al. 1999). Recently, more evidence has demonstrated that ribavirin treatment can modulate the expression of certain ISGs in a cell culture system of RSV and in patients undergoing treatment for the HCV

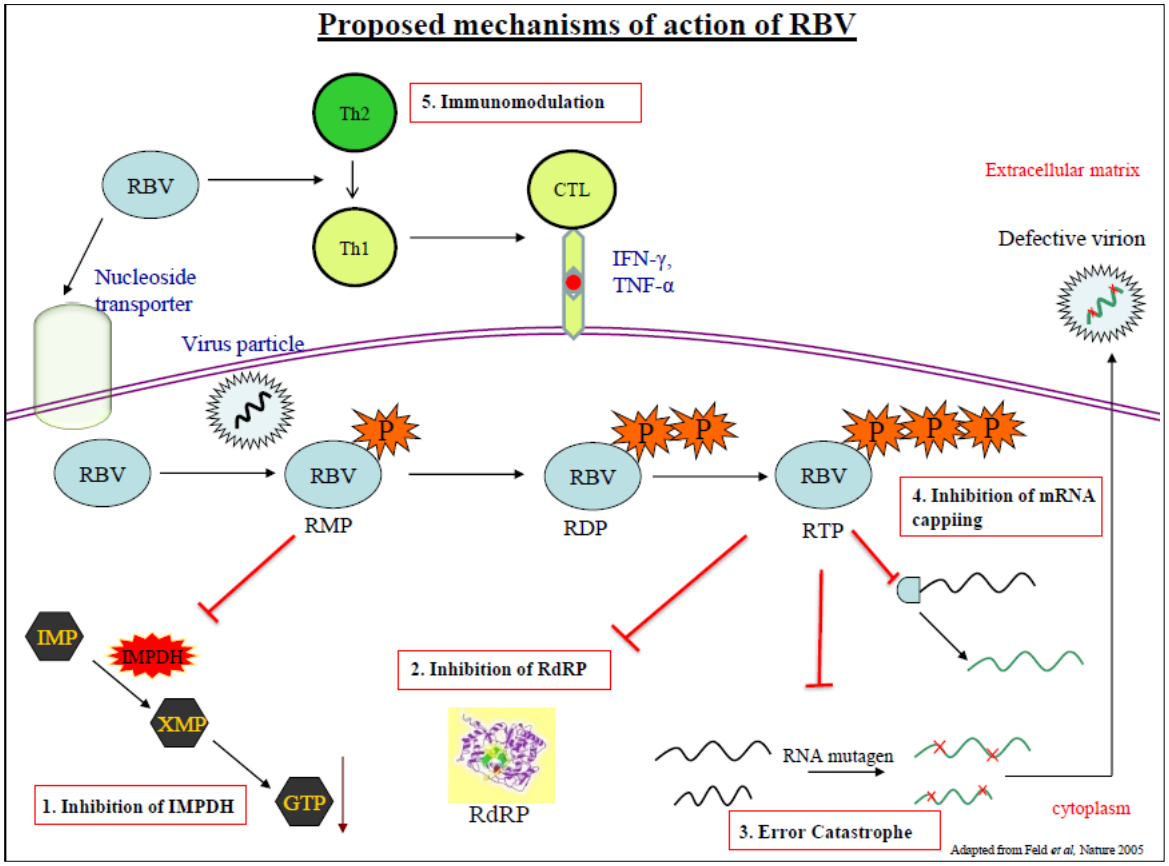

Figure 1. Proposed mechanisms of action of the antiviral action of ribavirin. Adapted from Feld *et al*. 2005.

Infection (Zhang, Jamaluddin et al. 2003; Thomas, Feld et al. 2010). Upregulation of ISGs in most cells occurs through binding of type I IFN onto IFN receptor. This results in the activation of Janus kinase1 (Jak1) and signal transducer and activator of transcription (STAT) signaling and results in the phosphorylation of STAT1, STAT2 and IRF9. This complex is known as ISGF3, translocates to the nucleus and acts as a transcription factor by binding onto IFN-sensitive response element (ISRE) to induce synthesis of ISGs. Since ribavirin-IFN treatment is significantly more successful than ribavirin mono therapy, the effect of ribavirin treatment in induction of various ISGs was recently evaluated. In a recent study a group of patients was given ribavirin prior to the ribavirin-IFN treatment and another group was given the normal ribavirin-IFN treatment. When the gene expression data was compared, the group that received ribavirin prior to the ribavirin-IFN showed more ISG induction. These results indicate that ribavirin treatment may augment the activity of IFN by interacting with an antiviral signaling pathway (Feld, Nanda et al. 2007; Thomas, Feld et al. 2010).

Although these proposed mechanisms came from the research performed over the last four decades, the exact mechanism of action of ribavirin is still unclear mainly due to its apparent pleiotropic nature (Vignuzzi, Stone et al. 2005; Dixit and Perelson 2006). The problems associated with ribavirin treatment and the future of this drug is discussed next.

Ribavirin resistance and future.

As mentioned earlier approximately 170 million people are infected with HCV worldwide. The majority acquire chronic infections and that can lead to HCC or liver

cirrhosis (Dixit and Perelson 2006). Because there is no vaccine currently available the ribavirin-IFN therapy is the only approved treatment at this time. The IFN-α monotherapy against HCV genotype 1infected patients had a limited success (~20%) in achieving sustained virological response (SVR). However, addition of ribavirin increased the SVR rate to ~50-60% (Feld and Hoofnagle 2005; Dixit and Perelson 2006; Shah, Sunderland et al. 2010).

It is evident that ribavirin has a key role in achieving SVR in HCV infections, however very little is known about factors involved in the resistance to ribavirin treatment. This is of utmost importance since, ~45-50% patients infected with HCV genotype 1 do not respond to ribavirin-IFN combination therapy and understanding the role of these factors can improve the therapeutic outcome of the ribavirin-IFN treatment. Many previous studies have thus far largely focused on the role of viral factors for the resistance to ribavirin treatment. Unlike other viruses like HIV or influenza, drug resistant mutations in HCV genome is very rare (Hofmann, Sarrazin et al. 2003; Sarrazin, Mihm et al. 2005; Johnson, Brun-Vezinet et al. 2007; Wohnsland, Hofmann et al. 2007). Recent studies have suggested that ribavirin resistance can be more influenced by host factors than viral determinants (Ibarra and Pfeiffer 2009; Thomas, Feld et al. 2010). A previous research demonstrated that most of the ribavirin resistance observed in a HCV replicon system was mainly due to the changes in the cell line (Pfeiffer and Kirkegaard 2005). Moreover, it was also confirmed that this observed resistance to ribavirin is attributed to lower uptake in that cell line (Pfeiffer and Kirkegaard 2005) (Ibarra and Pfeiffer 2009).

For ribavirin to function, it needs to be transported in the cell. This transportation is facilitated by host nucleoside transporters, which are divided into two categories: equilibrative nucleoside transporters (ENT) and concentrative nucleoside transporters (CNT) (Jarvis, Thorn et al. 1998). Nucleosides can be transported bidirectionally by ENTs, whereas CNTs transport the nucleoside against the concentration gradient. Both ENTs and CNTs are known to transport synthetic nucleosides into the cell, including ribavirin (Errasti-Murugarren, Pastor-Anglada et al. 2007; Zhang, Visser et al. 2007). A majority of proposed mechanisms explained earlier require the import of ribavirin into the cell. A recent publication provided the evidence that cells treated with ribavirin for several passages developed the resistance to ribavirin and supported robust replication of polio virus. This resistance is analogous to chemotherapy resistance observed in some cancers. The polio virus is a model RNA virus known for its sensitivity to ribavirin. ribavirin sensitive cells treated with the inhibitor of nucleoside transporter mimicked the ribavirin resistant phenotype (Ibarra and Pfeiffer 2009).

These results are clinically relevant and may explain the observed resistance in HCV patients with long exposure to ribavirin. However, they cannot explain why some patients completely do not respond to the treatment. We hypothesized that some cells are naturally resistant to ribavirin treatment even without any prior exposure to the drug and specific host factors and not viral determinants are responsible for such resistance. Our results clearly demonstrated that three out of seven chosen cell types were naturally resistant to ribavirin treatment without prior exposure to the drug against two prototypic NNS RNA viruses VSV and SeV. Both of these viruses were previously shown to be very sensitive to the ribavirin treatment (Sidwell, Khare et al. 1975; Larson, Stephen et al.

1976; Toltzis and Huang 1986/6; Toltzis, O'Connell et al. 1988/4; Cuevas, Sanjuan et al. 2005). Results of this study explain some treatment failures associated with ribavirin treatments. With the increased number of reported cases of HCV infections and next generation HCV inhibitors in the pipeline, it is extremely important to improve the antiviral efficacy of ribavirin. Future studies in search of ribavirin-like compounds to be used in combination with IFN remains to be the optimistic future for the HCV infected patients.

Overall, this study demonstrates the important of studying the host factors interacting with virus to reduce the cell based antiviral drug resistance and improve the antiviral therapy. On other hand it is also very important to study host factors which suppresses the viral infection and mechanism of resistance to virus infection. This is important because viruses are being used as therapeutic options for the treatment of cancer or as vaccine vectors or gene therapy vectors. The resistance of specific pancreatic cancer cells to oncolytic VSV infection is explained in detail in the next chapter of this dissertation.

2.2 Introduction

Ribavirin (ribavirin, also known as virazole), 1-ß-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, is the first synthetic, broad-spectrum antiviral nucleoside analog (Sidwell, Huffman et al. 1972), which has been shown to exhibit antiviral activity against many RNA and DNA viruses both in vitro and in vivo (Parker 2005; Vignuzzi, Stone et al. 2005; Dixit and Perelson 2006; Martin and Jensen 2008). ribavirin was originally approved for the treatment of respiratory syncytial virus (RSV) infection in children, and today is also used to treat Lassa fever and, most importantly, hepatitis C virus (HCV) infections of humans (Dixit and Perelson 2006). While ribavirin alone has little or no effect on viral replication in HCV patients (Wohnsland, Hofmann et al. 2007), it dramatically improves long-term antiviral response in many treated patients when used in combination with interferon (IFN) (Dixit and Perelson 2006; Martin and Jensen 2008). The mechanism of synergy between ribavirin and IFN (Buckwold, Wei et al. 2003; Zhang, Jamaluddin et al. 2003), which is critical for successful anti-HCV therapy, remains unclear (Dixit and Perelson 2006).

Despite these successes with ribavirin/IFN combination therapy a large portion of patients are ‘‘non-responders’’ to this treatment (detectable HCV RNA throughout the treatment period). The mechanism of non-response to ribavirin/IFN treatment is highly controversial and, unfortunately, no alternative therapies exist for non-responders so far.

The understanding of ribavirin treatment failures is complicated by an unclear mechanism of ribavirin action, partly due to its apparent pleiotropic nature (Dixit and Perelson 2006; Martin and Jensen 2008). Upon uptake, ribavirin is metabolized in vivo through 5′-phosphorylation by cellular kinases into ribavirin mono- (RMP), di- (RDP)

and triphosphate (RTP) (Willis, Carson et al. 1978; Balzarini, Karlsson et al. 1993; Wu, Larson et al. 2005). Six distinct mechanisms (which may work together) have been proposed for antiviral action of ribavirin against different viruses: (Parker 2005; Vignuzzi, Stone et al. 2005; Dixit and Perelson 2006; Martin and Jensen 2008) (i) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH) essential for the de novo synthesis of GTP; (ii) direct interaction of phosphorylated ribavirin with and inhibition of viral RNA polymerase, (iii) RNA chain termination as a result of incorporation of RTP (GTP analog) into replicating RNA strands by viral RNA polymerases; (iv) "error catastrophe" as a result of RTP incorporation into the viral genome paired with cytidine and uridine as a substitute for guanine and/or adenine, resulting in so called "lethal mutagenesis", a meltdown of genetic information; v) inhibition of mRNA capping; and (vi) immunomodulation of antiviral cellular responses such as the ability to induce a Th2 to Th1 shift in the immune response. Previous studies in search of explanations for ribavirin treatment failures were largely focused on the role of viral determinants of ribavirin resistance (Vignuzzi, Stone et al. 2005; Wohnsland, Hofmann et al. 2007), as any antiviral mechanism of ribavirin via direct interactions with the viral RNA polymerase can hypothetically be overcome by mutations in the viral RNA polymerase. Such an escape via a single mutation in the RNA-dependent RNA polymerase has been shown to confer resistance to ribavirin via increased polymerase fidelity in poliovirus (Pfeiffer and Kirkegaard 2003; Vignuzzi, Stone et al. 2005) and foot- and mouth disease virus (Airaksinen, Pariente et al. 2003; Sierra, Airaksinen et al. 2007).

While drug resistant viral mutants may explain at least some failures with ribavirin treatments, recent reports propose that cell-based resistance to ribavirin could be an important factor explaining the low antiviral activity of ribavirin in at least some experimental and clinical systems (Wohnsland, Hofmann et al. 2007). For example, Pfeiffer and Kirkegaard provided in vitro evidence that resistance of infected cells to ribavirin can be conferred not only via mutations in the viral genome (‘‘virus-based resistance’’) but also through changes in the ribavirin treated cells (‘‘cell-based resistance’’) (Pfeiffer and Kirkegaard 2003; Pfeiffer and Kirkegaard 2005). A recent study by Ibarra and Pfeiffer (Ibarra and Pfeiffer 2009) shows that the development of cell-based resistance to ribavirin treatment via decreased ribavirin uptake can greatly limit ribavirin antiviral activity. To examine whether certain cell types are naturally resistant to ribavirin even without prior drug exposure, we selected seven different cell lines from various hosts and compared them for the antiviral activities of ribavirin against two nonsegmented negative-strand RNA viruses (order Mononegavirales), vesicular stomatitis virus (VSV, family *Rhabdoviridae*) and Sendai virus (SeV, family *Paramyxoviridae*), which were previously shown to be highly sensitive to ribavirin treatment(Sidwell, Khare et al. 1975; Larson, Stephen et al. 1976; Toltzis and Huang 1986/6; Toltzis, O'Connell et al. 1988/4; Cuevas, Sanjuan et al. 2005). Our results show dramatic cell-type dependent differences in the antiviral activities of ribavirin, ranging from virtually no effect to very effective inhibition of viral replication, indicating that some cell types are naturally resistant to ribavirin treatment even without prior exposure to this drug. The data presented in this study shed light on the mechanisms of the

ribavirin activity against VSV and SeV, and may explain at least some of the reported failures with ribavirin treatments.

2.3 Materials and methods

Cell lines and viruses.

The following seven cell lines were used in this study: Syrian golden hamster kidney fibroblast cells (BHK21, ATCC# CCL-10); human cervical adenocarcinoma cells (HeLA, ATCC# CCL-2); human epithelial lung carcinoma cells (A549, ATCC# CCL-185), mouse mammary gland adenocarcinoma cells (4T1, ATCC# CRL-2539), human epidermal carcinoma cells (HEp2, ATCC# CCL-23); and African green monkey kidney cells (Vero, ATCC# CCL-81). In addition, we used BSRT7 cells which are derived from BHK21, constitutively express bacteriophage T7 polymerase and described by Buchholz et al.(Buchholz, Finke et al. 1999). Monolayer cultures of these cell lines were maintained in Minimum Essential Medium (Eagle's MEM, Cellgro) or Dulbecco's modified Eagle's medium (DMEM, Cellgro) supplemented with 9% fetal bovine serum (FBS, Gibco) in a 5% CO2 atmosphere at 37°C. VSV-GFP is a recombinant wild type (wt) VSV (Indiana serotype) encoding GFP as an extra gene between the G and L genes (Das SC, Nayak D et al. 2006), kindly provided by Dr. Asit K. Pattnaik (University of Nebraska). Recombinant SeV-GFP (Fushimi strain) encoding GFP upstream of the NP gene (Wiegand, Bossow et al. 2007) was kindly provided by Dr. Wolfgang J. Neubert (Max-Planck-Institute of Biochemistry, Germany). To grow VSV-GFP or SeV-GFP, BHK21 or Vero cells, respectively, were infected with viruses at a multiplicity of infection (MOI) of 0.05 CIU (cell infectious units) per cell in MegaVir HyQSFM4 serum-free medium (SFM, Hyclone) and incubated for 24–48 h at 34°C. This temperature (34°C) was chosen as it supported optimal replication of both viruses in the seven cell lines and all virus infections presented in this study were conducted at 34°C.

SeV-GFP was grown without acetylated trypsin in the medium as it has the wt monobasic trypsin-dependent cleavage site in the F protein mutated to an oligobasic cleavage site, allowing F activation in any cell type through an ubiquitous furin-like protease (Wiegand, Bossow et al. 2007).

Inhibitors.

Ribavirin was purchased from MP Biomedicals (cat. no. 196066); guanosine (cat. no. 101907), actinomycin D (ActD) (cat. no. 10465805) from MP Biomedicals and S-(4-Nitrobenzyl)-6-thioinosine (NBMPR, also known as NBTI, cat. no. N2255) from Sigma-Aldrich. Stock solution of ribavirin (0.1 M) was made in H2O, while ActD (2 mg/ml) was dissolved in 100% ethyl alcohol and guanosine (20 mM) and NBMPR (16.8 mM) in DMSO.

Virus infections in the presence of inhibitors.

Most experiments were conducted using 24-well tissue culture plates and nearly 100% confluent cells treated with drugs in SFM (or mock-treated with SFM) and infected with VSV-GFP or SeV-GFP(or mock-infected with SFM) at MOI of 3 CIU/cell. The MOI for each virus/cell type combination was calculated by infecting each cell line with VSV-GFP or SeV-GFP serial dilutions in SFM and counting infectious foci with the aid of fluorescence microscopy. Ribavirin was added to the cells at 24 h before infection. After absorption of virus for 1 h in the absence of drugs (to rule out an interference of drugs with virus attachment/entry), SFM containing unabsorbed virus was removed, cells were washed three times with phosphate-buffered saline (PBS), and 300 ml/well of SFM with the same concentrations of drugs as in the pretreatment was added to each well. The fluorescence and bright field photographs of cells at 10x magnification were captured 24

h post infection (p.i.) or 48 h p.i. using an Olympus DP70 digital camera mounted on an Olympus IX71 inverted fluorescent microscope and Olympus DP Controller software. To examine effect of ribavirin on virus production, SFM containing infectious particles was collected 24 or 48 h p.i., and viral titrations were performed in 96- well plate format by infecting BHK21 (for VSV) or Vero cells (for SeV) with serial virus dilutions. For SeV titration, cells were overlaid with 100 ml SFM containing 1.2% Avicel RC-581 (FMC BioPolymer, Philadelphia, PA) as previously described (Matrosovich, Matrosovich et al. 2006), while a 0.56 SFM/1% bactoagar mixture was used to overlay VSV infected cells. The effect of the exogenously added guanosine on VSV and SeV replication in the presence or absence of ribavirin was examined using confluent monolayers of cells in 96-well tissue culture plates (performed three times, done in triplicates). Cells were infected with either VSV-GFP or SeV-GFP (or mock-infected with SFM) at MOI of 3 CIU/cell. After 1 h p.i., virus was removed and cells were washed with PBS and mock-treated or treated with the SFM containing 500 μM ribavirin or 50 μM guanosine, or ribavirin together with guanosine. Guanosine was dissolved in DMSO and the final concentration of DMSO in the media added to all wells was 0.25%. The intensity of fluorescent signal at 18 h p.i for VSV and 24 h p.i for SeV was quantified using a Fluorescence Multi-Well Plate Reader CytoFluor 4000 (PerSeptive Biosystems, Inc., Framingham, MA) with the standard in built CytoFluor filter set (excitation wavelength at 485 and emission wavelength at 530 nm). Values were corrected for background fluorescence by subtracting the values of uninfected cells from the value of each infected well.

Plaque reduction assay to determine ribavirin inhibitory concentrations.

To estimate the 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) for ribavirin, antiviral screening was conducted by means of a plaque reduction assay using 24-well tissue culture plates. Cells were infected with VSV-GFP or SeV-GFP in SFM (or mock-infected with SFM) at an MOI producing about 100 virus plaques per well on each cell line in the absence of ribavirin. After absorption of virus for 1 h without ribavirin (to rule out an interference with virus attachment/entry), SFM containing unabsorbed virus was removed, cells were washed three times with phosphate-buffered saline (PBS), and overlaid with 200 ml/well of SFM containing 1.2% Avicel RC-581 and increasing concentrations of ribavirin. Cells were then incubated for 24 h (VSV) or 48 h (SeV). Plaques were counted with the aid of fluorescence and bright field microscopy, and the 50% (IC_{50}) and 90% (IC_{90}) inhibitory concentrations were calculated. Initial experiments were done using 0, 200, 500 or 1000 µM of ribavirin as it was done for virus infections at MOI 3 to determine the range of ribavirin activity for each virus/cell line combination. After that, all plaque reduction experiments were conducted using different ranges of ribavirin concentrations to more precisely determine the IC_{50} and IC_{90} values. Each of these experiments was performed at least twice (done in duplicates) and plaque numbers represent the mean 6 standard deviation of the mean.

Virus growth analysis.

The relative efficiency of the initiation of infection by VSV-GFP and SeV-GFP was measured by titrating viruses on the seven cell lines to determine the number of viral particles successfully initiating infection in a given cell line. For one-step growth kinetics analysis, confluent cell monolayers in 24-well plates were infected in parallel at an MOI

of 3 CIU/cell. At 1 h p.i., infection medium was aspirated, cells were washed three times with PBS (to minimize carryover of virions), and 300 ml of fresh SFM was added to each well. SFM from each well was collected at the specified time intervals, flash frozen at 280uC, and analyzed by titration as described above.

Ribavirin uptake assay.

Cell monolayers were prepared exactly as for virus infections using 12- or 24-well tissue culture plates. The $^{[3H]}$ribavirin uptake experiments were conducted essentially as in (Ibarra and Pfeiffer 2009) but with some modifications. Cells were plated the day prior to generate about 90% confluence on the day of the experiment. For ribavirin uptake in the presence or absence of NBMPR (15 or 100 µM), cells (in triplicates) on 24-well plates were pretreated with this nucleoside transporter inhibitor in DMSO (or with DMSO alone) for 15 minutes. Cells were then washed with PBS and treated with 100 ml of SFM (same medium used for infections but without virus) containing 50 µM ribavirin 1% of which was $^{[3H]}$ribavirin (ViTRax, Placentia, CA, cat. no. VT193, specific activity 5 Ci/mmol) for 15 minutes in a 5% CO2 atmosphere at 37°C. For the long-term accumulation of ribavirin, cells (in triplicates) on 12-well plates were washed with PBS and treated with 275 ml of SFM (same medium used for infections but without virus) containing the same concentration of ribavirin/$^{[3H]}$ribavirin (in the absence of NBMPR) as above but incubated for 1 h, 16 h or 24 h. To measure intracellular $^{[3H]}$ribavirin, cells were then placed on ice for 5 minutes (to stop an uptake) and washed 3 times with cold PBS. The cells were then trypsinized, pelleted at 200 x g for 4 minutes and cell pellets were frozen at -80°C. Nucleotide pool isolation was conducted as described in [26]. Specifically, tubes with frozen cell pellets were placed on ice and 75 ml of 1.3N cold

formic acid was added to each pellet, cell pellets were resuspended in formic acid and incubated for 1 h (tubes were vortexed every 15 minutes) on ice. After 1 h extraction period, the formic acid suspension was centrifuged at 17,000 x g, and the supernatant extracts (75 ml) were transferred to new tubes and quantified (15 ml) by scintillation counting for the intracellular [^{3}H] accumulation. Cell numbers (from separate plates) were counted by two separate methods. First, cells were trypsinized and cell number was determined using a hemocytometer. Cell numbers were independently confirmed by staining monolayers (from a separate plate) with blue-fluorescent Hoechst 33342 dye (Invitrogen), which selectively stains nuclei. At least 5 random fields were photographed using a fluorescence microscope and DAPI filter and nuclei were then counted. Uptake values were determined by dividing the counts per minute (CPM) by number of cells (CPM/cell) in a 24-well plate. For ribavirin uptake in the presence of ActD, cells were pretreated with 5 mg/ml ActD for 2 h, media was aspirated (without cell washing), and then ribavirin uptake assay was conducted as described above.

Cell viability assays.

Cellular toxicity of ribavirin was determined using about 80% confluent cells treated with increasing ribavirin concentrations (0, 200, 500 or 1000 µM) at 37°C and 5% CO2 for 24 h. After 24 h, all cells reached 100% confluence and were analyzed by the following three assays: i) MTT (Biotium, cat. no. 30006, 96-well plate format) cell viability assay; ii) CellTiter-Glo luminescent cell viability assay (Promega cat. no. G7570, 96-well plate format); and iii) cell counting using trypan blue dye exclusion as an indicator of live cells (24-well plate format). MTT assay was conducted according to the manufacturer's (Biotium) protocol. Briefly, after 24 h incubation with ribavirin, 10 ml of

MTT solution was added to each well and cells were incubated for 4 h at 37°C. Media was then removed and 200 ml of DMSO added to each well. OD values were measured using a Multiskan Ascent Microplate Photometer (Thermo Fisher Scientific) at a test wavelength of 570 nm and reference wavelength of 630 nm to determine the OD_{570}− OD_{630} signal. CellTiter-Glo assay was conducted according to the manufacturer's (Promega) protocol and using 96-well white opaque culture plates (PerkinElmer, cat. no. 6005680). After 24 h incubation with ribavirin, 100 ml of CellTiter-Glo reagent was added to each well. Plates were mixed for 2 minutes on orbital shaker to induce cell lysis, and incubated for 10 minutes to stabilize the luminescence signal. Luminescence was measured using Perkin Elmer TopCount NXT microplate luminescence counter. For trypan blue dye exclusion, 24-well plates were used. After 24 h incubation with ribavirin, cells were trypsinized and the number of viable cells was determined microscopically in a hemocytometer by trypan blue exclusion.

2.4 Results

Identification of ribavirin-resistant cell lines.

To determine whether ‘‘natural’’ (without pre-exposure to drug) resistance to ribavirin exists in some cell types, we selected seven commonly used cell lines (BHK21, BSRT7, HeLa, A549, 4T1, HEp2, and Vero) originated from various hosts and tissues, and compared them for the antiviral activity of ribavirin against VSV and SeV. To facilitate virus detection, we employed recombinant viruses containing an additionally inserted GFP gene (Fig. 2A). While such insertion results in a mild attenuation of VSV (Das, Nayak et al. 2006) and SeV (Wiegand, Bossow et al. 2007; Murphy AM and VZ 2009) both viruses replicate similarly to parental wt strains and, thus, serve as useful models for studying replication of wt viruses. Cells were treated with increasing concentrations of ribavirin added to the media 24 h before infection, and then infected with viruses at MOI of 3 CIU/cell with ribavirin treatment continued after virus absorption. The MOI for each cell line was calculated individually by titrating viruses on each of the seven cell lines as described in Materials and Methods and Table 2. Following ribavirin treatment and virus infection, pictures were taken 24 h post infection (p.i.) for VSV or 48 h p.i. for SeV using fluorescence and light microscopy. As shown in Figure 2B, GFP associated fluorescence attributable to viral replication was readily detectable in all tested cells lines infected with VSV or SeV when no ribavirin was added to the media, indicating that all cell lines were susceptible to infection by these two viruses. Consistent with previous studies demonstrating antiviral activity of ribavirin against VSV, ribavirin effectively inhibited VSV in BSRT7, HeLa and HEp2 cells even at the lowest (200 μM) tested drug concentration (Fig. 2B). However, ribavirin had a surprisingly mild effect on the VSV-driven GFP expression in BHK21, Vero and A549 cells even when used at 1000

μM concentration with a somewhat intermediate effect in 4T1 cells (Fig. 2B). In general, ribavirin inhibited SeV replication to a greater degree than VSV with markedly stronger inhibition in 4T1 cells. However, Figure 2B clearly shows a similar pattern of ribavirin resistance in BHK21, Vero and A549 cells for VSV and SeV, suggesting that cellular rather than virus-specific factors determine the dramatic differences between tested cell lines in their response to ribavirin. A similar pattern was also observed when ribavirin was added to the medium 6 h (rather than 24 h) before or 1 h after infection (without ribavirin pretreatment), although in general ribavirin was more effective when longer pretreatments were conducted. In addition, a similar pattern of ribavirin effect in the seven cell lines was observed when experiments were conducted at 37°C rather than at 34°C [34°C was chosen for experiments presented here as it supported optimal replication of both viruses in the seven cell lines or with cells of various passage level (3 to 20 passages) or confluence (70%), demonstrating that the observed effect was not determined by the state of the cells. To determine whether GFP levels correlated with the production of new infectious virus particles, the medium was collected and subjected to plaque assay on BHK21 (for VSV) or Vero (for SeV) cells. Virus titration analysis showed a clear correlation between GFP signal and the number of infectious virus particles produced in different cell lines under various treatment conditions (Fig. 2C). Next, we examined a possibility that a higher sensitivity of VSV and SeV to ribavirin in 4T1, BSRT7, HeLa and HEp2 was due to the increased cellular toxicity of ribavirin in these cell lines, which could result in the decreased ability of these cells to efficiently support viral replication. To address this issue, we used three different assays to measure cell viability using cells prepared and ribavirin treated the same way as for virus

infections shown in Figure 2: i) colorimetric MTT assay based on the reduction of the yellow tetrazolium salt MTT to the insoluble purple formazan crystals, which are solubilized by the addition of a detergent in metabolically active cells (Fig. 3A); ii) luminescent ‘‘CellTiter-Glo’’ assay based on quantitation of the intracellular ATP content as an indicator of metabolically active cells (Fig. 3B); iii) live cell counting using trypan blue dye exclusion as an indicator cell membrane integrity in the live cells (Fig. 3C). Using these three different methods (as described in Materials and Methods), we showed that ribavirin treatment even at 1000 μM concentration did not produce any statistically significant decrease in cell viability in any of the tested cell lines under our experimental conditions (Fig. 3), indicating that the observed pattern of ribavirin antiviral activity was not due to the differential ribavirin cytotoxicity in the tested cell lines (Fig. 3). To prepare cells for these assays, 80% confluent cells were treated with ribavirin for 24 h (same conditions used for virus infections in Figure 2). After 24 h treatment, all tested cell lines reached 100% confluence suggesting that ribavirin did not produce any substantial cytotoxicity that would prevent cell growth. However, we recognize that the cell viability assays conducted on 100% confluent cells may not be sensitive enough to detect all adverse effects of ribavirin on the host cell. Nevertheless, the absence of significant drop in cell viability by 3 independent assays were in good agreement with the lack of visible differences between ribavirin treated and untreated cells using light microscopy (Figure 2B). All infection experiments described above were conducted at MOI of 3 CIU/cell to achieve one-step replication of viruses in all tested cell lines. We also conducted additional experiments with cells infected at MOI 0.2, 0.5, 1, 10 or 20 in the presence of increasing concentrations of ribavirin (same range as above) and observed

a similar pattern of ribavirin resistance in Vero, BHK21 and A549, indicating that this effect was MOI independent. To further confirm the MOI-independent character of ribavirin resistance in Vero, BHK21 and A549 cells, we conducted a plaque reduction assay in the presence of ribavirin, which also allowed us to calculate the 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) of ribavirin for each virus/cell type combination, as described in Materials and Methods. As shown in Figure 4 and summarized in Table 3, the IC_{50} and IC_{90} values were in good agreement with our data using MOI 3 infections (Fig. 2). We find especially striking resistance of Vero cells to ribavirin with IC_{50}=2250 µM for VSV and 1550 µM for SeV and IC_{90}>3000 µM for both viruses. Compared to SeV, VSV was consistently more resistant to ribavirin in all tested cell lines, which might be associated with its markedly faster growth in all tested cell lines (addressed below). Nevertheless, the similar cell type dependent pattern of ribavirin resistance for VSV and SeV suggests that cellular determinants play a major role in ribavirin resistance.

Analysis of ribavirin uptake in different cell lines.

A recent study by Ibarra and Pfeiffer (2009) showed that the development of cell-based resistance to ribavirin treatment via decreased ribavirin uptake can greatly limit ribavirin antiviral activity. Therefore, we wanted to examine a possibility that the ribavirin resistance of Vero, BHK21 and A549 cells was a result of defective ribavirin uptake in these cell types, using methodology similar to that described previously (Ibarra and Pfeiffer 2009). To measure ribavirin short-term uptake, cells were treated with SFM (same media type used for infections but without virus) containing 50 µM ribavirin (1% of which was [^{3}H] ribavirin). After 15-minute incubation, cells were collected and measured for the level of [^{3}H]ribavirin uptake normalized to the number of cells as

described in Materials and Methods. As shown in Figure 5A (black bars), all tested cell lines showed somewhat similar levels of ribavirin import after 15-minute incubation, indicating that none of the tested cell lines was defective in ribavirin uptake. To confirm that the slightly lower [^{3}H]ribavirin counts presented in Figure 5A for BHK21, A549, and Vero cells reflect active uptake of ribavirin into the cells (rather than background counts), we also analyzed ribavirin uptake in cells pretreated with increasing concentrations of nitrobenzylthioinosine (NBMPR), a specific inhibitor of equilibrative nucleoside transport via ENT1 (inhibited at lower NBMPR concentrations) and ENT2 (inhibited at higher NBMPR concentrations) nucleoside transporters, which were (especially ENT1) previously shown to be primarily responsible for ribavirin import into the cells [28,29]. Our results clearly showed ribavirin uptake was inhibited in most cell lines at both lower (15 μM) and higher (100 μM) NBMPR concentrations (Fig. 5A), confirming that ENT play at least some role in the influx of ribavirin into all tested cell types. Interestingly, we were unable to see any additional decrease of ribavirin uptake in 4T1 cells at the higher NBMPR concentration (100 μM) where both ENT1 and ENT2 are inhibited (Ibarra and Pfeiffer 2009). However, a decrease was observed at 15 μM NBMPR concentration, suggesting that ENT1 is involved in the ribavirin uptake in this cell line. While our short-term uptake experiments did not reveal any defects in ribavirin import in the seven cell lines, we wanted to see whether long-term accumulation of [^{3}H]ribavirin, which depends on the ribavirin metabolism) was different in the seven cell lines. To test it, we conducted a similar uptake experiment described above but with cells treated with [^{3}H]ribavirin for 1 h, 16 h and 24h (instead of 15 minutes). As shown in Figure 5B, dramatic variations were observed in the long-term accumulation of ribavirin in different cell types.

Importantly, it correlated with the antiviral efficacy of ribavirin in the tested cell lines. Thus, all 3 ribavirin-resistant cell lines, BHK21, A549 and especially Vero showed markedly decreased levels of ribavirin accumulation suggesting that such the differences in the intracellular ribavirin metabolism may be responsible for natural resistance of BHK21, A549 and Vero cells to antiviral ribavirin treatment (Ibarra and Pfeiffer 2009).

Neutralizing effect of guanosine and actD on the antiviral activity of ribavirin

One of the major proposed mechanisms of ribavirin antiviral action is the inhibition of the host enzyme IMPDH essential for the de novo synthesis of GTP. Moreover, a recent study suggests that inhibition of IMPDH and the consequent decrease in the cellular GTP pool (but not interactions of ribavirin metabolites with viral polymerase) is the predominant mechanism of action of ribavirin against RSV (a paramyxovirus) (Leyssen, Balzarini et al. 2005). To examine whether ribavirin inhibits VSV and SeV in all seven tested cell lines primarily via depletion of the GTP pool, we analyzed the effect of exogenously added guanosine on the antiviral effect of ribavirin. If GTP depletion alone is sufficient for inhibition of viral replication, we expected complete neutralization of the ribavirin effect in cells treated with a combination of ribavirin (500 μM) and guanosine (50 μM). The selected 50 μM guanosine concentration should result in dramatic increase in the intracellular GTP levels. According to previous studies, even 10μM exogenous guanosine produces at least 4-fold excess of physiological GTP levels within Vero, HepG2, MDCK and other cell lines (Wray, Gilbert et al. 1985; Leyssen, Balzarini et al. 2005; Sun, Chung et al. 2007). Cells were infected with either VSV-GFP or SeV-GFP at MOI of 3 CIU/cell, and then mock treated or treated with the SFM containing ribavirin or guanosine, or ribavirin together with guanosine. The intensity of GFP-associated

fluorescence attributable to viral replication was quantified (as described in Materials and Methods) at 18 h p.i for VSV and 24 h p.i for SeV (Fig. 6). As expected, guanosine treatment alone had no significant effect on virus replication (Fig. 6) in most cell lines. It had also a clear neutralizing effect on ribavirin in BHK21 and A549 cells, already highly resistant to ribavirin (Fig. 6). Intriguingly, guanosine had an intermediate neutralizing effect in BSRT7 cells for VSV and a very small effect on ribavirin activity in the ribavirin sensitive HeLa, 4T1 and HEp-2 (and BSRT7 for SeV) cells (Fig. 6), although all tested cell lines had somewhat similar levels of [^{3}H]-guanosine uptake. The addition of 50 µM guanosine was unable to neutralize the ribavirin effect in these 4 cell lines even when the ribavirin concentration was lowered to 200 or 100 µM. Also, a similar result was obtained when 200µM guanosine was added to the medium. These data suggest that a decrease in the cellular GTP pool is not the predominant mechanism of ribavirin action against VSV and SeV in HeLa, 4T1, HEp-2 and BSRT7 cells, and that other mechanisms also contribute to ribavirin activity against these two viruses in those cell lines.

Previous studies showed that actinomycin D (ActD), an inhibitor of DNA-primed RNA synthesis (but not viral RNA-dependent RNA synthesis), was able to revert the antiviral effect of ribavirin against several RNA viruses, including VSV (Toltzis and Huang 1986/6), RSV(Smee and Matthews 1986), Sindbis virus (Malinoski and Stollar 1980) and rotavirus (Smee, Sidwell et al. 1982). Two mechanisms of such reversion were proposed including the stabilization of cellular GTP levels (Malinoski and Stollar 1980; Smee, Sidwell et al. 1982; Smee and Matthews 1986; Toltzis and Huang 1986/6) and inhibition of ribavirin triphosphate (RTP) production (Smee and Matthews 1986). To examine whether ribavirin neutralization by ActD can be also reproduced in case of SeV

and whether it is cell type dependent, we infected cells with VSV-GFP or SeV-GFP at MOI 3 CIU/cell and treated these cells with ActD (5 mg/ml) or ribavirin (500 μM) alone or with both drugs together at 1 h p.i. Photographs of infected cells were taken at 24 h p.i. and the media from each well was collected and titered to determine the number of new infectious particles produced. As shown in Figure 7 (A–C), ActD had a clear neutralizing effect on ribavirin in most cell lines, while it had a somewhat mild effect on viral replication when used alone in most cell lines with the strongest negative effect observed in HEp2 cells for SeV and HeLa cells for VSV. The tolerance of both viruses to ActD treatment is consistent with a relative independence of their exclusively cytoplasmic replication cycle on new mRNA synthesis by cellular RNA polymerase II, a target of ActD. To rule out a possibility that ActD treatment affected ribavirin import into the cells, all seven cell lines were treated with ActD (or mock-treated) for 2 h followed by a [^{3}H]ribavirin uptake experiment conducted as described in Materials and Methods. Our results showed that ActD treatment did not inhibit ribavirin uptake, but actually resulted in a slight increased uptake of ribavirin, demonstrating that the observed reversal of ribavirin antiviral action (Fig. 7) was not due to the interference of ActD with ribavirin uptake.

Resistance of cell lines to ribavirin and their ability to support viral replication

As noted, the seven cell lines used in this study were selected solely based on their ability to support replication of VSV and SeV. To assess any possible correlation between the general ability of these viruses to replicate in these cell lines and their resistance to ribavirin, we compared VSV and SeV for their ability to initiate infection and for their replication kinetics in these cell lines without ribavirin treatment. First, VSV-GFP or

SeV-GFP virus stocks were titrated in parallel on different cell lines and the relative ability of each virus to initiate virus infection was calculated by counting infectious foci generated on each cell line. As shown in Figure 8A (and Table 2 with the numbers calculated based on the Figure 8A data), Vero, BHK21 and A549 cells, all highly resistant to ribavirin, were among the four cell lines most susceptible to VSV infection. Consequently, for our MOI 3 infections described in Figures 2, 4, 6 and 7 to achieve VSV MOI 3 infection for each cell line, for each 3 ml of the VSV-GFP virus stock added to the ribavirin-resistant BHK21 cells (13.2 µl to A549, 23.7 µl to Vero), 227 µl of the same stock was added to the ribavirin-sensitive 4T1 and HEp2 and 132 µl to HeLa cells (Table 2). However, ribavirin-sensitive BSRT7 cell line was found to be as susceptible to VSV as the most ribavirin-resistant Vero cells (Figure 8A and Table 2). In case of SeV, most cell lines (except for 4T1) showed somewhat similar rates of viral infection initiation for SeV, without any strong correlation with ribavirin sensitivity (Figure 8A and Table 2).

We also conducted one-step growth kinetics analysis by infecting each cell type with VSV-GFP or SeV-GFP at MOI of 3 CIU/cell (MOI was calculated individually for each virus/cell type combination) and measuring production of new infectious particles by collecting medium from each well at specified time points and titrating it as described in Materials and Methods. While some correlation can be seen in SeV with its fastest growth kinetics (and highest titers) in BHK21, A549 and Vero cells (all three resistant to ribavirin), it is less apparent in the case of VSV, which grows relatively similarly in most cell lines (Fig. 8B). Together, all these results show no clear correlation between abilities of cell lines to support viral replication and their resistance to ribavirin, although the

abilities of cells to support robust virus replication may be an important factor that would allow successful replication in the presence of ribavirin as all three ribavirin-resistant cell lines supported high replication levels of both VSV and SeV. Nevertheless, our results show that virus growth phenotype alone (e.g., VSV in BSRT7) cannot be used to predict efficacy of ribavirin against VSV or SeV in a given cell line.

2.5 Discussion

In this study, we compared the antiviral activity of ribavirin against two prototypic members of the order Mononegavirales, VSV (a rhabdovirus) and SeV (a paramyxovirus), in seven different cell lines originated from various hosts and tissues. Previous studies showed that ribavirin can effectively inhibit replication of VSV (Toltzis and Huang 1986/6; Toltzis, O'Connell et al. 1988/4; Cuevas, Sanjuan et al. 2005) and SeV (Sidwell, Khare et al. 1975; Larson, Stephen et al. 1976) as well as other members of Mononegavirales (Hruska, Bernstein et al. 1980; Smee and Matthews 1986; Toltzis and Huang 1986/6; Toltzis, O'Connell et al. 1988/4; Jordan, Briese et al. 1999/9; Crotty, Cameron et al. 2002; Cuevas, Sanjuan et al. 2005; Leyssen, Balzarini et al. 2005; Elia, Belloli et al. 2008; Pelaez, Lyon et al. 2009). However, in most of these studies only one or two different cell lines were tested. The seven cell lines used in this study were selected solely based on their ability to support replication of both viruses. The two-virus approach allowed us to discriminate between virus specific and cell-based resistance to ribavirin treatment because, although both viruses belong to the same order Mononegavirales, they belong to different families and have noticeably different growth kinetics in these cell lines.

Our results show striking differences between cell lines, ranging from the extremely poor antiviral activity of ribavirin in Vero cells (e.g., IC_{50} = 2250 µM for VSV and 1550 µM for SeV; IC_{90} >3000 µM for both viruses), moderate activity in BHK21 and A549 cells, and very effective inhibition in HEp2, HeLa, 4T1 and BSRT7 cells (IC_{50}=10 µM, IC_{90}=40 µM for VSV in BSRT7; IC_{50}=16 µM, IC_{90}=40 µM for SeV in BSRT7).

This pattern was confirmed using various infection and ribavirin treatment conditions, with cells infected and treated at 34 or 37°C, high or low MOI, and with ribavirin treatment starting at 24 h before infection, 6 h before infection, or 1 h p.i. Using three different cell viability assays, we showed that ribavirin treatment even at 1000 μM concentration did not produce any significant cytotoxicity in any of the tested cell lines at our experimental conditions, nor did we observe any significant differences between tested cell types, indicating that the observed pattern of ribavirin resistance was not due to differences in ribavirin toxicity. It is important to emphasize that the median ribavirin plasma concentration in HCV patients at the peak of ribavirin therapy is between 6.6and 9 μM (Glue 1999; Aguilar Marucco, Gonzalez de Requena et al. 2008; Loustaud-Ratti, Alain et al. 2008; Maynard, Pradat et al. 2008). Therefore, the IC_{50} and IC_{90} values for Vero, BHK21 and A549 cells (Table 3) indicate extremely high resistance of these cell types to ribavirin.

Our data strongly argue that the observed resistance of VSV and SeV to ribavirin in Vero, BHK21 and A549 was not due to the generation of ribavirin-resistant mutants in these cells. Such ‘‘virus-based’’ resistance mechanism was previously described for several other RNA virus groups, including polioviruses (Pfeiffer and Kirkegaard 2003; Vignuzzi, Stone et al. 2005), foot-and mouth disease virus (Airaksinen, Pariente et al. 2003; Sierra, Airaksinen et al. 2007) and recently for HCV (Cuevas, Gonzalez-Candelas et al. 2009). However, even when our cells were treated with ribavirin starting as early as 24 h before infection (Fig. 2), we observed little effect of ribavirin on viral replication in ribavirin-resistant cells, ruling out any possibility of virus adaptation to ribavirin. In addition, when VSV was passed 10 to 15 times in HeLa, BSRT7 and BHK21 cells in the

presence of sub-inhibitory ribavirin concentrations, no viral adaptation to ribavirin was ever observed (N.R.S. and V.Z.G., unpublished data). These observations are consistent with a previous study by Cuevas et al. demonstrating that even after 100 generations under sub-inhibitory concentrations of ribavirin, resistance of VSV to ribavirin was not achieved, with selected populations generally less fit than the ancestral population both in the presence and absence of ribavirin (Cuevas, Sanjuan et al. 2005).

A recent study by Ibarra and Pfeiffer (Ibarra and Pfeiffer 2009) showed that the development of cell-based drug resistance after continuous ribavirin treatment via decreased drug uptake can greatly limit ribavirin efficacy. In addition, any potential antiviral mechanism absolutely relies on ribavirin entry into the cell. Therefore, we compared our seven cell lines for their ability to internalize ribavirin. Our results showed a similar ribavirin uptake in all tested cell lines after 15-minute treatment, indicating that none of the tested cell lines was defective in ribavirin uptake. In addition, using NBMPR, a specific inhibitor of equilibrative nucleoside transporters, we confirmed that ENT1 and possibly ENT2 transporters are involved in the ribavirin uptake (Jarvis, Thorn et al. 1998; Fukuchi, Furihata et al. 2010). A similar ribavirin uptake level by all tested cell lines is not surprising as ENTs are ubiquitously expressed in virtually all cell types (Endres, Moss et al. 2009). However, when we analyzed long-term ribavirin accumulation in cells after 16 h or 24 h treatment, a totally different picture was observed. Four cell lines sensitive to ribavirin (BSRT7, HeLa, HEp2 and 4T1) showed significantly higher levels of ribavirin accumulation compared to ribavirin-resistant BHK21, A549 and Vero. Vero cells had particularly low accumulation which may explain the highest resistance of this cell line to ribavirin treatment among all the cell lines tested in our study (Table 3).

It is important to note that while the 15-minute uptake say determines the ability of cells to internalize ribavirin, the long-term accumulation is dependent on the cellular metabolism of ribavirin. Neutral ribavirin molecule can be transported freely in and out of a cell via ENTs, but once it is phosphorylated, negative-charged RMP, RDP, or RTP are trapped inside the cells. A good illustration of the difference between the ribavirin uptake and its long term accumulation is ribavirin hyperaccumulation in erythrocytes resulting in hemolytic anemia in some ribavirin-treated patients. Similarly to nucleated cells, ribavirin is transported into erythrocytes via ENTs (Jarvis, Thorn et al. 1998) and converted into RMP, RDP and RTP. However, unlike nucleated cells, they lack the phosphatases needed to hydrolyze RMP/RDP/RTP into ribavirin [(Page and Connor 1990; Gish 2006; Endres, Moss et al. 2009). Recent study by Endres et al. (2009) directly showed that total radioactivity of ribavirin after long-term administration is predominantly attributed to RMP and RTP (Endres, Moss et al. 2009). Hyperaccumulation of these molecules, along with other factors, results in cellular toxicity of erythrocytes and subsequent anemia (Gish 2006).

While future studies are warranted to directly analyze ribavirin metabolism in the seven cell lines, our results indicate that these cell lines may significantly differ in their abilities to accumulate sufficient amounts of phosphorylated ribavirin metabolites required for effective ribavirin antiviral actions. RMP is believed to play the major antiviral role as a competitive inhibitor of the enzyme IMPDH essential for the de novo synthesis of GTP and is also capable of binding and inhibiting at least some viral polymerases (Parker 2005), including viral polymerase of VSV (Toltzis and Huang 1986/6; Toltzis, O'Connell et al. 1988/4; Cuevas, Sanjuan et al. 2005). RTP may also play

an important role in the inhibition of VSV and SeV replication via interaction with viral polymerase (shown for RTP and VSV (Toltzis, O'Connell et al. 1988/4)), ‘‘error catastrophe’’ or any other mechanisms which involves RTP as a substrate for viral RNA polymerase.

To examine whether ribavirin inhibits VSV and SeV primarily via depletion of the GTP pool, we treated VSV or SeV infected cells with ribavirin in the presence of extracellular guanosine which restores normal intracellular GTP level. Guanosine had a clear (almost 100%) neutralizing effect on ribavirin in BHK21, A549 and Vero cells, which are already highly resistant to ribavirin. However, very little effect was observed on the ribavirin activities in ribavirin-sensitive cells, especially HeLa, 4T1 and HEp-2 cells. Together, these data suggest that a decrease in the cellular GTP pool is not the predominant mechanism of ribavirin action against VSV and SeV in HeLa, 4T1, HEp-2 and BSRT7 cells, and that other mechanisms also contribute to ribavirin activity against these two viruses in these cell lines.

Unlike guanosine, ActD was able to effectively neutralize ribavirin in all tested cell lines. Previous studies showed that ActD neutralizes ribavirin effects via two mechanisms (likely not mutually exclusive). Malinoski and Stollar (1980) showed that ActD neutralized effect of ribavirin against Sindbis virus by maintaining the GTP pool size at its normal level (the mechanism of this stabilization is still unknown) (Malinoski and Stollar 1980). A similar effect of ActD on GTP pool stabilization was shown by Smee and Matthews (1986) in RSV-infected cells treated with ribavirin. However, they also analyzed the metabolism of RMP to its mono-, di-, and triphosphate derivatives in

uninfected and RSV-infected cells, and concluded that ActD also neutralized ribavirin effect via inhibition of RTP production (Smee and Matthews 1986).

Based on the ability of ActD (but not guanosine) to neutralize the effect of ribavirin in ribavirin-sensitive cell lines (HeLa, 4T1, HEp-2 and BSRT7), we hypothesize that ribavirin antiviral activity in these cell lines depends not only on the depletion of the GTP pool (can be restored by guanosine addition) but also on the successful 5'-phosphorylation of ribavirin into RMP/RDP/RTP (Willis, Carson et al. 1978; Balzarini, Karlsson et al. 1993; Wu, Larson et al. 2005) which were previously shown to inhibit VSV RNA synthesis in vitro (Toltzis, O'Connell et al. 1988/4). At the same time, we think that ribavirin acts in ribavirin-resistant cell types (BHK, A549 and Vero) primarily via depletion of GTP pool due to insufficient amounts of phosphorylated ribavirin molecules in these cells, explaining why the effect of ribavirin can be completely reversed in these cell lines by guanosine. Further experiments are planned to test this hypothesis and further investigate the mechanism of ribavirin neutralization by ActD.

Overall, our data point out to an interesting possibility that the mechanism of virus inhibition by ribavirin may be more dependent on cell type than we currently expect. This could explain numerous conflicting reports regarding the ‘‘true’’ mechanism of ribavirin action proposed by different research groups for the same virus (Parker 2005; Vignuzzi, Stone et al. 2005; Dixit and Perelson 2006; Martin and Jensen 2008). Furthermore, we anticipate that different results for other viruses might be obtained in the cell lines utilized here. For example, a recent study demonstrated an effective inhibition of canine distemper virus (CDV, family Paramyxoviridae, genus Morbillivirus) in Vero

cells [IC_{50}=20–50 μM, IC_{80}=40–110 μM] (Elia, Belloli et al. 2008). This result suggests that CDV and SeV might be inhibited by ribavirin via different mechanisms.

At present, we cannot explain dramatic differences between BHK21 and BSRT7 cells in their resistance to ribavirin and the long term ribavirin accumulation. BSRT7 cell line is derived from BHK21 and constitutively express bacteriophage T7 polymerase under control of the cytomegalovirus promoter and the neomycin resistance gene (Buchholz, Finke et al. 1999). Although we cannot explain why these two cell lines are so different in regard to ribavirin, we also noticed significant differences in cell appearance, cell growth kinetics, viral growth kinetics and the phenotype of infectious foci for VSV and SeV between BHK and BSRT7 cells, suggesting that some additional changes were introduced into BSRT7 when or since this recombinant cell line was generated, or that T7 polymerase expression may be responsible for some or all of those phenotypes.

We believe the very similar pattern of ribavirin activity against VSV and SeV in seven different cells lines may indicate that these two viruses are inhibited by ribavirin via the same mechanism. Although the mechanism of SeV (genus Respirovirus) inhibition by ribavirin has not been previously studied, a previous study on RSV (another member of the family Paramyxoviridae, but belongs to the genus Pneumovirus) suggests the predominant mechanism of action of ribavirin against RSV is inhibition of cellular IMPDH activity by RMP (and consequent decrease in the cellular GTP pool) rather than interactions of ribavirin metabolites with the viral polymerase (Leyssen, Balzarini et al. 2005). In contrast, a previous study using in vitro transcription reactions with purified VSV virions demonstrated that RMP, RDP and RTP significantly inhibited viral polymerase activity and hypothesized that these molecules reversibly inhibit an initiating

step of VSV RNA synthesis (Toltzis, O'Connell et al. 1988/4). Further experiments are needed to examine molecular mechanisms of VSV and SeV inhibition by ribavirin.

Overall, our data demonstrate the antiviral activity of ribavirin is naturally limited in many cell types which may explain at least some ribavirin treatment failures. Further studies aimed at the understanding molecular determinants responsible for cell-based resistance to ribavirin are warranted. This understanding may become an important tool for tailoring individualized treatments with ribavirin (and possibly other nucleoside analogs) against important viral pathogens. Future experiments are also needed to determine whether the observed differences between different cell lines are limited only to non-segmented negative-strand RNA viruses by analyzing effect of ribavirin on replication of positive-strand RNA or segmented negative-strand RNA viruses in these cell lines. Finally, our results strongly point out the importance of using multiple cell lines of different origin when antiviral efficacy and potency are examined for new as well as established drugs in vitro.

2.6 Figures

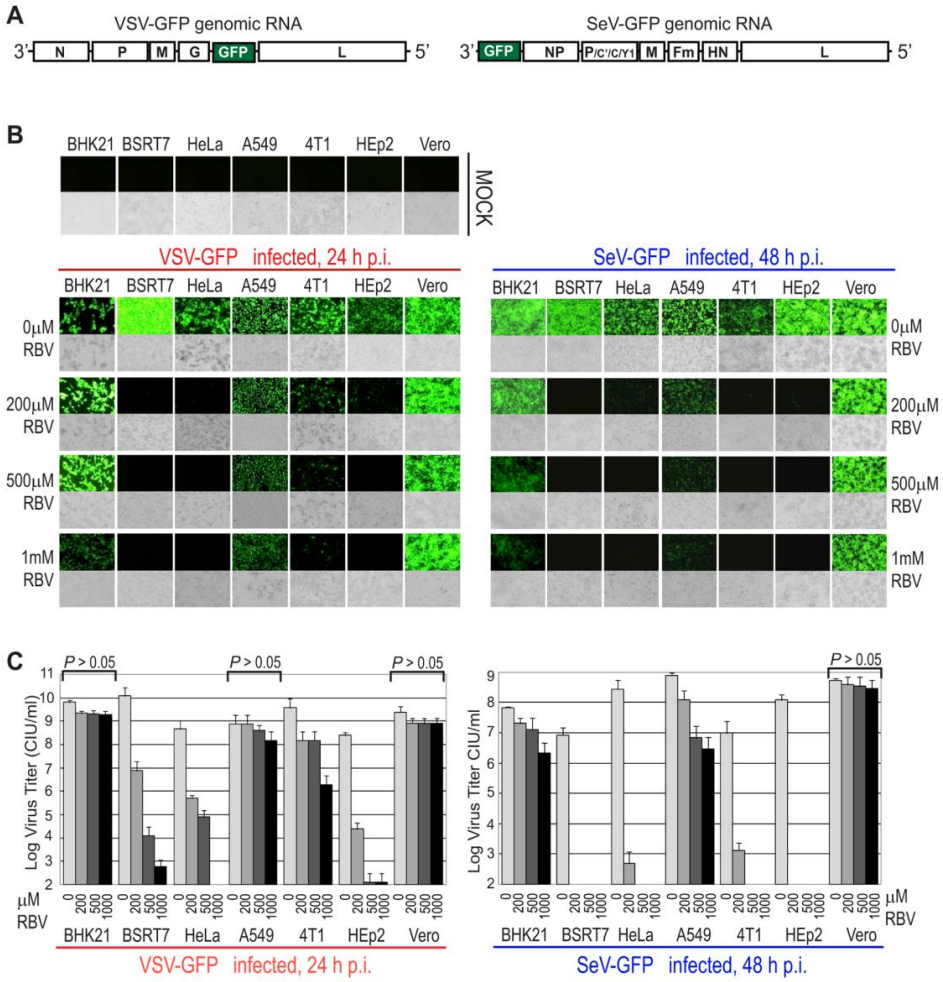

Figure 2: Effect of ribavirin on viral replication in seven cell lines. **(**A) The organization of the negative-sense RNA genomes of the recombinant viruses used in this study. (B) The panels show photographs of cells pretreated for 24 h with increasing concentrations of ribavirin as indicated (or mock-treated), infected with VSV-GFP (left) or SeV-GFP (right) at MOI 3 CIU/cell (or mock-infected, upper row), and then the same concentrations of drugs as in the pretreatment was added to each well after virus absorption. Fluorescence (upper panels) and light (lower panels) microscopy images were captured at 10× magnification. The photographs are typical representations of at least three independent experiments and an average field for each well is shown. (C) Media from the experiments described in B was collected at 24 h p.i for VSV (left) or at 48 h p.i for SeV (right) and virus titer was determined by standard plaque assay on BHK21 (for VSV) or Vero cells (for SeV). The data represent the mean ± standard deviation of two independent experiments (done in duplicates). Statistical analysis was done using one-way ANOVA with Tukey's post hoc test (GraphPad Prism 4, San Diego, CA). ribavirin treatments without significant decrease in viral titer at any tested ribavirin concentrations as compared to mock-treated cells ("0 μM ribavirin") are indicated as P>0.05.

2.6 Figures continued

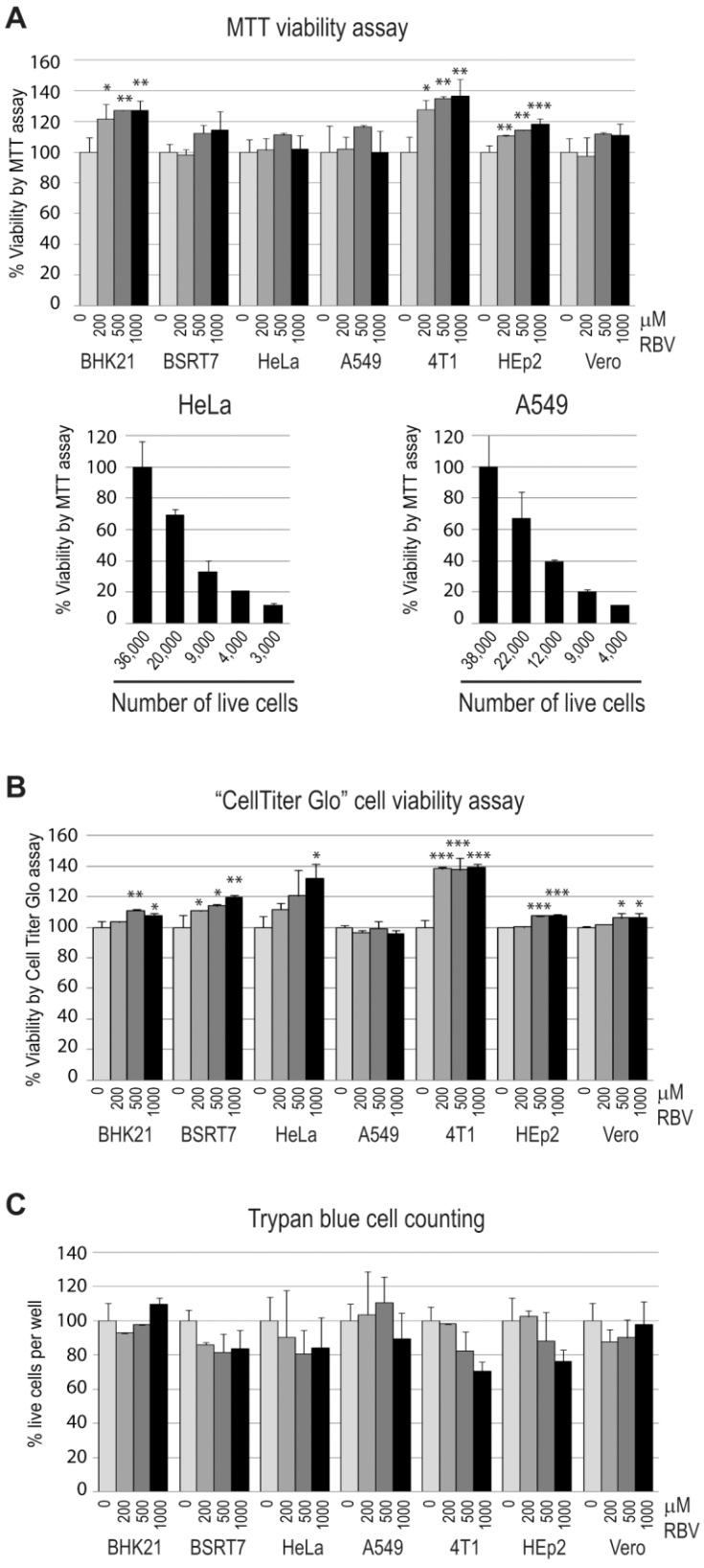

Figure 3. Effect of ribavirin on cell viability of seven cell lines.

To determine the relative toxicity of increasing concentrations of ribavirin in different cell lines, 80%-confluent uninfected cells were treated with ribavirin for 24 h and tested for viability using MTT cell viability assay (A) or CellTiter-Glo Luminescent Cell Viability Assay (B) or by cell counting using trypan blue dye exclusion as an indicator of live cells (C) as described in Materials and Methods. To determine the sensitivity of the MTT assay, serial dilutions of A549 and HeLa cells were plated [lower left and right graphs in (A)], grown for 24 h, cells from separate wells were trypsinized and counted using a hemocytometer), and MTT assay was conducted as described in Materials and Methods. (A–C) The data (done in triplicate) represent the mean ± standard deviation and are expressed as a percentage of the untreated control. Statistical analysis was done using one-way ANOVA with Tukey's post hoc test (GraphPad Prism 4, San Diego, CA). ***P<0.001, **P<0.01, *P<0.05, as compared to mock-treated cells (indicated as 0 µM ribavirin).

2.6 Figures continued

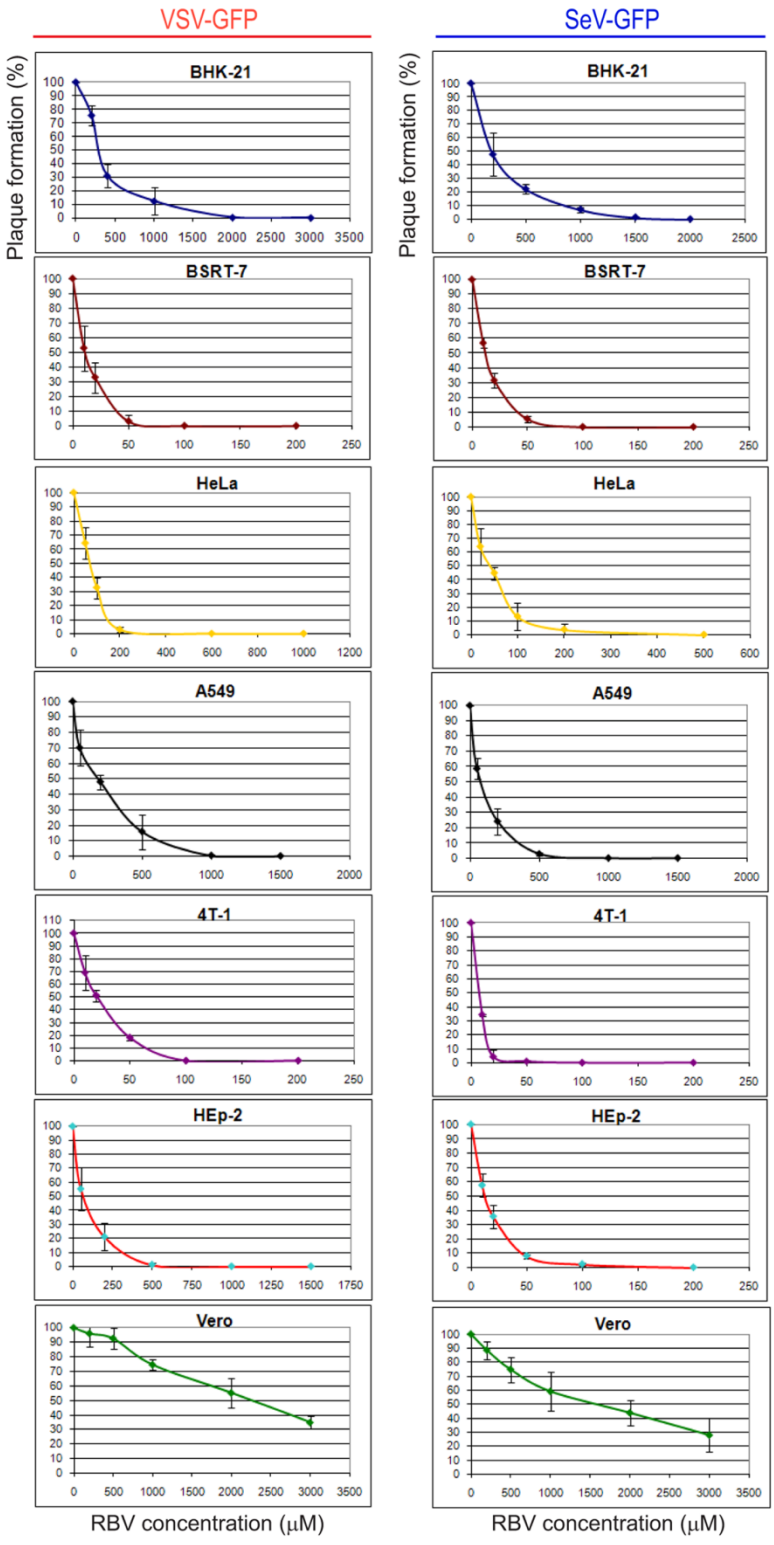

Figure 4. Plaque reduction assay to determine ribavirin inhibitory concentrations. Cell monolayers were infected with VSV-GFP or SeV-GFP (or mock-infected; 0 μM ribavirin) using virus dilutions producing about 100 virus ("100%") on each cell line in the absence of ribavirin, overlaid with SFM containing 1.2% Avicel RC-581 and increasing concentrations of ribavirin (note that different ribavirin concentrations were used for each virus-cell type combination). Cells were then incubated for 24 h (VSV) or 48 h (SeV), and plaques were counted with the aid of fluorescence and bright field microscopy. "0%" indicates that no fluorescent infectious foci were detected. Each experiment was performed at least twice (done in duplicates) and data points represent the mean ± standard deviation.

2.6 Figures continued

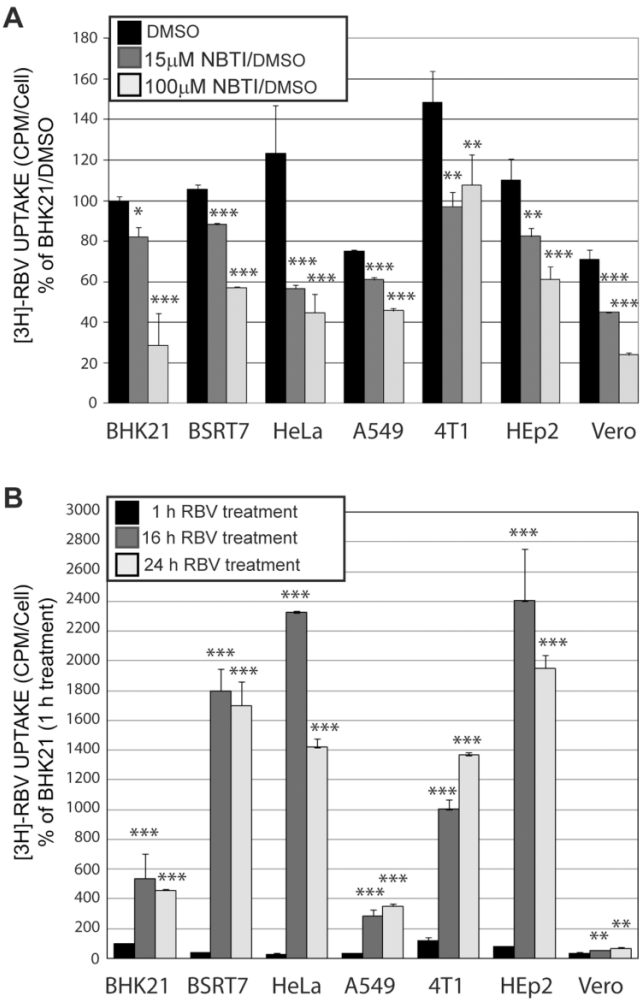

Figure 5. Ribavirin uptake and its inhibition in different cell lines. **(**A) Cell monolayers on 24-well plates (done in triplicates) were pretreated for 15 minutes with 15 or 100 μM NBMPR/DMSO or mock-treated with the same amount of DMSO as contained in the treated wells. Cells were then treated with SFM containing 50 μM ribavirin 1% of which was [^{3}H]ribavirin for 15 minutes at 37°C. Nucleotide pools were isolated and measured for [^{3}H]. Uptake values represent CPM divided by number of cells in a 24-well plate and normalized to the uptake by DMSO-treated BHK21 cells (defined as 100%). The mean ± standard deviation is shown for four independent experiments. (B) Cell monolayers (done in triplicates) on 12-well plates were treated with SFM containing 50 μM ribavirin 1% of which was [^{3}H]ribavirin at 37°C for 1 h, 16 h or 24 h. Nucleotide pools were isolated and measured for [^{3}H]. Uptake values represent CPM divided by number of cells in a 12-well plate and normalized to the uptake by BHK21 cells for 1 h (defined as 100%). (A–B) Statistical analysis was done using one-way ANOVA with Tukey's post hoc test (GraphPad Prism 4, San Diego, CA). ***P<0.001, **P<0.01, *P<0.05, as compared to ribavirin only treated cells (A) or cells treated with ribavirin for 1 h (B).

2.6 Figures continued

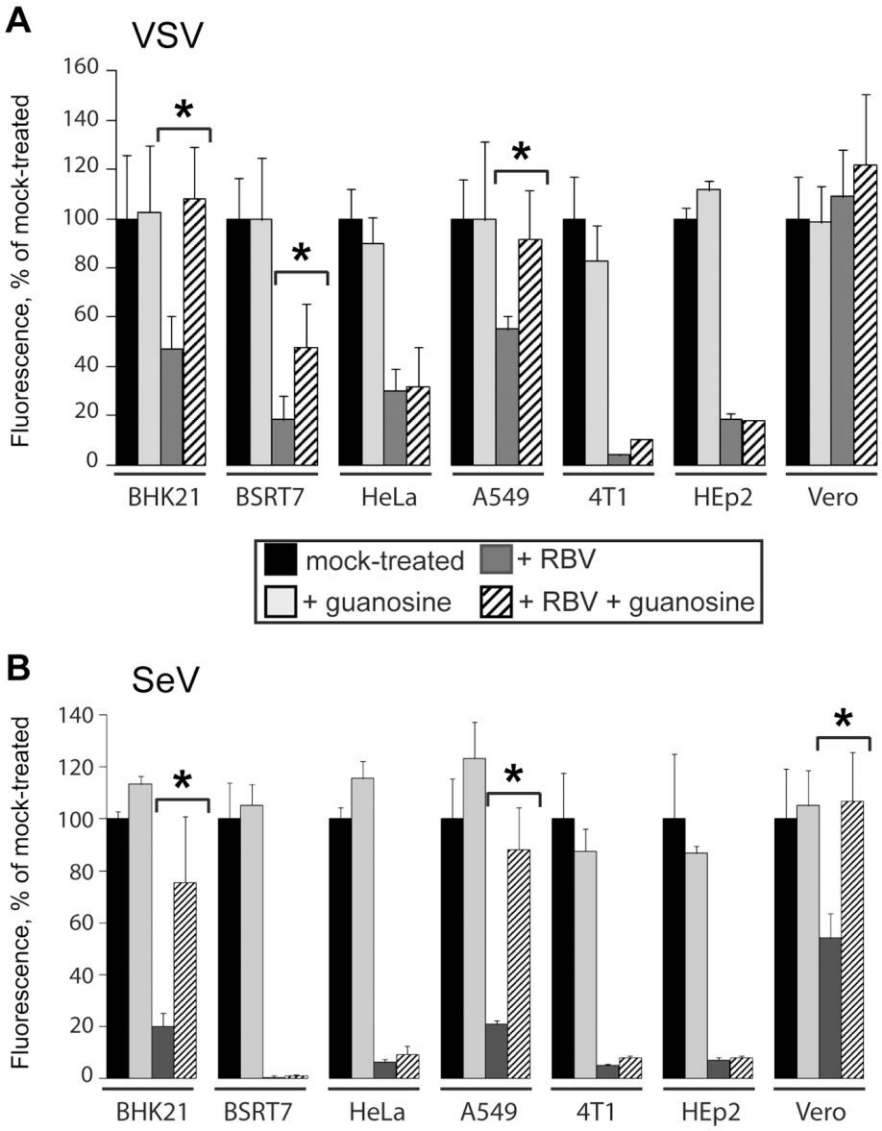

Figure 6. Effect of exogenously added guanosine on antiviral activity of ribavirin**.** Cells were mock infected or infected with either VSV-GFP or SeV-GFP at MOI of 3 CIU/cell, and then mock-treated or treated with SFM containing 500 µM ribavirin, 50 µM guanosine, or both. The intensity of GFP fluorescent signal at 18 h p.i for VSV (A) and 24 h p.i for SeV (B) was quantified using a 96-well plate reader, as described in Materials and Methods. Each of these experiments was performed twice (done in triplicates) and data points represent the mean ± standard deviation. (A–B) Statistical analysis was done using one-way ANOVA with Tukey's post hoc test (GraphPad Prism 4, San Diego, CA). ***P<0.001, **P<0.01, *P<0.05 are shown to compare ribavirin plus guanosine treatment against ribavirin treatment only.

2.6 Figures continued

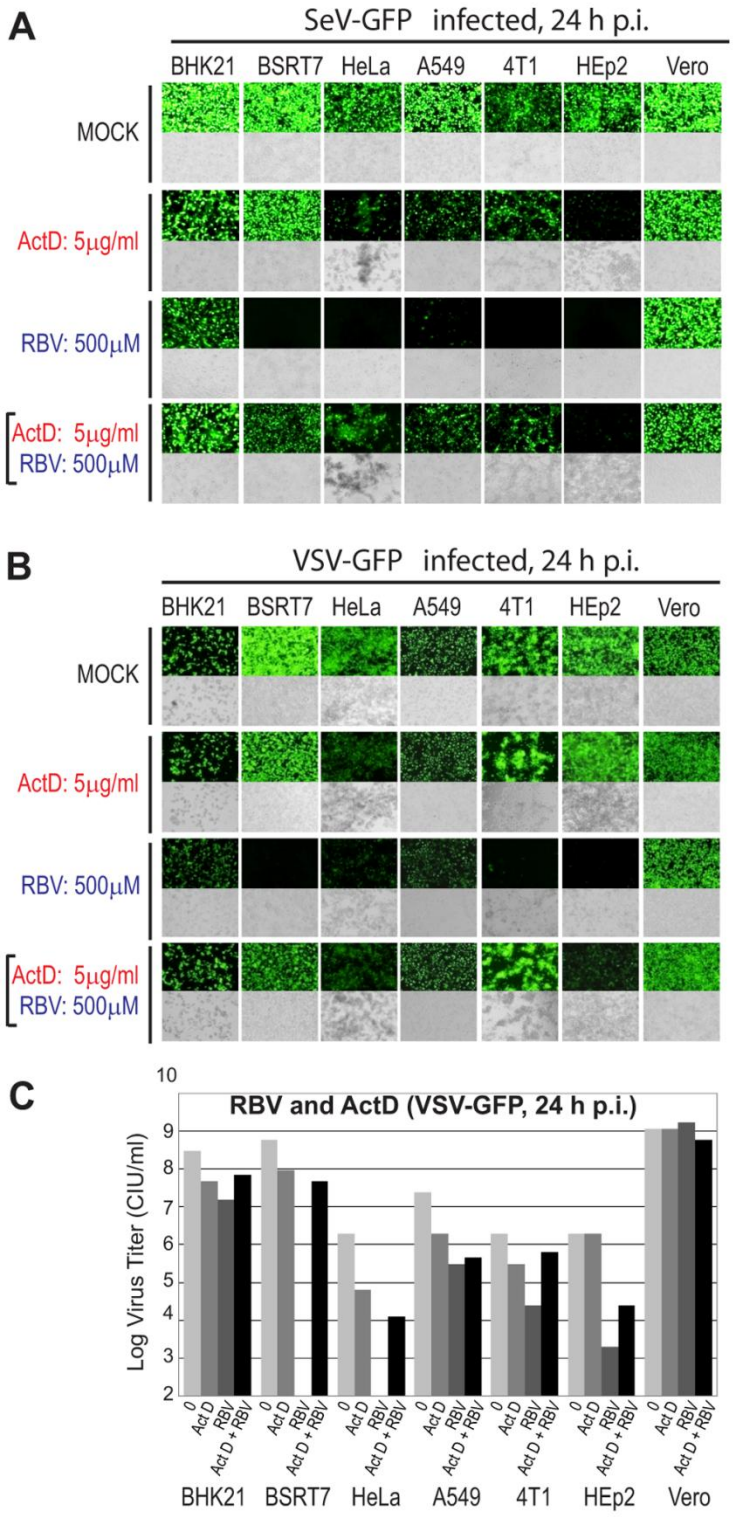

Figure 7. Effect of ActD on antiviral activity of ribavirin. Cell monolayers were infected with SeV-GFP (A) or VSV-GFP (B) at MOI 3 CIU/cell in the absence of drugs, or with 5µg/ml ActD, 500 µM ribavirin, or both. Fluorescence (upper panels) and light (lower panels) microscopy images were captured at 10× magnification. The photographs are typical representations of at least three independent experiments and an average field for each well is shown. (C) The number of new infectious VSV-GFP particles generated in the wells photographed in (B) was determined by analysis of SFM collected from each well by plaque assay on BHK21 cells (done in duplicates, average is shown).

2.6 Figures continued

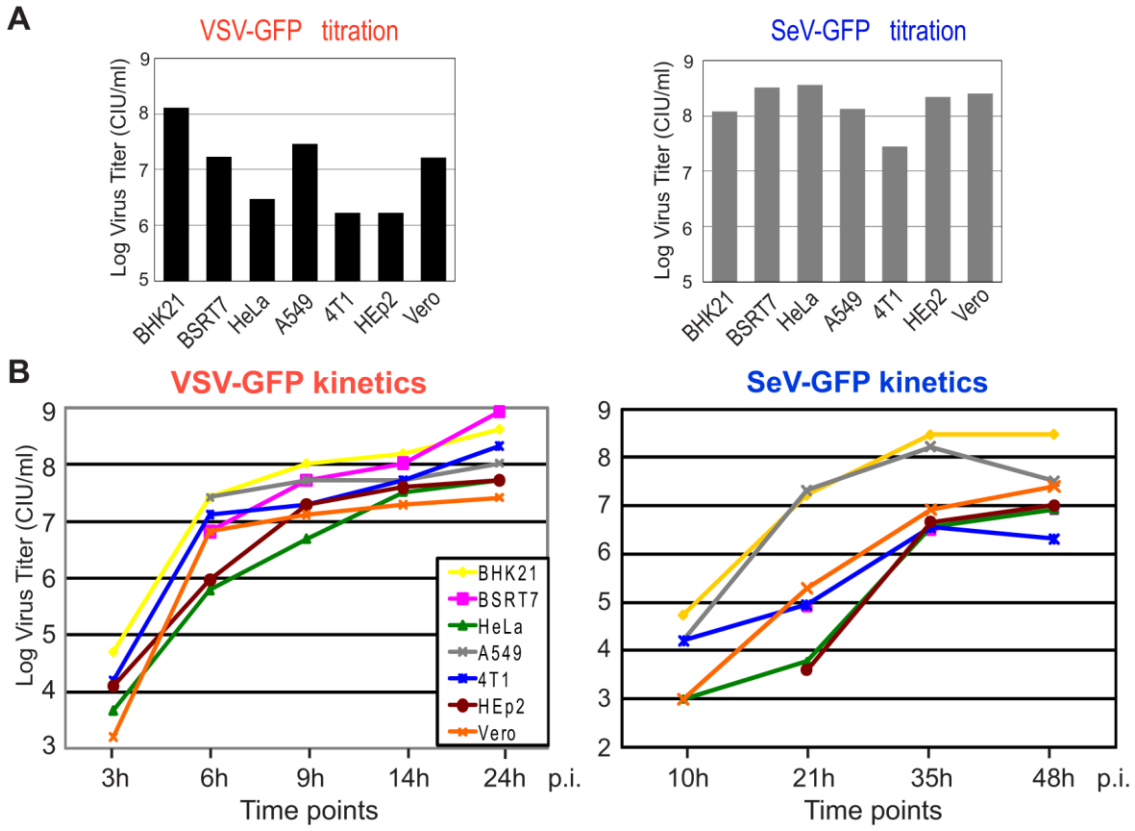

Figure 8. Viral infectivity and replication kinetics in the seven cell lines. (A) Cells were infected with serial dilutions of VSV-GFP (left) or SeV-GFP (right), and infectious foci were counted to calculate the infectivity of the viral stock for each cell line. (B) One-step kinetics of viral replication in seven cell lines. Cells were infected in parallel with VSV-GFP or SeV-GFP at MOI of 3 CIU/cell (1 h absorption), washed 3 times with PBS, and kept in SFM. The media containing newly generated virions was collected at the indicated time points and viral titrations were performed on BHK21 (for VSV) or Vero cells (for SeV).

2.7 Tables

	VSV	SeV
BHK21	3.0 CIU^{BHK}/cell	7.5 CIU^{HeLa}/cell
BSRT7	22.5 CIU^{BHK}/cell	3.2 CIU^{HeLa}/cell
HeLa	132.0 CIU^{BHK}/cell	3.0 CIU^{HeLa}/cell
A549	13.2 CIU^{BHK}/cell	8.1 CIU^{HeLa}/cell
4T1	227.0 CIU^{BHK}/cell	37.8 CIU^{HeLa}/cell
HEp2	227.0 CIU^{BHK}/cell	4.8 CIU^{HeLa}/cell
Vero	23.7 CIU^{BHK}/cell	4.3 CIU^{HeLa}/cell

Table 2. Relative number of infectious virus particles added to different cell lines to achieve MOI 3 for each virus/cell combination. Most experiments in this study were conducted using 24-well tissue culture plates and nearly 100% confluent cells treated with ribavirin in SFM (or mock- treated with SFM) and infected with VSV-GFP or SeV-GFP (or mock-infected with SFM) at MOI of 3 CIU/cell of the tested cell line. The MOI of 3 CIU/cell for each virus/cell type combination was calculated by infecting each cell line with serial dilutions of VSV-GFP or SeV-GFP virus stock in SFM and counting infectious foci with the aid of fluorescence microscopy (see Figure 8A). VSV CIU^{BHK} - number of cell infectious units (infectious particles) determined by titration of VSV-GFP virus stock on BHK21 cells. SeV CIU^{HeLa} - number of cell infectious units calculated by titration of SeV-GFP virus stock on HeLa cells.

2.7 Tables continued

Cell type	IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)
	VSV-GFP		SeV-GFP	
BHK21	275	1100	190	850
BSRT7	10	40	16	40
HeLa	70	150	40	110
A549	190	610	90	320
4T1	20	60	10	18
HEp2	70	310	12	45
Vero	2250	>3000	1550	>3000

Table 3. Antiviral activity (IC_{50} and IC_{90}) of ribavirin against VSV and SeV in different cell types. The 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) for ribavirin were estimated by means of the plaque reduction (Fig. 4) as described in Materials and Methods. Data are expressed as mean without standard error of mean that, however, never exceeded 20% of the mean values. Note that an extremely poor potency of ribavirin against VSV and SeV in Vero did not allow it to reach IC_{90} even at 3000 μM ribavirin concentration (">3000")

CHAPTER 3: RESISTANCE OF PANCREATIC CANCER CELLS TO ONCOLYTIC VESICULAR STOMATITIS VIRUS: ROLE OF TYPE I INTERFERON SIGNALING

3.1 Background

VSV as an anticancer agent against pancreatic ductal adenocarcinoma.

Pancreatic ductal adenocarcinoma (PDA) is the most common form of pancreatic cancer and accounts for more than 85% of all pancreatic malignancies. PDAs are highly invasive and rapidly metastasized to different tissues (Stathis A and Moore 2010). Due to poor prognosis the survival rate of patients for more than 5 years is less than 5%. A majority of patients die between 4-6 months after initial diagnosis (Jemal, Siegel et al. 2010). PDA initiates with alterations in oncogenes and tumor suppressor genes, which leads to a series of pre-invasive stages of PDA known as pancreatic intraepithelial neoplasias (PanINs). These PanINs eventually results in metastatic invasive PDA (Farrow B, Albo D et al. 2008). These lesions recruit different immune cells to these sites resulting in a massive local inflammatory response. This results in recruitment of regulatory T cells, myeloid derived suppressor cells (MDSCs) for immunosuppression and this immune-privilege leads to the establishment of devastating disease (Farrow B, Albo D et al. 2008).

A complete surgical resection of tumor is the only curative treatment available at this time for PDA. Even with surgical resection only 15–20% patients have the survival rate of greater than 5 years. Even though there has been important development in

understanding the molecular mechanisms of PDA regulation, alternative therapies are urgently needed (Jones, Zhang et al. 2008; Jemal, Siegel et al. 2010).

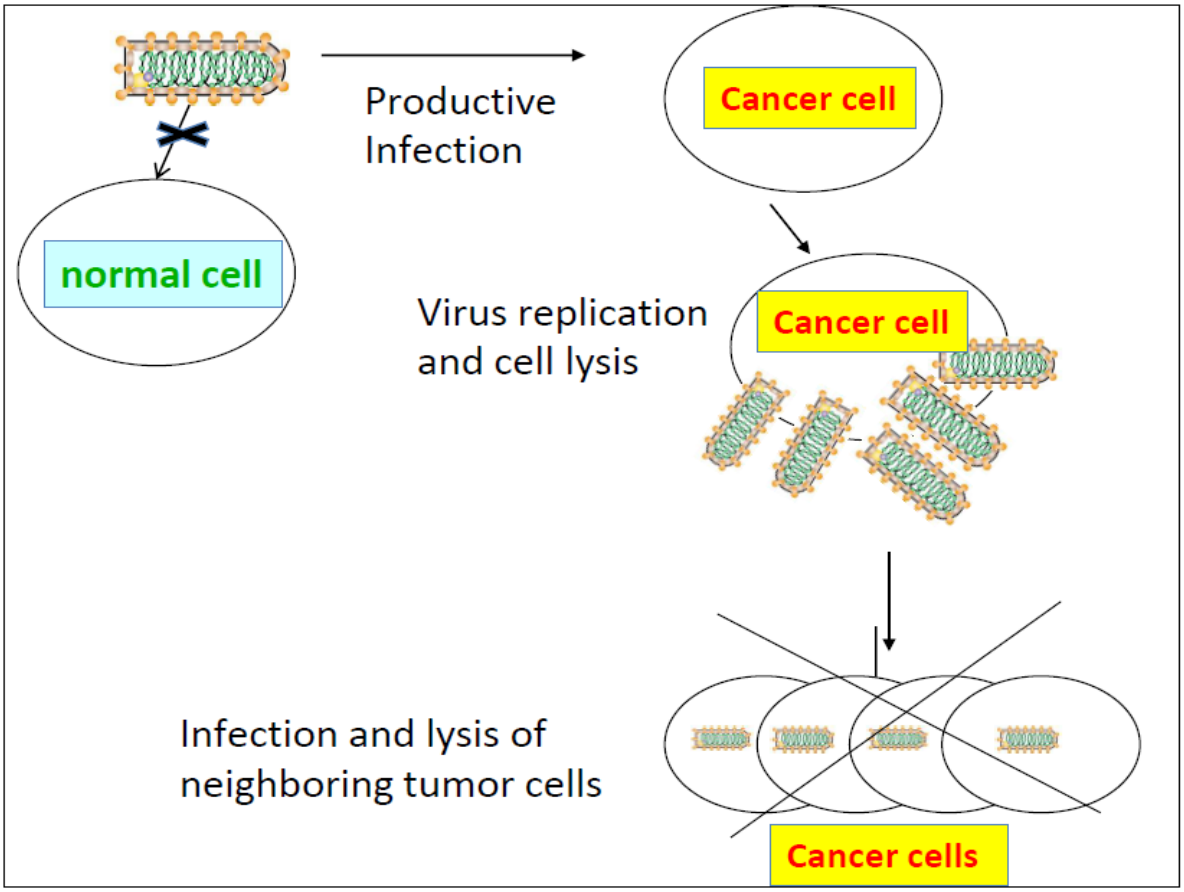

Figure 9. Schematics of a typical oncolytic virotherapy. (Figure is used with the permission from Dr. Andrea Murphy)

In 1893 it was first observed that some patients suffering from cancer infected with viruses exhibited signs of tumor regression (Kelly and Russell 2007; Sinkovics JG and and Horvath 2008). This observation led to scientific research into the development of oncolytic virus (OV) therapy. OV therapy is a relatively new emerging therapeutic option, where specifically tumor cells are being targeted and killed by viruses without infecting healthy cells. Such selectivity is achieved due to the fact that many cancer cells

have defective innate immune responses or deregulation of other signaling pathways to facilitate the replication of viruses selectively in cancer cells (Vaha_Koskela, Tuittila et al. 2003; Breitbach, Reid et al. 2010; Hastie and Grdzelishvili 2012). Defective innate immune responses, such as defects in type 1 IFN response is advantageous for tumor growth, however it makes them more susceptible for selective viral infection (Stojdl, Lichty et al. 2000; Naik and Russell 2009; Hastie and Grdzelishvili 2012; Murphy, Besmer et al. 2012).

Many viruses have been evaluated for their ability to specifically target cancer cells against various kinds of tumors including reovirus, Newcastle disease virus, mumps virus, measles virus, adenovirus, VSV, vaccinia virus, Herpes simplex virus etc. Viruses which require specific surface markers or nuclear transcription factors expressed exclusively by specific cancer cells can selectively kill those tumor cells. Alternatively, specific oncolytic viruses can be genetically engineered which can exclusively infect and kill various tumor cells by exploiting their defective antiviral immune responses (Russell, Peng et al. 2012).

Several characteristics of VSV make it a promising OV against various types of cancers. Compared with other viruses for oncolytic virotherapy, VSV has some obvious advantages. VSV biology is very well studied and VSV is relatively independent of any host receptor for infection and cell cycle. VSV can be easily propagated to very high titer and infect a majority of cell lines from various hosts and origin (Barber 2004). Moreover, VSV replicates within the cytoplasm, which prevents the risk of any host cell transformation. Human also do not possess any pre-existing immunity against VSV. The relatively small genome of VSV can be easily manipulated to make a better more

efficient and safer oncolytic VSV (Barber 2004; Hastie and Grdzelishvili 2012). Similar to other OVs, there are some limitations that need to be overcome to make VSV a successful candidate for future clinical successes.

In the past decade a great number of recombinant VSVs (rVSV) have been developed by different laboratories to make more efficient OVs to reduce tumor burden without infecting healthy cells of hosts. Currently, several approaches have been utilized to improve the safety and oncoselectivity of VSV as an OV. The most common and most popular approach employs mutating VSV M protein, which is required by VSV to evade the antiviral innate immune responses by non-cancerous normal host cells. VSV M protein mutants retain their ability to kill tumor cells by improving their oncoselectivity and safety. Most studies have used a VSV M mutant with mutation or deletion of methionine at 51^{st} position of the M protein. In our study we used the VSV with the deletion at 51^{st} position of M protein and the green fluorescent protein (GFP) in the viral genome for the easy monitoring of virus infection. This strain will be referred to as VSV-Δ-M51-GFP throughout the dissertation. This mutation results in the prevention of binding M protein with the Rae1-Nup98 mRNA complex. This interaction is critical for VSV to inhibit the transport of cellular mRNA from nucleus to cytoplasm (Petersen, Her et al. 2000) and a major mechanism through which VSV evaded the host innate immune responses (Hastie and Grdzelishvili 2012). VSV M mutants are unable to block this transport making them extremely susceptible to host antiviral responses and thus provide enhanced safety including lack of neurotoxicity *in vivo*. Moreover, this added feature makes VSV a better OV because a majority of tumors are known to have defective innate

immune responses and VSV-Δ-M51 can selectively kill tumor cells with improved safety.

Role of host type I IFN responses in resistance to OV therapy.

A previous study from our laboratory demonstrated for the first time that VSV is a promising oncolytic agent against PDA (Murphy, Besmer et al. 2012). In this study a majority of tested PDA cell lines were susceptible to VSV-Δ-M51-GFP infection and efficiently killed by virus infection. However, five PDA cell lines showed resistance to VSV-Δ-M51-GFP infection at least at low multiplicity of infection (MOI) (Murphy, Besmer et al. 2012). One of the resistance cells chosen for *in vivo* experiments exhibited the same phenotype as *in vitro* and tumor burden was not reduced compared to VSV-Δ-M51-GFP susceptible cell line. Resistance to oncolytic VSV infection has been reported with other cancer cell types including prostate cancer, bladder cancer, sarcoma, chronic lymphosytic leukemia (Carey, Ahmed et al. 2008; Tumilasci, Oliere et al. 2008; Zhang, Matsui et al. 2010; Janelle, Brassard et al. 2011; Paglino and van den Pol 2011). Many studies have provided evidences which suggest that resistance to OV therapy is mainly host driven and not virus driven. For example, a synovial sarcoma cell line was found to be resistant to three very different viruses including VSV-Δ-M51, Sindbis virus and cytomegalovirus (CMV) (Paglino and van den Pol 2011). Previous study from our laboratory also evaluated the oncolytic potential of variants of VSV, RSV, SeV and conditionally replicative adenoviruses (CRAds) in 13 PDA cells and found that some PDA cells are resistant to all of these viruses (Murphy, Besmer et al. 2012). These data suggests that host factors play a key role in determining the oncolytic potential and success of OV therapy.

There are various mechanisms of resistance to VSV oncolysis that have been noted, including intact type I IFN response or defective apoptosis pathway in tumor cells (Gaddy DF and and Lyles 2005; Monsurro, Beghelli et al. 2010; Paglino and van den Pol 2011). However, it is widely accepted that the sensitivity to type I IFN by tumor cells is the major mechanism of oncolytic VSV resistance (Le Boeuf, Diallo et al. 2010; Paglino and van den Pol 2011; Moerdyk-Schauwecker, Shah et al. 2012). Previously our lab demonstrated that compared to VSV susceptible PDA cells, VSV resistant PDA cells both produced and responded to type I IFN signaling (Murphy, Besmer et al. 2012). In contrast to these results it is commonly believed that most cancer cells have defective type I IFN responses, because intact type I IFN response negatively affects tumor development (Barber 2004; Lichty BD, Power AT et al. 2004; Murphy, Besmer et al. 2012). Type I IFN responses are usually anti-angiogenic, anti-proliferative and pro-apoptotic (Wang, Rahbar et al. 2011). Despite of these, various cancer cells have been shown to synthesize or respond to type I IFN including lymphomas (Sun, Pabon et al. 1998), melanomas (Linge, Gewert et al. 1995; Wong, Krauer et al. 1997) , bladder cancer (Matin, Rackley et al. 2001) , mesotheliomas (Saloura, Wang et al. 2010), renal cancer(Pfeffer, Wang et al. 1996) and probably other cancers (Stojdl DF 2003).

OVs targeting different signaling pathways to mount a strong attack against tumor cells can enhance the cancer therapy drastically. A number of studies with various cancers have shown that elevated levels of certain ISGs are directly responsible for the resistance to various viruses. These ISGs are usually upregulated through the type I IFN induced JAK-STAT signaling (Fig 10). Moreover, a few studies of OV resistant cell culture model of prostate cancer and sarcomas have demonstrated that reducing the level

of these ISGs can enhance the susceptibility to OV (Carey, Ahmed et al. 2008; Paglino and van den Pol 2011). The reduction of ISGs has shown to be achieved by pre-treating cells with the inhibitor of JAK-1 (JAK Inh I) (Paglino and van den Pol 2011), with histone deacetylase inhibitor valproic acid (VPA) (Hoti, Chowdhury et al. 2006; Paglino and van den Pol 2011; Watanabe, Hashimoto et al. 2012) or with vaccinia viral protein B18R (Le Boeuf, Diallo et al. 2010; Paglino and van den Pol 2011) . Treatment with JAK Inh. I, VPA or vaccinia viral protein B18R can attenuate the type I IFN signaling (Paglino and van den Pol 2011).

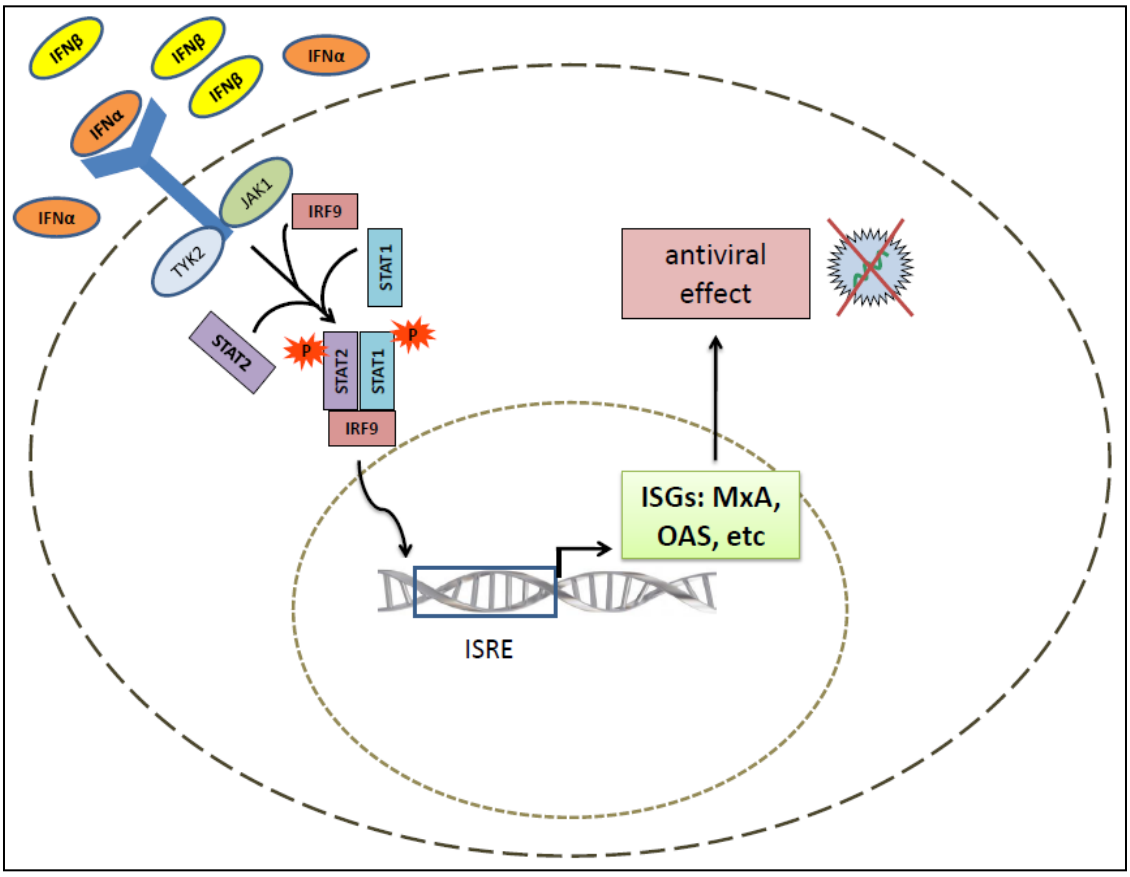

Figure 10. The type I IFN signaling cascade showing synthesis of ISGs.

In this study, we hypothesized that constitutive expression of specific ISGs is mainly responsible for the resistance of PDA to oncolytic VSV and the resistance can be overcome by selective inhibition of the type I IFN signaling. To test this hypothesis the role of type I IFN signaling in the resistance to oncolytic VSV has been investigated in many PDA cells. Moreover, we also determined whether the PDA cells resistant to VSV-Δ-M51-GFP can become more susceptible to infection when treated with inhibitor of type I IFN signaling. To overcome the resistance of specific PDA cells to oncolytic VSV infection, it is extremely important to find the host factors responsible for such phenotypes. Because, type I IFN response is mainly responsible for such resistance, 33 genes involved with type I IFN response were evaluated for the expression of mRNA. Our data clearly demonstrated that at least two host factors MxA and OAS (known antiviral genes) were constitutively expressed in only VSV-Δ-M51-GFP resistant cells (Fig. 10).

These results also correlated with the protein expression data for both genes. Our data also indicated that in virus infected cells the reduced levels of MxA and OAS correlate with the increased susceptibility to VSV-Δ-M51-GFP infection. Even though we demonstrated that a role of type I IFN responses in the resistance of PDA cells to VSV-Δ-M51-GFP infection, it is possible that other cellular factors may influence the susceptibility of VSV-Δ-M51-GFP to various PDA.

Overall, our results demonstrate the importance and practical implications of studying specific host-viral interactions to improve the oncolytic virotherapy.

3.2 Introduction

Oncolytic virus (OV) therapy utilizes viruses with naturally inherited or engineered properties enabling them to preferentially infect and kill cancer cells (Russell and Peng 2007; Vähä-Koskela MJ, Heikkilä JE et al. 2007; Breitbach, Reid et al. 2010). This approach utilizes common cancer characteristics such as defective innate immune responses or abnormalities in regulation of mRNA translation or cellular signaling pathways to provide the needed cancer specificity.

Vesicular stomatitis virus (VSV) has been successfully used as an OV in preclinical models of a number of malignancies [reviewed in (Barber 2004; Hastie and Grdzelishvili 2012)]. As a result, a clinical trial using VSV against hepatocellular carcinoma is currently in progress (Clinicaltrials.gov, 2012, Trial ID: NCT01628640). A number of oncolytic VSV recombinants have been developed to address safety concerns relating to the use of wild-type (wt) VSV. In one of these, VSV-ΔM51-GFP, a deletion of the methionine at amino acid position 51 of the matrix (M) protein prevents shut down of cellular gene expression (Ahmed, McKenzie et al. 2003), providing enhanced safety, including an absence of neurotoxicity in vivo, while still demonstrating good oncolytic potential (Stojdl DF 2003; Ebert O, Harbaran S et al. 2005; Goel, Carlson et al. 2007; Ahmed M 2008; Wu L 2008; Kelly EJ, Nace R et al. 2010; Wollmann G 2010).

We recently tested wild-type (wt) VSV and two non-neurotropic VSV recombinants (including VSV-ΔM51-GFP), as well as recombinant Sendai virus, recombinant respiratory syncytial virus and two recombinant adenoviruses against a panel of human pancreatic ductal adenocarcinoma (PDA) cell lines (Murphy, Besmer et

al.). PDAs are highly aggressive and metastatic (Stathis A and Moore 2010) and represent about 95% of pancreatic cancers. PDA is one of the most lethal abdominal malignancies (Lindsay TH, Jonas BM et al. 2005; Farrow B, Albo D et al. 2008), and current treatments are largely ineffective (Stathis A and Moore 2010). Our study demonstrated VSV is a promising oncolytic agent against PDA, as the majority of PDA cell lines tested were highly susceptible to infection and killing by VSV recombinants (Murphy, Besmer et al.). However, five PDA cell lines as well as the non-malignant HPDE cell line were resistant to most VSV recombinants, (wt VSV, VSV-ΔM51-GFP, and VSV-p1-GFP), at least at low multiplicities of infection (MOI), the expected scenario in vivo.

Unlike permissive PDA cell lines, most resistant PDA cell lines were able to both secrete and respond to type I interferon (IFN), suggesting intact type I IFN responses contributed to their resistance phenotype (Murphy, Besmer et al.). While other mechanisms have been noted (Barber 2004; Hastie and Grdzelishvili 2012), type I IFN sensitivity is believed to be a major factor contributing to VSV's oncoselectivity, as it is unable to efficiently infect healthy cells. In contrast, the majority of cancer cells are thought to be defective in type I IFN production and responses (Barber 2004; Lichty BD, Power AT et al. 2004; Hastie and Grdzelishvili 2012), as IFN responses are generally anti-proliferative, anti-angiogenic and pro-apoptotic (Wang, Rahbar et al. 2011), conditions unfavorable for tumor formation. However, some cancer cells are known to produce and/or respond to type I IFN (Stojdl, Lichty et al. 2000; Naik and Russell 2009), including some mesotheliomas (Saloura, Wang et al. 2010), melanomas (Linge, Gewert et al. 1995; Wong, Krauer et al. 1997), lymphomas (Sun, Pabon et al. 1998), bladder

cancers (Matin, Rackley et al. 2001), renal cancers (Pfeffer, Wang et al. 1996), and possibly other cancers (Stojdl DF 2003).

Here we further analyze a panel of 11 clinically relevant human PDA cell lines for the presence of type I IFN response, determine the functionality of that response in resistance to VSV-ΔM51-GFP and attempt to identify an RNA and/or protein which presence or absence was well correlated with resistance to this virus. The cell lines most resistant to VSV-ΔM51-GFP infection were shown to constitutively express at least some interferon stimulated genes (ISGs), including the antiviral genes MxA and OAS. Inhibition of the JAK/STAT signaling pathways reduced ISG expression and improved VSV-ΔM51-GFP infectivity, replication and oncolysis, implicating IFN responses in resistance.

Apart from type I IFN signaling, other mechanisms have been implicated to induce ISGs. For example ISGs can be upregulated by activation of NF-κB pathway (Basagoudanavar, Thapa et al. 2011), via chromatin modification (Paglino and van den Pol 2011) or through IFN-λ signaling. Our laboratory has planned to explore these other mechanisms of ISG stimulation in future. I am currently investigating the potential role of NF-κB activation in the induction of ISGs and conferring resistance of PDA cells to VSV-ΔM51-GFP.

3.3 Materials and Methods:

Cell lines.

The human PDA cell lines used in this study were: AsPC-1 (ATCC CRL-1682), Capan-1 (ATCC HTB-79), Capan-2 (ATCC HTB-80), CFPAC-1 (ATCC CRL-1918), HPAC (ATCC CRL-2119), HPAF-II (ATCC CRL-1997), Hs766T (ATCC HTB-134), MIA PaCa-2 (ATCC CRL-1420), Panc-1 (ATCC CRL-1469), Suit2 (Iwamura T, Katsuki T et al. 1987) and T3M4 (Okabe T, Yamaguchi N et al. 1983). In addition, a non-malignant human pancreatic duct epithelial (HPDE) cell line (Furukawa T, Duguid WP et al. 1996) was used and maintained in Keratinocyte-SFM (K-SFM, Gibco). This cell line was generated by introduction of the E6 and E7 genes of human papillomavirus 16 into normal adult pancreas epithelium, retains a genotype similar to pancreatic duct epithelium and is non-tumorigenic in nude mice (Furukawa T, Duguid WP et al. 1996). The mouse breast cancer cell line 4T1 (ATCC CRL-2539), baby hamster kidney BHK-21 fibroblasts (ATCC CCL-10) and African green monkey kidney Vero cells (ATCC CCL-81) were used to grow viruses and/or as controls. Capan-1, CFPAC-1, HPAC, MIA PaCa-2, Panc-1, Suit2, 4T1 and Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Cellgro). AsPC-1, Capan-2 and T3M4 cells were maintained in RPMI 1640 (HyClone). HPAF-II and BHK-21 cells were maintained in modified Eagle's medium (MEM, Cellgro). All cell growth media were supplemented with 9% fetal bovine serum (FBS, Gibco), 3.4 mM L-glutamine, 900 U/ml penicillin and 900 µg/ml streptomycin (HyClone). MEM was further supplemented with 0.3% glucose (w/v). K-SFM was never supplemented with serum. Cells were kept in a 5% CO_2 atmosphere at 37°C. For all experiments, PDA cell lines were passaged no more than 10 times

Viruses.

The following viruses were used in this study: VSV-ΔM51-GFP, vaccinia virus expressing T7 (VVT7) and herpes simplex virus type 1 (HSV-1) (MacIntyre strain; ATCC, VR-539). VSV-ΔM51-GFP has a deletion of methionine at amino acid position 51 of the M protein and the green fluorescent protein (GFP) ORF inserted at position 5 of the viral genome (Wollmann G 2010). VVT7 was created by integration of the bacteriophage T7 RNA polymerase into the vaccinia virus (strain Western Reserve) thymidine kinase gene (Fuerst, Niles et al. 1986/11). VSV-ΔM51-GFP was grown on BHK-21 while VVT7 and HSV-1 were grown on Vero. Viral titers were determined by standard plaque assay on 4T1 and/or Vero cells and expressed as cell infectious units (CIU) per ml.

RNA analysis.

Cells were either mock-treated, infected with VSV-ΔM51-GFP at MOI of 10 CIU per cell (based on 4T1) or treated with 5,000 U/ml IFN-α (Calbiochem, 407294). Total RNA was extracted from cells at 4 or 12 hours (h) post-infection (p.i.) using the Quick-RNA Mini Prep kit in accordance with manufacturer instructions (Zymo Research). 0.5 μg of total RNA per reverse transcription (RT) reaction using SMARTScribe reverse transcriptase (Clontech) was used for the cDNA synthesis as per manufacturer's protocol. PCR was carried out on this cDNA using the following conditions: denaturation at 94°C for 45 seconds (s), annealing at 57°C for 45 s, extension at 72°C for 45 s for either 25, 30 or 35 cycles and a finishing step at 72°C for 8 min. All primers for PCR are shown in the Table 7 and were designed to not amplify genomic DNA. PCR products were

electrophoresed on a 2% agarose gel with ethidium bromide and photographed using a GelDoc-It imager (UVP imaging, Upland, CA).

Western blot.

Cellular lysates were prepared from cells either mock-treated, infected with VSV-ΔM51-GFP at MOI 10 CIU/cell (based on 4T-1) or treated with 5,000 U/ml IFN-α. At 1 h p.i., virus was aspirated and cells were extensively washed and incubated in growth media containing 5% FBS. Cells were harvested at 12 h p.i. and lysed in lysis buffer (pH 7.5) containing 1% Triton-X-100, 20mM Hepes, 0.15 M NaCl, 2 mM EDTA and supplemented with c-inhibitor (2X, Roche) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich). Total protein concentration was determined by Bradford assay. Twenty µg of total protein was separated by electrophoresis on 10% SDS-PAGE gels and electroblotted to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked using 5% non-fat powdered milk in TBS-T [0.5 M NaCl, 20 mM Tris (pH 7.5), 0.1%Tween20]. Membranes were incubated with 1:5000 rabbit polyclonal anti-VSV antibodies (raised against VSV virions), 1:1000 rabbit anti-MX1 (Sigma-Aldrich, SAB1100070), 1:200 rabbit anit-OAS2 (Santa Cruz, sc-99097), 1:3000 mouse anti-GFP (Rockland, 600-301-215), and the following antibodies from Cell Signaling (1:1000): anti-STAT1 (9172), Stat1-P (9171), Stat2 (4594), Stat2-P (4441), IRF3 (4302), IRF3-P (4947), eIF2α (5324), and eIF2α-P (3398) in TBS-T with 5% BSA. Detection was with 1:2000 goat anti-rabbit or 1:5000 goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, 111-035-003 and 115-035-003, respectively) using the Enhanced Chemiluminescence Plus (ECL+) protein detection system (GE Healthcare).

Membranes were reprobed with mouse anti-actin antibody (clone C4) (Moyer, Baker et al. 1986/8) to verify sample loading.

JAK Inhibition.

For new infectious particle production, 6-well plates were seeded such that they were approximately 80% confluent at the time of inhibitor treatment. Cells were pretreated with 0.5 or 2.5μM JAK inhibitor I (Jak Inh I, Calbiochem) or vehicle (DMSO) only in cell culture media with 5% FBS (K-SFM was used without FBS) for 48 h prior to infection (media was removed and replaced with fresh drug/vehicle containing media after the first 24 h). Cells were then mock infected or infected with VSV-ΔM51-GFP in DMEM without FBS at an MOI of 10 CIU/cell (based on 4T1). Following a 1 h absorption period, the virus containing media was aspirated, cells were washed three times with PBS and growth media with 5% FBS containing either 0.5 or 2.5μM JAK Inh. 1 or vehicle was added to the wells. At 16 h p.i., media was collected and used for a standard plaque assay on BHK-21 cells. Cells treated and infected in the same manner were also used to prepare cellular lysates for western blotting as described above.

For plaque and cell viability assays, cells were seeded in 96-well plates such that they were approximately 80% confluent at the time of inhibitor treatment, and pretreated for 48 h with JAK Inh. I as described above. For the plaque assay, cells were then infected with 8-fold serial dilutions of VSV-ΔM51-GFP in DMEM without FBS. Following a 1 h absorption period, the virus containing media was aspirated and replaced with cell culture media containing 5% FBS and either JAK Inh I or vehicle. At 17 h p.i., fluorescent foci were visualized and counted using fluorescent microscopy. For the cell viability assay, following pretreatment, cells were mock infected or infected with VSV-

ΔM51-GFP in DMEM without FBS at a MOI of 1 CIU/cell (based on 4T1). Following a 1 h absorption period, the virus containing media was aspirated and replaced with growth media with 5% FBS containing either 0.5 or 2.5 μM JAK Inh. 1 or vehicle. GFP fluorescence was measured every 24 h using a CytoFluor multi-well plate reader (PerSeptive Biosystems) with excitation filter of 450/50nm, emission filter of 530/25nm and gain=50. At 5 days (d) p.i., cell viability was analyzed using a 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) cell viability assay (Biotium) in accordance with manufacturer instructions.

Permissiveness of cells to different viruses.

Cells were seeded in 24-well plates such that they were approximately 90% confluent at the time of infection. Cells were infected with 8-fold serial dilutions of VSV-ΔM51-GFP, VVT7, or HSV-1 in DMEM without FBS for 1 h. Virus was aspirated and cells were overlaid with the appropriate growth media containing 5% FBS and 0.5% BactoAgar. For VSV-ΔM51-GFP, fluorescent foci were visualized at 3 d p.i. by fluorescent microscopy. For all viruses, at 5 d p.i. cells were fixed by addition of 350 μl 10% neutral buffered formalin (Sigma-Aldrich) to each well. Following 1 h incubation at room temperature, the agar was gently removed and cells stained with 1% crystal violet in 20% methanol to allow visualization of plaques.

Cell viability following infection with different viruses.

Cells were seeded in 96-well plates such that they were approximately 80% confluent at the time of infection. Cells were mock infected or infected with VSV-ΔM51-GFP, VVT7, or HSV in SFM-MegaVir, at an MOI of 1 or 0.01 CIU/cell (based on Vero). Following a 1 h absorption period, the virus containing media was aspirated and

replaced with growth media containing 5% FBS. At 5 d p.i., cell viability was analyzed using an MTT cell viability assay (Biotium) in accordance with manufacturer instructions.

Statistical Analysis.

All statistical analyses were performed using GraphPad Prism, version 5.03 for Windows (GraphPad Software, San Diego, California). Comparisons within a cell line following treatment were made using a one-way ANOVA with Bonferroni post-test.

3.4 Results

Identification of antiviral genes constitutively expressed in PDA cells resistant to VSV.

Our recent evaluation of VSV against human PDA cell lines revealed substantial diversity in their susceptibility to VSV-mediated oncolysis, which correlated with permissiveness to VSV infection (Murphy, Besmer et al.). Most resistant PDA cell lines were both sensitive to IFN-α treatment (which strongly inhibited VSV infection) and capable of secreting IFN-β following VSV infection (Murphy, Besmer et al. 2012), suggesting resistant PDA cell lines may retain active type I IFN signaling. In this study, we assessed type I IFN related cellular responses to VSV-ΔM51-GFP infection on a molecular level in a panel of 11 clinically relevant human PDA cell lines (Table 4), and examined the role of the JAK/STAT signaling pathways in the observed resistance phenotypes.

Expression of 33 human genes (Table 7) responsible for sensing viral infection [e.g. retinoic-acid-inducible gene I (RIG-I)], activating production of type I IFNs [e.g. IFN-regulatory factor (IRF) 3], sensing IFNs [e.g. interferon alpha/beta receptor 1 (IFNAR1)] and inducing an antiviral state in cells [e.g. myxovirus (influenza) resistance 1 (MxA)] was assessed. Since we wanted to focus on a specific set of genes, this was done using semi-quantitative RT-PCR. Genes were selected based on their importance in type I IFN responses. Some of these genes, particularly ISGs, are expressed in response to type I IFNs, and are expected to be upregulated in cells with intact type I IFN signaling following VSV infection. Also, differential expression of RIG-I (Wilden, Fournier et al. 2009), myeloid differentiation primary response protein MyD88 (MyD88) (Wongthida, Diaz et al. 2011), IRF3 (Marozin, Altomonte et al. 2008; Wilden, Fournier et al. 2009),

IRF7 (Wilden, Fournier et al. 2009) IFNAR1/2 (Saloura, Wang et al. 2010; Zhang, Matsui et al. 2010), tyrosine-protein kinase JAK1 (JAK1) (Dunn, Sheehan et al. 2005), signal transducer and activator of transcription (STAT) 1 (Lee, Bluyssen et al. 1997; Sun, Pabon et al. 1998), IRF9 (Matin, Rackley et al. 2001; Saloura, Wang et al. 2010)**,** MxA (Monsurro, Beghelli et al. 2010; Paglino and van den Pol 2011), 2'-5'-oligoadenylate synthetase (OAS) (Monsurro, Beghelli et al. 2010; Saloura, Wang et al. 2010), and interferon-induced, double-stranded RNA-activated protein kinase (PKR) (Saloura, Wang et al. 2010) has been previously reported to correlate to or be responsible for the virus or IFN resistance phenotypes of various cancer cell types.

PDA cells were mock infected or infected with VSV-ΔM51-GFP for 4 h, and total RNA was isolated and analyzed for expression of candidate genes. As shown in Figure 11, most analyzed genes (including all additional genes listed in Table 7 but not shown in Figure 11) were expressed at similar levels in all cell lines regardless of the VSV resistance phenotype. However, several genes were clearly differentially expressed. Among genes associated with sensing viral infection, IRF7 was down regulated in Capan-1 and MIA PaCa-2, both highly susceptible to VSV-mediated cell death (Murphy, Besmer et al. 2012). In agreement with a key role for IRF7 in the expression of type I IFNs following viral infection (Honda, Yanai et al. 2005), these same two cell lines also lacked IFN-α and β gene expression even after VSV-ΔM51-GFP infection (Fig. 11). Interestingly, reduced expression of IRF-7 has recently been linked to increased metastasis in breast cancer, with disruption of IFN signaling identified as the primary cause (Bidwell, Slaney et al. 2012).

Although in our previous study only 4 cell lines (HPAF-II, HPAC, Hs766T and non-malignant HPDE) produced detectable levels of secreted IFN-β at 18 h p.i. (Murphy, Besmer et al. 2012), here VSV-ΔM51-GFP at 4 h p.i. induced production of IFN-β mRNA in all cell lines except Capan-1 and MIA PaCa-2 (Fig. 11). Most cell lines (again, with the exception of Capan-1 and MIA PaCa-2) also showed constitutive expression of IFN-α mRNA that was only marginally increased upon VSV-ΔM51-GFP infection at 4 h p.i. The highest constitutive levels of IFN-α mRNA were seen in three of the resistant cell lines, HPAF-II, Hs766T and HPAC. While differences in IRF7, IFN-α and IFN-β mRNA expression were observed, these alone could not discriminate between the phenotypes of PDA cells based on their permissiveness to VSV.

As shown in Figure 11, differences were not seen in the expression of the IFN-α/IFN-β receptor (IFNAR1 and IFNAR2), or the major components of IFN type I signal transduction (JAK1, STAT1, STAT2 and IRF9) at the transcriptional level. However, there was great variability in the expression of two ISGs, MxA and OAS. Importantly, there was a correlation between virus resistance phenotype and the levels of MxA and OAS mRNA. MxA and OAS were at the highest levels in the resistant cell lines HPAF-II, Hs766T, HPAC and non-malignant HPDE; at lower levels (especially in the absence of infection) in less resistant Suit2, T3M4 and CFPAC-1; and minimally produced in the remaining cell lines, all of which are highly susceptible to VSV-ΔM51-GFP infection. Interestingly, while MxA and OAS had variable mRNA expression in different PDA cell lines a 4 h p.i., a similar pattern was not observed with other antiviral ISGs such as PKR, RNAse L, ISG56 and viperin.

To confirm the association of MxA and OAS expression with the resistance of PDA cells to VSV-ΔM51-GFP, we conducted a similar experiment with a representative group of 7 cell lines mock-treated, treated with IFN-α, or infected with VSV-ΔM51-GFP for 12 h to allow for accumulation of activated proteins. In this experiment (Fig. 12), we conducted not only mRNA analysis (as in Figure 11), but also western blot analysis to determine protein levels of the analyzed genes. In addition to the non-malignant HPDE, which has intermediate resistance, we analyzed two highly resistant (HPAF-II, Hs766T), one intermediate (HPAC) and three susceptible cell lines (MIA PaCa-2, AsPC-1, Suit2). Susceptibility to viral infection was confirmed based on virus-directed GFP expression, which we have previously shown correlates well with viral protein synthesis (Murphy, Besmer et al. 2012). Importantly, among susceptible cell lines, we selected two cell lines (AsPC-1 and Suit2) previously shown to be responsive to IFN-α treatment and one cell line (MIA PaCa-2) which was completely resistant to IFN-α treatment (Murphy, Besmer et al. 2012). As shown in Figure 12, a correlation was observed between MxA and OAS accumulation (mRNA and protein) and the resistance phenotype of PDA cell lines.

Again, all resistant cell lines showed constitutive expression of MxA and OAS even in the absence of virus infection or IFN treatment. The expression of MxA and OAS is controlled via STAT1 and STAT2 activation. Although variations in mRNA levels were not observed for STAT1 and STAT2 (Fig. 11 and 12), we tested whether phosphorylation of the protein products differed in VSV permissive and resistant cell lines. As shown in Figure 12, although most cell lines had detectable levels of phosphorylated STAT1 protein following 12-h IFN-α treatment, the highest levels of STAT1 activation following VSV-ΔM51-GFP infection were observed in HPAF-II,

Hs766T and HPAC, all of which are resistant. Also, lower but detectable levels of phosphorylated STAT1 in untreated cells were detected in these three cell lines and in HPDE, which may at least partially explain the constitutive expression of MxA and OAS in these cell lines. Among the susceptible cell lines, STAT1 was activated in AsPC-1 and to a much lesser degree in Suit2 upon infection, but not in MIA PaCa-2, although it was strongly activated in MIA PaCa-2 upon IFN treatment. A similar pattern of protein phosphorylation was observed for STAT2 (Fig. 12).

To test whether PDA cell lines susceptible to VSV-ΔM51-GFP are able to detect VSV infection, we analyzed phosphorylation of IRF3. Interestingly, all tested PDA cell lines showed increased IRF3 phosphorylation in response to infection (Fig. 12), suggesting the upstream components of the RIG-I pathway are functional. In agreement with this observation, type III IFN-λ mRNA was expressed in all cell lines in response to VSV-ΔM51-GFP infection (Fig. 11 and 12). However, at 12 h p.i., MIA Paca-2 still failed to activated IFN-α and IFN-β mRNA expression.

Key differences in mRNA and protein expression are summarized in Table 4 and Table 5 respectively. Together, our gene expression data suggest all tested PDA cell lines are capable of sensing VSV infection, most produce type I IFNs, and most are capable of sensing IFN. However, resistant cell lines were characterized by constitutive expression of at least two ISGs, MxA and OAS, which possibly contribute to their resistance phenotype.

Improved oncolysis of resistant PDA cells by combination treatment with JAK inhibitor I.

Gene expression data showed a correlation between resistance to VSV-ΔM51-GFP infection and elevated expression of at least some ISGs. Therefore, a possible causative role for these and other ISGs in the resistance phenotype was tested by inhibition of the JAK/STAT signaling pathways responsible for activation of ISG expression, using a general inhibitor of JAKs, JAK Inh. I. First, the effectiveness of the JAK Inh I treatment was confirmed by western blot for p-STAT1, MxA and OAS. Twenty-four h treatment with 0.5 or 2.5μM JAK Inh I completely eliminated STAT1 phosphorylation and markedly reduced MxA and OAS protein levels. The same treatments for 48 h, followed by mock infection or infection with VSV-ΔM51-GFP at MOI 10 (based on 4T-1) for 16 h, further reduced MxA and OAS protein to below detectable levels in uninfected cells and sharply reduced them in infected cells (Figure 13). In general, MxA and OAS expression in mock treated cells was consistent with that seen in Figure 12, despite different treatment conditions, although some variation was noted (e.g., MxA levels in HPAC cells).

When new infectious virus particle production was determined following JAK Inh I treatment, by collecting supernatants and titering them on BHK-21, increases were seen for the resistant cell lines (CFPAC, HPAC, HPAF-II, HPDE and Hs766t) (Table 6). Consistent with the improvement in virus production, a robust increase in viral protein accumulation was seen for the resistant cell lines CFPAC, HPAC, HPDE and Hs766T and a slight increase was seen for the resistant cell line HPAF-II. Treatment also caused a modest increase in viral protein accumulation in Suit2, a cell line we classify as

susceptible. While titers were well correlated with viral protein as determined by western blot, this was not absolute, possibly due to low viral titers in some cell lines (especially HPAF-II) and cell line variations in virus replication kinetics and infectious/non-infectious particle ratios.

Our previous study showed a correlation between susceptibility of PDA cells to VSV-mediated cell death and the relative infectivity of VSV on different PDA cell lines (Murphy, Besmer et al. 2012). To determine if inhibition of JAK/STAT signaling improved VSV-ΔM51-GFP infectivity in resistant cells, VSV-ΔM51-GFP was titered on mock treated cells or cells treated with JAK Inh I for 48 h. Consistent with previous results (Murphy, Besmer et al. 2012), titers were lower on resistant cell lines (CFPAC, HPAC, HPAF-II, HPDE, Hs766T) than susceptible cell lines (AsPC-1, MIA PaCa-2, Suit2). This difference remained even with JAK Inh I treatment. However, some improvement in titer was observed on HPAC and HPAF-II with treatment (Figure 14), although it was statistically significant only for HPAF-II. We observed for all resistant cell lines, with the possible exception of HPDE, a clear increase in plaque size upon JAK Inh I treatment. An increase in plaque size was also observed on the susceptible AsPC-1 cell line at the highest inhibitor concentration. This data suggests that while activation of the JAK/STAT pathways contributes to resistance to VSV-ΔM51-GFP in most resistant cell lines, other factors may contribute to the differences in VSV titers in various PDA cell lines.

To determine if the improvement in new infectious particle production and plaque size translated into improved cell killing, cells were pretreated with JAK Inh I for 48 h and infected with VSV-ΔM51-GFP at MOI 1 (based on 4T-1). Virus-directed GFP

expression was measured every 24 h and cell viability was determined at the end of five days by MTT assay. GFP expression increased upon JAK Inh I treatment for all five resistant cell lines plus the susceptible cell lines AsPC-1 and Suit2 (Figure 15). No increase was seen for the susceptible cell line MIA PaCa-2. When cell viability was determined (Figure 16A), a statistically significant increase in VSV-mediated cell death was seen with inhibitor treatment for HPDE, the expected outcome for a "normal' (non-malignant) cell line. Importantly, a similar result was observed in the VSV-resistant CFPAC-1, HPAC and Hs766T cell lines at least at the highest concentration of inhibitor (2.5μM), suggesting an involvement of JAK/STAT signaling in the resistance phenotype of these cell lines. Treatment with JAK Inh. I alone generally did not cause a loss in cell viability, as measured by MTT, although a statistically significant decrease was seen for Hs766T at the lowest concentration only, HPDE at the highest concentration only and Suit2 at both concentrations. Consistent with the possible cytotoxic effects of JAK Inh I on Suit2 and HPDE, virus-directed expression of GFP dropped at the higher concentration (2.5 μM) compared to the lower concentration (0.5 μM), although it still exceeded expression in untreated cells (Figure 15). Interestingly, in some cases, most notably HPAF-II, JAK Inh I caused a significant increase in MTT signal in uninfected cells. This may be due to an increase in the number of viable cells or due to an increase in mitochondrial activity (the actual parameter measured by the MTT assay), which can be caused by stress. To more specifically look at this question, the experiment was repeated with HPAF-II with a parallel plate used for cell counting. While less dramatic than in the previous experiment, treatment with 2.5μM JAK Inh I alone did cause a statistically significant increase in MTT signal (Figure 16B). However, this treatment did

not change the number of viable cells number (Figure 16B). This experiment also confirmed that treatment of HPAF-II with 2.5 μM JAK Inh. I caused an increase in cell killing by VSV-ΔM51-GFP.

Susceptibility of PDA cells to other viruses.

The ability of VSV-ΔM51-GFP to initiate infection on the PDA and HPDE cell lines was assessed by performing a plaque assay using serial dilutions of virus Permissiveness was expressed as a ratio of the viral titer on PDA cell line to the titer on Vero cells with higher numbers indicating greater permissiveness and cell lines listed in order of permissiveness (Figure 17). In confirmation of our previous results, the five resistant cell lines (CFPAC, HPAC, HPAF-II, HPDE and Hs766T) showed the least susceptibility to VSV-ΔM51-GFP, in terms of both plaque size and number, and formed a distinct cluster from the susceptible cell lines. All of these highly resistant cells constitutively express at least some ISGs, including MxA and OAS, not seen in the more susceptible cell lines. In contrast, the two PDA cell lines where VSV-ΔM51-GFP formed the largest plaques (MIA PaCa-2 and Capan-1) are the only cell lines that fail to express both IFN-α and β following VSV-ΔM51-GFP infection (Figure 11 and 12). Previous studies showed that VSV is highly sensitive to type I IFN responses, an effect even more pronounced in VSV-ΔM51-GFP as a result of the methionine 51 deletion in the M protein (Coulon, Deutsch et al. 1990; Black, Rhodes et al. 1993; Stojdl DF 2003). If indeed the susceptibility profile of these cells is at least in part determined by their type I IFN status, it would be expected that viruses capable of evading the host type I IFN response would display a different profile. To test this hypothesis, we used two large DNA viruses unrelated to VSV, recombinant vaccinia virus VVT7 (a poxvirus) and HSV-1 (a

herpesvirus), both of which have been shown to evade type I IFN antiviral responses (Paladino and Mossman 2009; Perdiguero and Esteban 2009). While the specific VV and HSV-1 viruses used in this experiment are not used as OVs, other recombinants based on VV and HSV have been developed for that purpose. When VVT7 and HSV-1 were titered on PDA cell lines, there was not a correlation between the permissiveness of cells to these two viruses (Figure 17) and their type I IFN status (Figure 11 and 12), as indicated by the different ordering of these cells lines by permissiveness as compared to VSV-ΔM51-GFP. This is consistent with the greater abilities of VVT7 and HSV-1 to evade this pathway. The degree of curvature of the graphs in Figure 17 indicates the variability in cell line permissiveness to the viruses. The variability in cell line permissiveness was similar for VSV-ΔM51-GFP and HSV-1 although they differed in which cell lines were susceptible or resistant. In contrast, the range much smaller for VVT7 with the exception of highly resistant (to VVT7) Capan-2

To determine if susceptibility of the PDA cells to these three viruses extended to cell killing, cell viability was determined by MTT assay at 5 d p.i. following infection with VSV-ΔM51-GFP, VVT7 or HSV-1 at MOI 1 or 0.01 as determined by titration on Vero cells that support robust replication of all three viruses (Fig. 18). For VSV-ΔM51-GFP, results closely mimicked those reported in our previous study (Murphy, Besmer et al. 2012). However, all VSV-resistant cell lines (CFPAC-1, HPAC, Hs766T, HPAF-II and HPDE), were more effectively killed by HSV-1 and VVT7 than VSV-ΔM51-GFP at MOI 1, and this was also true for most of those cell lines at MOI 0.01. Importantly, HSV-1 and VVT7 did not demonstrate superior oncolytic abilities in all cell lines (eg. AsPC-1 and Capan-2 at MOI 0.01). Although we cannot rule out that other (IFN-

unrelated) factors influenced cell susceptibility to these three viruses, the results are consistent with type I IFN responses being at least a factor in determining resistance to VSV-ΔM51-GFP infection.

3.5 Discussion

In this study, we demonstrated on a molecular level that the resistant cell lines tested have functional type I IFN responses similar to those observed in the non-malignant HPDE cell line. Importantly, we found that, unlike susceptible PDA cells, resistant cell lines constitutively expressed high levels of MxA and OAS, two important IFN-stimulated antiviral proteins.

We previously showed that while the majority of tested PDA cell lines were highly susceptible to VSV-ΔM51-GFP, five of these cell lines (BxPC-3, CFPAC-1, HPAC, HPAF-II and Hs766T) and the non-malignant pancreatic duct epithelial cell line HPDE were at least somewhat resistant to VSV-ΔM51-GFP mediated oncolysis (Murphy, Besmer et al. 2012). The same pattern of resistance was also observed for wt VSV and VSV-p1-GFP, with a minor deviation for wt VSV in that HPDE and CFPAC were more susceptible than AsPC-1 and T3M4 (Murphy, Besmer et al. 2012). These phenotypes were confirmed here for VSV-ΔM51-GFP (Fig. 17 and 18). However, it should be noted that BxPC-3, previously shown to be highly resistant to VSV (Murphy, Besmer et al. 2012), was omitted from this study as it displayed an unstable phenotype, being generally resistant but occasionally highly susceptible to VSV-ΔM51-GFP. Even though passage numbers were limited for all tested cell lines, we observed that increased passage number tended to correlate with increased susceptibility of BxPC-3 to VSV-ΔM51-GFP suggesting alterations in cell biology may be responsible for this variability. BxPC-3 cells in culture have been shown to undergo an epithelial to mesenchymal transition (Roy, Sahraei et al. 2011). Interestingly, our previous study showed BxPC-3 differed from the other highly resistant cell lines in that secretion of IFN-β was not

detectable following VSV infection (Murphy, Besmer et al. 2012). Further studies are needed to better understand the BxPC-3 phenotype.

In order to mount an effective IFN mediated antiviral response, cells must first detect the virus. For RNA viruses replicating in the cytoplasm, such as VSV, detection occurs primarily through binding of single or double stranded viral RNA to RIG-I or melanoma differentiation associated gene 5 (MDA5) (Nakhaei, Genin et al. 2009; Shmulevitz, Pan et al. 2010). This initiates a signaling cascade resulting in phosphorylation of IRF 3 and 7 and formation of homo- and heterodimeric transcription factors necessary for expression of the type I IFNs α and β. Both of these secreted IFNs bind to the IFNAR1/2 receptor of the infected as well as surrounding non-infected cells, resulting in phosphorylation of the receptor by the Janus kinases JAK1 and TYK2. This results in recruitment and phosphorylation of STAT1 and STAT2 which together with IRF9 form a transcription factor, ISGF3, which recognizes IFN-stimulated response elements (ISRE) leading to transcription of ISGs, many of which have direct antiviral functions or contribute to the formation of an antiviral state. Several of these ISGs, including ISG15, MxA, OAS and PKR, have been shown to effectively inhibit replication of rhabdoviruses such as VSV (Sadler and Williams 2008).

The high sensitivity of VSV to type I IFN responses is a major factor determining VSV's oncoselectivity, as it is believed most cancer cells are defective in type I IFN responses (Stojdl, Lichty et al. 2000; Naik and Russell 2009). However, some cancers retain the ability to produce and/or respond to type I IFN (Stojdl, Lichty et al. 2000; Naik and Russell 2009). For example, PC3 prostate cancer cells (Ahmed M, Cramer SD et al. 2004; Carey, Ahmed et al. 2008), SW982 human sarcoma cells (Paglino and van den Pol

2011), RT-4 and RT112 bladder cancer cells (Zhang, Matsui et al. 2010), and multiple mesothelioma cells lines (Saloura, Wang et al. 2010) have been shown to be resistant to VSV infection at least in part due to IFN responsiveness and/or constitutive ISG expression. Furthermore, constitutive ISG expression was shown to be predictive of permissiveness of several PDA cell lines to adenovirus infection (Monsurro, Beghelli et al. 2010). Given this and our previous data demonstrating many resistant PDA cell lines both produce and respond to type I IFN (Murphy, Besmer et al. 2012), as does the "normal" non-malignant HPDE cell line, we examined the responses of these cell lines to VSV-ΔM51-GFP at the molecular level. All tested cell lines appeared to be able to sense VSV-ΔM51-GFP as seen by production of IFN-λ mRNA and an increase in IRF3 phosphorylation following infection, even in cell lines where IFN-α and/or β transcription is not induced (Fig. 11 and 12).

STAT1 phosphorylation was detected in response to VSV-ΔM51-GFP infection in the resistant cell lines and in the susceptible cell lines AsPC-1 and Suit2, although the response in Suit2 was not robust. In the susceptible Mia PaCa-2 cells, STAT1 was phosphorylated in response to exogenously added IFN-α but not to VSV-ΔM51-GFP, suggesting the inability of this cell line to produce type I IFN (possibly due to poor IRF7 expression).

Importantly, MxA was constitutively expressed at both the mRNA and protein level in all resistant cell lines but in none of the susceptible cell lines. MxA has broad antiviral activity against a wide range of RNA and even some DNA viruses, regardless of subcellular site of replication (Haller and Kochs 2011). It is believed that MxA GTPases recognize viral RNP complexes and form oligomeric rings around them, thereby blocking

their function. MxA has also been implicated in the hyperphosphorylation of VSV P protein, which may interfere with its function (Schuster, Johnston et al. 1996/6/1). Constitutive expression of MxA (270-fold over baseline) was observed in SW982 sarcoma cell line resistant to VSV (Paglino and van den Pol 2011). Similarly, increased expression of MxA in PDA cells was recently associated with resistance to adenovirus-based OV (Monsurro, Beghelli et al. 2010). Furthermore, OAS was constitutively expressed in all the resistant cell lines as well as a few susceptible cell lines, at least at the mRNA level, although the highest levels were detected in resistant cell lines. OAS converts adenosine triphosphate into a series of 20-50 oligoadenylates (2-5A), which activate the latent ribonuclease (RNaseL). The activated OAS-RNaseL system promotes apoptosis, attenuates proliferation, degrades viral and cellular RNA, and inhibits protein synthesis (Justesen, Hartmann et al. 2000; Mandal, Abebe et al. 2011). Previous studies showed increased expression of OAS in PDA cells resistant to adenoviruses (Monsurro, Beghelli et al. 2010), and human mesothelioma cells resistant to VSV (Saloura, Wang et al. 2010). As both MxA and OAS have been shown to have antiviral activity against VSV, they almost certainly contribute to the resistance phenotype of the PDA cells studied here. However, as there are hundreds of ISGs, a number of which also have known antiviral functions (Sadler and Williams, 2008), resistance is likely to involve more than just these two proteins.

It is unclear why these proteins are expressed constitutively at high levels. While low levels of phosphorylated STAT1 were detected in some uninfected resistant cell lines, it is uncertain whether these levels were sufficient for the observed expression of MxA and other ISGs in uninfected cells. However, the strong reduction in MxA and OAS

protein levels upon inhibition of JAK/STAT signaling (Fig. 13) would argue the effect is at least partially mediated through this mechanism. It is also not clear why not all resistant cell lines constitutively express all of the ISGs tested, since the ISGs are all under the control of ISREs. One possibility is that alternative regulatory mechanisms influence expression of some of these genes. For example, RelA (p65 subunit of NF-κB) has been shown to regulate a subset of ISGs (Basagoudanavar, Thapa et al. 2011). IFN-λ is also known to regulate ISG expression, although it regulates those genes through the same mechanisms as type I IFN and the resulting ISG expression profile is thought to be nearly identical (Donnelly and Kotenko 2010). Expression of these genes may also be influenced by chromosome modification as treatment with a histone deacetylase inhibitor was shown to decrease ISG expression and increase VSV infectivity in the SW982 human sarcoma cell line (Paglino and van den Pol 2011). Since the NF-κB signaling regulates several pathways and has been implicated to be involved in carcinogenesis the possible role of its activation in ISG induction is further investigated, however not discussed in this dissertation.

In our study, five of the 11 human PDA cell lines tested showed constitutive expression of the ISGs MxA and OAS. While this collection of cell lines is likely not representative of the clinical situation, a recent study showed that a significant subset of the bulk PDA tissues and xenografted primary PDA cells tested had an mRNA expression profile typical of an inflamed state including upregulation of ISGs such as MxA (Monsurro, Beghelli et al. 2010), demonstrating the existence of this phenotype in the patient population. In addition, we have identified a number of cell lines able to respond to type I IFN (Murphy, Besmer et al.) as well as produce type I IFN in response

to VSV-ΔM51-GFP. While all of these cell lines were susceptible to VSV-ΔM51-GFP in vitro, results presented here suggest that the type I IFN responsiveness of these cells may lead to suboptimal VSV-ΔM51-GFP oncolysis. For example, virus-directed GFP expression increased in both AsPC-1 and Suit2 upon JAK Inh. I treatment (Figure 15) and AsPC-1 plaque size increased when the higher concentration of inhibitor was used.

Given these phenotypes, our results suggest that high constitutive expression of ISGs may be useful biomarkers in identifying PDAs, and possibly other cancers, resistant to OV therapy with VSV or other viruses highly sensitive to IFN. In our study, the ISGs MxA and OAS were particularly well correlated with this phenotype. While PDAs with this profiles are unlikely to be successfully treated with IFN sensitive OVs such as VSV, use of alternative OVs (e.g. vaccinia virus or HSV-1), better equipped to evade IFN responses could be a better option. Alternatively, treatment with more than one OV (combined virotherapy) could also lead to enhanced oncolysis as was previously shown for VSV in combination with vaccinia virus (Le Boeuf, Diallo et al. 2010). Furthermore, any future OV therapy will likely involve a combination of OV(s) and chemotherapy (Ottolino-Perry, Diallo et al. 2010).

While we have demonstrated a role for type I IFN responses in the resistance of PDA cells to VSV-ΔM51-GFP infection, we cannot rule out the possible influence of other factors on susceptibility and/or oncolysis. For example, VSV has been shown to cause cell death via apoptosis (Gaddy DF and and Lyles 2005; Gaddy DF and and Lyles 2007; Sharif-Askari, Nakhaei et al. 2007; Cary, Willingham et al. 2011), and inhibition of apoptosis, a signature of many cancer cells (Hamacher, Schmid et al. 2008), has the potential of limiting/delaying cell death following VSV infection. Studies are in progress

examining the role apoptosis and other factors may have in contributing to the resistance of some PDAs to oncolytic VSV.

3.6 Figures

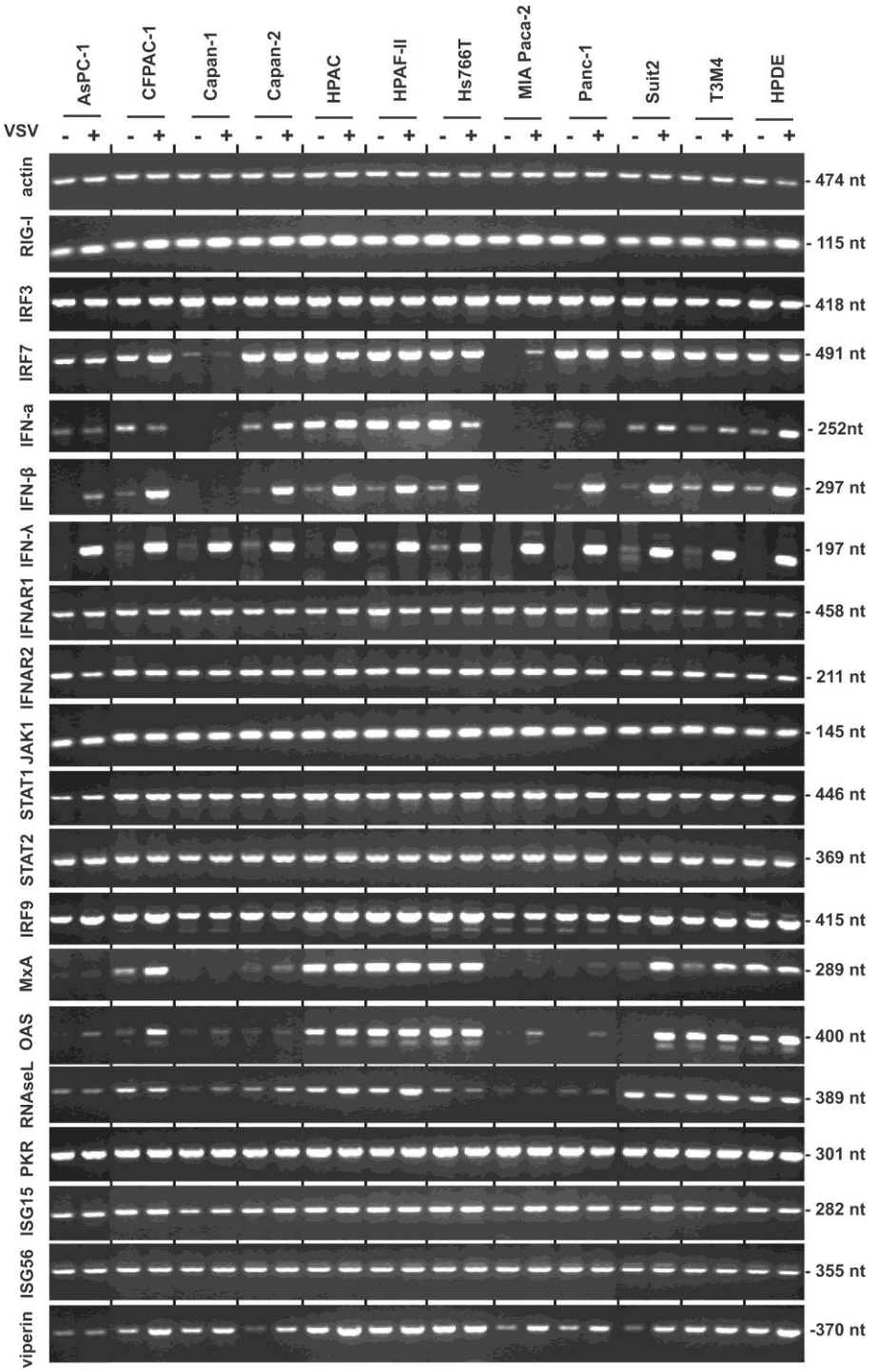

Figure 11. mRNA expression of IFN related genes. Cells were mock infected (-) or infected (+) with VSV-ΔM51-GFP at MOI 10 CIU/cell and harvested at 4 h p.i. Extracted mRNA was reverse transcribed and then analyzed by semi-quantitative PCR for the indicated genes. PCR product sizes are indicated on the right.

3.6 Figures continued

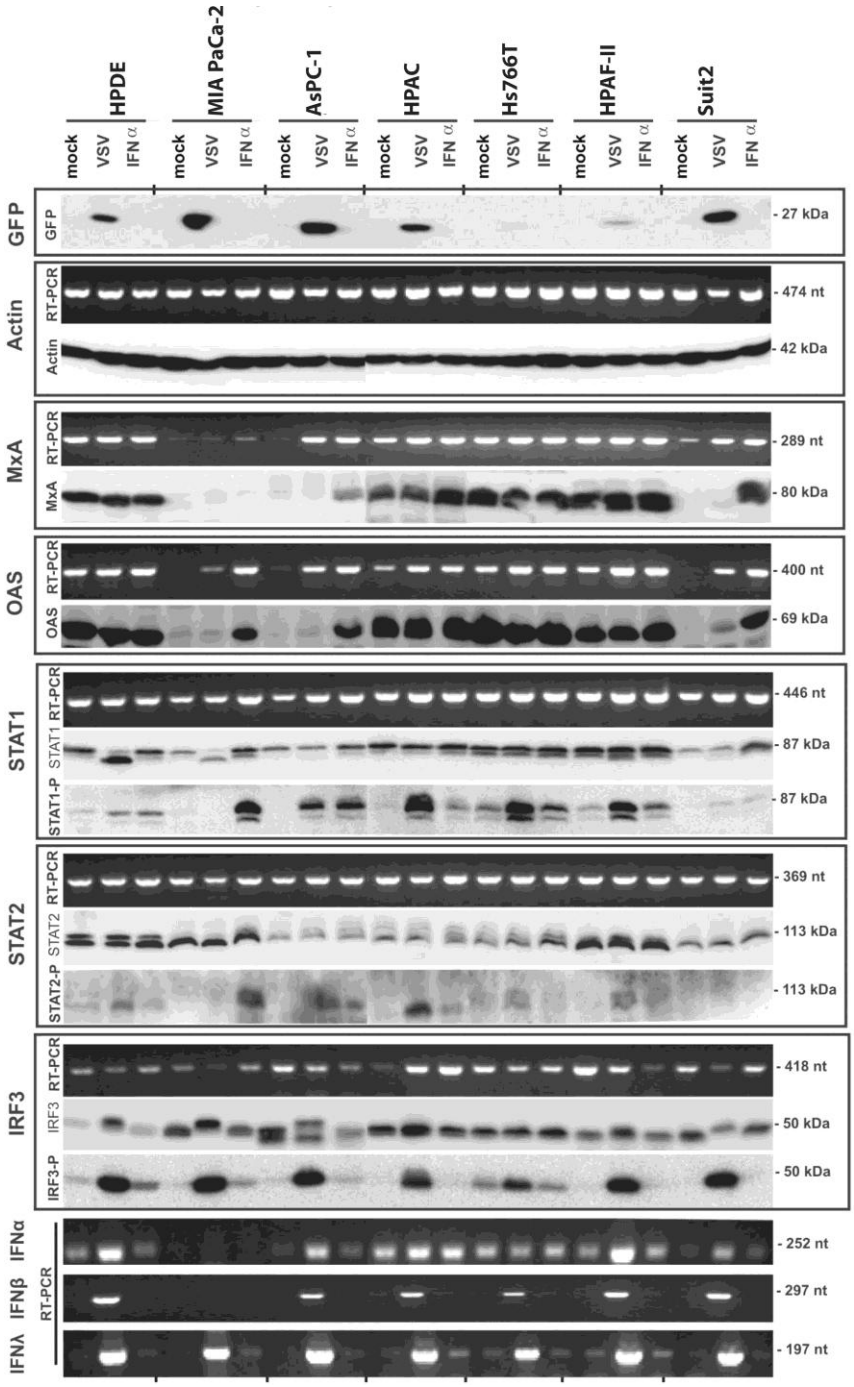

Figure 12. mRNA and protein expression of IFN related genes. Cells were mock infected, infected with VSV-ΔM51-GFP (indicated as VSV) at MOI 10 CIU/cell or treated with 5,000 U/ml IFN-α. Cells were harvested at 12 h p.i. and mRNA was reverse transcribed and analyzed by semi-quantitative PCR or cell lysates were prepared and analyzed by western blot for the indicated protein. PCR (nt) and protein (kDa) product sizes are indicated on the right.

3.6 Figures continued

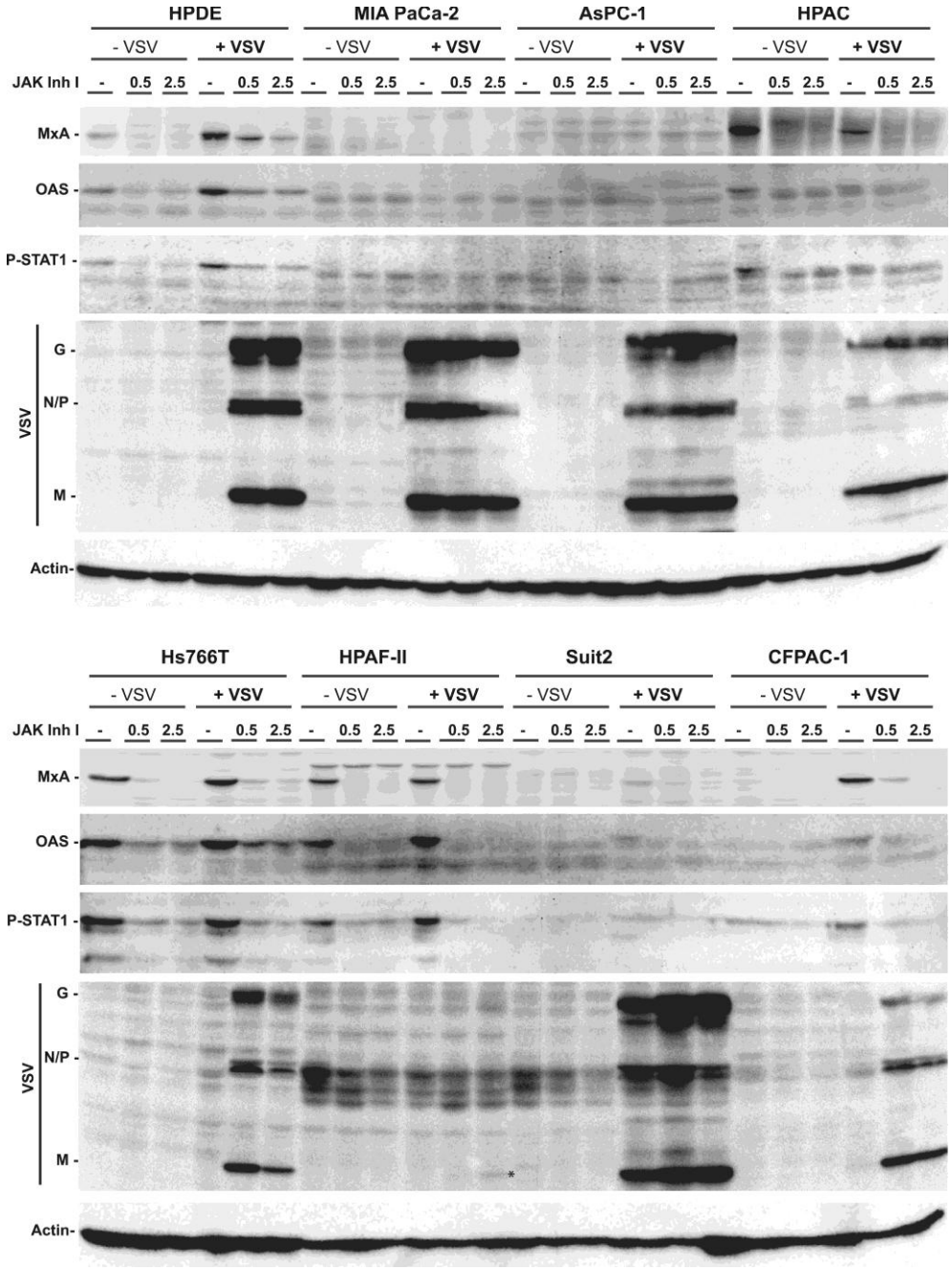

Figure 13. Effect of JAK/STAT signaling inhibition on p-STAT1, MxA and OAS expression. Cells were mock (DMSO) treated or treated with 0.5 or 2.5μM JAK Inh I for 48 h prior to infection with VSV-ΔM51-GFP at MOI 10 CIU/cell. Cells were harvested at 16h p.i. and cell lysates were prepared and analyzed by western blot for the indicated proteins. *, position of M protein in JAK Inh I treated HPAF-II cells

3.6 Figures continued

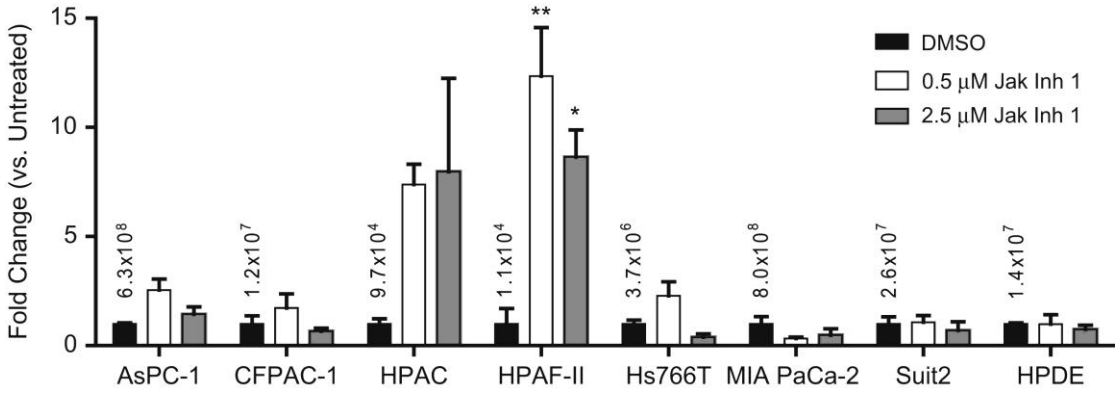

Figure 14. Effect of JAK/STAT signaling inhibition on PDA cell susceptibility to VSV-ΔM51-GFP. Cells were mock (DMSO) treated or treated with 0.5 or 2.5μM JAK Inh I for 48 h prior to infection with serial dilutions of VSV-ΔM51-GFP. At 17hpi, VSV-ΔM51-GFP fluorescent foci were counted to determine viral titers. Titers are expressed as a ratio to the mock treated titer, with the mock treated titer indicated. Titers were done in duplicate and data represent the mean ± standard error of mean. Treatments were compared using a 1-way ANOVA followed by the Bonferroni posttest for multiple comparisons. *=$p<0.05$; **=$p<0.01$

3.6 Figures continued

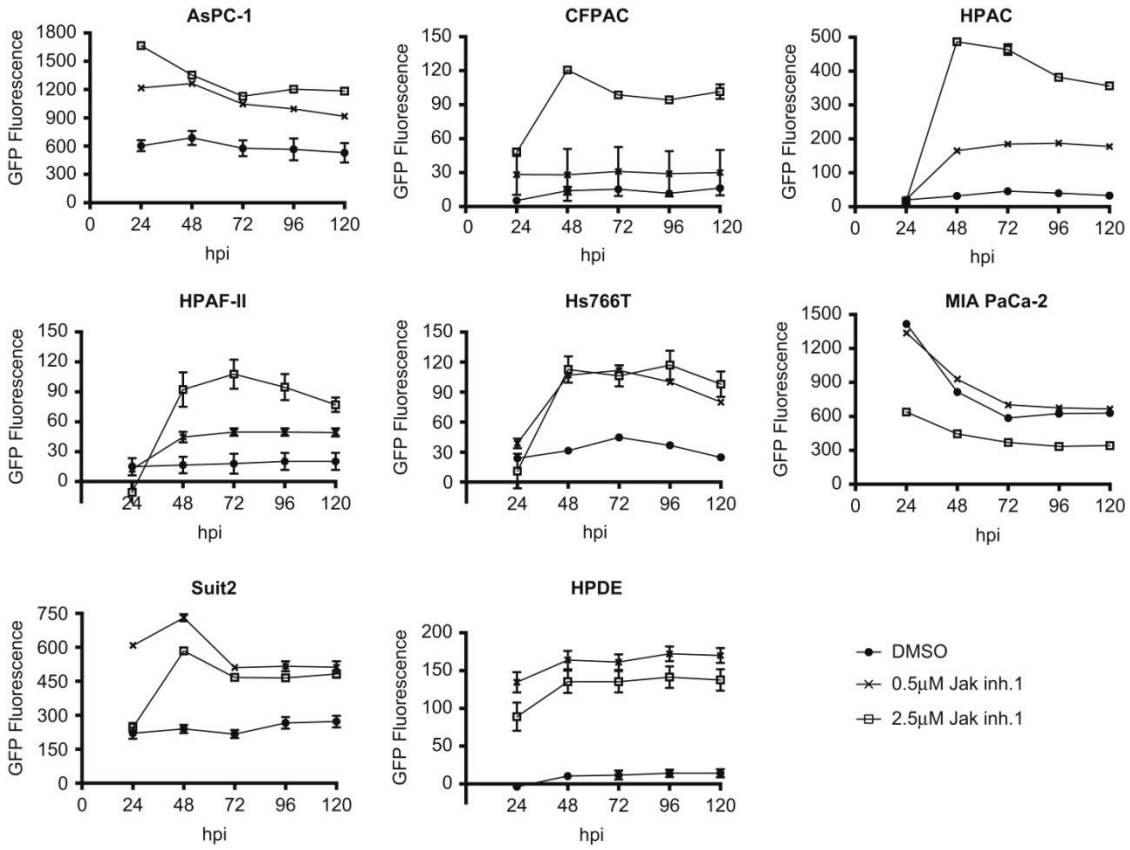

Figure 15. Effect of JAK/STAT signaling inhibition on virus-directed GFP expression. Cells were mock (DMSO) treated or treated with 0.5 or 2.5µM JAK Inh. I for 48 h prior to infection with VSV-ΔM51-GFP at MOI 1 CIU/cell. GFP fluorescence following infection was measured at 24 h intervals.

3.6 Figures continued

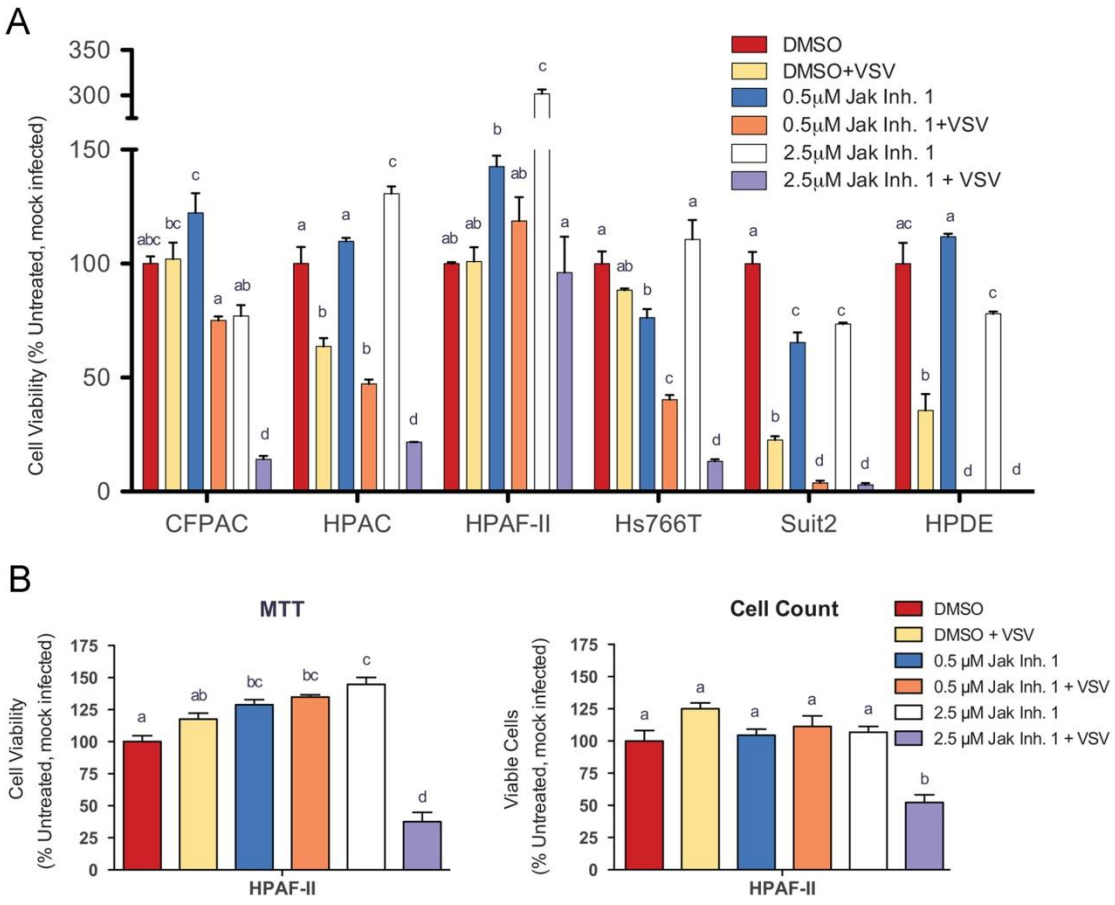

Figure 16. Effect of JAK/STAT signaling inhibition on PDA cell viability following infection. (A) Cells were mock (DMSO) treated or treated with 0.5 or 2.5μM JAK Inh. I for 48 h prior to infection with VSV-ΔM51-GFP at MOI 1 CIU/cell. Cell viability was analyzed by MTT assay at 5 d p.i. and is expressed as a percent of the DMSO only (mock) control. (B) The same assay was also performed in parallel with viable cell counts. The assays were done in triplicate and data represent the mean ± standard error of mean. Treatments were compared using a 1-way ANOVA followed by the Bonferroni posttest for multiple comparisons. Within each cell line, the presence of the same letter above a bar indicates treatments are not statistically different (cut-off $p<0.05$). For example, a bar marked "ab" does not differ from one marked "a" or "b" but is significantly different from one marked "c".

3.6 Figures continued

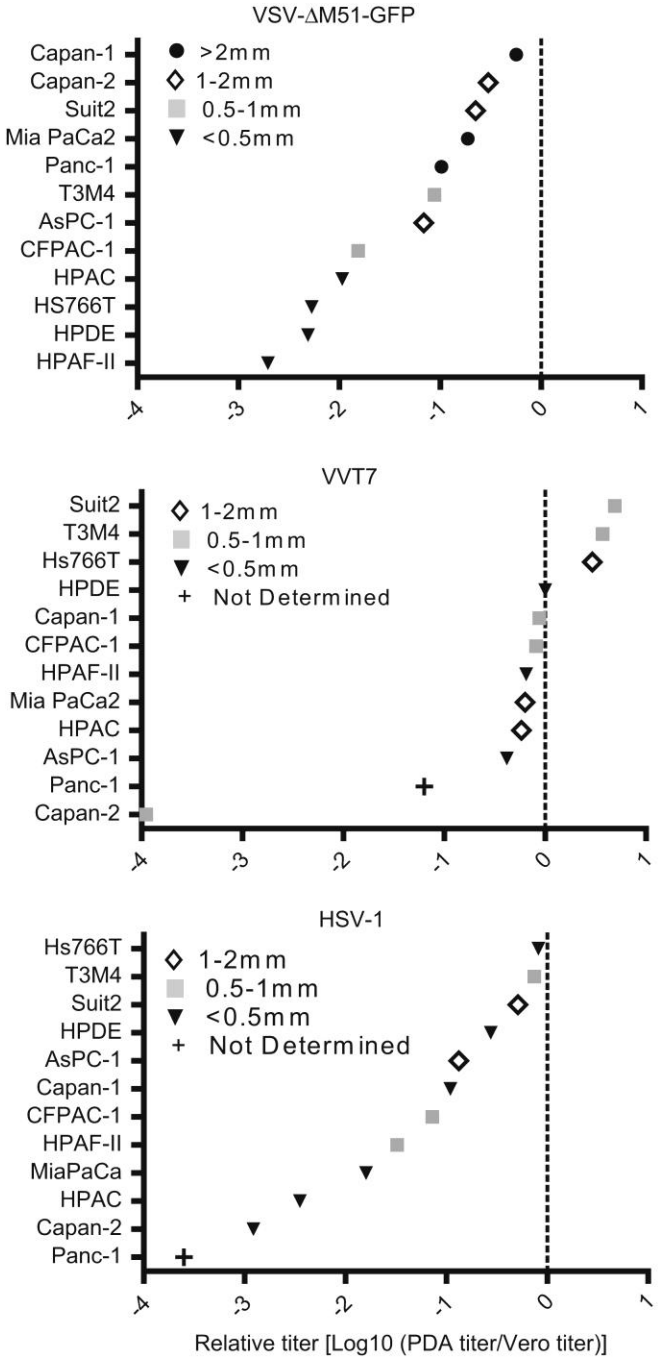

Figure 17. Permissiveness of PDA cell lines to VSV-ΔM51-GFP, VVT7 and HSV-1. Cells were infected with serial dilutions of virus and, after a 1 h absorption, overlayed with media containing 0.5% BactoAgar. VSV-ΔM51-GFP foci were counted by fluorescent microscopy at 3 d p.i. VVT7 and HSV-1 plaques were counted after staining with crystal violet at 5 d p.i. Titers are expressed relative to those on Vero cells. A relative yield of 0 indicates that the PDA cell line and Vero are equally permissive to the virus, while negative numbers indicate reduced permissiveness on the PDA cell line. Plaque size was determined for all viruses at 5 d p.i. following crystal violet staining.

3.6 Figures continued

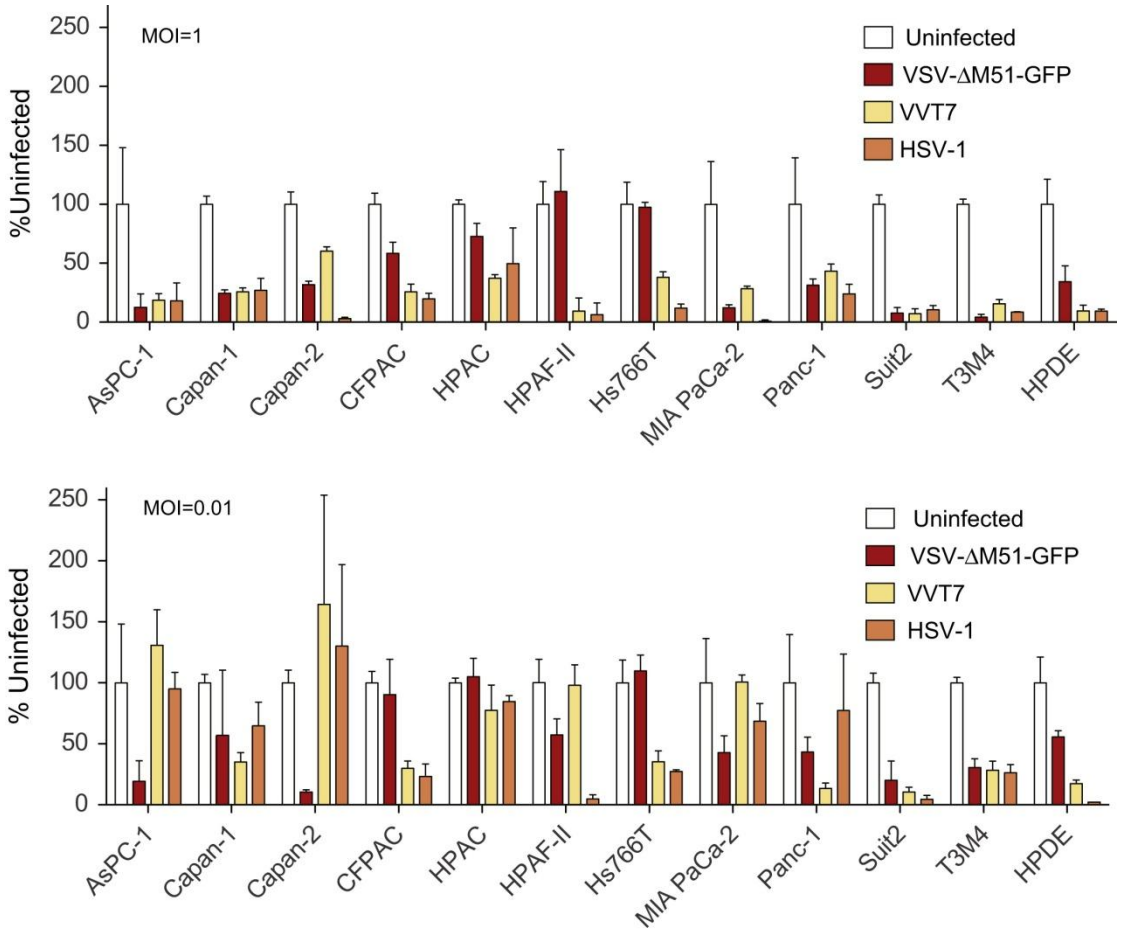

Figure 18. PDA cell viability following infection with VSV-ΔM51-GFP, VVT7 and HSV-1. Cells were mock infected or infected with VSV-ΔM51-GFP, VVT7 or HSV-1 at MOI of 1 (A) or 0.01 (B) CIU/cell. Cell viability was analyzed by an MTT assay at 5 d p.i. and expressed as a percent of mock-infected controls. MTT assays were done in triplicate and the data represent the mean ± standard error of mean.

3.7 Tables

Human cell line	Origin[a]	VSV-ΔM51 resistance[c]	IRF7			IFN-α			IFN-β			IFN-λ			MxA			OAS		
			Low[d]	High[e]	VSV[f]	Low	High	VSV	Low	High	VSV	Low	High	VSV	Low	High	VSV	Low	High	VSV
Capan-1	Liver metastasis	–	x			x			x			x		x	x			x		
MIA PaCa-2	Primary PDA	–	x		x	x			x			x		x	x			x		
Panc-1	Primary PDA	–		x		x			x		x	x		x	x			x		
Capan-2	Primary PDA	–		x			x		x		x	x		x	x			x		
AsPC-1	Ascites	–		x			x		x		x	x		x	x			x		x
Suit2	Liver metastasis	–		x			x		x		x	x		x		x	x	x		x
T3M4	LN metastasis[b]	–		x			x		x		x	x		x		x	x		x	
HPDE	Non-malignant	+		x			x		x		x	x		x		x			x	
CFPAC-1	Primary PDA	++		x			x		x		x	x		x		x	x		x	
HPAC	Primary PDA	++		x			x		x		x	x		x		x			x	
Hs766T	LN metastasis	+++		x			x		x		x	x		x		x			x	
HPAF-II	Primary PDA	+++		x			x		x		x	x		x		x			x	

Table 4. Human pancreatic cell lines used in this study and a summary of VSV-ΔM51-GFP susceptibility and expression of select mRNAs.

[a] All cell lines (except for non-malignant HPDE) have PDA origin
[b]LN, lymph node
[c] -, susceptible; +, intermediate; ++, resistant; +++ highly resistant
[d] mRNA expression undetectable or barely detectable
[e] mRNA robustly expressed
[f] mRNA expression increases upon VSV-ΔM51-GFP infection

3.7 Tables continued

Human cell line	VSV-ΔM51 resistance[a]	IRF3-p				STAT1-p				MxA				OAS			
		Low[b]	High[c]	VSV[d]	IFN-α[e]	Low	High	VSV	IFN-α	Low	High	VSV	IFN-α	Low	High	VSV	IFN-α
MIA PaCa-2	–	x		x		x			x	x				x			x
AsPC-1	–	x		x		x		x	x	x			x	x			x
Suit2	–	x		x		x		x	x	x			x	x		x	x
HPDE	+	x		x		x		x	x		x				x		
HPAC	++	x		x		x		x	x		x		x		x		
Hs766T	+++	x		x			x	x	x		x				x		
HPAF-II	+++	x		x			x	x	x		x				x		

Table 5. Summary of VSV-ΔM51-GFP susceptibility and expression of selected proteins.

[a] -, susceptible; +, intermediate; ++, resistant; +++ highly resistant

[b] protein expression undetectable or barely detectable

[c] protein robustly expressed

[d] protein expression increases upon VSV-ΔM51-GFP infection

[e] protein expression increases upon IFN-α treatment

3.7 Tables continued

PDA Cells	Treatment[a]	Yield[b]	Ratio[c]
AsPC-1	VSV	8.4×10^7	1
	VSV + 0.5 μM JAK Inh. I	4.2×10^8	5
	VSV + 2.5 μM JAK Inh. I	2.6×10^8	3
CFPAC-1	VSV	1.3×10^6	1
	VSV + 0.5 μM JAK Inh. I	2.6×10^7	20
	VSV + 2.5 μM JAK Inh. I	2.1×10^7	16
HPAC	VSV	2.6×10^6	1
	VSV + 0.5 μM JAK Inh. I	3.1×10^7	12
	VSV + 2.5 μM JAK Inh. I	7.9×10^6	3
HPAF-II	VSV	2.0×10^4	1
	VSV + 0.5 μM JAK Inh. I	1.3×10^6	64
	VSV + 2.5 μM JAK Inh. I	5.2×10^6	256
Hs766T	VSV	4.1×10^4	1
	VSV + 0.5 μM JAK Inh. I	2.1×10^7	512
	VSV + 2.5 μM JAK Inh. I	1.0×10^7	256
MIA PaCa-2	VSV	4.2×10^8	1
	VSV + 0.5 μM JAK Inh. I	5.0×10^8	1.2
	VSV + 2.5 μM JAK Inh. I	2.1×10^8	0.5
Suit2	VSV	2.1×10^8	1
	VSV + 0.5 μM JAK Inh. I	1.3×10^8	0.6
	VSV + 2.5 μM JAK Inh. I	8.4×10^7	0.4
HPDE	VSV	6.1×10^4	1
	VSV + 0.5 μM JAK Inh. I	1.3×10^8	2048
	VSV + 2.5 μM JAK Inh. I	1.3×10^8	2048

Table 6. New infectious virus particle production at 16 h p.i. after 48 h pre-treatment with JAK Inh I.

[a] "VSV" indicates VSV-ΔM51-GFP
[b] Virus was collected from the indicated cell line and titer was determined on BHK-21 cells
[c] Ratio of virus yield on JAK Inh. I treated cells to mock treated cells

3.7 Tables continued

Gene name	Primer name	Nucleotide Sequence (5' to 3')[a]	Product size, bp	NIH GenBank accession number[b]
MxA	VG 138	(+) GCTACACACCGTGACGGATATGG	289	M30817
	VG 139:	(-) CGAGCTGGATTGGAAAGCCC		
PKR	VG 140:	(+) GCCTTTTCATCCAAATGGAATTC	301	M35663
	VG 141:	(-) GAAATCTGTTCTGGGCTCATG		
OAS2	VG 142:	(+) TCAGAAGAGAAGCCAACGTGA	400	M87434a
	VG 143:	(-) CGGAGACAGCGAGGGTAAAT		
ISG15	VG 144:	(+) CATGGGCTGGGACCTGACGGTGAAG	282	M13755a
	VG 145:	(-) CTGCTGCGGCCCTTGTTATT		
IFN-α	VG 146:	(+) GGAGGAGAGGGTGGGAGAAAC	252	NM_024013.1
	VG 147:	(-) GAAAGCGTGACCTGGTGTATGAG		
IFN-β	VG 148:	(+) GACGCCGCATTGACCATCTA	297	NM_002176.2
	VG 149:	(-) CCTTAGGATTTCCACTCTGACT		
β-actin	VG 150:	(+) GCAAAGACCTGTACGCCAACA	474	NM_001101a
	VG 151:	(-) CCTCGGCCACATTGTGAAC		
IRF3	VG 154:	(+) CGAAGTGGGAGACAGGACGC	418	NM_001571.5
	VG 155:	(-) CGTGGGCACAACCTTGACCA		
IRF7	VG 156:	(+) GGCAGAGCCGTACCTGTCAC	491	NM_001572.3 NM_004029.2 NM_004031.2
	VG 157:	(-) TGGTATAGCGTGGGGAGCCA		

Table 7. List of target genes and oligonucleotides used in the study.

3.7 Tables continued

MAVS	VG 158:	(+) AGTCTCGTTTCCTCTCAGTCC	175	NM_020746.4 NR_037921.1 NM_001206491.1
	VG 159:	(-) CTGTGAGGCAGGGCAGGTAAG		
LGP2	VG 160:	(+) GGCCCTGTTCGATGACCGCA	153	NM_024119.2
	VG 161:	(-) CTTTGGCGGGTGCGGGTGAA		
ISG56	VG 162:	(+) GGAGCCTGGCTAAGCAAAACC	355	NM_001548.3
	VG 163:	(-) AAAGTTGCCCCAGGTCACCA		
STAT1	VG 164:	(+) AGCAAGACTGGGAGCACGCTG	446	NM_007315.3
	VG 165:	(-) TGCCACACCATTGGTCTCGTGT		
STAT2	VG 166:	(+) CCAGGCCAAAGGGAAGACACCC	369	NM_005419.3
	VG 167:	(-) GCGTTGCGTAGGTCCACCCC		
JAK1	VG 168:	(+) CCAGCCCAAGCCCCGAGAAAT	145	NM_002227.2
	VG 169:	(-) GCCTCTCCCAAGGTGCTCGC		
JAK2	VG 170:	(+) GGGTGTTCGCGTCGCCACTT	379	NM_004972.3
	VG 171:	(-) AGAACATTTGCCGTCGCGGG		
PSMA7	VG 172	(+) TTTGCAGGCCTCACCGCCGA	289	NM_002792.2
	VG 173	(-) GCACTGACTTGGCACCCCGA		
Viperin	VG 176:	(+) TGCCACAATGTGGGTGCTTACAC	370	NM_080657.4
	VG 177:	(-) GTTGATCTTCTCCATACCAGCTTCC		
MDA5	VG 178:	(+) TCACGGACTTGCCCTCTCCA	166	NM_022168.2
	VG 179:	(-) GCAGCAATCCGGTTTCTGTCT		
IFN-λ	VG 180:	(+) CCAGCCCCTTCACACCCTGCACC	197	NM_172140.1
	VG 181:	(-) GGTCTCGCGTGAGGAGGCGGAAG		

Table 7 continued. List of target genes and oligonucleotides used in the study.

3.7 Tables continued

IFNAR1	VG 182:	(+) AGTTCAGTGGCTCCACGCCT	458	NM_000 629.2
	VG 183:	(-) TGGTGTGTGCTCTGGCTTTCA		
IFNAR2	VG 184:	(+) TTCTCATGGTGTATATCAGCC	211	NM_000 874.3
	VG 185:	(-) GCACAGTTCTTAACCACCTTC		
IRF9	VG 186	(+) GTGCAAAGCCTGGATTGCCG	415	NM_006 084.4
	VG 187:	(-) AGGCCTGCTCCATCTTCACTG		
MYD88	VG 192:	(+) TCTCGGAAAGCGAAAGCCGGCG	183	NM_001 172567.1 NM_002 468.4 NM_001 172569.1 NM_001 172568.1 NM_001 172566.1
	VG 193:	(-) TTGAGAGCAGCCAGGGGAAGGG		
RNase L	VG 194:	(+) CCACGTGCACAGCGGGAAGT	389	NM_021 133.3
	VG 195:	(-) ACCAGCCGTCCAAGGTCCTCT		
NF-κB	VG 196:	(+) ATCTTCCCGGCAGAGCAGCCCA	161	L19067.1
	VG 197:	(-) ATGCGCACTGTCCCTGGTCCT		
TYK2	VG 198:	(+) TGGGCGAGGGTCACTTCGGC	206	NM_003 331.4
	VG 199:	(-) CTCGCCTTGGTCCTCGCAGC		
IKBKE	VG 200:	(+) ACTCTGGAAGTGGCAAGGACAT	234	NM_014 002.3
	VG 201:	(-) TACCTGATCCCGGCTCTTCACCA		
TNF-α	VG 202:	(+) CCCCAGAGGGAAGAGTTCCCCA	152	NM_000 594.2
	VG 203:	(-) CGGCGGTTCAGCCACTGGAG		
STAT3	VG 204:	(+) TGGCCCAATGGAATCAGCTACAGC	145	NM_139 276.2
	VG 205:	(-) TGCTGGCCGCATATGCCCAAT		

Table 7 continued. List of target genes and oligonucleotides used in the study.
[a] (+) indicates primer has mRNA polarity, (-) indicates primer is complimentary to mRNA.
[b] Except for MAVS, Myd88, NF- κB, IKBKE, SOCS1 and RIG-I, at least one primer in each pair was selected to span an exon-intron boundary

CHAPTER 4: DISSERTATION SUMMARY AND CONCLUSION

In Summary, my first study demonstrated that certain cell types are naturally resistant to ribavirin treatment without any prior exposure to the drug. Our data also indicated that differences in the intracellular RBV metabolism among cell types may be responsible for the natural resistance. Further experiments are required to identify specific cellular factors responsible for defective ribavirin metabolism in ribavirin resistant cell types. These results may explain some treatment failures with ribavirin therapy. Targeting these factors may improve the therapeutic outcome of ribavirin therapy in virus-infected patients.

In the second study, specific genes involved in type I IFN signaling are evaluated for their role in resistance of specific PDA cells to oncolytic VSV-ΔM51-GFP. We demonstrated that ISGs such as MxA and OAS may be useful biomarkers to identify PDAs susceptible for VSV-ΔM51-GFP mediated oncolysis. Further research can lead to a better understanding of resistance, which can help us to design newer drugs to target ISGs responsible for viral resistance to enhance oncolysis.

4.1 Ribavirin resistance

Ribavirin was chemically synthesized more than 40 years ago and approved to use in humans against respiratory syncytial virus (RSV) 20 years ago. Later it is also approved against Lassa fever virus infection and in combination with IFN-α against HCV infections. Moreover, ribavirin has been shown to be effective in preclinical models against various RNA and DNA viruses. Despite of these successes, some patients exhibit resistance to ribavirin treatment and the exact mechanism of this resistance is not fully understood (Liuzzi, Mason et al. 2005; Ibarra and Pfeiffer 2009). For example, ribavirin treatment in combination with IFN fails to induce sustained virological response (SVR) in ~45% patients with chronic HCV infection (Liu, Su et al. 2007; Thomas, Feld et al. 2010). Moreover, ribavirin has very limited success when used against respiratory syncytial virus (RSV) infection in children and Lassa fever virus infection. Importantly, increased concentration of ribavirin within the cell is linked to increased antiviral response (Feld and Hoofnagle 2005). Since majority of previous studies focused on the generation of ribavirin resistant virus mutant, we wanted to study the host based resistance of ribavirin using cell culture system. Even though longer exposure to ribavirin treatment may result in the development of drug resistance (Ibarra and Pfeiffer 2009), we aim to identify cell types which can be naturally resistant to ribavirin treatment and factors responsible for such resistance.

Several studies have shown that ribavirin resistant mutant viruses can be developed with long term exposure of ribavirin treatment (Airaksinen, Pariente et al. 2003; Pfeiffer and Kirkegaard 2003; Vignuzzi, Stone et al. 2005; Sierra, Airaksinen et al. 2007; Cuevas, Gonzalez-Candelas et al. 2009). However, this has not been established

with VSV (Cuevas, Sanjuan et al. 2005). In addition, ribavirin resistant cells infected with VSV or SeV exhibited no signs of virus adaptation. When VSV was passed for 15 passages in HeLa, BSRT7 or in BHK cells in the presence of inhibitory ribavirin concentrations no increase in viral titer or infectivity was observed to suggest any possibility of virus adaptation. A previous study could not find a ribavirin resistant VSV mutant even after 100 generations in the presence of sub-inhibitory concentrations of ribavirin (Cuevas, Sanjuan et al. 2005).

In this study we analyzed the antiviral activity of ribavirin in the panel of seven different cell types from various hosts against VSV and SeV. Though, both viruses belong to the same order, they belong to two different families having different morphology and replication kinetics. Previously it has been shown that replication of both of these viruses can be effectively inhibited by ribavirin treatment both *in vitro* and *in vivo* (Sidwell, Khare et al. 1975; Larson, Stephen et al. 1976; Toltzis and Huang 1986/6; Toltzis, O'Connell et al. 1988/4). Also, ribavirin has been shown to be very effective against various viruses of order *Mononegavirales* (Wray, Gilbert et al. 1985; Leyssen, Balzarini et al. 2005; Sun, Chung et al. 2007) (Malinoski and Stollar 1980; Smee, Bray et al. 2001). However, all these studies utilized only one or two cell lines to test the efficacy of ribavirin against a particular virus. We tested the inhibitory effect of ribavirin on seven different cell types against two different viruses. Our results demonstrated that specific cell types such as Vero (IC_{50}=2250 µM for VSV and 1550 µM for SeV) were highly resistant to ribavirin whereas, A549 and BHK cells showed moderate resistance to ribavirin treatment. The other tested cell types including HeLa, HEp2, 4T1 and BSRT7 inhibited both VSV and SeV at much lower ribavirin

concentration than resistance cells. The observed ribavirin resistance was irrespective of virus or host type. We hypothesized that resistance of RBV against VSV and SeV is mainly attributed to cellular factors and not viral factors and RBV metabolism might play a key role for this observed resistance. We demonstrated that both VSV and SeV replication kinetics over the period of 24 h and 48 h are very different and the conferred resistance of RBV is not due to the toxicity of ribavirin.

A recent study showed that cell-based ribavirin resistance can be developed after continuous exposure with ribavirin and it's mainly because of limited uptake through nucleoside transporters (Ibarra and Pfeiffer 2009). When we tested all of our seven cell lines for the ribavirin uptake, we observed that none of our tested cell lines were defective in ribavirin uptake. We also used NBMPR a specific inhibitor of equilibrative nucleoside transporter (ENT) and demonstrated that ENT1 or ENT2 are mainly responsible for ribavirin uptake. However, when we compared the accumulation of ribavirin after 24 h post treatment, we found clear correlation between the ribavirin resistance and low levels of ribavirin accumulation. In other words, cells most sensitive to ribavirin treatment (HeLa, HEp2, BSRT7 and 4T1) had the highest levels of intracellular ribavirin and vice versa. As mentioned earlier in chapter two, it is known that intracellular uptake of ribavirin results in the phosphorylation of ribavirin and once phosphorylated, ribavirin is trapped inside the cell (Endres, Moss et al. 2009). Thus it can be assumed that ribavirin resistant cells in some capacity are defective in ribavirin metabolism and this defect is at least partially responsible for the resistance.

The mechanism of action of ribavirin is still highly controversial mainly due to its pleiotropic nature. One of the most common mechanisms of action of ribavirin is via

inhibition of IMPDH which results into the depletion of cellular GTP pool. This reduced GTP levels negatively affects viral replication. However, the GTP levels can be restored if the cells are co-treated with exogenous guanosine. We did not observe any drastic reversal in the activity of RBV in any of the VSV or SeV infected ribavirin sensitive cell types (HeLa, HEp2, BSRT7 or 4T1). In few previous studies a general inhibitor of transcription ActD has been shown to reverse the antiviral action of ribavirin either by stabilization of GTP levels or by inhibition of RTP synthesis (Malinoski and Stollar 1980; Smee and Matthews 1986). Our results demonstrated that ActD but not guanosine reversed the antiviral effect of ribavirin. These results led us to further hypothesize that effect of ribavirin is dependent on the synthesis of ribavirin mono-, di- or tri- phosphates (RMP/RDP/RTP). In addition, recently it has been shown that ribavirin treatment-induced specific interferon stimulated genes (ISG), may be important for the inhibition of viral replication (Feld and Hoofnagle 2005; Thomas, Feld et al. 2010). Since ActD inhibits the general cellular transcription, it can be hypothesized that ActD neutralizes the effect of ribavirin by inhibiting the transcription of specific ISGs, which are required for its activity.

Together, our data suggested that IMPDH inhibition is not the primary mechanism of action against VSV and SeV at least in the cell types tested and other mechanisms (explained above) alone or together might contribute for the antiviral activity of ribavirin.

Very similar pattern of ribavirin activity was observed against both VSV and SeV, suggests that it is possible that both viruses are being inhibited by the same mechanism even though they belong to different families. Previously, mechanism of

ribavirin's antiviral action has not been studied for SeV. However, for another paramyxovirus RSV, IMPDH inhibition and depletion of GTP levels has been shown as the primary mechanism of action of ribavirin (Leyssen, Balzarini et al. 2005). In contrast, in a previous study utilizing *in vitro* transcription reaction with purified VSV indicated that RMP, RDP and RTP significantly inhibited VSV RNA polymerase activity and RNA synthesis (Toltzis and Huang 1986/6). To better understand the molecular mechanisms of the antiviral activity of ribavirin against VSV and SeV, we plan to investigate the metabolism of ribavirin in ribavirin resistant and sensitive cell types. This will help us to identify, cellular factors responsible for defective ribavirin metabolism. In order to understand the molecular mechanism of action of ribavirin, we plan to investigate the expression profile of ribavirin resistant and sensitive cell types when treated with ribavirin. This experiment is important, since ribavirin treatment has been suggested to modulate the expression of specific ISGs and this expression profile may contribute to the antiviral activity of ribavirin. We plan to isolate total RNA and determine the mRNA expression of several genes regulated by ribavirin treatment using Affymatrix microarray.

Overall, our study provided the first evidence that some cells can be naturally resistant to ribavirin. Our results may explain some of these failures associated with the treatment of ribavirin. In future, experiments can be aimed to study efficacy of ribavirin-IFN combined treatment in several cell types against various viruses. These studies could identify the specific cellular factors responsible for such resistance. Once these cellular targets are found, several ribavirin-like compounds, which are better to overcome cell based resistance can be synthesized and tested for improved efficacy. Furthermore, the most efficacious compounds can be tested *in vivo* to study their pharmacokinetics and

dosage. Moreover, liver tissue samples from patients with chronic HCV infection can be tested for the specific cellular targets to predict the success of ribavirin-IFN therapy.

4.2 New approaches to overcome oncolytic VSV resistance in cancer cells

Previous study from our laboratory analyzed for the first time VSV as an oncolytic agent against PDA and observed great heterogeneity among different cell lines in the susceptibility to VSV. Out of 13 tested PDA cell lines, five cell lines (BxPC-3, CFPAC-1, HPAC, HPAF-II and Hs766t) and a non-malignant HPDE cells were at least partially resistant to VSV-ΔM51-GFP (Murphy, Besmer et al. 2012). This study also revealed that all the VSV resistant cell types both secreted and responded to Type I IFN. Type I IFN includes IFN-α and IFN-β. Both IFN-α/β, provide an essential host defense against various viruses by triggering the innate antiviral responses in cells. Once synthesized IFNs are secreted and can work in autocrine or paracrine manner to exert its antiviral activity. All the members of the type I IFN family can be recognized by a single receptor known as IFNAR. This receptor is attached with Janus kinases Jak1 and Tyk2. Jak1 and Tyk2 activate STAT1 and STAT2 upon binding of type I IFN onto IFNAR. Transcription factors STAT1 and STAT2 subsequently get dimerized to form a complex with IRF9. This complex is known as ISGF3 and binds to ISRE site on DNA and leads to the activation of hundreds of genes. Many of these genes are interferon stimulated genes (ISGs) and known to have direct antiviral activity against various viruses including VSV.

The basis of VSV oncoselectivity is based on the fact that majority of cancer cells are defective in Type I IFN responses (Stojdl, Lichty et al. 2000; Naik and Russell 2009). However, some cancer cells have been found to have intact type I IFN response (Carey, Ahmed et al. 2008; Paglino and van den Pol 2011). A few recent studies have indicated that constitutive expression of specific ISGs can predict the susceptibility to OV (Monsurro, Beghelli et al. 2010).

Based on our previous data and recent publications we hypothesized that the constitutive expression of specific ISGs in specific PDA cells might contribute to the VSV-ΔM51-GFP resistance. To test this hypothesis we examined 12 PDA cells and one non-malignant HPDE cell line at molecular levels for several genes involved in type I IFN responses. Our data showed that MxA was expressed constitutively at both RNA and protein level in all VSV resistant cell lines but not in any of the sensitive cell lines. MxA is an interferon-induced dynamin like GTPases. MxA exhibited antiviral activity against a variety of RNA viruses by blocking the transport of viral nucleocapsids into the nucleus and thereby preventing transcription of the viral genome. In addition, OAS was also found to be constitutively expressed in all resistant cell types and in few susceptible cell types at least at mRNA level. We also observed the low levels of pSTAT1 in some of the resistant cell types. Also, susceptibility to VSV increases in all resistant cells, when MxA and OAS expression is reduced by the inhibitor of Jak/STAT signaling. Together, this data suggest at least partial role of pSTAT1 in constitutive expression of ISGs.

Here we showed that expression of MxA and OAS can be used as a biomarker of resistance against PDA cells for the treatment with oncolytic VSV. In this study we utilized the general inhibitor of type I IFN signaling to downregulate the expression of MxA and OAS. However, this inhibitor may exhibit its inhibitory effect against other ISGs. To further demonstrate that, this resistance is mainly due to the constitutive expression of MxA and OAS, we are going to use the specific sh-RNA targeting MxA and OAS mRNAs. If the treatment with specific sh-RNA against MxA and OAS results in increased virus replication and cell death in resistant PDA cells, that will further confirm the major role of these two proteins in PDA resistance.

In this study we clearly demonstrated the role of type I IFN responses in the resistance of PDA cells to VSV-ΔM51-GFP. However, it is possible that other mechanisms are involved with these resistance phenotypes. For example NF-κB activation has been implicated to regulate the transcription of IRF3/7 (Hiscott, Grandvaux et al. 2003). These findings indicate that it is possible that up-regulation of specific ISGs such as MxA and OAS is NF-κB driven. Also, defects in cellular apoptotic pathway has been shown to acquire resistance to oncolytic VSV in glioblastoma cells(Gaddy DF and and Lyles 2007).

For future, two independent projects are in progress to evaluate the possible involvement of these other mechanisms with VSV resistant phenotype of PDA cells. The first project is to analyze the possible role of NF-κB activation in resistance of specific PDA cells OV therapy. NF-κB is a transcription factor known to regulate several pathways of cell survival, proliferation, inflammation, angiogenesis and differentiation (Shishodia and Aggarwal 2002) and shown to be constitutively active in various cancers including PDA (Baldwin 1996) . To identify the NF-κB inhibitor which can reverse the VSV-ΔM51-GFP resistance of PDA cells, a panel of known NF-κB inhibitors targeting the NF-κB activation through different pathways is being screened. In this screening experiment we have observed that a specific NF-κB inhibitor have increased the VSV replication and increased PDA cell killing. Our data demonstrated that the expression of specific ISGs (MxA and OAS) are at least partially responsible of VSV resistance. Previously, NF-κB activation has been implicated to induce specific ISGs. To determine the exact molecular mechanism of this NF-κB inhibitor in increasing oncolytic VSV replication and increased PDA cell killing, we plan to investigate the mRNA expression

profile of VSV resistant cells, mock treated, treated with the effective NF-κB inhibitor or with JAK inh. I using Affymatrix microarray. This experiment will be able to identify all ISGs which are constitutively expressed only in VSV resistant cells and could potentially be targeted to overcome this resistance. Moreover, experiments are also in progress to determine which of these inhibitors can down regulate MxA and OAS expression and simultaneously improves oncolysis. Pretreatment of this inhibitor is able to reverse the VSV resistance in resistant PDA cells. To show that these results can be translated *in vivo*, we have planned to test the efficacy of this effective NF-κB inhibitor in athymic nude mouse model system to potentially reduce tumor burden. Further, we aim to evaluate the expression of specific ISGs such as MxA and OAS in clinical samples from various pancreatic cancer patients to demonstrate the clinical significance of this study.

VSV kill cells by inducing apoptosis and known to induce apoptosis via several pathways (Gaddy DF and and Lyles 2007). Many cancer cells are known to have defect in some of the apoptotic pathway. These defects may lead to the resistance of cancer cells to VSV. Another project in our laboratory is investigating the possible role of defective apoptotic signaling in PDA cells resistance to VSV.

My studies demonstrated that the expression of specific ISGs, MxA and OAS might contribute to the VSV-ΔM51-GFP resistance in specific PDA cells. Also, susceptibility to VSV-ΔM51-GFP can be increased in all resistant cells, when MxA and OAS expression is reduced by the inhibition of Jak/STAT signaling. This information can be used in future to develop an assay to identify PDA cells or other cancer cells likely to get treated with VSV-ΔM51-GFP. Cancer cell types showing constitutive expression of ISGs can be treated with alternative oncolytic viruses which are better in evading cellular

type I IFN response. Alternatively, treatment with more than one OV (combined virotherapy) could also lead to enhanced oncolysis as was previously shown for VSV in combination with vaccinia virus (Le Boeuf, Diallo et al. 2010). In addition, combination of oncolytic virus and chemotherapeutic drugs can be used to enhance oncolysis in different cancer cells (Ottolino-Perry, Diallo et al. 2010).

REFERENCES

Aguilar Marucco, D., D. Gonzalez de Requena, et al. (2008). "The use of trough ribavirin concentration to predict sustained virological response and haematological toxicity in HIV/HCV-co-infected patients treated with ribavirin and pegylated interferon." J Antimicrob Chemother 61(4): 919-924.

Ahmed M, Cramer SD, et al. (2004). "Sensitivity of prostate tumors to wild type and M protein mutant vesicular stomatitis viruses." Virology 330(1): 34-49.

Ahmed, M., M. O. McKenzie, et al. (2003). "Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis." J Virol 77(8): 4646-4657.

Ahmed M, M. T., Puckett S, Kock ND, Lyles DS. (2008). "Immune response in the absence of neurovirulence in mice infected with m protein mutant vesicular stomatitis virus." Journal of Virology 82(18): 9273-9277.

Airaksinen, A., N. Pariente, et al. (2003). "Curing of foot-and-mouth disease virus from persistently infected cells by ribavirin involves enhanced mutagenesis." Virology 311(2): 339-349.

Asselah, T., E. Estrabaud, et al. (2010). "Hepatitis C: viral and host factors associated with non-response to pegylated interferon plus ribavirin." Liver Int 30(9): 1259-1269.

Balachandran, S., M. Porosnicu, et al. (2001). "Oncolytic activity of vesicular stomatitis virus is effective against tumors exhibiting aberrant p53, Ras, or myc function and involves the induction of apoptosis." J Virol 75(7): 3474-3479.

Baldwin, A. S., Jr. (1996). "The NF-kappa B and I kappa B proteins: new discoveries and insights." Annu Rev Immunol 14: 649-683.

Baltimore, D. (1971). "Expression of animal virus genomes." Bacteriol Rev 35(3): 235-241.

Balzarini, J., A. Karlsson, et al. (1993). "Eicar (5-ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide). A novel potent inhibitor of inosinate dehydrogenase activity and guanylate biosynthesis." J Biol Chem 268(33): 24591-24598.

Barber, G. N. (2004). "Vesicular stomatitis virus as an oncolytic vector." Viral Immunology 17(4): 516-527.

Basagoudanavar, S. H., R. J. Thapa, et al. (2011). "Distinct roles for the NF-kappa B RelA subunit during antiviral innate immune responses." J Virol 85(6): 2599-2610.

Bidwell, B. N., C. Y. Slaney, et al. (2012). "Silencing of Irf7 pathways in breast cancer cells promotes bone metastasis through immune escape." Nat Med.

Black, B. L., R. B. Rhodes, et al. (1993). "The role of vesicular stomatitis virus matrix protein in inhibition of host-directed gene expression is genetically separable from its function in virus assembly." J.Virol. 67(8): 4814-4821.

Bougie, I. and M. Bisaillon (2003). "Initial binding of the broad spectrum antiviral nucleoside ribavirin to the hepatitis C virus RNA polymerase." J Biol Chem 278(52): 52471-52478.

Bougie, I. and M. Bisaillon (2004/5/21). "The broad spectrum antiviral nucleoside ribavirin as a substrate for a viral RNA capping enzyme." J.Biol.Chem. 279(21): 22124-22130.

Breitbach, C. J., T. Reid, et al. (2010). "Navigating the clinical development landscape for oncolytic viruses and other cancer therapeutics: no shortcuts on the road to approval." Cytokine Growth Factor Rev 21(2-3): 85-89.

Buchholz, U. J., S. Finke, et al. (1999). "Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter." J Virol 73(1): 251-259.

Buckwold, V. E., J. Wei, et al. (2003). "Synergistic in vitro interactions between alpha interferon and ribavirin against bovine viral diarrhea virus and yellow fever virus as surrogate models of hepatitis C virus replication." Antimicrob Agents Chemother 47(7): 2293-2298.

Cameron, C. E. and C. Castro (2001). "The mechanism of action of ribavirin: lethal mutagenesis of RNA virus genomes mediated by the viral RNA-dependent RNA polymerase." Curr Opin Infect Dis 14(6): 757-764.

Carey, B. L., M. Ahmed, et al. (2008). "Early steps of the virus replication cycle are inhibited in prostate cancer cells resistant to oncolytic vesicular stomatitis virus." J Virol 82(24): 12104-12115.

Cary, Z. D., M. C. Willingham, et al. (2011). "Oncolytic Vesicular Stomatitis Virus Induces Apoptosis in U87 Glioblastoma Cells by a Type II Death Receptor Mechanism and Induces Cell Death and Tumor Clearance In Vivo." J Virol 85(12): 5708-5717.

Chen, M., T. Ogino, et al. (2007). "Interaction of vesicular stomatitis virus P and N proteins: identification of two overlapping domains at the N terminus of P that are involved in N0-P complex formation and encapsidation of viral genome RNA." J Virol 81(24): 13478-13485.

Conzelmann, K. K. (2004). "Reverse genetics of mononegavirales." Curr Top Microbiol Immunol 283: 1-41.

Coulon, P., V. Deutsch, et al. (1990). "Genetic evidence for multiple functions of the matrix protein of vesicular stomatitis virus." The Journal of general virology 71 (Pt 4): 991-996.

Crotty, S., C. Cameron, et al. (2002). "Ribavirin's antiviral mechanism of action: lethal mutagenesis?" J Mol Med 80(2): 86-95.

Crotty, S., C. E. Cameron, et al. (2001). "RNA virus error catastrophe: direct molecular test by using ribavirin." Proc Natl Acad Sci U S A 98(12): 6895-6900.

Crotty, S., D. Maag, et al. (2000). "The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen." Nat Med 6(12): 1375-1379.

Cuevas, J. M., F. Gonzalez-Candelas, et al. (2009). "Effect of ribavirin on the mutation rate and spectrum of hepatitis C virus in vivo." J Virol 83(11): 5760-5764.

Cuevas, J. M., R. Sanjuan, et al. (2005). "Mode of selection and experimental evolution of antiviral drugs resistance in vesicular stomatitis virus." Infect Genet Evol 5(1): 55-65.

Cureton, D. K., R. H. Massol, et al. (2010). "The length of vesicular stomatitis virus particles dictates a need for actin assembly during clathrin-dependent endocytosis." PLoS Pathog 6(9): e1001127.

Das SC, Nayak D, et al. (2006). "Visualization of intracellular transport of vesicular stomatitis virus nucleocapsids in living cells." Journal of Virology 80(13): 6368-6377.

Das, S. C., D. Nayak, et al. (2006). "Visualization of intracellular transport of vesicular stomatitis virus nucleocapsids in living cells." J Virol 80(13): 6368-6377.

Dixit, N. M. and A. S. Perelson (2006). "The metabolism, pharmacokinetics and mechanisms of antiviral activity of ribavirin against hepatitis C virus." Cell Mol Life Sci 63(7-8): 832-842.

Donnelly, R. P. and S. V. Kotenko (2010). "Interferon-lambda: a new addition to an old family." J Interferon Cytokine Res 30(8): 555-564.

Dunn, G. P., K. C. Sheehan, et al. (2005). "IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression." Cancer Res 65(8): 3447-3453.

Ebert O, Harbaran S, et al. (2005). "Systemic therapy of experimental breast cancer metastases by mutant vesicular stomatitis virus in immune-competent mice." Cancer Gene Therapy 12(4): 350-358.

Elia, G., C. Belloli, et al. (2008). "In vitro efficacy of ribavirin against canine distemper virus." Antiviral Res 77(2): 108-113.

Endres, C. J., A. M. Moss, et al. (2009). "The role of the equilibrative nucleoside transporter 1 (ENT1) in transport and metabolism of ribavirin by human and wild-type or Ent1-/- mouse erythrocytes." J Pharmacol Exp Ther 329(1): 387-398.

Eriksson, B., E. Helgstrand, et al. (1977). "Inhibition of influenza virus ribonucleic acid polymerase by ribavirin triphosphate." Antimicrob Agents Chemother 11(6): 946-951.

Errasti-Murugarren, E., M. Pastor-Anglada, et al. (2007). "Role of CNT3 in the transepithelial flux of nucleosides and nucleoside-derived drugs." J Physiol 582(Pt 3): 1249-1260.

Farrow B, Albo D, et al. (2008). "The role of the tumor microenvironment in the progression of pancreatic cancer." Journal of Surgical Research 149(2): 319-328.

Feld, J. J. and J. H. Hoofnagle (2005). "Mechanism of action of interferon and ribavirin in treatment of hepatitis C." Nature 436(7053): 967-972.

Feld, J. J., S. Nanda, et al. (2007). "Hepatic gene expression during treatment with peginterferon and ribavirin: Identifying molecular pathways for treatment response." Hepatology 46(5): 1548-1563.

Fuerst, T. R., E. G. Niles, et al. (1986/11). "Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase." Proc.Natl.Acad.Sci.U.S.A 83(21): 8122-8126.

Fukuchi, Y., T. Furihata, et al. (2010). "Characterization of ribavirin uptake systems in human hepatocytes." J Hepatol 52(4): 486-492.

Fukuhara N, Huang C, et al. (2002). "Mutational analysis of the Sendai virus V protein: importance of the conserved residues for Zn binding, virus pathogenesis, and efficient RNA editing." Virology 299(2): 172-181.

Furuichi, Y., A. LaFiandra, et al. (1977). "5'-terminal structure and mRNA stability." Nature (London) 266: 235-239.

Furukawa T, Duguid WP, et al. (1996). "Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16." The American Journal of Pathology 148(6): 1763-1770.

Gaddy DF and D. S. and Lyles (2005). "Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways." Journal of Virology 79(7): 4170-4179.

Gaddy DF and D. S. and Lyles (2007). "Oncolytic vesicular stomatitis virus induces apoptosis via signaling through PKR, Fas, and Daxx." Journal of Virology 81(6): 2792-2804.

Gallois-Montbrun, S., Y. Chen, et al. (2003). "Structural analysis of the activation of ribavirin analogs by NDP kinase: comparison with other ribavirin targets." Mol Pharmacol 63(3): 538-546.

Garcin, D., T. Pelet, et al. (1995). "A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus." Embo J 14(24): 6087-6094.

Giedlin, M. A., D. N. Cook, et al. (2003). "Vesicular stomatitis virus: an exciting new therapeutic oncolytic virus candidate for cancer or just another chapter from Field's Virology?" Cancer Cell 4(4): 241-243.

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

Glue, P. (1999). "The clinical pharmacology of ribavirin." Semin Liver Dis 19 Suppl 1: 17-24.

Goel, A., S. K. Carlson, et al. (2007). "Radioiodide imaging and radiovirotherapy of multiple myeloma using VSV(Delta51)-NIS, an attenuated vesicular stomatitis virus encoding the sodium iodide symporter gene." Blood 110(7): 2342-2350.

Goswami, B. B., E. Borek, et al. (1979). "The broad spectrum antiviral agent ribavirin inhibits capping of mRNA." Biochem Biophys Res Commun 89(3): 830-836.

Green, T. J., S. Macpherson, et al. (2000). "Study of the assembly of vesicular stomatitis virus N protein: role of the P protein." J Virol 74(20): 9515-9524.

Green, T. J., X. Zhang, et al. (2006). "Structure of the vesicular stomatitis virus nucleoprotein-RNA complex." Science 313(5785): 357-360.

Haller, O. and G. Kochs (2011). "Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity." J Interferon Cytokine Res 31(1): 79-87.

Hamacher, R., R. M. Schmid, et al. (2008). "Apoptotic pathways in pancreatic ductal adenocarcinoma." Mol Cancer 7: 64.

Hastie, E. and V. Grdzelishvili (2012). "Vesicular stomatitis virus as a flexible platform for oncolytic virotherapy against cancer." Journal of General Virology.

Hastie, E. and V. Z. Grdzelishvili (2012). "Vesicular stomatitis virus as a flexible platform for oncolytic virotherapy against cancer." J Gen Virol 93(Pt 12): 2529-2545.

Hiscott, J., N. Grandvaux, et al. (2003). "Convergence of the NF-kappaB and interferon signaling pathways in the regulation of antiviral defense and apoptosis." Ann N Y Acad Sci 1010: 237-248.

Hofmann, W. P., C. Sarrazin, et al. (2003). "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 187(6): 982-987.

Honda, K., H. Yanai, et al. (2005). "IRF-7 is the master regulator of type-I interferon-dependent immune responses." Nature 434(7034): 772-777.

Hoofnagle, J. H. (2002). "Course and outcome of hepatitis C." Hepatology 36(5 Suppl 1): S21-29.

Horikami, S. M. and S. A. Moyer (1995/8/20). "Alternative amino acids at a single site in the Sendai virus L protein produce multiple defects in RNA synthesis in vitro." Virology 211(2): 577-582.

Hoti, N., W. Chowdhury, et al. (2006). "Valproic acid, a histone deacetylase inhibitor, is an antagonist for oncolytic adenoviral gene therapy." Mol Ther 14(6): 768-778.

Hruska, J. F., J. M. Bernstein, et al. (1980). "Effects of ribavirin on respiratory syncytial virus in vitro." Antimicrob Agents Chemother 17(5): 770-775.

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

Iwamura T, Katsuki T, et al. (1987). "Establishment and characterization of a human pancreatic cancer cell line (SUIT-2) producing carcinoembryonic antigen and carbohydrate antigen 19-9." Jpn J Cancer Res. 78(1): 54-62.

Janelle, V., F. Brassard, et al. (2011). "Mutations in the glycoprotein of vesicular stomatitis virus affect cytopathogenicity: potential for oncolytic virotherapy." J Virol 85(13): 6513-6520.

Jarvis, S. M., J. A. Thorn, et al. (1998). "Ribavirin uptake by human erythrocytes and the involvement of nitrobenzylthioinosine-sensitive (es)-nucleoside transporters." Br J Pharmacol 123(8): 1587-1592.

Jemal, A., R. Siegel, et al. (2010). "Cancer statistics, 2010." CA Cancer J Clin 60(5): 277-300.

Johnson, V. A., F. Brun-Vezinet, et al. (2007). "Update of the drug resistance mutations in HIV-1: 2007." Top HIV Med 15(4): 119-125.

Jones, M. E. (1980). "Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis." Annu Rev Biochem 49: 253-279.

Jones, S., X. Zhang, et al. (2008). "Core signaling pathways in human pancreatic cancers revealed by global genomic analyses." Science 321(5897): 1801-1806.

Jordan, I., T. Briese, et al. (1999/9). "Inhibition of Borna disease virus replication by ribavirin." J.Virol. 73(9): 7903-7906.

Justesen, J., R. Hartmann, et al. (2000). "Gene structure and function of the 2'-5'-oligoadenylate synthetase family." Cell Mol Life Sci 57(11): 1593-1612.

Kaplan, B. (2006). "Mycophenolic acid trough level monitoring in solid organ transplant recipients treated with mycophenolate mofetil: association with clinical outcome." Curr Med Res Opin 22(12): 2355-2364.

Kato, A., C. Cortese-Grogan, et al. (2004/7). "Characterization of the amino acid residues of sendai virus C protein that are critically involved in its interferon antagonism and RNA synthesis down-regulation." J.Virol. 78(14): 7443-7454.

Kelly, E. and S. J. Russell (2007). "History of oncolytic viruses: genesis to genetic engineering." Molecular therapy : the journal of the American Society of Gene Therapy 15(4): 651-659.

Kelly EJ, Nace R, et al. (2010). "Attenuation of vesicular stomatitis virus encephalitis through microRNA targeting." Journal of Virology 84(3): 1550-1562.

Kirsi, J. J., J. A. North, et al. (1983). "Broad-spectrum antiviral activity of 2-beta-D-ribofuranosylselenazole-4-carboxamide, a new antiviral agent." Antimicrob Agents Chemother 24(3): 353-361.

Lamb, R. A. (1993). "Paramyxovirus fusion: a hypothesis for changes." Virology 197(1): 1-11.

Lamb, R. A., B. W. Mahy, et al. (1976). "The synthesis of sendai virus polypeptides in infected cells." Virology 69(1): 116-131.

Lamb, R. A. and G. D. Parks (2007). Paramyxoviridae: The viruses and their replication. Fields Virology. e. In: D.M.Knipe and P.M.Howley, Lippincott Williams & Wilkins, Philadelphia, 5th edition.: 1449-1496.

Lamb, R. A., R. G. Paterson, et al. (2006). "Paramyxovirus membrane fusion: lessons from the F and HN atomic structures." Virology 344(1): 30-37.

Lanford, R. E., D. Chavez, et al. (2001). "Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes." J Virol 75(17): 8074-8081.

Larson, E. W., E. L. Stephen, et al. (1976). "Therapeutic effects of small-particle aerosols of ribavirin on parainfluenza (sendai) virus infections of mice." Antimicrob Agents Chemother 10(4): 770-772.

Lawson, N. D., E. A. Stillman, et al. (1995). "Recombinant vesicular stomatitis viruses from DNA." Proc.Natl.Acad.Sci.U.S.A 92(10): 4477-4481.

Le Boeuf, F., J. S. Diallo, et al. (2010). "Synergistic interaction between oncolytic viruses augments tumor killing." Mol Ther 18(5): 888-895.

Lederman, M. M. (1995). "Host-directed and immune-based therapies for human immunodeficiency virus infection." Ann Intern Med 122(3): 218-222.

Lee, C. K., H. A. Bluyssen, et al. (1997). "Regulation of interferon-alpha responsiveness by the duration of Janus kinase activity." J Biol Chem 272(35): 21872-21877.

Lenard, J. and R. Vanderoef (1990). "Localization of the membrane-associated region of vesicular stomatitis virus M protein at the N terminus, using the hydrophobic, photoreactive probe 125I-TID." J Virol 64(7): 3486-3491.

Letchworth, G. J., L. L. Rodriguez, et al. (1999). "Vesicular stomatitis." Vet J 157(3): 239-260.

Leyssen, P., J. Balzarini, et al. (2005). "The predominant mechanism by which ribavirin exerts its antiviral activity in vitro against flaviviruses and paramyxoviruses is mediated by inhibition of IMP dehydrogenase
The anti-yellow fever virus activity of ribavirin is independent of error-prone replication." J Virol 79(3): 1943-1947.

Lichty BD, Power AT, et al. (2004). "Vesicular stomatitis virus: re-inventing the bullet." TRENDS in Molecular Medicine 10(5): 210-216.

Lindsay TH, Jonas BM, et al. (2005). "Pancreatic cancer pain and its correlation with changes in tumor vasculature, macrophage infiltration, neuronal innervation, body weight and disease progression." Pain 119(1-3): 233-246.

Linge, C., D. Gewert, et al. (1995). "Interferon system defects in human malignant melanoma." Cancer Research 55(18): 4099-4104.

Liu, W. L., W. C. Su, et al. (2007). "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 196(3): 425-434.

Liuzzi, M., S. W. Mason, et al. (2005). "Inhibitors of respiratory syncytial virus replication target cotranscriptional mRNA guanylylation by viral RNA-dependent RNA polymerase." J Virol 79(20): 13105-13115.

Loustaud-Ratti, V., S. Alain, et al. (2008). "Ribavirin exposure after the first dose is predictive of sustained virological response in chronic hepatitis C." Hepatology 47(5): 1453-1461.

Maag, D., C. Castro, et al. (2001). "Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin." J Biol Chem 276(49): 46094-46098.

Malinoski, F. and V. Stollar (1980). "Inhibition of Sindbis virus replication in Aedes albopictus cells by virazole (ribavirin) and its reversal by actinomycin: a correction." Virology 102(2): 473-476.

Mandal, S., F. Abebe, et al. (2011). "2 '-5 ' oligoadenylate synthetase 1 polymorphism is associated with prostate cancer." Cancer 117(24): 5509-5518.

Marozin, S., J. Altomonte, et al. (2008). "Inhibition of the IFN-beta response in hepatocellular carcinoma by alternative spliced isoform of IFN regulatory factor-3." Mol Ther 16(11): 1789-1797.

Marroqui, L., A. Estepa, et al. (2008). "Inhibitory effect of mycophenolic acid on the replication of infectious pancreatic necrosis virus and viral hemorrhagic septicemia virus." Antiviral Res 80(3): 332-338.

Martin, P. and D. M. Jensen (2008). "Ribavirin in the treatment of chronic hepatitis C." J Gastroenterol Hepatol 23(6): 844-855.

Matin, S. F., R. R. Rackley, et al. (2001). "Impaired alpha-interferon signaling in transitional cell carcinoma: lack of p48 expression in 5637 cells." Cancer Research 61(5): 2261-2266.

Matrosovich, M., T. Matrosovich, et al. (2006). "New low-viscosity overlay medium for viral plaque assays." Virol J 3: 63.

Maynard, M., P. Pradat, et al. (2008). "Prediction of sustained virological response by ribavirin plasma concentration at week 4 of therapy in hepatitis C virus genotype 1 patients." Antivir Ther 13(4): 607-611.

Mire, C. E., A. D. Miller, et al. (2012). "Recombinant vesicular stomatitis virus vaccine vectors expressing filovirus glycoproteins lack neurovirulence in nonhuman primates." PLoS Negl Trop Dis 6(3): e1567.

Moerdyk-Schauwecker, M., N. R. Shah, et al. (2012). "Resistance of pancreatic cancer cells to oncolytic vesicular stomatitis virus: Role of type I interferon signaling." Virology.

Monsurro, V., S. Beghelli, et al. (2010). "Anti-viral state segregates two molecular phenotypes of pancreatic adenocarcinoma: potential relevance for adenoviral gene therapy." J Transl Med 8: 10.

Mosmann, T. R. and S. Sad (1996). "The expanding universe of T-cell subsets: Th1, Th2 and more." Immunol Today 17(3): 138-146.

Moyer, S. A., S. C. Baker, et al. (1986/8). "Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs." Proc.Natl.Acad.Sci.U.S.A 83(15): 5405-5409.

Muller, W. E., A. Maidhof, et al. (1977). "Virazole (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; a cytostatic agent." Biochem Pharmacol 26(11): 1071-1075.

Murphy AM and G. VZ (2009). "Identification of sendai virus L protein amino acid residues affecting viral mRNA cap methylation." Journal of Virology 83(4): 1669-1681.

Murphy, A. M., D. M. Besmer, et al. (2012). "Vesicular stomatitis virus as an oncolytic agent against pancreatic ductal adenocarcinoma." J Virol 86(6): 3073-3087.

Naik, S. and S. J. Russell (2009). "Engineering oncolytic viruses to exploit tumor specific defects in innate immune signaling pathways." Expert Opin Biol Ther 9(9): 1163-1176.

Nakhaei, P., P. Genin, et al. (2009). "RIG-I-like receptors: sensing and responding to RNA virus infection." Semin Immunol 21(4): 215-222.

Obuchi M, Fernandez M, et al. (2003). "Development of recombinant vesicular stomatitis viruses that exploit defects in host defense to augment specific oncolytic activity." Journal of Virology 77(16): 8843-8856.

Okabe T, Yamaguchi N, et al. (1983). "Establishment and characterization of a carcinoembryonic antigen (CEA)-producing cell line from a human carcinoma of the exocrine pancreas." Cancer 51(4): 662-668.

Ottolino-Perry, K., J. S. Diallo, et al. (2010). "Intelligent design: combination therapy with oncolytic viruses." Mol Ther 18(2): 251-263.

Page, T. and J. D. Connor (1990). "The metabolism of ribavirin in erythrocytes and nucleated cells." Int J Biochem 22(4): 379-383.

Paglino, J. C. and A. N. van den Pol (2011). "Vesicular stomatitis virus has extensive oncolytic activity against human sarcomas: rare resistance is overcome by blocking interferon pathways." J Virol 85(18): 9346-9358.

Paladino, P. and K. L. Mossman (2009). "Mechanisms employed by herpes simplex virus 1 to inhibit the interferon response." J Interferon Cytokine Res 29(9): 599-607.

Parker, W. B. (2005). "Metabolism and antiviral activity of ribavirin." Virus Res 107(2): 165-171.

Pelaez, A., G. M. Lyon, et al. (2009). "Efficacy of oral ribavirin in lung transplant patients with respiratory syncytial virus lower respiratory tract infection." J Heart Lung Transplant 28(1): 67-71.

Perdiguero, B. and M. Esteban (2009). "The interferon system and vaccinia virus evasion mechanisms." J Interferon Cytokine Res 29(9): 581-598.

Petersen, J. M., L. S. Her, et al. (2000). "The matrix protein of vesicular stomatitis virus inhibits nucleocytoplasmic transport when it is in the nucleus and associated with nuclear pore complexes." Molecular and cellular biology 20(22): 8590-8601.

Pfeffer, L. M., C. Wang, et al. (1996). "Human renal cancers resistant to IFN's antiproliferative action exhibit sensitivity to IFN's gene-inducing and antiviral actions." J Urol 156(5): 1867-1871.

Pfeiffer, J. K. and K. Kirkegaard (2003). "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 100(12): 7289-7294.

Pfeiffer, J. K. and K. Kirkegaard (2005). "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 79(4): 2346-2355.

Pringle, C. R. (1999). "Virus taxonomy--1999. The universal system of virus taxonomy, updated to include the new proposals ratified by the International Committee on Taxonomy of Viruses during 1998." Arch Virol 144(2): 421-429.

Rahmeh, A. A., A. D. Schenk, et al. (2010). "Molecular architecture of the vesicular stomatitis virus RNA polymerase." Proc Natl Acad Sci U S A 107(46): 20075-20080.

Rankin, J. T., Jr., S. B. Eppes, et al. (1989/1). "Studies on the mechanism of the antiviral activity of ribavirin against reovirus." Virology 168(1): 147-158.

Robins, R. K., G. R. Revankar, et al. (1985). "The importance of IMP dehydrogenase inhibition in the broad spectrum antiviral activity of ribavirin and selenazofurin." Adv Enzyme Regul 24: 29-43.

Rodriguez, L. L. (2002). "Emergence and re-emergence of vesicular stomatitis in the United States." Virus Res 85(2): 211-219.

Roy, L. D., M. Sahraei, et al. (2011). "MUC1 enhances invasiveness of pancreatic cancer cells by inducing epithelial to mesenchymal transition." Oncogene 30(12): 1449-1459.

Russell, S. J. and K. W. Peng (2007). "Viruses as anticancer drugs." Trends Pharmacol Sci 28(7): 326-333.

Russell, S. J., K. W. Peng, et al. (2012). "Oncolytic virotherapy." Nat Biotechnol 30(7): 658-670.

Sadler, A. J. and B. R. Williams (2008). "Interferon-inducible antiviral effectors." Nat Rev Immunol 8(7): 559-568.

Saloura, V., L. C. Wang, et al. (2010). "Evaluation of an attenuated vesicular stomatitis virus vector expressing interferon-beta for use in malignant pleural mesothelioma: heterogeneity in interferon responsiveness defines potential efficacy." Hum Gene Ther 21(1): 51-64.

Sarrazin, C., U. Mihm, et al. (2005). "Clinical significance of in vitro replication-enhancing mutations of the hepatitis C virus (HCV) replicon in patients with chronic HCV infection." J Infect Dis 192(10): 1710-1719.

Scheidel, L. M., R. K. Durbin, et al. (1989/12). "SVLM21, a Sindbis virus mutant resistant to methionine deprivation, encodes an altered methyltransferase." Virology 173(2): 408-414.

Scheidel, L. M. and V. Stollar (1991). "Mutations that confer resistance to mycophenolic acid and ribavirin on Sindbis virus map to the nonstructural protein nsP1." Virology 181: 490-499.

Schuster, A., I. C. Johnston, et al. (1996/6/1). "Expression of the human MxA protein is associated with hyperphosphorylation of VSV P protein in human neural Cells." Virology 220(1): 241-245.

Schwegmann, A. and F. Brombacher (2008). "Host-directed drug targeting of factors hijacked by pathogens." Sci Signal 1(29): re8.

Severson, W. E., C. S. Schmaljohn, et al. (2003). "Ribavirin causes error catastrophe during Hantaan virus replication." J Virol 77(1): 481-488.

Shah, N. R., A. Sunderland, et al. (2010). "Cell type mediated resistance of vesicular stomatitis virus and Sendai virus to ribavirin." PLoS ONE 5(6): e11265.

Sharif-Askari, E., P. Nakhaei, et al. (2007). "Bax-dependent mitochondrial membrane permeabilization enhances IRF3-mediated innate immune response during VSV infection." Virology 365(1): 20-33.

Shatkin, A. J. (1985/2). "mRNA cap binding proteins: essential factors for initiating translation." Cell. 40(2): 223-224.

Shiffman, M. L. (2009). "What future for ribavirin?" Liver Int 29 Suppl 1: 68-73.

Shishodia, S. and B. B. Aggarwal (2002). "Nuclear factor-kappaB activation: a question of life or death." J Biochem Mol Biol 35(1): 28-40.

Shmulevitz, M., L. Z. Pan, et al. (2010). "Oncogenic Ras promotes reovirus spread by suppressing IFN-beta production through negative regulation of RIG-I signaling." Cancer Research 70(12): 4912-4921.

Shuman, S. (1982). "RNA capping by HeLa cell RNA guanylyltransferase. Characterization of a covalent protein-guanylate intermediate." Journal of Biological Chemistry 257(12): 7237-7245.

Sidwell, R. W., J. H. Huffman, et al. (1972). "Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide." Science 177(50): 705-706.

Sidwell, R. W., G. P. Khare, et al. (1975). "In vitro and in vivo effect of 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) on types 1 and 3 parainfulenza virus infections." Chemotherapy 21(3-4): 205-220.

Sierra, M., A. Airaksinen, et al. (2007). "Foot-and-mouth disease virus mutant with decreased sensitivity to ribavirin: implications for error catastrophe." J Virol 81(4): 2012-2024.

Sinkovics JG and J. C. and Horvath (2008). "Natural and genetically engineered viral agents for oncolysis and gene therapy of human cancers." Arch. Immun. Ther. Exp. 56: 3s-59s.

Smallwood, S., T. Hovel, et al. (2002/12/5). "Different substitutions at conserved amino acids in domains II and III in the Sendai L RNA polymerase protein inactivate viral RNA synthesis." Virology 304(1): 135-145.

Smee, D. F., M. Bray, et al. (2001). "Antiviral activity and mode of action studies of ribavirin and mycophenolic acid against orthopoxviruses in vitro." Antivir Chem Chemother 12(6): 327-335.

Smee, D. F. and T. R. Matthews (1986). "Metabolism of ribavirin in respiratory syncytial virus-infected and uninfected cells." Antimicrob Agents Chemother 30(1): 117-121.

Smee, D. F., R. W. Sidwell, et al. (1982). "Inhibition of rotaviruses by selected antiviral substances: mechanisms of viral inhibition and in vivo activity." Antimicrob Agents Chemother 21(1): 66-73.

Stanifer, M. L., D. K. Cureton, et al. (2011). "A recombinant vesicular stomatitis virus bearing a lethal mutation in the glycoprotein gene uncovers a second site suppressor that restores fusion." Journal of virology 85(16): 8105-8115.

Stathis A and M. J. Moore (2010). "Advanced pancreatic carcinoma: current treatment and future challenges." Nature Reviews in Clinical Oncology 7: 163-172.

Stojdl DF, L. B., tenOever BR, Paterson JM, Power AT, Knowles S, Marius R, Reynard J, Poliquin L, Atkins H, Brown EG, Durbin RK, Durbin JE, Hiscott J, Bell JC. (2003). "VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents." Cancer Cell 4(4): 263-275.

Stojdl, D. F., B. Lichty, et al. (2000). "Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus." Nat Med 6(7): 821-825.

Streeter, D. G., J. T. Witkowski, et al. (1973). "Mechanism of action of 1- -D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent." Proc Natl Acad Sci U S A 70(4): 1174-1178.

Sun, W. H., C. Pabon, et al. (1998). "Interferon-alpha resistance in a cutaneous T-cell lymphoma cell line is associated with lack of STAT1 expression." Blood 91(2): 570-576.

Sun, Y., D. H. Chung, et al. (2007). "Activity of ribavirin against Hantaan virus correlates with production of ribavirin-5'-triphosphate, not with inhibition of IMP dehydrogenase." Antimicrob Agents Chemother 51(1): 84-88.

Swinteck, B. D. and D. S. Lyles (2008). "Plasma membrane microdomains containing vesicular stomatitis virus M protein are separate from microdomains containing G protein and nucleocapsids." Journal of virology 82(11): 5536-5547.

Takimoto, T., J. L. Hurwitz, et al. (2005). "Recombinant Sendai virus as a novel vaccine candidate for respiratory syncytial virus." Viral Immunol 18(2): 255-266.

Tam, R. C., B. Pai, et al. (1999). "Ribavirin polarizes human T cell responses towards a Type 1 cytokine profile." J Hepatol 30(3): 376-382.

Tam, R. C., K. Ramasamy, et al. (2000). "The ribavirin analog ICN 17261 demonstrates reduced toxicity and antiviral effects with retention of both immunomodulatory activity and reduction of hepatitis-induced serum alanine aminotransferase levels." Antimicrob Agents Chemother 44(5): 1276-1283.

Tan, S. L., G. Ganji, et al. (2007). "Systems biology and the host response to viral infection." Nat Biotechnol 25(12): 1383-1389.

Thomas, E., J. J. Feld, et al. (2010). "Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models." Hepatology.

Toltzis, P. and A. S. Huang (1986/6). "Effect of ribavirin on macromolecular synthesis in vesicular stomatitis virus-infected cells." Antimicrob.Agents Chemother. 29(6): 1010-1016.

Toltzis, P., K. O'Connell, et al. (1988/4). "Effect of phosphorylated ribavirin on vesicular stomatitis virus transcription." Antimicrob.Agents Chemother. 32(4): 492-497.

Tumilasci, V. F., S. Oliere, et al. (2008). "Targeting the apoptotic pathway with BCL-2 inhibitors sensitizes primary chronic lymphocytic leukemia cells to vesicular stomatitis virus-induced oncolysis." J Virol 82(17): 8487-8499.

Vähä-Koskela MJ, Heikkilä JE, et al. (2007). "Oncolytic viruses in cancer therapy." Cancer Letters 254(2): 178-216.

Vaha_Koskela, M. J., M. T. Tuittila, et al. (2003). "A novel neurotropic expression vector based on the avirulent A7(74) strain of Semliki Forest virus." Journal of Neurovirology 9(1): 1-15.

Vignuzzi, M., J. K. Stone, et al. (2005). "Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications." Virus Res 107(2): 173-181.

Vo, N. V., K. C. Young, et al. (2003). "Mutagenic and inhibitory effects of ribavirin on hepatitis C virus RNA polymerase." Biochemistry 42(35): 10462-10471.

Wang, B. X., R. Rahbar, et al. (2011). "Interferon: current status and future prospects in cancer therapy." J Interferon Cytokine Res 31(7): 545-552.

Watanabe, Y., Y. Hashimoto, et al. (2012). "Enhanced antitumor efficacy of telomerase-specific oncolytic adenovirus with valproic acid against human cancer cells." Cancer Gene Ther 19(11): 767-772.

Whelan, S. P., L. A. Ball, et al. (1995). "Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones." Proc.Natl.Acad.Sci.U.S.A 92(18): 8388-8392.

Whelan, S. P. and G. W. Wertz (1999/1). "Regulation of RNA synthesis by the genomic termini of vesicular stomatitis virus: identification of distinct sequences essential for transcription but not replication." J.Virol. 73(1): 297-306.

Wiegand, M. A., S. Bossow, et al. (2007). "De novo synthesis of N and P proteins as a key step in Sendai virus gene expression." J Virol 81(24): 13835-13844.

Wilden, H., P. Fournier, et al. (2009). "Expression of RIG-I, IRF3, IFN-beta and IRF7 determines resistance or susceptibility of cells to infection by Newcastle Disease Virus." Int J Oncol 34(4): 971-982.

Willis, R. C., D. A. Carson, et al. (1978). "Adenosine kinase initiates the major route of ribavirin activation in a cultured human cell line." Proc Natl Acad Sci U S A 75(7): 3042-3044.

Wohnsland, A., W. P. Hofmann, et al. (2007). "Viral determinants of resistance to treatment in patients with hepatitis C." Clin Microbiol Rev 20(1): 23-38.

Wollmann G, R. V., Simon I, Rose JK, van den Pol AN. (2010). "Some attenuated variants of vesicular stomatitis virus show enhanced oncolytic activity against human glioblastoma cells relative to normal brain cells." Journal of Virology 84(3): 1563-1573.

Wong, L. H., K. G. Krauer, et al. (1997). "Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma." J Biol Chem 272(45): 28779-28785.

Wongthida, P., R. M. Diaz, et al. (2011). "VSV oncolytic virotherapy in the B16 model depends upon intact MyD88 signaling." Mol Ther 19(1): 150-158.

Wray, S. K., B. E. Gilbert, et al. (1985). "Mode of action of ribavirin: effect of nucleotide pool alterations on influenza virus ribonucleoprotein synthesis." Antiviral Res 5(1): 29-37.

Wu, J. Z., G. Larson, et al. (2005). "Phosphorylation of ribavirin and viramidine by adenosine kinase and cytosolic 5'-nucleotidase II: Implications for ribavirin metabolism in erythrocytes." Antimicrob Agents Chemother 49(6): 2164-2171.

Wu L, H. T., Meseck M, Altomonte J, Ebert O, Shinozaki K, García-Sastre A, Fallon J, Mandeli J, Woo SL. (2008). "rVSV(M Delta 51)-M3 is an effective and safe oncolytic virus for cancer therapy." Human Gene Therapy 19(6): 635-647.

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

Zhang, K. X., Y. Matsui, et al. (2010). "Down-regulation of type I interferon receptor sensitizes bladder cancer cells to vesicular stomatitis virus-induced cell death." International Journal of Cancer. Journal International Du Cancer 127(4): 830-838.

Zhang, Y., M. Jamaluddin, et al. (2003). "Ribavirin treatment up-regulates antiviral gene expression via the interferon-stimulated response element in respiratory syncytial virus-infected epithelial cells." J Virol 77(10): 5933-5947.

Zhou, S., R. Liu, et al. (2003). "The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA." Virology 310(2): 333-342.

Zimmerman, T. P. and R. D. Deeprose (1978). "Metabolism of 5-amino-1-beta-D-ribofuranosylimidazole-4-carboxamide and related five-membered heterocycles to 5'-triphosphates in human blood and L5178Y cells." Biochem Pharmacol 27(5): 709-716.