Mechanism of Action of Ribavirin in the Treatment of Chronic Hepatitis C

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Abstract: The current standard treatment for chronic hepatitis C consists of the combination of pegylated interferon and ribavirin. Although interferon is known to have potent antiviral, immunomodulatory, and anti-inflammatory activities against hepatitis C, the mechanism of ribavirin action against this virus is not clearly understood. This article will review proposed mechanisms of ribavirin activity, along with their supporting data, covering ribavirin's roles in the direct inhibition of hepatitis C viral replication, the inhibition of inosine monophosphate dehydrogenase, the induction of mutagenesis to the threshold of error catastrophe, and immunomodulation.

Keywords
Ribavirin, hepatitis C, inosine, ribonucleoside

Hepatitis C virus (HCV) incurs a major disease burden in the United States and worldwide. Nearly 4 million Americans have been exposed to HCV, and approximately 2.7 million harbor chronic HCV.[1,2] Although the incidence of newly acquired infection has been declining, the number of identified cases continues to rise as public awareness and screening for HCV increase; this trend is not expected to reach its peak until 2015.[2] In addition, the number of HCV complications such as end-stage liver disease, hepatocellular carcinoma, and death has also increased with the age of the infected population. Consequently, hepatitis C–related cirrhosis and hepatocellular carcinoma have become the leading indications for liver transplantation in the United States.

Significant advances have been achieved in the treatment for chronic HCV over the past three decades. In the early 1990s, a 6-month course of interferon alfa (IFN-alfa) monotherapy, the first approved therapy for chronic HCV, resulted in sustained virologic response rates (SVR) of 6–12%. The extension of therapy duration to 12 months improved SVR to 16–20%.[3] The addition of ribavirin to IFN-alfa significantly enhanced SVR to 35–40%.[4] More recently, the pegylation of IFN-alfa, when used concomitantly with ribavirin, has further enhanced SVR to 54–56%.[5-7] This combination regimen is currently the standard of care for chronic HCV and will likely remain the foundation of HCV therapy in the near future.

IFN-alfa has potent antiviral activity, mainly due to its ability to induce IFN-stimulated genes, which encode proteins that inhibit various stages of viral replication.[8] In addition, IFN-alfa also has an immunomodulatory effect, interacting with both the adaptive and innate immune response of the host. IFN-alfa promotes T-helper (Th)1 cell differentiation of the T-lymphocytes over Th2 cells, leading to increased production of interleukin (IL)-2 and IFN-gamma. In addition, IFN-alfa produces an anti-inflammatory effect by inhibiting the synthesis of various cytokines, including tumor necrosis factor (TNF) and IL-1.[9] However, the mechanism of action of ribavirin in conjunction with IFN-alfa is not clearly understood. This article will review proposed mechanisms of ribavirin activity and their supporting data.

Ribavirin

Discovered in 1972 by Witkowski and coworkers,[10] ribavirin is a guanosine analogue that produces broad-spectrum activity against several RNA and DNA viruses.[11] Although originally approved only for the treatment of severe respiratory syncytial virus (RSV) infection in children,[12] ribavirin has been used in the treatment of Lassa fever virus infection,[13,14] influenza A and B,[15] and other viruses. In the early 1990s, ribavirin was studied for the treatment of HCV. As a single agent, ribavirin had no significant effect on HCV RNA levels, despite observations of improvements in serum aminotransferase levels[16] and liver histology.[17] Prolonging the course of treatment did not add any benefit in terms of virologic clearance, either.[18] Therefore, ribavirin has been used for treatment of chronic HCV only in combination with IFN-alfa. This clinical data suggests that ribavirin monotherapy may not directly or adequately inhibit viral replication in patients.

Understanding the molecular basis of ribavirin's antiviral activity against HCV has been impeded by the lack of available and efficient HCV culture systems and HCV replication animal models. Utilizing results from studies conducted on other RNA viruses as well as the limited data available on HCV itself, ribavirin's antiviral ability as a single agent has been postulated to result from four pathways, namely (1) direct inhibition of HCV replication, (2) inhibition of host inosine monophosphate dehydrogenase (IMPDH) enzyme, (3) mutagenesis induction to drive a rapidly replicating virus beyond the threshold to error catastrophe, and (4) immunomodulation by inducing a Th1 immune response (Figure 1). These mechanisms are not mutually exclusive. It is not known which mechanism predominates, particularly during ribavirin's synergistic action with IFN-alfa.

Direct Inhibition of HCV Replication Including Inhibition of HCV RNA Polymerase

Ribavirin is converted intracellularly into ribavirin monophosphate (RMP) by adenosine kinase and then into dephosphorylated and triphosphorylated forms by nucleoside monophosphate and diphosphate kinases.[19,20] In most cell types, ribavirin triphosphate (RTP) forms dominate, with concentrations as high as 20–100 times that of RMP.[21] Binding of RTP to the nucleotide binding site of the RNA polymerase prevents the binding of the correct nucleotides, leading to a reduction in viral replication or to the production of defective virions. Competitive inhibition of viral RNA polymerase by RTP has been reported in the influenza virus,[22] the vesicular stomatitis virus,[23,24] and the LaCrosse virus.[25] In recent years, the development of a novel primer-extension assay for the RNA-dependent RNA polymerase (RdRP) employing end-labeled primers has allowed the study of ribavirin incorporation into viral RNA. Using this assay, the RdRP-catalyzed incorporation of ribavirin into poliovirus RNA opposite uridine and cytidine has been demonstrated at equivalent efficiency, albeit at a substantially slower rate and with a weaker binding affinity, than the incorporation of guanine or adenine nucleotides.[26]

The ability of the HCV RNA polymerase located in the NS5B region to utilize RTP as a substrate and incorporate it into HCV RNA has been shown by Maag and associates.[27] Again, the utilization of RTP opposite uridine and cytidine occurred at equivalent efficiency and templates containing ribavirin appeared to cause a block to HCV RNA elongation. Through this pathway, ribavirin has been presumed to cause premature termination of nascent HCV RNA and increase mutagenesis by producing defective virions.[26,27] In an in vitro study of ribavirin's effect on a full-length HCV RNA polymerase, the inhibition of RNA elongation was observed via formation of stalled elongation complexes.[28] In addition, when RMP was present in the RNA template, cytosine or uridine pairing occurred at an exceedingly low catalytic efficiency (ie, 200–300 times slower) than that of adenosine or guanosine template pairing with these natural nucleotides. Consequently, a template containing RMP significantly reduced the efficiency of viral RNA synthesis. Nevertheless, the rate of ribavirin incorporation into HCV RNA was much slower (50- to 100-fold lower) than that of a natural nucleotide.[28] In cell culture studies, ribavirin inhibits HCV replication. Ribavirin-resistant HCV mutants have been isolated, suggesting a direct effect of ribavirin on the HCV replication complex. Interestingly, these mutants map to both NS5B (the RdRP) and NS5A.[29,30] The role of NS5A in HCV replication and ribavirin resistance is

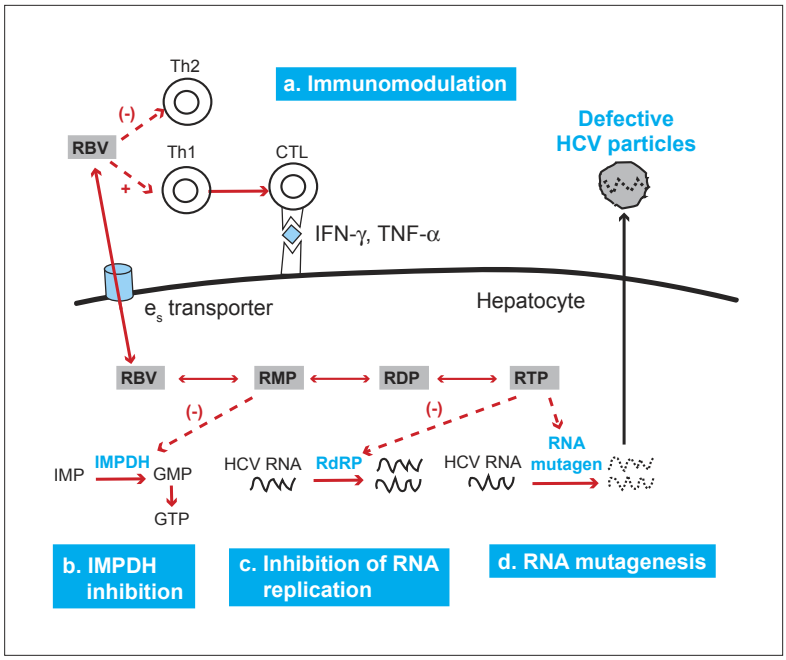

Figure 1. Proposed sites of ribavirin action against hepatitis C virus (HCV), including a) induction of a shift from Th2 to Th1 immune response, b) inhibition of IMPDH leading to GTP pool depletion, c) direct inhibition of HCV replication, and (d) induction of mutagenesis, leading to production of defective viral particles.

RBV=ribavirin; Th=T-helper cell; IFN-γ= interferon-gamma; TNF-α=tumor necrosis factor-alfa; RMP=ribavirin monophosphate; RDP=ribavirin diphosphate; RTP=ribavirin triphosphate; IMP=inosine monophosphate; IMPDH=inosine monophosphate dehydrogenase; RdRP=RNA-dependent RNA polymerase; CTL=cytotoxic T-lymphocyte; e_s=equilibrative nucleoside transporter; GMP=guanosine monophosphate; GTP=guanosine triphosphate.

Adapted from Feld and Hoofnagle.[71]

unclear, but NS5A binds RNA and as such may be an accessory factor of RdRP in HCV replication.

The posttranslational capping of the 5' end of viral mRNA has been proposed as a site of ribavirin action against several RNA viruses. Following the translation of several viral mRNA, guanine pyrophosphate is formed at the 5' end by mRNA guanylyltransferase, a modification called capping. This cap protects the mRNA from degradation by the host enzymes and promotes cap-dependent translation of the mRNA. The incorporation of ribavirin at the 5' end in place of guanosine hinders the methylation step, therefore impeding mRNA translation.[31] Competition between guanosine and ribavirin for the 5' terminal guanylation has been described by Goswami and associates in the vaccinia virus.[32] Moreover, resistance to ribavirin in a mutant Sindbis virus has been attributed to mutations in the guanylyltransferase enzyme, suggesting that this enzyme may be involved in the antiviral activity of ribavirin.[33] However, this mechanism would not apply to HCV, as this virus does not form capped mRNA.

Inhibition of IMPDH

IMPDH inhibition was proposed as the mechanism of action of ribavirin shortly after its discovery. Ribavirin monophosphate mimics inosine 5'-monophosphate (Figure 2) and is a competitive inhibitor of IMPDH, an enzyme involved in the de novo synthesis of guanine nucleotides. It is the rate-limiting enzyme in the conversion of inosine 5'-monophosphate to xanthine monophosphate, which can be converted into guanosine monophosphate (GMP) by adding an amine. Guanosine monophosphate can be further phosphorylated into guanosine triphosphate (GTP), one of the building blocks for viral RNA replication. Ribavirin has been shown to inhibit de novo synthesis of guanine nucleotides and to decrease intracellular GTP pools via IMPDH inhibition in vitro.[34,35] Decreasing the intracellular GTP pools has been shown to improve the activity of nucleoside analogues, presumably by increasing the incorporation of the nucleoside analogue into the nascent viral RNA. This may lead to a decline in viral protein synthesis and limit replication of viral genomes.

The inhibition of influenza virus replication via the inhibition of the IMPDH pathway by ribavirin has been demonstrated by Wray and colleagues.[36] Intracellular GTP pools were depleted to approximately half the baseline levels with ribavirin treatment, without significant effects on other nucleotide levels. Furthermore, the addition of exogenous guanosine partially restored viral RNA synthesis. In GB virus-B, a virus closely related to HCV and used as a surrogate to study HCV replication, ribavirin also affected a reduction in intracellular GTP levels and lowered viral RNA levels. This inhibitory effect was attenuated by the addition of guanosine, consistent with the theory of IMPDH inhibition.[37] Such an inhibitory effect has also been demonstrated in an HCV subgenomic replicon system, where ribavirin decreased the colony

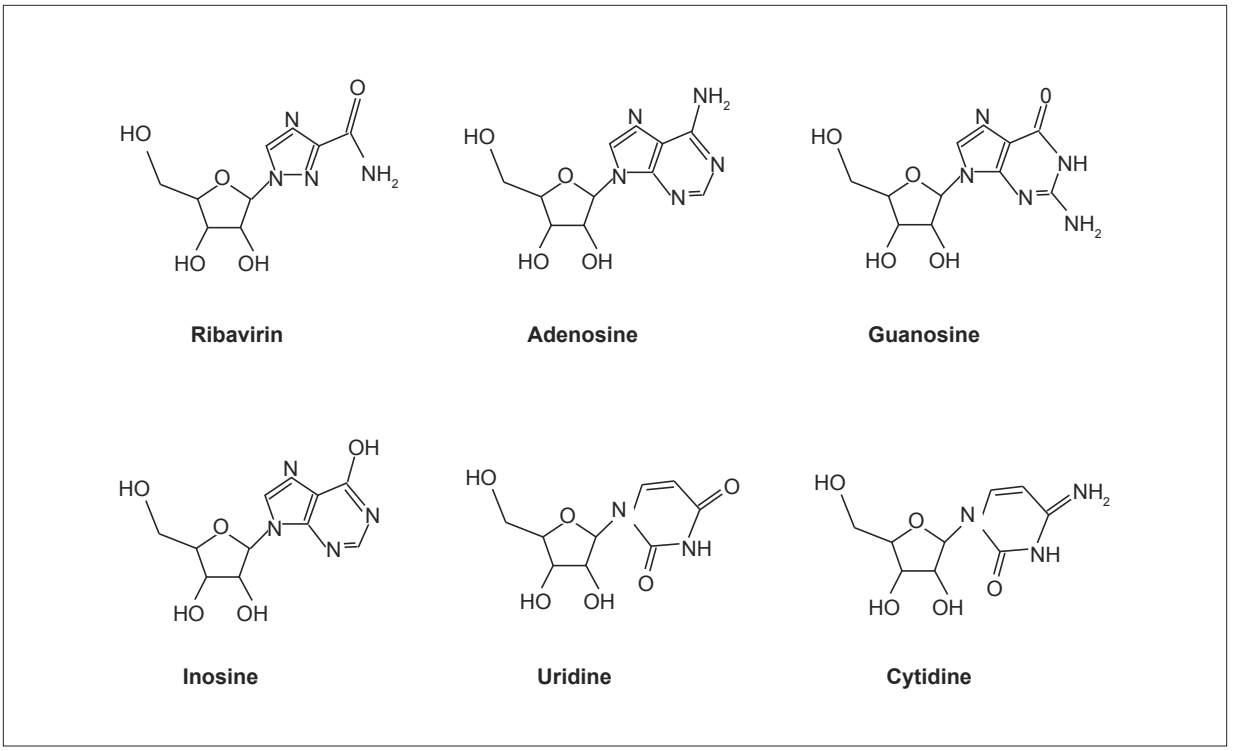

Figure 2. Chemical structures of ribavirin, inosine, and ribonucleosides.

formation efficiency of the replicon HCV RNA in a dose-dependent fashion. This effect was further enhanced by ribavirin combination treatment with other potent, non-nucleoside IMPDH inhibitors such as mycophenolic acid (MPA) or VX-497, and was again reversed by the addition of guanosine with the treatment.[38]

However, despite the dose-dependent reduction in GTP pools caused by ribavirin, the inhibition of influenza virus replication was not fully reversed by complete restoration of the guanosine stores.[36] In addition, the use of other IMPDH inhibitors (MPA or VX-497) alone had only minimal effect on the colony-forming efficiency of HCV replicon RNA.[38] There was also no significant antiviral effect seen with MPA on the GB virus-B.[37] Therefore, IMPDH inhibition alone cannot account for the full anti-HCV action of ribavirin.

Mutagenesis and Error Catastrophe

The existence of HCV as many quasispecies is a testimony to the low fidelity and the lack of proofreading and repair capabilities of HCV RNA polymerase. Quasispecies diversity is a result of the high frequency of mutations that occur during viral replication, which produces virions with minor genomic differences. Such a high mutation rate allows viral subspecies to escape detection by the host immune system or to develop resistance to antiviral therapies. However, a high mutation frequency is also dangerous, because the genetic integrity can survive only a limited amount of variability. It is hypothesized that viral populations have an upper limit of variability. Ribavirin has been postulated to act as a mutagen, increasing the frequency of viral mutation to the point of pushing the virus beyond the threshold of error catastrophe, leading to viral extinction.[39]

Once RTP is incorporated into viral RNA, it forms a template for pairing with cytidine triphosphate or uridine triphosphate (with equal efficiency), increasing the frequency of G→A and A→G transitions in the viral genome, and therefore, promoting mutagenesis.[31] In vitro studies conducted with poliovirus,[26] West Nile virus,[40] GB virus-B36, and Hantaan virus[41] showed the mutagenic nature of ribavirin. In the poliovirus model, viral replication was inhibited by the presence of 2 mM guanidine due to the inhibition of the ATPase activity of the viral *2C* gene product, a protein which is essential for viral genome replication. A single transition mutation in the *2C* gene can produce guanidine-resistant virus, and this mutation occurs at a frequency of 10^{-5} in the wild-type poliovirus population. In the presence of ribavirin,

an increase in the frequency of guanidine-resistant virus was observed.[26] In addition, direct sequencing of the VP1 capsid-coding sequence of the virus grown in the absence of ribavirin had an average of 1.5 mutations per genome, but this rate increased to 15 mutations per genome when 1,000 μM ribavirin was present.[26] Furthermore, a 4-fold increase in the mutation led to a 10-fold decrease in the infectivity of the viral RNA, and a 10-fold increase in the mutation frequency was sufficient to reduce the viable viral population to 0.05% of the normal population. The investigators therefore concluded that poliovirus exists on the threshold of error catastrophe and that ribavirin increases the mutation rate beyond the error threshold.[39] One caveat to this conclusion is that the concentrations of ribavirin used in this study were 20 times higher than clinically relevant doses.

The sequencing of viral genome segments of the West Nile virus treated with ribavirin also revealed significant increases in nucleotide transition mutations. Additionally, the relative infectivity of the viral RNA synthesized in the presence of ribavirin was decreased in comparison to untreated controls, and extinction of the virus was seen after 4 passages of the HeLa cell culture with 47 μg/mL of ribavirin.[40] In the GB virus-B model, specific infectivity was significantly reduced when the virus was grown in the presence of ribavirin. Ribavirin led to a 4-log reduction in viral RNA levels; this reduction was partially reversed by the addition of guanosine, suggesting that the depletion of the GTP pool by IMPDH inhibition augmented the mutagenic effect of ribavirin.[37] In the Hantaan virus, ribavirin treatment led to increased mutations in viral RNA and the subsequent synthesis of nonfunctional viral proteins as well.[41]

The mutagenic effect of ribavirin on HCV has been demonstrated in various cell propagation systems. In the HCV subgenomic replicon system, the sequencing of the neo gene segment demonstrated that ribavirin, in combination with the IMPDH inhibitors MPA or VX-497, increased the replicon error rate by approximately 2-fold.[37,38] In a cell culture using a binary HCV expression system, Contreras and colleagues[42] reported that the clinically relevant dose of ribavirin at 50 μM induced mutations across the viral genome, with error generation being more significant in the core region of NS5A and NS5B. However, the rate of mutagenesis was lower than that reported in the poliovirus by Crotty and coworkers,[39] and the rate did not rise with increasing ribavirin doses. The investigators therefore speculated that ribavirin increased the mutagenesis rate just to the brink of the error catastrophe level for the poliovirus but not to a level adequate to extinguish HCV RNA synthesis completely. This mutagenic effect of ribavirin on HCV RNA has been confirmed in other HCV replicon models.[43,44]

Data on the mutagenic ability of ribavirin on HCV in humans have been controversial. Young and associates[30] demonstrated a modest increase in mutations in the NS5B region of HCV in patients treated with ribavirin monotherapy but not at a level adequate to induce error catastrophe. More recently, Asahina and colleagues reported significant increases in mutation rates in the NS5A and NS5B regions of HCV in patients treated with ribavirin monotherapy, and these mutations during ribavirin monotherapy correlated with virologic response to subsequent IFN-alfa and ribavirin combination therapy.[45] However, Chevaliez and associates did not find an increase in the mutation rates in the NS3 protease and NS5A proteins in patients treated with ribavirin monotherapy or IFN-alfa and ribavirin combination therapy.[46]

Nevertheless, the incorporation of RTP into viral RNA occurs at a slower rate than that of natural nucleotides, and the frequency of mutagenesis in HCV has not been consistently high enough to induce error catastrophe. In a cell culture study, the exposure of HCV RNA to 1,000 μM ribavirin led to a frequency of mutations of, at most, 1 per 7,000 nucleotides or approximately 1 per genome per replication cycle.[27] Therefore, error catastrophe may be only one of several mechanisms by which ribavirin exerts its antiviral action.

Immunomodulation

The host adaptive immune response against viruses consists of virus-specific CD4+ Th cells and CD8+ cytotoxic (CTL) cells. CD4+ Th cells help CD8+ CTL cells and B-cells, whereas CD8+ CTL cells mediate the cytotoxic killing of virus-infected cells. During viral infections, both T-cell subsets that produce type 1 cytokines such as interferon-gamma (IFN-gamma), tumor necrosis factor-alfa (TNF-alfa) and IL-2 are activated first in an attempt to clear the virus (type 1 or Th1 response). Subsequently, the T-cells that produce type 2 cytokines such as IL-4, IL-5, and IL-10 are activated to regulate Th1 cells and to stimulate the humoral response, which confers immunity toward the virus.

Clearance of HCV has been demonstrated to correlate with an early, robust Th1 immune response, whereas chronic infection has been attributed to an early Th2 predominance.[47,48] In acute HCV infection, spontaneous clearance of the virus was seen more frequently in patients with a Th1 response activation, whereas a Th2 response was associated with chronicity.[49] Patients with persistent HCV viremia were found to have fewer peripheral blood mononuclear cells (PBMC) possessing a Th1 response to HCV core protein than patients with self-limited HCV infection did.[50] Indeed, a higher level of the Th2 cytokines IL-4 and IL-10 has been detected in the sera of several

patients with chronic HCV infection.[51] A predominance of IL-4 and IL-10 and a lack of IL-2 production by NS3 antigen-specific T-cells from patients with chronic HCV was demonstrated in vitro.[52] Lastly, a decline in HCV viral levels in response to IFN therapy was associated with a reduction in Th2 cytokine response.[53]

Ribavirin has been demonstrated to have the immunomodulatory effect of shifting a Th2 response in favor of a Th1 phenotype. Antibody production and humoral response were inhibited by ribavirin administration in plaque-forming cells in vivo.[54] Ribavirin treatment of RSV infection not only eradicated the virus but also reduced the prevalence of hyperactive airway disease, a phenomenon that has been attributed to a polarized Th2 response.[55] When isolated human T-lymphocytes were activated with phorbol ester plus ionomycin, ribavirin enhanced a type 1 (Th1) cytokine response with IL-2, IFN-gamma, and TNF-alfa while suppressing the type 2 (Th2) response with IL-4, IL-5, and IL-10 at the levels of both mRNA and protein expression.[56]

Ribavirin's immunomodulatory action has also been shown in several animal models. Immunization of ribavirin-treated mice with hepatitis B virus (HBV) core antigen or HBV e antigen led to amplification of the core antigen- or e antigen-specific IL-2 and IFN-gamma levels (a Th1 response) compared to untreated controls. Similarly, ribavirin treatment of a HBV e antigen transgenic mouse model led to a dose-dependent shift to Th1 over Th2 phenotype.[57] Ribavirin also increased macrophage activation and promoted Th1 cytokine production at the expense of Th2 cytokine production in mice infected with murine hepatitis strain 3.[58,59] Moreover, ribavirin increased Th1 cytokine production, core-specific cytotoxic T-cell activity as well as IL-2 and IL-12 levels when given to mice immunized with the HCV core antigen.[60] Interestingly, in vitro and in vivo induction of Th1 cytokines was observed with the ribavirin L-enantiomer, levovirin, despite the absence of antiviral activity from this drug.[61]

In humans, ribavirin and levovirin have both been seen to enhance HCV-specific Th1 response by increasing IFN-gamma and TNF-alfa production by PBMC in patients with chronic HCV.[62] Ribavirin-induced augmentation of HCV-specific T-cell reactivity with increased IFN-gamma and decreased IL-10 mRNA expression was observed in PBMC of patients with chronic HCV.[63] Additionally, ribavirin increased Th1 cytokine production (IFN-gamma, IL-2, and IL-12) and TNF-alfa production in PBMC of patients with chronic HCV. In the same study, IFN-alfa tended to suppress IL-2, IL-4, and IL-12 secretion but enhanced TNF-alfa, IFN-gamma, and IL-10 production. When combined with ribavirin, IFN-alfa countered ribavirin's effect on IL-2, IL-4, and IL-12, but both drugs upregulated IFN-gamma and IL-10 expression.[64] Finally, patients treated with combination IFN-alfa and ribavirin therapy were reported to have lower IL-10 production, as compared with those treated with IFN-alfa alone, lending further support to the role of ribavirin as an immunomodulator.[65] This immunomodulatory effect of ribavirin may partially account for its synergistic action with IFN-alfa against HCV.

IFN-alfa and Ribavirin Interaction

So far, the mechanism of the synergistic effects of IFN-alfa and ribavirin against HCV beyond the summation of their individual efficacies is unclear. Studies of viral kinetics demonstrating the effect of IFN-alfa alone, and in combination with ribavirin, on HCV RNA levels at various times during therapy may lend support to the theories of ribavirin's role in immunomodulation and lethal mutagenesis. Herrmann and coworkers noted a faster decline in viral load during the third phase of therapy (beyond Day 28) in patients treated with pegylated IFN-alfa and ribavirin combination therapy, as compared with those treated with pegylated IFN-alfa alone.[66] This slope in the third phase was predictive of SVR and was interpreted to represent treatment-enhanced infected cell loss, which was attributed to enhanced immune response and, perhaps, increased mutagenesis. In another kinetics model, Dixit and associates demonstrated that ribavirin affects the second phase (beyond 2 days of therapy) of viral decay in patients treated with IFN-alfa and ribavirin combination therapy.[67] They also proposed that this antiviral effect may be mediated by increased mutation frequencies in viral RNA, leading to noninfectious virions, which are then cleared by the immune system. Ribavirin upregulates several IFN-stimulated response genes during RSV infection.[68] This may be the molecular basis for the synergism of ribavirin's immunomodulatory activity with IFN-alfa against HCV.

In the clinical setting, ribavirin improves the prevention of relapse after therapy has been completed. In a large international trial, treatment with pegylated IFN-alfa alone led to an end-of-treatment response (ETR) of 59%, as compared with an ETR of 69% for the combination of pegylated IFN-alfa and ribavirin. At 6-month follow-up, however, SVR had dropped to 29% for the monotherapy group and 56% for the combination group.[5] Thus, the modest improvement in ETR with the addition of ribavirin to pegylated IFN-alfa was magnified in SVR, ie, ribavirin improved SVR by reducing relapse rates. Furthermore, ribavirin dosing early in the course of therapy seems to be most predictive of treatment outcomes. In another large multicenter study where previous IFN nonresponders received combination therapy with pegylated IFN-alfa and ribavirin, reducing the ribavirin

dose from at least 80% to less than 60% during the first 20 weeks of treatment compromised SVR by half, whereas reducing the dose beyond 20 weeks of treatment did not affect SVR.[69] These observations, along with the observations from the kinetics studies, suggest that ribavirin could decrease relapse rate by accelerating viral clearance early in the treatment course due to the above mechanisms.

Among the HCV genotypes, a higher dose of ribavirin and a longer course of therapy have been established as requirements for successful therapy of genotype 1 HCV infection, as compared to genotypes 2 and 3.[6] High doses of ribavirin in the range of 1,600–3,600 mg daily (mean of 2,500 mg daily) were recently reported to improve SVR in genotype 1-infected patients with high viral loads.[70] These studies suggest that increased ribavirin levels can compensate for the nonresponse to IFN that is frequently observed in genotype 1-infected patients. As such, ribavirin appears less sensitive to HCV genotype differences than IFN-alfa.

Conclusion

Ribavirin has proven invaluable as a broad-spectrum antiviral drug against many infections, including hepatitis C. Although it has little antiviral power against HCV by itself, combining it with IFN-alfa releases its lethal prowess. Yet the mechanism responsible for ribavirin's increased efficacy during concomitant administration with IFN is not well established. Its immunomodulatory effect cannot account for its antiviral activity in cell cultures, and direct effects on HCV RNA levels are not observed in patients on ribavirin monotherapy. IMPDH inhibition alone does not inhibit HCV replication in cell cultures, and clinically relevant concentrations of ribavirin have not generated HCV error catastrophe in cell cultures. Perhaps the success of ribavirin is attributable to its multivalent nature as a purine analogue involved in multiple cellular pathways. Elucidation of the interplay between different ribavirin mechanisms of action and between interferon is not only important in understanding how the current therapy for HCV works, but also in defining the role of ribavirin as a component of future combination therapies in order to maximize the chances at HCV extinction.

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