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REVIEW

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Ribavirin's antiviral mechanism of action: lethal mutagenesis?

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Abstract Ribavirin, an antiviral drug discovered in 1972, is interesting and important for three reasons: (a) it exhibits antiviral activity against a broad range of RNA viruses; (b) it is currently used clinically to treat hepatitis C virus infections, respiratory syncytial virus infections, and Lassa fever virus infections; and (c) ribavirin's mechanism of action has remained unclear for many years. Here we recount the history of ribavirin and review recent reports regarding ribavirin's mechanism of action, including our studies demonstrating that ribavirin is an RNA virus mutagen and ribavirin's primary antiviral mechanism of action against a model RNA virus is via lethal mutagenesis of the RNA virus genomes. Implications for the development of improved versions of ribavirin and for the development of novel antiviral drugs are discussed.

Keywords Antiviral drug · Hepatitis C virus · Respiratory syncytial virus · Error catastrophe · Virus evolution

Abbreviations *HCV:* Hepatitis C virus · *HIV:* Human immunodeficiency virus · *IMPDH:* Inosine monophosphate dehydrogenase · *RSV:* Respiratory syncytial virus

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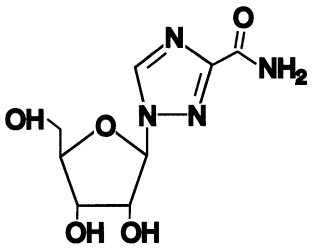
Introduction

Ribavirin, the nucleoside analog 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Fig. 1), known by the trade name Virazole (also known as Rebetron in combination with interferon-α), exhibits antiviral activity against a variety of RNA viruses in cell culture [1, 2, 3] including RNA viruses from the families of paramyxoviruses [4, 5], flaviviruses [6, 7, 8], picornaviruses [9, 10], orthomyxoviruses [11, 12, 13], arenaviruses [14, 15], reoviruses [16], and bunyaviruses [17, 18]. Ribavirin also exhibits activity against some DNA viruses in cell culture [2, 19]. In animal model systems ribavirin is effective against a more limited set of viruses, all RNA viruses [11, 14, 18, 20, 21, 22, 23]. In humans ribavirin is currently used in combination with interferon-α to treat hepatitis C virus (HCV) infections [24, 25], and ribavirin is used as monotherapy for Lassa fever virus infections [26] and severe respiratory syncytial virus (RSV) infections [27, 28]. Ribavirin is currently unique among antiviral drugs as a therapeutic agent that is clinically effective against unrelated viruses from three diverse families, as HCV, Lassa fever virus, and RSV are very different RNA viruses with virtually no sequence homology. These characteristics have made ribavirin a drug of substantial research interest. However, the fact that clinical treatment with ribavirin requires high drug doses with significant side effects and is efficacious in only a mi-

Fig. 1 Chemical structure of ribavirin



nority of HCV patients in combination with interferon-α (currently 30–40% sustained response [24, 25, 29], and the new pegylated interferon-α is expected to increase this percentage to approx. 50% [30]) has generated substantial interest in the development of better versions of ribavirin. Unfortunately, the uncertain nature of ribavirin's antiviral mechanism of action has long been a major stumbling block to understanding the drug and developing more efficacious derivatives for clinical use.

Ribavirin is an IMPDH inhibitor

Shortly after the discovery of the broad-spectrum antiviral activity of ribavirin in 1972 by ICN Pharmaceuticals [2], it was suggested by the same group that ribavirin's antiviral activity is via inhibition of the cellular protein inosine monophosphate dehydrogenase (IMPDH) [31]. Ribavirin is a nucleoside analog (Fig. 1). Ribavirin monophosphate inhibits IMPDH in vitro and in cell culture [31]. Ribavirin's inhibition of IMPDH causes a decrease in the intracellular concentration of GTP in cell culture experiments [3, 13, 31, 32, 33]. It was therefore proposed that the decrease in intracellular GTP levels due to ribavirin's inhibition of IMPDH would stop the growth of RNA viruses by diminishing viral protein synthesis and limiting RNA replication of viral genomes [31]. This proposal was appealing because it potentially explained the broad-spectrum antiviral activity of ribavirin. However, multiple problems with this theory have since accumulated which suggest that inhibition of IMPDH is not ribavirin's primary antiviral mechanism of action.

A variety of studies proposed that inhibition of IMPDH was not sufficient for antiviral activity [3, 28, 32, 34, 35]. In one series of cell culture studies Wray et al. [13] demonstrated that ribavirin caused a reduction in intracellular GTP levels, but that reduction in GTP (maximum of approx. 35–40% reduction) was saturated at 25 μM ribavirin (Fig. 2A). Although 25 μM ribavirin exhibited antiviral activity against influenza in those experiments, the effect was modest (1.6- to 2.0-fold). Importantly, ribavirin's most substantial antiviral effect (20-fold) was seen at concentrations higher than 25 μM, where no additional effects on GTP levels were observed (Fig. 2A) [13]. This led the authors to conclude that ribavirin possesses two antiviral activities: the inhibition of IMPDH and a second uncharacterized antiviral activity, which is in fact the more potent activity [13]. Those results were corroborated in a similar study that concluded that the IMPDH inhibitory activity of ribavirin is likely secondary and "self-potentiates" a separate, more important, antiviral activity of ribavirin [32]. Related experiments observed that, at concentrations of ribavirin necessary to cause a 100-fold reduction in infectious poliovirus production, ribavirin had little effect on poliovirus translation or RNA replication [9], indicating again that the reduced intracellular levels of GTP are not ribavirin's primary antiviral effect (Fig. 2B).

The studies in the literature exploring ribavirin's inhibition of IMPDH as a possible antiviral mechanism of action have not demonstrated that this is necessary and/or sufficient for antiviral activity. Published studies have not shown that a reduction in intracellular GTP levels is directly responsible for the antiviral effect observed, or even that the intracellular GTP concentrations are directly correlated with the antiviral effect. (Some studies have concluded this based on the reversal of the antiviral effect by addition of supplementary GTP to the medium. However, this is not a sufficient control for such experiments, since ribavirin is a guanosine analog and large amounts of GTP competitor could at least partly counteract most any antiviral activity of ribavirin that has been posited.)

Lanford and colleagues [36] have recently reported that ribavirin has a potent antiviral activity against GB virus B (the closest known relative of HCV) in cell culture (Fig. 3). They then went on to demonstrate that mycophenolic acid, a potent IMPDH inhibitor, exhibits no antiviral activity against GB virus B, indicating that ribavirin's primary mechanism of action against GB virus is not via IMPDH [36] (Fig. 3).

Given the problems with the IMPDH model of ribavirin's antiviral activity, several other mechanisms of action have been proposed over the years but not fully explored, including ribavirin monophosphate inhibition of guanylyltransferase activity [37] and inhibition of viral transcription [17, 38] among others [16, 39]. These studies did not include mechanistic analysis of the antiviral activity in vivo or in cell culture. It has therefore been of interest to explore new mechanism of action hypotheses that might be able to explain ribavirin's broad spectrum antiviral activity against most RNA viruses.

Ribavirin has immunomodulatory properties

Ribavirin monotherapy is effective in Lassa fever virus [14, 26] and severe RSV infections [27], but not in HCV chronically infected patients. Why? Ribavirin monotherapy in HCV chronically infected patients does not lead to viral clearance and has only a modest [40] if any [41, 42] effect on viral load as measured by RNA genomes/ml serum. Nevertheless, ribavirin monotherapy is therapeutic, as liver damage (measured by serum alanine aminotransferase activity) is markedly reduced.

Tam and colleagues [43] approached this conundrum by considering whether ribavirin exhibits immunomodulatory effects. They demonstrated that ribavirin can af-

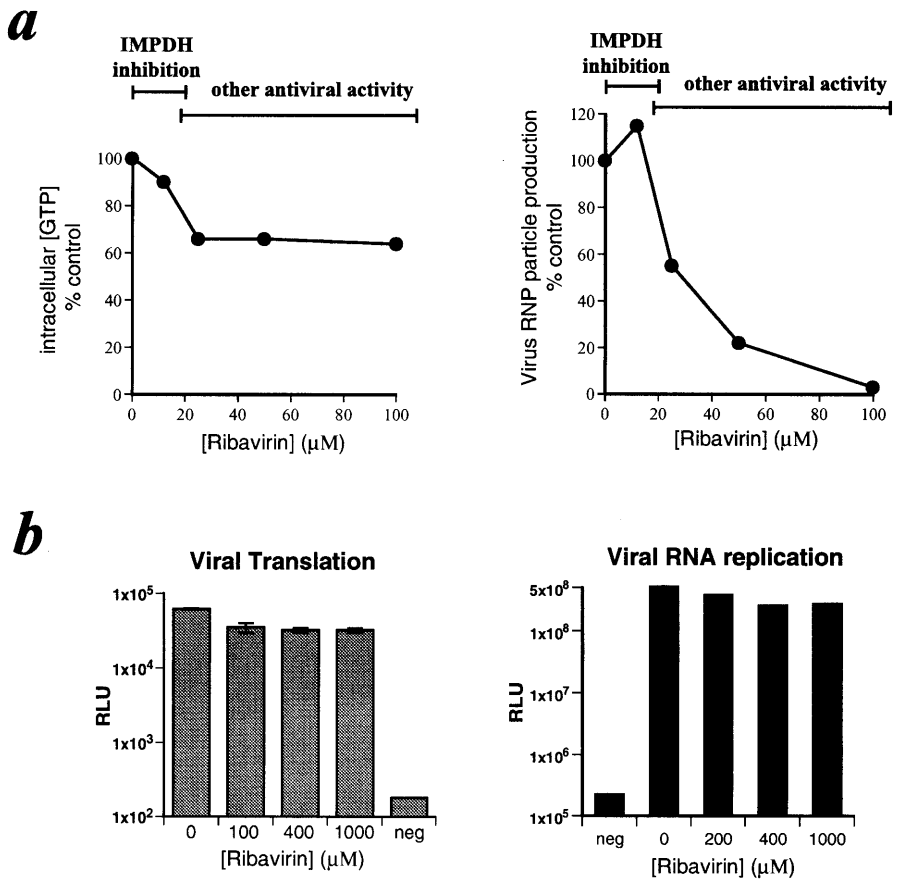


Fig. 2a, b Ribavirin's inhibition of IMPDH in vivo is an ineffectual antiviral activity. **a** Ribavirin-treated Madin-Darby canine kidney (MDCK) cells exhibit a decreasing intracellular GTP pool by HPLC (*left*), which stabilizes at 65% normal GTP level upon treatment with 25 μM ribavirin. Higher concentrations of ribavirin do not further affect the intracellular GTP pool. The majority of the antiviral effect seen against influenza in these ribavirin treated cells is seen at concentrations of ribavirin higher than 25 μM, indicating that there is another ribavirin activity that is the more important antiviral effect. (Modified from [13]) **b** Minor inhibition of poliovirus translation and replication in the presence of ribavirin. The poliovirus replicon, PolioLuc, has the capsid-coding sequence replaced by the luciferase gene [84], allowing rapid analysis of poliovirus replication and translation levels in cell culture under various conditions [48, 85]. Translation experiments (*left*) were performed in the presence of 2 μg/ml brefeldin A, which completely blocks poliovirus RNA replication [86], thus permitting direct analysis of the translation of the input replicon RNA. *RLU* Relative light units; *neg* untransfected cells. Ribavirin had only a minor effect of poliovirus translation. RNA replication experiments were performed using the PolioLuc replicon and increasing concentration of ribavirin (*right*). These results show that ribavirin's inhibition of IMPDH has a minimal effect on poliovirus translation or RNA replication, even at high concentrations of drug, demonstrating that although ribavirin has antiviral activity against the virus, ribavirin's inhibition of IMPDH is ineffectual against this virus, similar to the results of Wray et al. with influenza. (Modified from [9])

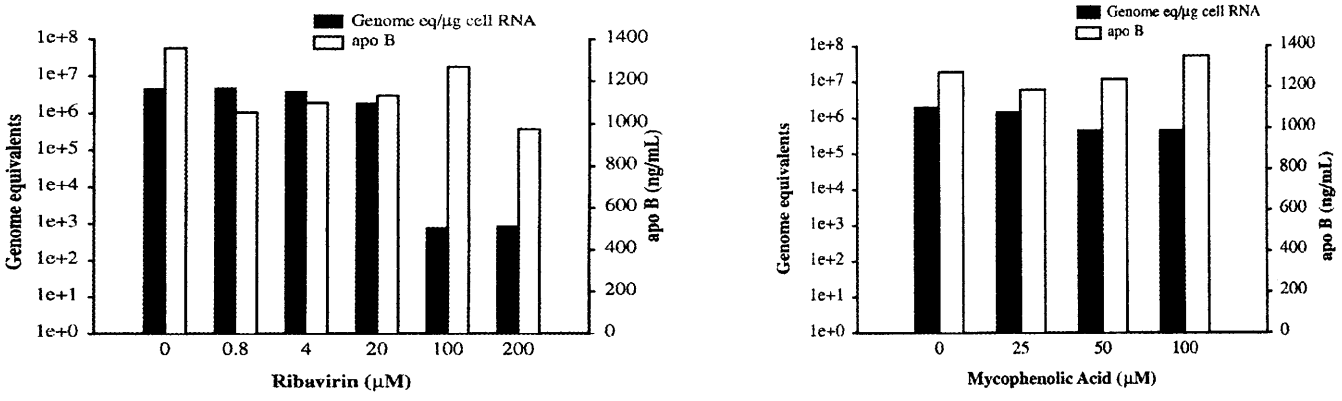


Fig. 3 Ribavirin inhibits GB virus. Ribavirin (*left*) but not the IMPDH inhibitor mycophenolic acid (*right*) exhibits potent antiviral activity against the RNA virus GB virus B (the closest known relative to HCV) in cell culture. These data indicate that inhibition of IMPDH is not sufficient for antiviral activity against GB virus B. Apolipoprotein B cellular protein levels are shown as a control for the health of the cells. (Reproduced with permission from [36])

fect the expression of the cytokine interleukin 10 in vivo in a mouse contact hypersensitivity model. Surprisingly, ribavirin suppressed interleukin 10 expression in BALB/c mice, but enhanced interleukin 10 expression in C57BL/6 mice. This led to greater inflammation in the ribavirin treated BALB/c mice and reduced inflammation in the ribavirin treated C57BL/6 mice [43]. However, in a separate study, ribavirin was capable of suppressing hepatic inflammation and injury in BALB/c mice [44].

Ribavirin may have immunomodulatory activity, but this cannot explain the fundamental antiviral activity of ribavirin. Ribavirin has a potent antiviral effect against many RNA viruses in cell culture. Since there is no immunomodulation in such systems, and since there is a correlation between the viruses ribavirin inhibits in cell culture and in small animal studies, the basic antiviral effect of ribavirin is not immunomodulation. Immunomodulation may be a supplementary activity. Interestingly, the L-enantiomer of ribavirin does not have antiviral activity in cell culture but does retain the immunomodulatory properties of ribavirin [44]. The L-enantiomer then provides the opportunity for a detailed examination of the comparative relevance of the direct antiviral activity of ribavirin versus the immunomodulatory activity of ribavirin in vivo. In vivo infection experiments using the ribavirin L-enantiomer as treatment could address the issue of whether the immunomodulatory activity of the drug has therapeutic antiviral effects in vivo. The results of such a study are difficult to predict, given the mouse strain and tissue specificities reported for the ribavirin immunomodulatory effect observed, and such studies warrant further exploration.

Ribavirin is an RNA virus mutagen

Ribavirin triphosphate accumulates in cells after treatment with the nucleoside [45]. Therefore we explored the possibility that ribavirin's antiviral effect requires direct incorporation into viral RNA. Poliovirus was used for those studies, as poliovirus is a well characterized model RNA virus with excellent genetic and biochemistry systems available [46]. Using a newly developed in vitro poliovirus polymerase ($3D^{pol}$) system capable of assessing the kinetics and thermodynamics of nucleotide incorporation [47, 48], it was demonstrated that, indeed, ribavirin triphosphate can be incorporated by the poliovirus polymerase as a nucleotide analog [9]. The poliovirus RNA-dependent RNA polymerase incorporated ribavirin in vitro as either a GTP analog or ATP analog [9]. Given these data, the expectation was that ribavirin would function as a chain terminator, as several classes of effective nucleoside analog antiviral drugs function as chain terminators to block viral replication (e.g., the acyclovir class of herpes virus antiviral drugs [49], and the human immunodeficiency virus (HIV) dideoxy reverse transcriptase inhibitors such as azidothymidine [50]). However, ribavirin is not a chain terminator of the poliovirus polymerase [9], leaving the mechanism of action still unclear.

Table 1 Ribavirin is an RNA virus mutagen: mutation frequencies in ribavirin treated poliovirus populations (data from [51])

	G→A	C→T	Total mutation frequency[a]
Normal population	0.5	1.2	2.1
100 μM ribavirin	–	1.3	2.5
400 μM ribavirin	4.4	5.0	9.3
1000 μM ribavirin	6.8	12.0	20.8

[a] Mutations per 10,000 nt sequenced

The observation that ribavirin could be incorporated as either a GTP or an ATP analog indicated that ribavirin might be an RNA virus mutagen in vivo. In subsequent experiments it was then discovered that ribavirin is a potent mutagen of poliovirus in cell culture (Table 1) [9, 51]. Importantly, the mutagenic activity of ribavirin correlated directly with its antiviral activity [9]. This led us to propose that ribavirin's mechanism of action is via lethal mutagenesis of viral RNA genomes.

Lethal mutagenesis, quasispecies, and error catastrophe

RNA viruses live as quasispecies, creating extraordinary genetic diversity through mutation. The rapid evolution of RNA viruses is apparently powered by the high mutation frequency in RNA virus populations [52, 53, 54, 55, 56, 57].

The quasispecies theory states that an RNA virus population does not consist of a single "wild-type" genotype, but instead is an ensemble of related genotypes [58, 59, 60, 61, 62]. This quasispecies is then capable of very rapid evolution in new environments, due to the large number of potentially beneficial mutations already present within the population. Maintaining such a high mutation frequency, however, is dangerous for the virus. There is an intrinsic limit to the maximum variability in viral genetic information before it loses meaning [60, 63], and if an RNA virus quasispecies goes beyond this mutation limit, the population is no longer viable. The phenomenon that occurs when the loss of genetic fidelity results in a lethal accumulation of errors has been termed "error catastrophe" (Fig. 4) [54, 60]. Most uni- and multicellular organisms have evolved a number of sophisticated processes to maintain their genetic information with high fidelity and stay far away from the threshold of error catastrophe. In contrast, Holland and Domingo [54, 64] have proposed that, due to their high mutation rate, RNA viruses exist on the threshold of error catastrophe, and that a moderate increase in mutation rate can kill a RNA virus population by causing a "genetic meltdown" [53, 65, 66].

Several indirect lines of evidence have been presented that support the existence of such an error catastrophe [9,

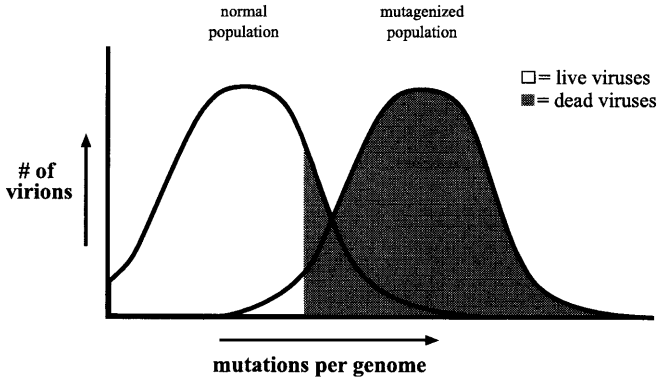


Fig. 4 Model of error catastrophe. The majority of viruses in a normal picornavirus population are viable [87]. However, a small increase in mutation frequency is predicted to push the virus population into error catastrophe (the mutagenized population on the right), where the number of errors per viral genome is sufficiently high to lethally mutate a majority of the virus population. This is predicted to be the case for most RNA viruses. *White* Live virus; *grey* dead virus

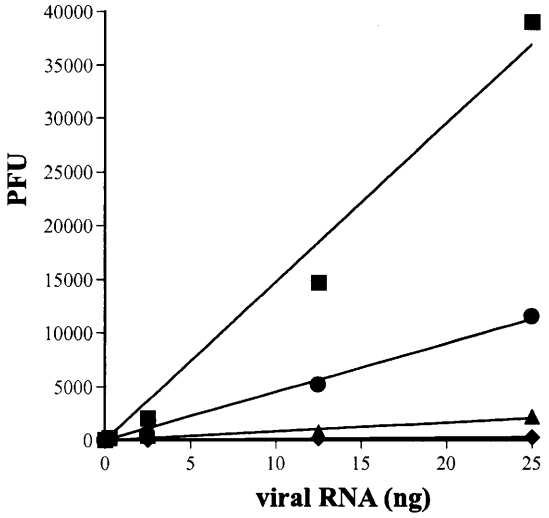


Fig. 5 Ribavirin's lethal mutagenesis: large reductions in specific infectivity of ribavirin mutagenized RNA virus genomes. Genomic poliovirus RNA from untreated cells (*squares*), 100 μM ribavirin-treated cells (*circles*), 400 μM ribavirin-treated cells (*triangles*), and 1000 μM ribavirin-treated cells (*diamonds*) was tested for specific infectivity by RNA transfection in a series of infectious center assays. Data are shown with a linear curve fit for each series. Viral RNA genomes isolated from cells treated with ribavirin exhibited large losses in specific infectivity. (Reproduced from [51])

65, 66, 67, 68]. The first experimental evidence of error catastrophe was observed in a bacteriophage model system [56]. Holland and Domingo [65] later used the RNA mutagens 5-azacytidine and 5-fluorouracil to examine what would happen if an animal RNA virus was experimentally mutagenized. To control for species-specific effects they carried out their experiments with both vesicular stomatitis virus and poliovirus. Their surprising observation was that vesicular stomatitis virus and poliovirus exhibit substantial reductions in titer in the presence of concentrations of mutagen that caused only a twofold increase in mutation frequency [65]. Sierra et al. [68] later extended these observations to foot-and-mouth disease virus. Loeb et al. [67] made the similar observation that HIV is sensitive to lethal mutagenesis. In their study, since HIV is an RNA virus that goes through a DNA intermediate, a DNA nucleoside analog mutagen was used. Although the change in mutation frequency was not determined, Loeb et al. [67] observed an impressive antiviral effect, frequently observing extinction of the HIV population after multiple cell culture passages in the presence of the mutagen.

Although these studies provided tantalizing evidence that error catastrophe exists, they were unable to provide direct proof that the antiviral effect of any of the drugs tested is exerted via its mutagenic effects on the viral genetic material and not via secondary effects of the drug on cellular physiology or viability, or via inhibition of other aspects of the virus life cycle.

Ribavirin's primary antiviral mechanism of action is via lethal mutagenesis of the viral RNA genomes

We endeavored to provide the first direct demonstration of error catastrophe/lethal mutagenesis [51]. To demonstrate that lethal mutagenesis is the primary antiviral mechanism of action of ribavirin against poliovirus, it was necessary to show that the majority of the antiviral effect observed is due to the direct effect of ribavirin on the viral RNA genetic material. If lethal mutagenesis is indeed the primary mechanism of action, a much higher percentage of the viral genomes produced in the presence of ribavirin should be inviable, i.e., noninfectious. Therefore RNA genomes produced in the presence of increasing concentrations of ribavirin were tested for their infectivity, by transfection of the naked RNA into susceptible cells. A striking decrease in RNA specific infectivity (plaque forming units/μg viral RNA genomes) was observed [51]. A 95% reduction in genome specific infectivity was seen with poliovirus RNA genomes produced in the presence of 400 μM ribavirin (Fig. 5).

By combining genome sequencing data with the genome infectivity data a graphic demonstration of the existence of error catastrophe could be obtained (Fig. 6). The graph in Fig. 6 illustrates that poliovirus appears to have evolved to exist near the edge of error catastrophe, as small increases in mutation frequency above the normal levels result in a large decline in viral infectivity, with a 95% loss in genome infectivity upon a fourfold increase in mutation frequency [51].

Existing at the edge of error catastrophe is predicted to optimize the evolutionary fitness of the RNA virus quasispecies population by maximizing genetic variation without sacrificing viability [69]. This recent data on ribavirin demonstrates that high genetic variability, a biological property that is normally a major advantage for an RNA virus, can be exploited against the virus by increasing that mutation rate beyond tolerable levels and causing a genetic meltdown.

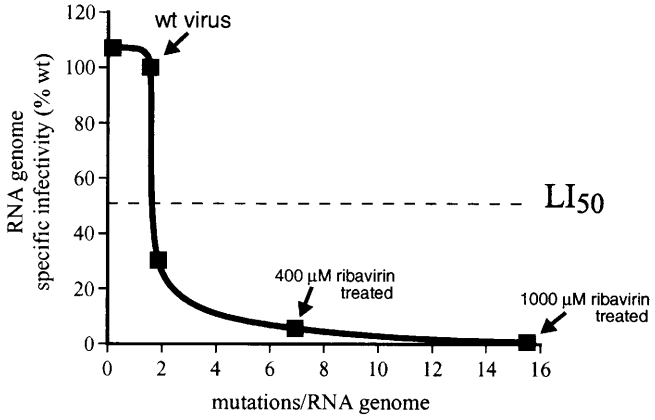


Fig. 6 Error catastrophe. Relationship of mutation frequency to genomic RNA infectivity. Specific infectivity of normal poliovirus RNA was set to 100%. The graph shows that poliovirus populations exist near the edge of error catastrophe, as there is a rapid decline in RNA genome infectivity at levels of mutagenesis only slightly higher than normal. The LI_{50} (50% loss of specific infectivity) is defined as the mutation frequency at which 50% of the viral genomes are lethally mutated (*dashed line*). Wild-type (*wt*) poliovirus genomes contain an average of approx. 1.5 mutations/genome. Poliovirus genomes from cells treated with 100 μM ribavirin contain an average of approx. 1.9 mutations/genome. Poliovirus genomes from cells treated with 400 μM ribavirin contain an average of approx. 6.9 mutations/genome. Poliovirus genomes from cells treated with 1000 μM ribavirin contain an average of approx. 15.5 mutations/genome. (Modified from [51]

Clinical implications of ribavirin's viral lethal mutagenesis mechanism of action

Ribavirin's activity as a nucleoside analog incorporated by the viral polymerase can explain the surprisingly broad spectrum action of ribavirin against members of most RNA virus families under laboratory cell culture conditions [2], and clinical activity against three virus infections from diverse families, as the one common feature of RNA viruses is that they possess an RNA-dependent RNA polymerase. A high mutation frequency appears to also be a common trait of most RNA viruses [54, 64, 69, 70].

The effectiveness of ribavirin in vivo as an RNA virus mutagen may be dependent on pharmacokinetics, i.e., the accumulation of ribavirin and ribavirin triphosphate in some tissues (such as liver when administered orally, or respiratory tract epithelium when administered as an aerosol) but not others. Alternatively, differences in efficacy may be due to different rates of ribavirin incorporation by the RNA-dependent RNA polymerases of different viruses. It is also possible that ribavirin may have different mechanisms of actions against different RNA viruses.

Ribavirin's lethal mutagenesis of poliovirus is probably enhanced by, or even predicated on, the ability of ribavirin monophosphate to inhibit IMPDH and thereby decrease cellular GTP pools. The decrease in cellular GTP pools likely increases the frequency of ribavirin incorporation as a mutagenic GTP analog.

Currently the mutation frequencies of all animal RNA virus species tested under laboratory conditions have been high, in the range of $1–5\times10^{-4}$/nt [51, 54, 64, 68, 70]. However, it is plausible that some RNA viruses have low mutation frequencies and are therefore less susceptible to error catastrophe. If such virus species are identified, it will be fascinating to explore the implications of their altered evolutionary strategy.

One of the interesting features of the clinical use of ribavirin is the results from trials using ribavirin as monotherapy in chronically infected HCV patients. Patients were given high levels of ribavirin and tracked for 24 weeks. No substantial decrease in viral loads was observed, but a statistically significant reduction in liver damage (serum alanine aminotransferase levels) was seen [40, 41, 42]. No good explanation was available for these results, and since ribavirin failed to control HCV by the virological assay (total RNA viral genomes in serum), monotherapy is considered ineffective. However, since ribavirin plus interferon-α results in viral clearance in significantly more patients than interferon-α alone [24, 25, 30], clearly ribavirin has a beneficial antiviral effect.

Our observation that ribavirin's primary mechanism of action against a model RNA virus is lethal mutagenesis [51] provides a plausible, although speculative, theory for the monotherapy clinical trial results. There is no cell culture system available to measure HCV growth, and therefore virological analysis of HCV from patients has been limited to bDNA and quantitative reverse transcriptase polymerase chain reaction analysis of HCV genomes per milliliter of serum [40, 41, 42]. In a chronic HCV infection, ribavirin monotherapy may reduce the levels of infectious HCV produced in the patient, but it may not significantly affect the production of total HCV RNA. Therefore it is possible that many of the HCV RNA genomes in ribavirin-treated patients are noninfectious. Inactive HCV genomes would of course not appear different from infectious genomes in reverse transcriptase polymerase chain reaction or bDNA assays, and therefore such a therapeutic effect would not be identified by the virological assays available. Additionally, if ribavirin monotherapy substantially lowered the burden of infectious HCV virus to a new steady state level, this might reduce the levels of liver damage (alanine aminotransferase levels). It should be emphasized that this proposal is speculative.

Note that the mutagenesis theory cannot be resolved by sequencing HCV genomes from ribavirin-treated versus untreated patients [71], as the complexities of the interactions of the quasispecies with the host and the innate differences in the initial viral genomes infecting the host make it impossible to accurately quantify changes in mutation frequencies due to ribavirin mutagenesis. If a robust HCV cell culture system is developed (see [72] for developments in this area, and [73, 74] for the initial descriptions of HCV replicons as a hopeful first step towards developing an HCV infectious virus system), it should then be possible to partially test the lethal muta-

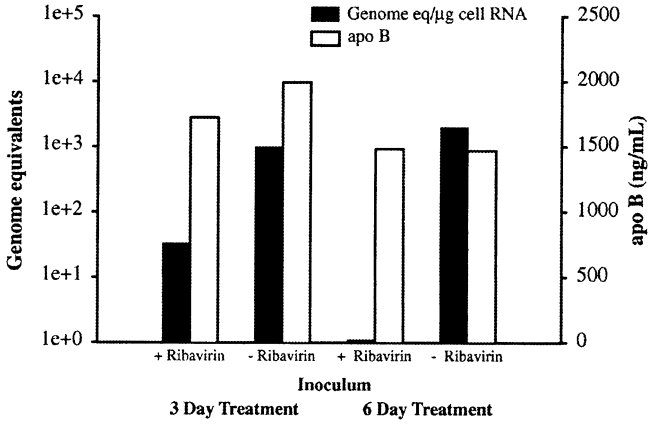


Fig. 7 Lethal mutagenesis of GB virus B by ribavirin treatment. GB virus B produced in the presence of 100 µM ribavirin has low specific infectivity. GB virus B was grown in cell culture with or without ribavirin, and secreted virus was harvested on day 3 or day 6 postinfection. Virus stocks grown under each of those conditions were adjusted to contain identical genome equivalents (based on TaqMan reverse transcriptase polymerase chain reaction), and each of those stocks were tested for infectivity by inoculation on fresh cells in culture. Virus infected cultures were harvested 7 days postinfection and the levels of GB virus B infection were measured by quantifying the amount of viral RNA genomes present in the infected cells (genome equivalents per microgram of cellular RNA). The vast majority of virus produced in the presence of ribavirin was noninfectious (Reproduced with permission from [36])

genesis of HCV hypothesis in humans by comparing the ratio of infectious to noninfectious HCV virions in the serum of ribavirin-treated versus untreated patients.

Alternatively, cell culture experiments using ribavirin on flaviviruses closely related to HCV, such as bovine viral diarrhea virus [75] and GB virus [76], should be able to address the lethal mutagenesis hypothesis in the flavivirus family. Indeed, Lanford and colleagues [36] have recently published compelling data that ribavirin has a pronounced antiviral effect on GB virus in cell culture (Fig. 3), and that antiviral effect is due to error prone viral replication in the presence of ribavirin, resulting in striking reductions in the specific infectivity of the virus (Fig. 7). GB virus is the closest known relative to HCV. This study by Lanford et al. corroborates the work from our laboratory on the ribavirin lethal mutagenesis hypothesis and extends it to include the most relevant HCV surrogate model known. (It should be noted that, similarly to the situation with HCV, ribavirin monotherapy does not appear to be effective against GB virus B in vivo, using tamarin monkeys, the presumed natural host of GB virus B [36].)

The fact that ribavirin as HCV monotherapy fails to result in viral clearance cannot be ignored. Why does ribavirin monotherapy work for Lassa fever virus and RSV but not HCV? The HCV polymerase may simply incorporate the mutagenic ribavirin nucleoside triphosphate less frequently. Alternatively, another major difference between these three infections is that HCV is a chronic infection, while Lassa fever virus and RSV are acute infections. Viruses that establish chronic or prolonged infections frequently cause substantial disturbances to the normal functioning of the immune system, preventing an immune response that would result in viral clearance. In the absence of an effective immune response to complement the antiviral drug monotherapy an additional antiviral factor is required to bring about viral clearance. This role is fulfilled by interferon-α, which is effective as monotherapy in a subset of cases [77, 78, 79], and interferon-α is known to exhibit potent direct antiviral activity against HCV replicons in cell culture [73, 80, 81].

Future drug development strategies

Several RNA virus mutagens other than ribavirin are known [65, 66, 82, 83], but they generally exhibit substantial cellular toxicity (as nucleoside analogs presumably incorporated by cellular DNA and RNA polymerases) and are unacceptable for use in humans at the necessary doses. (As a technical aside, conclusions regarding ribavirin's toxicity and efficacy in the current ribavirin literature have been complicated by the fact that ribavirin from Sigma contains an uncharacterized contaminant that is both cytotoxic in cell culture and poisonous in vivo. Therefore pure stocks from ICN, Schering-Plough, or academic sources are required for accurate analysis, and generally exhibit minimal toxicity at >500 µM on many cell lines.) In the interest of developing new antiviral drugs that exhibit activity against multiple RNA virus human pathogens, it may be possible to identify mutagenic nucleoside analogs that are highly specific for incorporation by viral RNA-dependent RNA polymerases. Such a drug development strategy should be plausible given the success in identifying and developing nucleoside analog anti-HIV therapies that are capable of specifically inhibiting that virus's RNA-dependent DNA polymerase without detrimentally affecting normal cellular processes.

Concluding remarks

Ribavirin has been demonstrated to have three activities in vivo or in cell culture: inhibition of IMPDH, immunomodulatory effects, and incorporation as a mutagenic nucleoside by the viral RNA polymerase. All three activities may play an antiviral role in vivo. However, it is only the third activity– mutagenesis of RNA virus genomes– that has been directly shown to have a direct potent antiviral effect [51]. Therefore, given this body of data, we propose that (a) lethal mutagenesis is the primary antiviral mechanism of action of ribavirin against RNA viruses, (b) the lethal mutagenesis is likely enhanced by the inhibition of IMPDH, and (c) identification of novel RNA virus mutagens is a promising new area of antiviral drug development.

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References

1. De Clercq E (1993) Antiviral agents: characteristic activity spectrum depending on the molecular target with which they interact. Adv Virus Res 42:1
2. Sidwell RW, Huffman JH, Khare GP, Allen LB, Witkowski JT, Robins RK (1972) Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1:2,4-triazole-3-carboxamide. Science 177:705
3. Smith RA, Kirkpatrick W (1980) Ribavirin, a broad spectrum antiviral agent. Academic, New York
4. Hruska JF, Bernstein JM, Douglas RG Jr, Hall CB (1980) Effects of ribavirin on respiratory syncytial virus in vitro. Antimicrob Agents Chemother 17:770
5. Povey RC (1978) In vitro antiviral efficacy of ribavirin against feline calicivirus, feline viral rhinotracheitis virus, and canine parainfluenza virus. Am J Vet Res 39:175
6. Neyts J, Meerbach A, McKenna P, De Clercq E (1996) Use of the yellow fever virus vaccine strain 17D for the study of strategies for the treatment of yellow fever virus infections. Antiviral Res 30:125
7. Jordan I, Briese T, Fischer N, Lau JY, Lipkin WI (2000) Ribavirin inhibits West Nile virus replication and cytopathic effect in neural cells. J Infect Dis 182:1214
8. Neyts J, Leyssen P, De Clercq E (1999) Infections with flaviviridae. Verh K Acad Geneeskd Belg 61:661
9. Crotty S, Maag D, Arnold JJ, Zhong W, Lau JY, Hong Z, Andino R, Cameron CE (2000) The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat Med 6:1375
10. Torre JC de la, Alarcon B, Martinez-Salas E, Carrasco L, Domingo E (1987) Ribavirin cures cells of a persistent infection with foot-and-mouth disease virus in vitro. J Virol 61: 233
11. Durr FE, Lindh HF (1975) Efficacy of ribavirin against influenza virus in tissue culture and in mice. Ann N Y Acad Sci 255:366
12. Oxford JS (1975) Inhibition of the replication of influenza A and B viruses by a nucleoside analogue (ribavirin). J Gen Virol 28:409
13. Wray SK, Gilbert BE, Noall MW, Knight V (1985) Mode of action of ribavirin: effect of nucleotide pool alterations on influenza virus ribonucleoprotein synthesis. Antiviral Res 5: 29
14. Jahrling PB, Hesse RA, Eddy GA, Johnson KM, Callis RT, Stephen EL (1980) Lassa virus infection of rhesus monkeys: pathogenesis and treatment with ribavirin. J Infect Dis 141: 580
15. Andrei G, De Clercq E (1990) Inhibitory effect of selected antiviral compounds on arenavirus replication in vitro. Antiviral Res 14–5:287
16. Rankin JT Jr, Eppes SB, Antczak JB, Joklik WK (1989) Studies on the mechanism of the antiviral activity of ribavirin against reovirus. Virology 168:147
17. Cassidy LF, Patterson JL (1989) Mechanism of La Crosse virus inhibition by ribavirin. Antimicrob Agents Chemother 33:2009
18. Sidwell RW, Huffman JH, Barnett BB, Pifat DY (1988) In vitro and in vivo Phlebovirus inhibition by ribavirin. Antimicrob Agents Chemother 32:331
19. Markland W, McQuaid TJ, Jain J, Kwong AD (2000) Broad-spectrum antiviral activity of the IMP dehydrogenase inhibitor VX-497: a comparison with ribavirin and demonstration of antiviral additivity with alpha interferon. Antimicrob Agents Chemother 44:859
20. Hruska JF, Morrow PE, Suffin SC, Douglas RG Jr (1982) In vivo inhibition of respiratory syncytial virus by ribavirin. Antimicrob Agents Chemother 21:125
21. Weissenbacher MC, Calello MA, Merani MS, McCormick JB, Rodriguez M (1986) Therapeutic effect of the antiviral agent ribavirin in Junin virus infection of primates. J Med Virol 20:261
22. Peters CJ, Jahrling PB, Liu CT, Kenyon RH, McKee MT Jr, Barrera Oro JG (1987) Experimental studies of arenaviral hemorrhagic fevers. Curr Top Microbiol Immunol 134:5
23. von Herrath MG, Berger DP, Homann D, Tishon T, Sette A, Oldstone MB (2000) Vaccination to treat persistent viral infection. Virology 268:411
24. Davis GL, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH, Albrecht J (1998) Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N Engl J Med 339:1493
25. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK (1998) Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 339:1485
26. McCormick JB, King IJ, Webb PA, Scribner CL, Craven RB, Johnson KM, Elliott LH, Belmont-Williams R (1986) Lassa fever. Effective therapy with ribavirin. N Engl J Med 314:20
27. Hall CB, Walsh EE, Hruska JF, Betts RF, Hall WJ (1983) Ribavirin treatment of experimental respiratory syncytial viral infection. A controlled double-blind study in young adults. JAMA 249:2666
28. Wyde PR (1998) Respiratory syncytial virus (RSV) disease and prospects for its control. Antiviral Res 39:63
29. Cummings KJ, Lee SM, West ES, Cid-Ruzafa J, Fein SG, Aoki Y, Sulkowski MS, Goodman SN (2001) Interferon and ribavirin vs interferon alone in the re-treatment of chronic hepatitis C previously nonresponsive to interferon: a meta-analysis of randomized trials. JAMA 285:193
30. Anonymous (2000) Phase III peg-intron plus Rebetol results released. AIDS Read 10:702
31. Streeter DG, Witkowski JT, Khare GP, Sidwell RW, Bauer RJ, Robins RK, Simon LN (1973) Mechanism of action of 1-beta-D-ribofuranosyl-1:2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. Proc Natl Acad Sci USA 70:1174
32. Robins RK, Revankar GR, McKernan PA, Murray BK, Kirsi JJ, North JA (1985) The importance of IMP dehydrogenase inhibition in the broad spectrum antiviral activity of ribavirin and selenazofurin. Adv Enzyme Regul 24:29
33. Lee HJ, Pawlak K, Nguyen BT, Robins RK, Sadee W (1985) Biochemical differences among four inosinate dehydrogenase inhibitors, mycophenolic acid, ribavirin, tiazofurin, and selenazofurin, studied in mouse lymphoma cell culture. Cancer Res 45:5512
34. Gilbert BE, Knight V (1986) Biochemistry and clinical applications of ribavirin. Antimicrob Agents Chemother 30:201
35. Smith RA, Knight K, Smith JAD (1984) Clinical applications of ribavirin. Academic, Orlando
36. Lanford RE, Chavez D, Guerra B, Lau JY, Hong Z, Brasky KM, Beames B (2001) Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes. J Virol 75:8074
37. Goswami BB, Borek E, Sharma OK, Fujitaki J, Smith RA (1979) The broad spectrum antiviral agent ribavirin inhibits capping of mRNA. Biochem Biophys Res Commun 89:830
38. Eriksson B, Helgstrand E, Johansson NG, Larsson A, Misiorny A, Noren JO, Philipson L, Stenberg K, Stening G, Stridh S, Oberg B (1977) Inhibition of influenza virus ribonucleic acid polymerase by ribavirin triphosphate. Antimicrob Agents Chemother 11:946
39. Borowski P, Mueller O, Niebuhr A, Kalitzky M, Hwang LH, Schmitz H, Siwecka MA, Kulikowsk T (2000) ATP-binding

domain of NTPase/helicase as a target for hepatitis C antiviral therapy. Acta Biochim Pol 47:173
40. Dusheiko G, Main J, Thomas H, Reichard O, Lee C, Dhillon A, Rassam S, Fryden A, Reesink H, Bassendine M, Norkrans G, Cuypers T, Lelie N, Telfer P, Watson J, Weegink C, Sillikens P, Weiland O (1996) Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. J Hepatol 25:591
41. Bodenheimer HC Jr, Lindsay KL, Davis GL, Lewis JH, Thung SN, Seeff LB (1997) Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C: a multicenter trial. Hepatology 26:473
42. Di Bisceglie AM, Conjeevaram HS, Fried MW, Sallie R, Park Y, Yurdaydin C, Swain M, Kleiner DE, Mahaney M, Hoofnagle JM (1995) Ribavirin as therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 123:897
43. Tam RC, Lim C, Bard J, Pai B (1999) Contact hypersensitivity responses following ribavirin treatment in vivo are influenced by type 1 cytokine polarization, regulation of IL-10 expression, and costimulatory signaling. J Immunol 163:3709
44. Tam RC, Ramasamy K, Bard J, Pai B, Lim C, Averett DR (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 44:1276
45. Miller JP, Kigwana LG, Streeter DG, Robins RK, Simon LN, Roboz J (1977) The relationship between the metabolism of ribavirin and its proposed mechanism of action. Ann N Y Acad Sci 284:211
46. Rueckert RR (1996) Picornaviridae: the viruses and their replication, 3rd edn. In: Fields BN, Knipe DM, Howley PM (eds) Fields virology, vol. 1. Lippincott-Raven, Philadelphia
47. Arnold JJ, Cameron CE (2000) Poliovirus RNA-dependent RNA polymerase (3D (pol)). Assembly of stable, elongation-competent complexes by using a symmetrical primer-template substrate (sym/sub). J Biol Chem 275:5329
48. Gohara DW, Crotty S, Arnold JJ, Yoder JD, Andino R, Cameron CE (2000) Poliovirus RNA-dependent RNA Polymerase ($3D^{pol}$): structural, biochemical, and biological analysis of conserved structural motifs A and B. J Biol Chem 275: 25523
49. De Clercq E (1988) Clinical use of antiviral drugs. Developments in medical virology, 3. Nijhoff Kluwer, Boston
50. De Clercq E (2001) Antiretroviral therapy. ASM, Washington
51. Crotty S, Cameron CE, Andino R (2001) RNA virus error catastrophe: direct molecular test by using ribavirin. Proc Natl Acad Sci USA 98:6895
52. Torre JC de la, Wimmer E, Holland JJ (1990) Very high frequency of reversion to guanidine resistance in clonal pools of guanidine-dependent type 1 poliovirus. J Virol 64:664
53. Domingo E (2000) Viruses at the edge of adaptation. Virology 270:251
54. Domingo E, Holland JJ (1994) Mutation rates and rapid evolution of RNA viruses. In: Morse SS (ed) The evolutionary biology of viruses. Raven, New York
55. Domingo E, Holland JJ, Ahlquist P (1988) RNA genetics. CRC, Boca Raton
56. Domingo E, Sabo D, Taniguchi T, Weissmann C (1978) Nucleotide sequence heterogeneity of an RNA phage population. Cell 13:735
57. Eggers HJ, Tamm I (1965) Coxsackie A9 virus: mutation from drug dependence to drug resistance. Science 148:97
58. Domingo E, Holland JJ, Biebricher C, Eigen M (1995) Quasispecies: the concept and the word. In: Gibbs AJ, Calisher CH, Garcia-Arenal F (eds) Molecular basis of virus evolution. Cambridge University Press, Cambridge
59. Domingo E, Menéndez-Arias L, Quiñones-Mateu ME, Holguín A, Gutiérrez-Rivas M, Martínez MA, Quer J, Novella IS, Holland JJ (1997) Viral quasispecies and the problem of vaccine-escape and drug-resistant mutants. Prog Drug Res 48:99
60. Eigen M, Biebricher C (1988) Sequence space and quasispecies distribution. In: Domingo E, Holland JJ, Ahlquist P (eds) RNA genetics. CRC, Boca Raton
61. Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S (1982) Rapid evolution of RNA genomes. Science 215:1577
62. Holland JJ, de la Torre JC, Steinhauer DA (1992) RNA virus populations as quasispecies. Curr Top Microbiol Immunol 176:1
63. Eigen M (1971) Selforganization of matter and the evolution of biological macromolecules. Naturwissenschaften 58:465
64. Domingo E, Holland JJ (1997) RNA virus mutations and fitness for survival. Annu Rev Microbiol 51:151
65. Holland JJ, Domingo, de la Torre JC, Steinhauer DA (1990) Mutation frequencies at defined single codon sites in vesicular stomatitis virus and poliovirus can be increased only slightly by chemical mutagenesis. J Virol 64:3960
66. Lee CH, Gilbertson DL, Novella IS, Huerta R, Domingo E, Holland JJ (1997) Negative effects of chemical mutagenesis on the adaptive behavior of vesicular stomatitis virus. J Virol 71:3636
67. Loeb LA, Essigmann JM, Kazazi F, Zhang J, Rose KD, Mullins JI (1999) Lethal mutagenesis of HIV with mutagenic nucleoside analogs. Proc Natl Acad Sci USA 96:1492
68. Sierra S, Davila M, Lowenstein PR, Domingo E (2000) Response of foot-and-mouth disease virus to increased mutagenesis: influence of viral load and fitness in loss of infectivity. J Virol 74:8316
69. Eigen M (1993) The origin of genetic information: viruses as models. Gene 135–2:37
70. Schrag SJ, Rota PA, Bellini WJ (1999) Spontaneous mutation rate of measles virus: direct estimation based on mutations conferring monoclonal antibody resistance. J Virol 73:51
71. Lee JH, von Wagner M, Roth WK, Teuber G, Sarrazin C, Zeuzem S (1998) Effect of ribavirin on virus load and quasispecies distribution in patients infected with hepatitis C virus. J Hepatol 29:29
72. Grakoui A, Hanson HL, Rice CM (2001) Bad time for Bonzo? Experimental models of hepatitis C virus infection, replication, and pathogenesis. Hepatology 33:489
73. Blight KJ, Kolykhalov AA, Rice CM (2000) Efficient initiation of HCV RNA replication in cell culture. Science 290: 1972
74. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110
75. Mendez E, Ruggli N, Collett MS, Rice CM (1998) Infectious bovine viral diarrhea virus (strain NADL) RNA from stable cDNA clones: a cellular insert determines NS3 production and viral cytopathogenicity. J Virol 72:4737
76. Beames B, Chavez D, Guerra B, Notvall L, Brasky KM, Lanford RE (2000) Development of a primary tamarin hepatocyte culture system for GB virus-B: a surrogate model for hepatitis C virus. J Virol 74:11764
77. Davis GL, Balart LA, Schiff ER, Lindsay K, Bodenheimer HC Jr, Perrillo Rp, Carey W, Jacobson IM, Payne J, Dienstag JL, et al (1989) Treatment of chronic hepatitis C with recombinant interferon alfa. A multicenter randomized, controlled trial. Hepatitis Interventional Therapy Group. N Engl J Med 321: 1501
78. Di Bisceglie AM, Martin P, Kassianides C, Lisker-Melman M, Murray L, Waggoner J, Goodman Z, Banks SM, Hoofnagle JH (1989) Recombinant interferon alfa therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. N Engl J Med 321:1506
79. Poynard T, Bedossa P, Chevallier M, Mathurin P, Lemonnier C, Trepo C, Couzigou P, Payen JL, Sajus M, Costa JM (1995) A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. N Engl J Med 332:1457
80. Frese M, Pietschmann T, Moradpour D, Haller O, Bartenschlager R (2001) Interferon-alpha inhibits hepatitis C virus subgenomic

RNA replication by an MxA-independent pathway. J Gen Virol 82:723
81. Guo JT, Bichko VV, Seeger C (2001) Effect of alpha interferon on the hepatitis C virus replicon. J Virol 75:8516
82. Pringle CR (1970) Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil 5-azacytidine, and ethyl methane sulfonate. J Virol 5:559
83. Burge BW, Pfefferkorn ER (1966) Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virology 30:204
84. Herold J, Andino R (2000) Poliovirus requires a precise 5′ end for efficient positive-strand RNA synthesis. J Virol 74:6394
85. Herold J, Andino R (2001) Poliovirus RNA replication requires genome circularization through a protein-protein bridge. Mol Cell 7:581–591
86. Maynell LA, Kirkegaard K, Klymkowsky MW (1992) Inhibition of poliovirus RNA synthesis by brefeldin A. J Virol 66: (1985)
87. Belsham GJ, Bostock CJ (1988) Studies on the infectivity of foot-and-mouth disease virus RNA using microinjection. J Gen Virol 69:265