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REVIEW

Mechanisms of action of ribavirin against distinct viruses

Jason D. Graci and Craig E. Cameron*

Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania, USA

SUMMARY

The nucleoside analogue ribavirin has antiviral activity against many distinct viruses both *in vitro* and *in vivo*. Five distinct mechanisms have been proposed to explain the antiviral properties of ribavirin. These include both indirect mechanisms (inosine monophosphate dehydrogenase inhibition, immunomodulatory effects) and direct mechanisms (interference with RNA capping, polymerase inhibition, lethal mutagenesis). Recent concerns about bioterrorism have renewed interest in exploring the antiviral activity of ribavirin against unique viruses. In this paper, we review the proposed mechanisms of action with emphasis on recent discoveries, as well as the implications of ribavirin resistance. Evidence exists to support each of the five proposed mechanisms of action, and distinct virus/host combinations may preferentially favour one or more of these mechanisms during antiviral therapy. Copyright © 2005 John Wiley & Sons, Ltd.

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INTRODUCTION

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, also known as Virazole) is a synthetic purine nucleoside analogue first synthesised by Sidwell and colleagues in 1972 (Figure 1) [1,2]. It is of particular interest because it was the first synthetic nucleoside to exhibit broad-spectrum antiviral activity, and is one of few antiviral drugs in clinical use effective against agents other than HIV and herpesviruses. X-ray crystallography indicated that the carboxamide moiety of the pseudobase causes ribavirin to resemble guanosine (or inosine) [3].

Ribavirin exhibits antiviral activity against a broad range of both DNA and RNA viruses *in vitro*. The initial report indicated activity against 16 DNA and RNA viruses in cell culture and in mice [2]. In some cases, this inhibition has transferred into clinical applications. Most notably, ribavirin is used in combination with interferon-α for treatment of HCV infection, although the success rate of this therapy is limited [4–6] but improved in non-genotype 1 HCV infection with pegylated interferon [7]. Ribavirin aerosol is used for treatment of paediatric infection by respiratory syncytial virus [8]. Ribavirin has also been used experimentally against a number of other viral infections, including Lassa fever virus infection [9] and other haemorrhagic fever virus infections [10].

More than 30 years since its discovery, the mechanism of action of ribavirin still remains controversial. A number of distinct mechanisms have been suggested depending on the particular virus being studied. Broadly, there are five primary mechanisms of action proposed for ribavirin. Indirect mechanisms include reduction in cellular guanosine triphosphate (GTP) pools via inosine monophosphate dehydrogenase (IMPDH) inhibition, and an immunomodulatory effect in which a T-helper type 1 (antiviral) immune response is maintained. Direct mechanisms include inhibition of RNA capping activity, direct inhibition of

*Corresponding author: Dr. Craig E. Cameron, 201 Althouse Lab, University Park, PA, USA.
E-mail: cec9@psu.edu

Abbreviations used

ALT, alanine aminotransferas; CTP, cytidine triphosphate; CPE, cytopathic effect; HCV, hepatitis C virus; EICAR, 5-ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide; eIF4E, eukaryotic initiation factor 4E; FMDV, foot-and-mouth disease virus; GBV-B, GB virus B; GMP, guanosine monophosphate; GTP, guanosine triphosphate; IMPDH, inosine monophosphate dehydrogenase; MPA, mycophenolic acid; PV, poliovirus; RdRp, RNA-dependent RNA polymerase; RMP, ribavirin monophosphate; RTP, ribavirin triphosphate; SARS, severe acute respiratory syndrome; UTP, uridine triphosphate; WNV, West Nile virus; XMP, xanthosine monophosphate

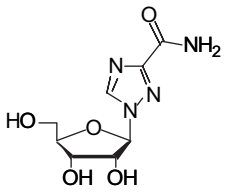

Figure 1. Ribavirin is a synthetic nucleoside analogue with a structure similar to guanosine

viral polymerases, and increased mutation frequency via incorporation of ribavirin into newly synthesised genomes leading to error catastrophe. Here, we review the proposed mechanisms of action for ribavirin and the evidence for each from a variety of studies with distinct viruses, with an emphasis on recent discoveries.

METABOLISM AND CELLULAR EFFECTS OF RIBAVIRIN

Ribavirin is clinically administered as the nucleoside. Adenosine kinase is the cellular enzyme responsible for conversion to ribavirin monophosphate (RMP). Cells deficient in adenosine kinase activity accumulate only small amounts of the phosphorylated forms of ribavirin [11,12]. Subsequent phosphorylation of RMP yields the di- and triphosphorylated nucleotides [12,13]. Phosphorylation is rapid, with half-maximal levels of metabolites being reached within a few hours of exposure to cultured cells [12]. Although the relative concentration of the mono-, di- and triphosphorylated forms varies by cell type, ribavirin triphosphate (RTP) is generally the predominant metabolite [12]. GTP pools are reduced approximately two-fold by ribavirin treatment, with a concurrent increase in cellular CTP and UTP [13,14]. These changes in nucleotide pools are due to the ability of RMP to act as an inhibitor of IMPDH (see following section). Deoxynucleotide forms of ribavirin have not been detected intracellularly, suggesting that ribavirin diphosphate is not a substrate for cellular ribonucleotide reductase. However, cellular deoxynucleotide pools are generally much smaller than ribonucleotide pools. Thus, ribavirin deoxynucleotides may be present in very low concentrations that have not been detected.

The metabolism of ribavirin is cell-specific. Smee and colleagues demonstrated a reduction in ribavirin metabolites in Vero 76 cells as compared to 3T3 cells, with approximately 13-fold less RMP accumulating in these cells [15]. The half-life of ribavirin metabolites is relatively short in cultured fibroblasts and lymphoblasts, although the nucleotides are much more stable in erythrocytes [12]. This accumulation of ribavirin in erythrocytes is responsible for the reversible haemolytic anaemia that is a side effect of clinical ribavirin therapy [16].

Ribavirin has profound effects upon treated cells. It is a cytostatic agent and causes a reduction in synthesis of DNA, RNA and proteins in exposed cells [14]. Although ribavirin triphosphate accumulates to significant levels in treated cells, ribavirin has not been detected in cellular RNA or DNA [17]. However, this may be due to a low rate of incorporation that is below the limit of detection for cell-based assays.

RIBAVIRIN IS AN INHIBITOR OF INOSINE MONOPHOSPHATE DEHYDROGENASE

The first unique step in the *de novo* cellular synthesis of guanine nucleotides is catalysed by the enzyme IMPDH (Figure 2). This reaction is NAD^+-dependent and converts inosine monophosphate (IMP) to xanthosine monophosphate (XMP). XMP can then be aminated to guanosine monophosphate (GMP) by the enzyme GMP synthase. GMP is further converted to guanine metabolites such as GTP and dGTP, which are essential as precursors for RNA and DNA synthesis, respectively. Additionally, GTP plays important roles in energy

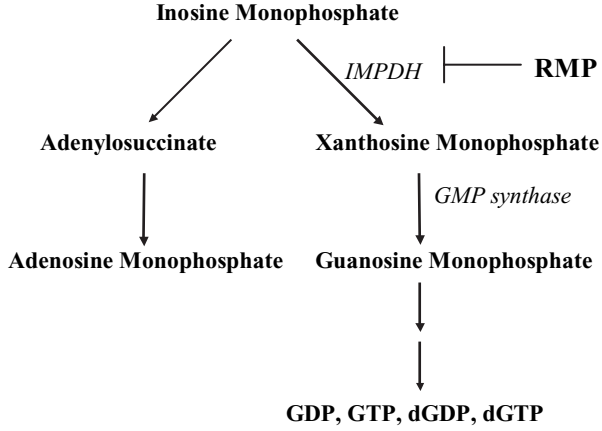

Figure 2. Schematic of the de novo pathway of guanine nucleotide biosynthesis. Conversion to XMP is the first committed step in synthesis of guanine nucleotides. This step is inhibited by ribavirin monophosphate. Enzymes shown in italics. IMPDH, inosine monophosphate dehydrogenase; RMP, ribavirin monophosphate

storage, intracellular signalling, translation by ribosomes and glycoprotein synthesis.

Due to its structural similarity to GMP, RMP is a potent competitive inhibitor of IMPDH [18]. RMP inhibits type I and type II isoforms of human IMPDH with a K_i of 650 nM and 390 nM, respectively [19]. An X-ray crystal structure of RMP in complex with the core domain from human IMPDH type II has been solved [20]. This structure revealed that RMP binds in the active site substrate pocket of IMPDH and is an excellent mimic of the natural substrate IMP.

Inhibition of IMPDH has been suggested as a possible mechanism for the antiviral properties of ribavirin. GTP pools have been demonstrated to be reduced approximately two-fold in ribavirin-treated cells [13]. GTP and dGTP are essential for translation, transcription and RNA and DNA replication. As such, reduction in available GTP was hypothesised to inhibit these integral processes of the viral life cycle, thus explaining the antiviral properties of ribavirn. Since this is a non-specific, cellular effect, IMPDH inhibition could also explain the broad-spectrum activity of ribavirin.

The antiviral activities of ribavirin, 5-ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide (EICAR), and mycophenolic acid (MPA) have been compared using a flavivirus (yellow fever virus) and paramyxoviruses (human parainfluenza virus 3, respiratory syncytial virus) [21]. EICAR is a structural analogue of ribavirin, and the 5′-monophosphate is also a potent inhibitor of IMPDH [22]. MPA is a potent uncompetitive inhibitor of IMPDH and does not need to be metabolically activated (reviewed in [23]). The effects of these three IMPDH inhibitors on Vero and HeLa cell GTP pools were analysed. For all three compounds, a linear correlation was noted between GTP pool inhibition and antiviral activity as measured by viral RNA synthesis, as well as antiviral effect measured by reduction in CPE, suggesting that IMPDH inhibition is the primary mechanism of antiviral activity for all three compounds. However, the measured GTP pool reduction in this case was much more dramatic than that previously reported by other researchers.

In contrast, there are many instances in which IMPDH inhibition alone has not been sufficient to explain the antiviral activity of ribavirin. In a study of the antiviral effect of ribavirin against influenza virus, it was found that the reduction in intracellular GTP pools was saturated at a relatively low concentration (25 μM) of ribavirin [24]. However, ribavirin had a more potent antiviral effect at higher concentrations, although cellular GTP pools were not diminished further. Studies with poliovirus (PV) have shown that the potent antiviral effect induced by ribavirin was accompanied by only small reductions in translation and RNA synthesis [25]. Furthermore, not all IMPDH inhibitors have antiviral activity [25,26]. Thus, IMPDH inhibition may not be the primary mechanism of antiviral activity in most cases.

Nevertheless, IMPDH inhibition can be an important contributor to the antiviral activity of ribavirin. By reducing levels of competing GTP, ribavirin can be more effective as a polymerase inhibitor, capping inhibitor or lethal mutagen (described below). Ribavirin has also been used as an IMPDH inhibitor to increase the potency of other antiviral nucleosides used to treat HIV [27–30], hepatitis B virus [31] and herpes simplex virus type 1 [32]. Yet, ribavirin has also demonstrated antagonism in combination with some nucleoside drugs [27,33].

RIBAVIRIN IS AN IMMUNOMODULATORY AGENT

Ribavirin has also been postulated to act via another indirect antiviral mechanism, by enhancing the host T-cell response. This conclusion stems from observations in HCV-infected patients that ribavirin can reduce serum alanine aminotransferase (ALT) levels (a marker of liver damage) without significantly reducing levels of circulating HCV RNA as determined via PCR [34]. Ribavirin has been suggested to act in combination therapy by maintaining the response to interferon treatment.

Ribavirin is thought to induce a switch in T-helper cell phenotype from type 2 to type 1 [35]. The T-helper type 1 response is associated with cellular immunity and is associated with expression of IL-2, gamma-interferon, and tumour necrosis factor-alpha [36,37]. The T-helper 2 response promotes humoural immunity and is associated with expression of IL-4, IL-5 and IL-10. It has been suggested that an ineffective host immune response is an important factor in chronic infections. A T-helper 2 response has been associated with the development of chronic disease in HCV infection [38]. Ribavirin has indeed been shown to modulate cytokine expression in human

T-cells [39]. Low levels (5–10 μM) of ribavirin inhibited a Type-2 response and promoted a Type-1 response in both CD4+ and CD8+ human T-cells *in vitro*.

Data obtained for the L-enantiomer of ribavirin (ICN 17261, also known as levovirin) support this hypothesis. This compound had similar efficiency in inducing Type-1 cytokine responses and reducing serum ALT levels in a murine model [40]. However, levovirin did not exhibit direct antiviral activity *in vitro* against viruses sensitive to ribavirin. As stereochemical differences likely preclude intracellular phosphorylation [41], this molecule should not be able to be converted to the phosphorylated metabolites, which are necessary for the direct antiviral effects of ribavirin. Clinical trials with levovirin in HCV patients were unsuccessful, suggesting that the immunomodulation observed in vitro is insufficient to produce clinical effects [42]. However, the mechanism by which ribavirin stimulates the immune response is not understood. Interestingly, recent results have suggested that ribvavirin monotherapy may indeed have an antiviral effect [43]. Furthermore, monitoring viral load by PCR-based methods may overlook antiviral activities that result in production of non-infectious genomes.

Mathematical modelling has been used to approximate the contribution of immunomodulatory effects towards ribavirin antiviral activity. The effect of interferon-α and ribavirin therapy on viral load decay has been modelled [44]. For this model, the assumption was used that ribavirin is able to reduce the specific infectivity of new virions (via lethal mutagenesis, see below). The model predicted that, in patients with high interferon effectiveness, ribavirin has little impact on viral load decay. However, in cases of low interferon effectiveness, ribavirin should make an important contribution to viral load reduction. Thus, this model was able to reconcile conflicting data about ribavirin effectiveness, by suggesting that a patient's responsiveness to interferon may be critical. Furthermore, this model ruled out an immunomodulatory effect of ribavirin.

This model was applied to clinical data from 17 patients and provided excellent fits to viral load data. Patients with high interferon effectiveness agreed with a model in which ribavirin has little influence. However, for patients with low interferon effectiveness, the observed loss rate of infected cells during combination therapy was lower than that predicted by interferon alone, indicating that an additional antiviral effect is likely provided by ribavirin.

However, the assumptions made in design of this model can be challenged. First, evidence demonstrating a reduction of HCV infectivity during clinical ribavirin treatment is lacking. Furthermore, the authors assume a progressive effect of ribavirin, with a plateau at 28 days, due to the slow accumulation of ribavirin in plasma, although this is not universally accepted. Finally, it would be informative to compare these predictions to larger samples of clinical data.

Nevertheless, Dixit *et al*. [43] have provided an interesting hypothesis which should be investigated further. Their conclusions suggest that ribavirin therapy can be targeted to those patients in which it will have a beneficial effect, and avoided in those who will have little benefit, reducing the side effects associated with combination therapy.

RIBAVIRIN IS AN INHIBITOR OF RNA CAPPING

The 5′-end of most cellular RNAs and some viral RNAs contains a 7-methylguanosine cap structure essential for RNA stability and translation. As a guanosine nucleotide analogue, ribavirin has the potential to interact with enzymes responsible for 'capping' cellular mRNAs and viral genomic RNAs. Generally, the cap structure is synthesised via three enzymatic reactions (Figure 3): (1) an

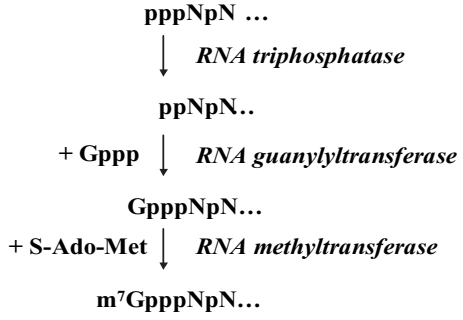

Figure 3. The 5′ 7-methylguanosine cap structure of RNA is produced by three enzymatic reactions. First, an RNA triphosphatase cleaves the 5′ end of the RNA, leaving a diphosphate. Next, GMP is added to the end of the RNA via a 5′-to-5′ linkage by an RNA guanylyltransferase. Finally, the terminal guanosine is methylated at the N-7 position by an RNA methyltransferase, utilising S-adenosylmethionine. Additional methylation can occur on the nucleotides located 3′ to the phosphate bridge of the cap

RNA triphosphatase cleaves the 5′ triphosphate of the RNA to a diphosphate; (2) an RNA guanylyltransferase catalyses the addition of GMP to this 5′ terminus via a unique 5′-to-5′ linkage; (3) an RNA (guanine-7-) methyltransferase catalyses the addition of a methyl group from S-adenosylmethionine to the terminal guanosine at the N-7 position (reviewed in [45]).

The translation initiation factor eIF4E is an oncogene whose overexpression is implicated in a variety of human tumours [46,47]. eIF4E interaction with the 5′ 7-methylguanosine cap of cellular mRNAs is essential for initiation of cap-dependent translation. Overexpression of eIF4E increases translation of a subset of sensitive transcripts, which can lead to malignancy.

Kentsis and colleagues investigated the ability of ribavirin to mimic the 7-methylguanosine cap [48]. Ribavirin triphosphate was shown to bind at the 7-methylguanosine cap binding site of eIF4E with the same affinity as 7-methylguanosine. Ribavirin was also shown to disrupt eIF4E nuclear bodies and to inhibit transport and translation of eIF4E-regulated mRNAs. Furthermore, low micromolar concentrations of ribavirin were able to suppress eIF4E-mediated transformation and tumour growth *in vitro* and *in vivo*. Beyond the cellular implications, these findings also suggest a mechanism of action against viruses which utilise cellular eIF4E or 7-methylguanosine, such as Lassa fever virus [49] and SARS coronavirus [50]. However, recent biochemical data suggest that RTP may not interfere with the interaction between eIF4E and 7-methylguanosine [51].

Some RNA viruses employ a 7-methylguanosine cap structure on the 5′ end of the genomic RNA [45]. Because eukaryotic mRNA capping occurs in the nucleus, virus-encoded enzymes are needed to catalyse the capping reaction for viruses replicating in the cytoplasm. As a guanosine analogue, ribavirin may have the capacity to inhibit the enzymes involved in this pathway.

Scheidel and Stollar isolated a Sindbis virus variant which showed resistance to both ribavirin and the noncompetitive IMPDH inhibitor MPA [52]. The cross-resistance implicates reduction in GTP pools to be at least partially responsible for ribavirin's mechanism of action against this virus. Further investigation indicated that resistance mapped to nsP1, the virus-encoded enzyme that mediates guanylyltransferase activity [53]. Although direct biochemical evidence was not obtained, this finding suggested that ribavirin inhibits the capping of RNA genomes, either by interfering with the guanylyltransferase or methyltransferase activities (both of which are thought to be encoded by nsP1) or potentially by being incorporated as a cap analogue, which may impact translation of the RNA.

It has been suggested that the mechanism of action of ribavirin against vaccinia virus is due to inhibition of the capping reaction [54]. RTP was found to be a potent inhibitor of the vaccinia virus mRNA guanylyltransferase, suggesting that ribavirin acts by producing mRNAs that are not competent for translation. Bougie and Bisallion investigated the interaction of ribavirin with the N-terminal fragment of the vaccinia virus D1 protein, which has both triphosphatase and guanylyltranseferase activity [55]. RTP was found to serve as a substrate for this enzyme, and formation of a covalent RMP-enzyme complex was demonstrated. Transfer of RMP to RNA was also shown biochemically. RNAs capped with ribavirin were more stable to degradation than uncapped RNAs. However, the 7-methyl group found on the normal cap structure is necessary for translation by interaction with eIF4E. The vaccinia virus capping machinery was unable to add 7-methyl group to ribavin; thus, RNAs capped by ribavirin were not efficiently translated. Although ribavirin was an inefficient competitor to GTP in a guanylyltransferase assay, IMPDH inhibition by ribavirin may potentiate this effect *in vivo*.

Benarroch and colleagues investigated the interaction of ribavirin triphosphate with the Dengue virus NS5 RNA 2′-*O*-methyltransferase domain ($NS5MTase_{DV}$) [56]. RTP inhibited the RNA 2′-*O*-methyltransferase activity in vitro with an IC_{50} of 100 μM. Furthermore, RTP was shown to compete for the GTP-binding site of $NS5MTase_{DV}$, with an apparent K_d in the range of 50 μM. An X-ray crystal structure of RTP bound to the enzyme revealed that RTP was located in the GTP-binding site but with an unexpected orientation. RTP mimics GTP in this case by a rotation that superimposes the NH_2 group of ribavirin with the NH_2 group at the 2-position of GTP. This orientation is unique relative to previous models that suggest ribavirin mimics the 1- and 6-position of guanosine (for instance, the crystal structure of ribavirin complexed with IMPDH or models of base-pairing with cytidine).

The observations discussed above suggest that ribavirin may exert its antiviral activity by interaction with RNA capping machinery. The activity may derive from either inhibition of the enzymes involved in adding a 7-methylguanosine cap to viral RNA, or by incorporation of ribavirin as the 5′cap, causing the molecule to be non-functional for translation. This mechanism may function against viruses utilising capped transcripts or genomes. However, it fails to explain the broad-spectrum activity of ribavirin, as many viruses sensitive to ribavirin do not utilise a cap structure during infection.

RIBAVIRIN IS A POLYMERASE INHIBITOR

The primary intracellular metabolite of ribavirin is RTP. It is possible that this nucleotide can interact with viral polymerases and inhibit nucleic acid synthesis. Accumulation of RTP in cells may allow efficient competition with essential GTP or ATP pools. Eriksson and coworkers demonstrated inhibition of the influenza virus RNA polymerase *in vitro* with RTP [57]. Neither ribavirin nor RMP showed any inhibitory activity. RTP was shown to act as a competitive inhibitor with respect to both ATP and GTP. RTP has also been suggested to specifically inhibit RNA synthesis by reovirus [17]. Inhibition was apparently independent of the concentration of the natural nucleotides included in an *in vitro* assay, suggesting inhibition was not by a competitive mechanism. The authors suggested that RTP might bind at a site close to the active site, changing the conformation.

There has also been demonstration of reduced elongation activity by the HCV polymerase *in vitro* when ribavirin was present in the template [58,59]. Polymerase inhibition has also been noted with vesicular stomatitis virus [60,61].

Ribavirin was also investigated as an inhibitor of HIV-1 reverse transcriptase [30]. RTP and RDP both inhibited elongation by HIV-RT in an *in vitro* extension assay, although RDP caused about 40% greater inhibition than RTP in this assay. No chain termination was detected. It is worth noting that this is a measure of DNA synthesis inhibition by a ribonucleotide.

RIBAVIRIN IS A LETHAL MUTAGEN OF RNA VIRUS GENOMES

The finding that RTP was the primary intracellular metabolite of ribavirin caused speculation as to whether the activity of ribavirin could be due to incorporation of the nucleotide into RNA by cellular or viral RNA polymerases. However, early experiments using radiolabelled ribavirin failed to detect any significant ribavirin incorporation into RNA molecules [17].

In 2000, Crotty and colleagues were able to provide strong evidence for ribavirin incorporation into RNA during virus replication [25]. An *in vitro* primer-extension assay was used to measure the kinetics of incorporation of ribavirin triphosphate by the poliovirus RNA-dependent RNA polymerase (RdRp). Ribavirin was incorporated slowly, at approximately the rate of an incorrect nucleotide, which should result in an average of one or two molecules incorporated per 7500-nucleotide RNA genome. Of greater importance was the discovery that ribavirin was templated by either uridine or cytidine with equal efficiency. Furthermore, the presence of ribavirin in the RNA template was able to direct incorporation of either CMP or UMP. Thus, ribavirin is capable of ambiguous basepairing, mimicking either of the natural purines (guanosine normally base-pairs with cytidine while adenosine normally base-pairs with thymidine [or uracil]). This ambiguous basepairing capacity is likely due to rotation of the carboxamide moiety of the pseudobase, generating hydrogen bond acceptor/donor sites favourable for interaction with either of the pyrimidine bases (Figure 4).

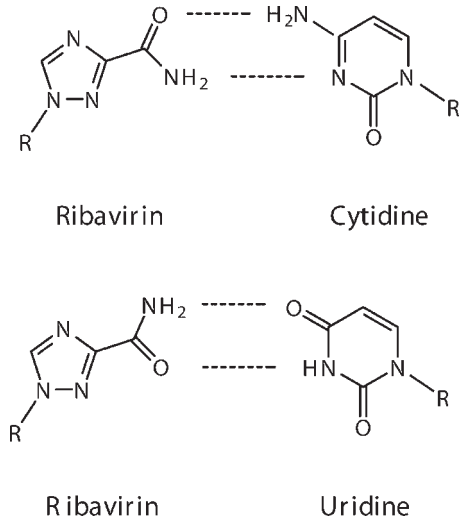

Figure 4. Ribavirin is an ambiguously hydrogen-bonding purine mimic. Rotation of the carboxamide moiety of the pseudobase can result in two distinct basepairing orientations, allowing incorporation opposite either of the naturally occurring pyrimidines

The fact that ribavirin can act as an ambiguous purine base analogue suggested that it has the potential to be mutagenic to RNAs into which it is incorporated. The *in vitro* incorporation studies suggested that ribavirin should induce transition mutations (A-to-G and C-to-U). To investigate this possibility, Crotty and colleagues sequenced the capsid-coding regions of poliovirus grown in the presence of varying concentrations of ribavirin. Sequencing revealed an increase in mutations, particularly the predicted transition mutations. Furthermore, ribavirin treatment resulted in only a minimal decrease in the levels of translation and RNA synthesis. Thus, the antiviral effect of ribavirin seemed to be mediated primarily by inducing mutations into the RNA genome.

RNA viruses have an extraordinarily high mutation frequency (10^{-3} to 10^{-5} per replication cycle), thought to be due to lack of a polymerase proofreading activity [62,63]. As a result, RNA viruses have been hypothesised to exist as a quasispecies, a heterogeneous population which hovers around a consensus or master sequence [64,65]. The genome variability present in such a population may be beneficial in allowing more rapid adaptation to environmental changes, including tropism, immune response or antiviral therapy. However, quasispecies theory predicts the existence of an upper limit to genome variability, known as the error threshold, beyond which additional mutations would be deleterious to the population. The term lethal mutagenesis has been coined to describe an antiviral strategy in which the population would be forced beyond the error threshold [66].

Crotty and colleagues demonstrated the existence of an error threshold by investigating the effect of mutation frequency on poliovirus viability [67]. Poliovirus was grown in the presence of a range of ribavirin concentrations, and the purified RNA was sequenced and assayed for infectivity. The specific infectivity of poliovirus RNA was found to decrease precipitously when the number of mutations per genome was increased only modestly beyond that of untreated viral RNA (Figure 5). Natural poliovirus populations have an average of approximately 1.5 mutations per genome. When this was increased to two mutations per genome, RNA specific infectivity was reduced to 50%. A four-fold increase resulted in only 5% genome infectivity. Furthermore, the antiviral effect of ribavirin was fully explainable by only the reduction in specific infectivity and a mild decrease in RNA synthesis.

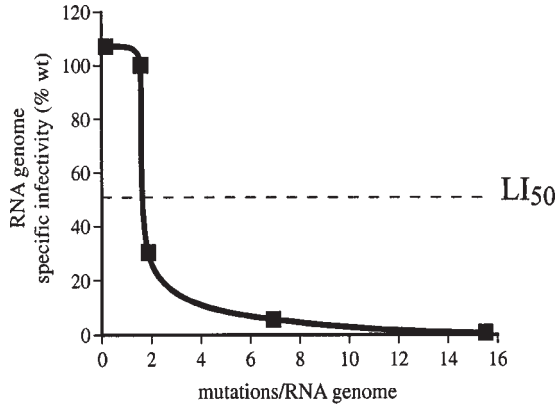

Figure 5. Poliovirus exists at the edge of error catastrophe. Poliovirus replication results in an average of 1.5 mutations per genome. Genome specific infectivity declines rapidly with only mild increase in mutagenesis. The LI_{50} (50% loss of specific infectivity) is the mutation frequency at which 50% of genomes are non-viable. Figure reproduced by permission of PNAS [67]

The question as to whether ribavirin acts as a mutagen during clinical treatment of HCV has been difficult to answer due to the lack of a suitable cell culture system. Although recent advances have made the investigation of HCV infection in cell culture possible [68–70], all evidence to date has relied upon the use of *in vitro* systems. Biochemical analysis of an HCV NS5B derivative indicated that this polymerase can incorporate ribavirin opposite either of the natural pyrimidines [58]. Mutagenesis induced by ribavirin was shown in a full-length HCV replication system [71]. Ribavirin was also shown to increase mutation frequency and reduce viability of HCV replicons [72, 73]. Ribavirin-induced mutagenesis of GB virus B (GBV-B), a surrogate model for HCV, was observed in tamarin hepatocytes [26]. Ribavirin had a potent antiviral effect on GBV-B, while MPA had no demonstrable antiviral effect. Furthermore, replication in the presence of ribavirin caused a reduction in the specific infectivity of GBV-B virions, suggesting induction of error-prone replication. However, ribavirin monotherapy had no effect *in vivo* against GBV-B-infection in tamarins, possibly because critical levels of ribavirin could not be reached in infected cells.

Recent work has implicated lethal mutagenesis as the mechanism for the antiviral effect of ribavirin against Hantaan virus. Although there are no

FDA-approved therapies for Hantaan virus infection, ribavirin has been shown to reduce the severity of haemorrhagic fever with renal syndrome in experimental use [74]. Severson and colleagues have shown that ribavirin can increase the mutation frequency of Hantaan virus [75]. It was subsequently suggested that ribavirin incorporation might reduce the stability of viral mRNA species as well [76].

Ribavirin also acts as a lethal mutagen against foot-and-mouth disease virus (FMDV) [77]. BHK-21 cells could be 'cured' of persistent FMDV infection by treatment with ribavirin. Ribavirin had a greater antiviral effect than MPA at concentrations causing an equivalent reduction in GTP pools, suggesting that IMPDH inhibition was not the sole antiviral mechanism. Ribavirin was found to be mutagenic to FMDV based on sequence analysis, and this effect was detectable even in the presence of added guanosine. The IMPDH inhibitor MPA was also found to induce an increase in mutation rate (although less than ribavirin), but this effect was abolished by the addition of exogenous guanosine, suggesting that this mutagenesis was the result of alteration of cellular nucleotide pool balance through IMPDH inhibition.

Ribavirin has also been shown to induce mutagenesis in West Nile virus (WNV) during infection of HeLa cells [78]. WNV was driven to extinction after four to five passages in the presence of less than 200 μM ribavirin, with a concurrent effect on genome infectivity. Sequence data indicated an increase in transition mutations. G → A transitions were the most common mutation, followed by C → U. The authors suggest that the particular observed mutations are due to ribavirin acting primarily as a GTP analogue during replication, and that there is a bias towards incorporation into the genome itself. Ribavirin incorporation as a GTP analogue would be expected to produce G → A transitions if incorporated into genomic RNA and C → U if incorporated into the antisense replication intermediate. Preferential incorporation as a GTP analogue could be explained by the reduced intracellular GTP pools caused by RMP inhibition of IMPDH.

VIRUS-ACQUIRED RESISTANCE TO RIBAVIRIN

A clinical concern in the development of antivirals is whether the target virus can develop resistance to the antiviral agent in question, and what impact that resistance will have on disease progression and pathogenicity of the virus population. The recent discovery of ribavirin-resistant virus populations has begun to shed light on these issues and provides some insight into the mechanism of action of ribavirin.

Ribavirin-resistant viruses were isolated from Sindbis virus populations grown in the presence of MPA[52]. This virus was shown to be cross-resistant to ribavirin treatment, and genetic analysis mapped resistance to the virus-encoded nsP1, presumed to mediate guanylyltransferase (capping) activity.

Young and coworkers isolated the first clinical ribavirin-resistant variant of HCV from patients treated with ribavirin monotherapy [79]. An amino acid substitution in the NS5B RdRp (F415Y in NS5B) was detected in all treated patients. After cessation of treatment, reversion of this residue to phenylalanine was observed in some patients. An HCV replicon containing the 415F variation was more susceptible to ribavirin in cell culture than 415Y. However, this resistance did not emerge in the replicon during cell culture passage. Importantly, while 415F is the consensus residue for genotype 1a, 415Y is found in all other genotypes. This observation suggests that ribavirin treatment may be more effective for patients infected with only genotype 1a. Furthermore, resistance occurring in the polymerase is consistent with ribavirin acting as a lethal mutagen, although sequencing of clinical isolates revealed only a moderate increase in genomic mutation frequency. The biochemical mechanism by which ribavirin resistance is derived should be investigated further, especially in light of recent data suggesting that resistance conferred by 415Y may not be robust [80]. Additionally, mutations in NS5A have been shown to confer resistance to ribavirin in HCV replicons propagated in cell culture [80].

Recently, ribavirin-resistant poliovirus was isolated by two independent groups [81,82]. Interestingly, resistance in each case was due to an identical glycine to serine mutation in the RdRp (G64S), indicating that there may be a limited number of solutions to overcoming lethal mutagenesis induced by ribavirin treatment. Resistance was mediated by increased fidelity of the PV polymerase. Presumably, an increase in replication

fidelity would restrict the breadth of the virus quasispecies and distance of the population from the error threshold, thus reducing the possibility of lethal mutagenesis. G64S PV was much less fit than wild-type PV as shown by competition experiments [81]. Interestingly, this virus population was more susceptible to inhibitors of uncoating, demonstrating the utility of the quasispecies in evasion of antiviral therapy [81].

RNA virus populations have likely evolved to exist as quasispecies in order to exploit the increased adaptability available to diverse populations. Thus, restricting the quasispecies through increased fidelity may have detrimental effects on the replicative ability or pathogenesis of high-fidelity viruses *in vivo*.

The fitness and pathogenicity of G64S PV has been investigated (M. Vignuzzi, J.K. Stone, J.J. Arnold, C.E. Cameron and R. Andino, in submission). The higher-fidelity polymerase of this virus resulted in fewer average mutations per genome, but with significant consequences. The resistant variant was less fit than wild-type virus in a competition assay in the absence of ribavirin, and was less able to adapt to adverse growth conditions. Infection of susceptible mice revealed that G64S has an attenuated phenotype and restricted tissue tropism. Furthermore, diversifying the population through chemical mutagenesis was able to restore tropism and pathogenesis. These observations suggest that quasispecies diversity is integral to pathogenesis and spread to distinct tissues *in vivo*.

CONCLUSIONS

The broad-spectrum antiviral activity of ribavirin can potentially be attributed to its multiple mechanisms of action. As a purine analogue, it can function in multiple cellular and viral processes. An important aspect of the antiviral activity of ribavirin may stem from the ability to act via multiple mechanisms simultaneously. Ribavirin can potentially act on numerous steps of the virus life cycle: inhibition of translation due to reduction in cellular GTP pools or incorporation as a cap analogue which inhibits translation; inhibition of genome or transcript capping, by suppression of GTP synthesis or direct competition; inhibition of RNA synthesis directly via active-site binding or reduction of GTP synthesis; ambiguous incorporation into RNA causing increased mutation and production of non-viable genomes; or enhancement of the antiviral immune response, preventing spread and pathogenesis. Furthermore, inhibition of IMPDH can also potentiate the direct effects of ribavirin by reducing the concentration of intracellular competitors, that is GTP.

The recent characterisation of ribavirin-resistant viruses suggests that ribavirin may be especially potent in an antiviral 'cocktail' in cases where ribavirin is presumed to act via lethal mutagenesis. Ribavirin-induced mutagenesis should select for high-fidelity replication, reducing the number of variants in the virus population. This should reduce the ability of the population to adapt to simultaneous antiviral therapy targeting an unrelated mechanism.

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