The effect of ribavirin and IMPDH inhibitors on hepatitis C virus subgenomic replicon RNA

Sifang Zhou, Rong Liu, Bahige M. Baroudy, Bruce A. Malcolm, and Gregory R. Reyes*

Antiviral Therapy, Schering–Plough Research Institute, Kenilworth, NJ 07033, USA

Received 12 December 2002; returned to author for revision 10 February 2003; accepted 12 February 2003

Abstract

The recent development of in vitro hepatitis C virus (HCV) RNA replication systems has provided useful tools for studying the intracellular anti-HCV activity of ribavirin. Ribavirin has been shown to: (1) induce “error catastrophe” in poliovirus (Crotty et al., 2001, Proc. Natl. Acad. Sci. USA 98, 6895–6900), (2) be a pseudo-substrate of the HCV RNA-dependent RNA polymerase (RdRp) in vitro (Maag et al., 2001, J. Biol. Chem. 276, 46094–46098), and (3) increase mutations in HCV RNA in the binary T7 polymerase/HCV cDNA replication system (Contreras et al., 2002, J. Virol. 76, 8505–8517). These findings have led to the hypothesis that ribavirin may also induce error catastrophe in HCV. However, the functional relevance of ribavirin-induced HCV RNA mutagenesis is unclear. By use of a colony formation assay, in which RNA is isolated from the HCV subgenomic replicon system following treatment, the impact of ribavirin, inosine-5′-monophosphate dehydrogenase (IMPDH) inhibitors, and the combination was assessed. Ribavirin reduced HCV replicon colony-forming efficiency (CFE) in a dose-dependent fashion, suggesting that ribavirin may be misincorporated into replicon RNA and result in an anti-replicon effect analogous to error catastrophe. This effect was markedly suppressed by addition of exogenous guanosine. Combination treatment with ribavirin and mycophenolic acid (MPA) or VX-497, both potent, nonnucleoside IMPDH inhibitors, and the combination was assessed. Ribavirin reduced HCV replicon colony-forming efficiency (CFE) in a dose-dependent fashion, suggesting that ribavirin may be misincorporated into replicon RNA and result in an anti-replicon effect analogous to error catastrophe. This effect was markedly suppressed by addition of exogenous guanosine. Combination treatment with ribavirin and mycophenolic acid (MPA) or VX-497, both potent, nonnucleoside IMPDH inhibitors, led to a greatly enhanced anti-replicon effect. This enhancement was reversed by inclusion of guanosine with the treatment. In contrast, MPA or VX-497 alone had only marginal effects on both the quantity and quality (CFE) of replicon RNA, suggesting that although IMPDH inhibition is an important contributing factor to the overall ribavirin anti-HCV replicon activity, IMPDH inhibition by itself is not sufficient to exert an anti-HCV effect. Sequencing data targeting the neo gene segment of the HCV replicon indicated that ribavirin together with MPA or VX-497 increased the replicon error rate by about two-fold. Taken together these results further suggest that lethal mutagenesis may be an effective anti-HCV strategy. The colony formation assay provides a useful tool for evaluating mutagenic nucleoside analogs for HCV therapy. Finally, the data from combination treatment indicate potential therapeutic value for an enhanced anti-HCV effect when using ribavirin in combination with IMPDH inhibition.

Keywords: Hepatitis C virus; Ribavirin; Mutagenesis; Inosine-5′-monophosphate dehydrogenase inhibitor; Combination therapy; Replicon

Introduction

Hepatitis C virus (HCV) infection is a major global health issue, with an estimated 3% of the world’s population being chronically infected (Wasley and Alter, 2000). Among them, about 20–30% eventually progress to chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma (Alter and Seeff, 2000). Currently, the standard treatment for HCV infection involves either interferon-α (IFNα) or PEG-interferon-α, in combination with ribavirin (1-β-D-ribofurano-syl-1,2,4-triazole-3-carboxamide) (Fried et al., 2002; Lauer and Walker, 2001). Ribavirin, when co-administered with IFNα, substantially improves the efficacy of the therapy, achieving 40% sustained virological response (SVR) in previously untreated patients, in contrast to ~13% with IFNα alone. In relapsed patients who had been treated with IFNα monotherapy, the SVR improvement was even more signif-
The underlying mechanism(s) for improvement in SVR from ribavirin in combination therapy is not yet fully understood. No pharmacokinetic interactions between IFNα and ribavirin have been detected (Khakoo et al., 1998). Nor is it clear whether ribavirin itself has any direct anti-HCV activity due to the lack of an efficient HCV culture system. Nevertheless, several possible antiviral mechanisms of action for ribavirin have been proposed based on findings from biochemical and virological assays using other RNA and DNA viral infection models (reviewed in Cameron and Castro, 2001; Reyes, 2001). To date, ribavirin has been shown to directly inhibit viral polymerases, such as the vesicular stomatitis virus (VSV) RNA polymerase (Toltzis et al., 1988), La Crosse encephalitis virus polymerase (Cassidy and Patterson, 1989), roovirus transcriptase (Rankin et al., 1989), influenza virus polymerase (Erikkson et al., 1977), and HCV RNA polymerase (Z. Hong et al., 50th American Association for the Study of Liver Diseases, Dallas, TX, USA, Abstract 773, 1999). A second proposed mechanism was that ribavirin monophosphate (RMP) acts as a competitive inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH) ($K_i \sim 250$ nM), a cellular enzyme involved in the rate-limiting step of the de novo synthesis of guanine nucleotides (Streeter et al., 1973; Malinowski and Stollar, 1981). The inhibition of IMPDH by ribavirin led to a reduction in intracellular guanosine triphosphate (GTP) pools in Madin–Darby canine kidney (MDCK) cells and thereby blocked influenza virus replication (Wray et al., 1985). Support for this mechanism of action also comes from reversal of ribavirin’s antiviral effect on influenza virus by exogenous addition of guanosine (Wray et al., 1985).

More recently, several lines of evidence have suggested that ribavirin may serve an immunomodulatory role, as an inducer of the helper-T-cell (CD4+)-type 1 (Th1) cytokine response and a suppressor of the type 2 (Th2) cytokine phenotype (reviewed in Tam et al., 2001). Although ribavirin’s immunomodulatory effect may have a role in vivo, this cannot account for its antiviral activities against a variety of DNA and RNA viruses in cell culture (De Clercq et al., 1991; Sidwell et al., 1972).

Another recently proposed mechanism of action for ribavirin is that it may act as a mutagen and push RNA viruses beyond a critical mutation rate threshold, thereby driving the virus population into “error catastrophe” (Crotty et al., 2000). This mechanism is consistent with the observation that many RNA viruses exist as highly heterogeneous “quasispecies” due to high mutation frequencies (reviewed in Domingo, 2000). The extremely high error rate of RNA viruses (due to the low fidelity of the viral replicases) is proposed to be an evolutionary advantage, but results in a viral population close to the edge of “error catastrophe,” i.e., a small increase in the error rate may lead to a drastic loss of genome viability/infectivity (Eigen, 1971; Holland et al., 1990; Domingo, 2000). In support of this theory, Crotty and co-workers have shown that ribavirin can be incorporated by the poliovirus-encoded RNA-dependent RNA polymerase into viral transcripts. The incorporated ribavirin is capable of base pairing equally well with either uridine monophosphate (UMP) or cytidine monophosphate (CMP), thereby potentially causing transition mutations (Crotty et al., 2000). It was subsequently shown that the anti-poliovirus activity of ribavirin correlated directly with this mutagenic effect (Crotty et al., 2001), suggesting that the primary mechanism of action in this system may be “error catastrophe.”

The contribution of each of the above postulated mechanisms to the clinical effect of ribavirin on HCV is still unknown. Additional support for ribavirin’s action as a mutagen in HCV therapy comes from a recent report that ribavirin is a substrate for an engineered form of the RNA-dependent RNA polymerase (RdRp) (Maag et al., 2001), and a second study showing that ribavirin treatment resulted in a two- to four-fold increase in the overall mutation frequency of HCV RNA in a binary, T7 polymerase-driven HCV replication system (Contreras et al., 2002). However, direct evidence for ribavirin-induced error catastrophe in HCV is still missing, as no viral infection system is available for measurement of HCV genome infectivity. In the very closely related HCV surrogate system using GB virus B (GBV-B), ribavirin treatment led to dramatic reduction in the infectivity of progeny virions (Lanford et al., 1993), have been shown to suppress and enhance, respectively, the effect of ribavirin despite the fact that neither alone had any effect on virus replication (Lanford et al., 2001).

In this report, the recently developed HCV subgenomic replicon system (Lohmann et al., 1999; Blight et al., 2000) has been used to examine ribavirin’s in vitro anti-HCV effect and possible underlying mechanisms of action (MOAs).

**Results**

*Effect of ribavirin on HCV replicon copy number*

Taking advantage of the recently developed HCV subgenomic replicon system, initial studies examined whether ribavirin would decrease replication. The replicon-bearing cells were treated with various concentrations of ribavirin.
Increase in the cell doubling time from $/H11011$ is important to note that replicon replication strongly correlated with an EC$_{50}$ of $80 \mu M$ and an EC$_{90}$ of $640 \mu M$ (Fig. 1). However, this anti-replicon activity closely correlated, or overlapped, with the cytotoxicity profile of the compound. The inhibitory effect of ribavirin in this replicon system is reproducible, but indicates a very narrow therapeutic window between activity and cellular toxicity. Cytotoxicity of ribavirin was evaluated in two ways: MTS assay and reduction in cellular GAPDH RNA as measured by real-time RT-PCR. Fifty percent cytotoxicity concentration, or CC$_{50}$, was $200 \mu M$ ribavirin as estimated by GAPDH reduction and $>640 \mu M$ by MTS assay (Fig. 1). This difference suggested that ribavirin is a cytostatic but not a cytotoxic agent. Replicon cells that have undergone high-dose ribavirin treatment ($100 \mu M$) are morphologically larger than untreated cells and grow more slowly (e.g., $200 \mu M$ ribavirin treatment for 2 weeks resulted in a five-fold increase in the cell doubling time from $21$ to $100$ hours, data not shown). These cells regained their normal growth rate on removal of ribavirin from the culture medium (data not shown).

Although a small therapeutic window appears to exist for ribavirin, based on replicon copy number in this system, it is important to note that replicon replication strongly correlates with host cell metabolic status (Pietschmann et al., 2001). Ribavirin’s cytostatic activity may therefore compromise reduction in replicon copy number as an in vitro measurement of drug efficacy due to the global inhibition of cellular metabolism. The observation that ribavirin has only weak anti-HCV replicon activity in vitro is consistent with clinical data from ribavirin monotherapy indicating no significant impact on HCV viral load in serum (Lauer and Walker, 2001).

Ribavirin reduces the colony-forming efficiency of HCV replicon RNA

In an effort to evaluate the anti-replicon activity of ribavirin independent of its cytostatic effect, colony formation experiments (analogous to reinfection studies) were performed. Following treatment of replicon cells with ribavirin in G418-free medium for 10 days, the total cellular RNA (including replicon RNA) was extracted and used to transfect naïve Huh7 cells. Three weeks later, the G418-resistant colonies were counted and normalized to the replicon copy number in the input RNA, which had been determined by real-time PCR using an HCV replicon standard curve, to obtain the colony-forming efficiency (CFE). Integrity of the replicon RNA recovered from these treatments was confirmed by performing qPCR targeting the 5’ UTR, neo, NS3, and NS5B regions, respectively. The data (not shown) yielded comparable quantification suggesting no significant degradation of replicon RNA had occurred. As transfection involves only RNA, and not any associated viral or host proteins, CFE is a direct assessment of the viability (replicative integrity) of the input replicon genomes.

To control for transfection efficiency, the same amount of total RNA ($8 \mu g$) was used in each transfection. The linear range of input genome RNA—for obtaining similar CFE—was determined by serially diluting replicon RNA from untreated cells with Huh7 RNA (1:1, 1:4, and 1:16). RNA diluted 1:4 (from 80 million to 20 million replicon copies) did not change CFE significantly; the 1:16 dilution (from 80 million to 5 million replicon copies) reduced CFE approximately fourfold (data not shown), suggesting a linear range between 20 and 80 million genome copies/transfection. About 580 colonies were obtained from 80 million copies wild-type HCV replicon RNA, giving a CFE of approximately $10^6$ colonies/µg of replicon RNA which is comparable to previous reports (Krieger et al., 2001).

Ribavirin reduced the replicon RNA’s ability to generate G418-resistant colonies in a dose-dependent manner: 50 µM ribavirin reduced CFE by ∼2-fold, 100 µM ribavirin reduced CFE by ∼4-fold, and 300 µM ribavirin reduced CFE by ∼24-fold (Fig. 2). Ribavirin at 10 µM did not result in a statistically significant change in CFE (Fig. 2). These data suggested that ribavirin was not an artifact of input copy number. With the 300 µM ribavirin treatment, the replicon copy number dropped 11-fold (to 7 million); however the CFE reduction (24-fold) was still significant even allowing for the 4-fold drop in transfection efficiency predicted by the wild-type genome titration study (i.e., an additional 6-fold reduction was still observed). It is noteworthy that replicon cells treated with up to 100 µM ribavirin for up to 10 days still showed only marginal reduction of replicon copy number (less than 0.16, 0.63, 2.5, 10, 40, 160, and 640 µM) in a 96-well plate format. Three days later the level of replicon RNA was determined by real-time RT-PCR, using GAPDH as a control for cellular RNA (Fig. 1). Ribavirin appeared to reduce the HCV replicon copy number in a dose-dependent manner, with an EC$_{50}$ of ∼$80 \mu M$ and an EC$_{90}$ of ∼$640 \mu M$ (Fig. 1). However, this anti-replicon activity closely correlated with an EC$_{50}$ of ∼$80 \mu M$ and an EC$_{90}$ of ∼$640 \mu M$ (Fig. 1). However, this anti-replicon activity closely correlated, or overlapped, with the cytotoxicity profile of the compound. The inhibitory effect of ribavirin in this replicon system is reproducible, but indicates a very narrow therapeutic window between activity and cellular toxicity. Cytotoxicity of ribavirin was evaluated in two ways: MTS assay and reduction in cellular GAPDH RNA as measured by real-time RT-PCR. Fifty percent cytotoxicity concentration, or CC$_{50}$, was $200 \mu M$ ribavirin as estimated by GAPDH reduction and $>640 \mu M$ by MTS assay (Fig. 1). This difference suggested that ribavirin is a cytostatic but not a cytotoxic agent. Replicon cells that have undergone high-dose ribavirin treatment ($100 \mu M$) are morphologically larger than untreated cells and grow more slowly (e.g., $200 \mu M$ ribavirin treatment for 2 weeks resulted in a five-fold increase in the cell doubling time from $21$ to $100$ hours, data not shown). These cells regained their normal growth rate on removal of ribavirin from the culture medium (data not shown).

Although a small therapeutic window appears to exist for ribavirin, based on replicon copy number in this system, it is important to note that replicon replication strongly correlates with host cell metabolic status (Pietschmann et al., 2001). Ribavirin’s cytostatic activity may therefore compromise reduction in replicon copy number as an in vitro measurement of drug efficacy due to the global inhibition of cellular metabolism. The observation that ribavirin has only weak anti-HCV replicon activity in vitro is consistent with clinical data from ribavirin monotherapy indicating no significant impact on HCV viral load in serum (Lauer and Walker, 2001).
Fig. 2. Ribavirin reduces the colony formation efficiency of the HCV replicon. Replicon cells were treated with 0, 10, 50, 100, or 300 μM ribavirin for 10 days in the absence of G418 selection. Replicon RNA was then extracted and used to transfect naïve Huh7 cells followed by G418 selection (see Materials and methods). The input replicon RNA copy number, presented in the first row of each panel, was independently determined by real-time RT-PCR using an HCV replicon RNA standard curve. Total number of colonies is presented in the second row of each panel. Colony formation efficiency (colony number normalized by HCV copy number) is given in the third row. R, ribavirin.

2-fold) (Fig. 2), consistent with the data from the 3-day dosing experiments (Fig. 1).

Exogenous guanosine suppresses ribavirin’s anti-replicon activity

Ribavirin and its monophosphate metabolite RMP have been shown to directly inhibit cellular IMPDH and decrease the intracellular pools of GTP (Wray et al., 1985). One explanation for the observed reduction in replicon CFE by ribavirin is that ribavirin, being a guanosine analog, may be misincorporated into the replicon genome; to do so, ribavirin must compete with cellular GTP. To test whether a decrease in GTP pool (induced by ribavirin) contributes to the replicon CFE reduction, exogenous guanosine, which would replenish GTP pools via the salvage pathway, was co-administered with ribavirin. As shown in Fig. 3, 10 μM exogenous guanosine (~30- or 100-fold excess of physiological GTP levels within HepG2 and MDCK cells, respectively (Z. Hong et al., unpublished data; Wray et al., 1985)) appeared to partially relieve the ribavirin-induced CFE reduction (from 200 to 440; no drug control, 730). Addition of 10 μM adenosine had no effect (Fig. 3). These data suggested that IMPDH inhibition and subsequent lowering of GTP pools may contribute to the observed reduction in CFE. The observation that addition of guanosine failed to completely reverse ribavirin’s adverse effect on CFE further suggested that the reduction of the GTP pool alone cannot account for all of the observed anti-replicon activity.

Neither MPA nor VX-497 alone has any effect on replicon RNA level and only a modest effect on CFE

To further address whether IMPDH inhibition (i.e., reduction of GTP pools alone) is sufficient to reduce replicon RNA copy number and CFE, two specific IMPDH inhibitors, mycophenolic acid (MPA) ($K_i \sim 20$ nM) (Carr et al., 1993), and VX-497 ($K_i \sim 10$ nM) (Markland et al., 2000), were evaluated for their anti-replicon effect. Replicon cells were treated with various concentrations of MPA (0–40 μM), but no significant effect on replicon quantity was observed (Fig. 4A), even though MPA began to show obvious cytotoxicity above 2 μM (data not shown). Similarly, VX-497, from 10 nM to 10 μM, did not inhibit replication (Fig. 4B). The EC$_{50}$ of VX-497 against a variety of RNA viruses including BVDV and EMCV ranges from 1 to 12 μM in tissue culture (Markland et al., 2000). VX-497 at concentrations above 10 μM showed significant cytotoxicity to replicon-bearing Huh-7 cells (data not shown). The effect of MPA and VX-497 on replicon CFE was examined by RNA retransfection assays. As shown in Fig. 5, 2 μM MPA ($\sim 100 \times K_i$, the highest tolerated dose for the 10-day assay) reduced replicon CFE only minimally (~2-fold), and as in the 3-day assay MPA did not reduce the total replicon copy number (Fig. 5, bottom, and Table 1). Similarly, VX-497 (100 nM, $\sim 10 \times K_i$) by itself, reduced the replicon CFE by only ~2-fold, without having any effect on the copy number (Fig. 6, middle). These data suggest that IMPDH inhibition and reduction of cellular GTP pools alone have only modest effects on CFE.

Table 1

Reduction in HCV subgenomic replicon CFE induced by different treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold reduction in CFE$^a$</th>
<th>N</th>
<th>$p^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>1</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>10 μM Rbv</td>
<td>1 ± 0.2</td>
<td>5</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>50 μM Rbv</td>
<td>2 ± 0.2</td>
<td>5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>100 μM Rbv</td>
<td>4 ± 0.6</td>
<td>5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>300 μM Rbv</td>
<td>20 ± 6</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 μM MPA</td>
<td>2 ± 0.3</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 μM MPA + 10 μM Rbv</td>
<td>4 ± 0.7</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 μM MPA + 100 μM Rbv</td>
<td>20 ± 4</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.1 μM VX-497</td>
<td>2 ± 0.1</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.1 μM VX-497 + 10 μM Rbv</td>
<td>5 ± 2</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.1 μM VX-497 + 100 μM Rbv</td>
<td>19 ± 8</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

$^a$ Values are means ± SD. CFE, colony-forming efficiency.

$^b$ Based on pairwise two-tailed t test, using average tabled t value for both.
Reduction of CFE by ribavirin is enhanced by IMPDH inhibitors

Reasoning that if ribavirin was incorporated at all into the replicon genome it must compete with intracellular GTP (Maag et al., 2001), IMPDH inhibitors were co-administered with ribavirin in an effort to facilitate its incorporation. Replicon cells were treated with a combination of ribavirin and either MPA (Fig. 5) or VX-497 (Fig. 6). MPA at 2 μM greatly potentiated the effect of ribavirin on replicon CFE: the reduction in CFE was promoted from 1- to 4-fold with 10 μM ribavirin, and from 4- to 20-fold with 100 μM ribavirin, compared with ribavirin monotherapy (P < 0.05) (Fig. 5, Table 1). The effects from combination treatments were also significantly higher than that from MPA treatment alone (P < 0.05) (Table 1).

This enhancement phenomenon was confirmed by replacing MPA with VX-497. The VX-497/ribavirin combination dramatically reduced the CFE of the replicon by up to ~20 fold, in contrast to ribavirin (4-fold, P < 0.05) or VX-497 (2-fold, P < 0.05) alone (Fig. 6, middle, and Table 1). The reduction in CFE with combination treatment was evident even with 10 μM ribavirin (4-fold reduction in CFE). Consistent with the MPA data, the replicon copy number from the VX-497/ribavirin 10-day treatment was reduced only 1- to 3-fold (Fig. 6, middle).

To further confirm the reduction of cellular GTP levels as the underlying mechanism for the observed enhancement, 10 μM guanosine was added to the ribavirin/VX-497 dosing regimen. As shown at the bottom of Fig. 6, exogenous guanosine restored the colony-forming ability to wild-type level. (Combining the outcome from the two independent studies yields an apparent CFE level 0.9 ± 0.3 that of the wild-type.) These data, as well as those from MPA/ribavirin treatment, suggest that the activity of ribavirin on replicon quality (CFE) could be enhanced by co-administration of IMPDH inhibitors.

Ribavirin/IMPDH inhibitor combination increases mutation frequency in replicon neo gene

To determine whether ribavirin, IMPDH inhibitors, or a combination, can increase the mutation frequency of the replicon genome, the sequences of the NS5A and neo genes, before and after drug treatment, were analyzed by extracting replicon RNA and PCR amplifying/cloning the target into the bacterial vector. Inserts from independent bacterial colonies were sequenced and analyzed for mutation frequency. The effects of ribavirin (100 μM), MPA (2 μM), and VX-497 (0.1 μM) on mutation of either the NS5A or neo gene were not statistically significant (Table 2 and Table 3);
Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NS5A mutation rate (×10⁻³)</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>6 ± 2</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td>100 µM Rbv</td>
<td>7 ± 2</td>
<td>15</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>300 µM Rbv</td>
<td>8 ± 2</td>
<td>9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>2 µM MPA</td>
<td>7 ± 2</td>
<td>13</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>2 µM MPA + 100 µM Rbv</td>
<td>9 ± 4</td>
<td>7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>0.1 µM VX-497</td>
<td>7 ± 2</td>
<td>5</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>0.1 µM VX-497 + 100 µM Rbv</td>
<td>7 ± 2</td>
<td>9</td>
<td>&gt;0.2</td>
</tr>
</tbody>
</table>

a Values are means ± SD.
b Based on pairwise two-tailed t test, using average tabled t value for both.

c Colony formation efficiency of the replicon RNA by transfection assays. Treatment with ribavirin at, or below, cytotoxic levels (50% IC₅₀) for up to 10 days had only minimal effects on intracellular replicon RNA level (Figs. 1, 2, 6), consistent with the clinical observation that ribavirin monotherapy is not effective in reducing HCV viremia (Lauer and Walker, 2001). Higher concentrations of ribavirin considerably lowered the HCV replicon RNA level; however, a clear conclusion is difficult to draw as this effect may largely result from the cytostatic effect of ribavirin. The effect of ribavirin on the CFE of the replicon RNA seemed to be more pronounced, with 50 µM ribavirin reproducibly decreasing the replicon CFE by about 2-fold, and higher doses decreasing it up to 10-fold (Fig. 2, Table 1). Moreover, by separating drug treatment from readout, the colony formation assay makes possible evaluation of high levels of drug without obfuscation by the cytotoxicity issue. Without a true HCV culture system this approach may be the best surrogate for assessing the impact of mutagens on viral “infectivity.”

Discussion

The development of the HCV subgenomic replicon (Lohmann et al., 1999) (Blight et al., 2000), in addition to the binary HCV full-length replication system (Chung et al., 2001), has allowed for the first time a detailed study of the mechanisms of action underlying ribavirin’s intracellular anti-HCV activity. The effect of ribavirin on the HCV subgenomic replicon has been examined in two ways: by measuring the HCV replicon copy number (quantity) using quantitative real-time RT-PCR; and by assessing the colony formation efficiency (quality) of the replicon RNA by transfection assays. Treatment with ribavirin at, or below, cytotoxic levels (50 µM) for up to 10 days had only minimal effects on intracellular replicon RNA level (Figs. 1, 2, 6), consistent with the clinical observation that ribavirin monotherapy is not effective in reducing HCV viremia (Lauer and Walker, 2001). Higher concentrations of ribavirin considerably lowered the HCV replicon RNA level; however, a clear conclusion is difficult to draw as this effect may largely result from the cytostatic effect of ribavirin. The effect of ribavirin on the CFE of the replicon RNA seemed to be more pronounced, with 50 µM ribavirin reproducibly decreasing the replicon CFE by about 2-fold, and higher doses decreasing it up to 10-fold (Fig. 2, Table 1). Moreover, by separating drug treatment from readout, the colony formation assay makes possible evaluation of high levels of drug without obfuscation by the cytotoxicity issue. Without a true HCV culture system this approach may be the best surrogate for assessing the impact of mutagens on viral “infectivity.”

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>neo mutation rate (×10⁻³)</th>
<th>N</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>4 ± 4</td>
<td>24</td>
<td>—</td>
</tr>
<tr>
<td>100 µM Rbv</td>
<td>7 ± 4</td>
<td>8</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>2 µM MPA</td>
<td>6 ± 3</td>
<td>10</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>2 µM MPA + 100 µM Rbv</td>
<td>8 ± 5</td>
<td>10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>0.1 µM VX-497</td>
<td>4 ± 4</td>
<td>13</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>0.1 µM VX-497 + 100 µM Rbv</td>
<td>9 ± 4</td>
<td>7</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

a Values are means ± SD.
b Based on pairwise two-tailed t test, using average tabled t value for both.
While confirming in principle the results of Chung et al. (i.e., that ribavirin leads to mutations in the HCV genome) (Contreras et al., 2002), these studies differ in several aspects and offer several additional insights into ribavirin’s mechanism of action. First, the subgenomic system used in this study may be more comparable to true HCV replication, as the replicon RNA comes solely from autonomous replication based on the RdRp; that is, the interpretation of the data is not complicated by the involvement of a second DNA-dependent RNA polymerase (i.e., the T7 RNA polymerase) as in the binary T7/HCV DNA viral replication system. Furthermore, the replicon-bearing cells can be cultured virtually indefinitely, rendering flexibility of drug treatment duration, whereas dosing and drug exposure in the binary HCV replication system are limited, as the host cells must be harvested 24 h after T7 polymerase delivery, due to the cytopathic effect of the vaccinia viral vector (Chung et al., 2001). Second, since the tolerability of the HCV genome to enhanced mutation rates (i.e., the “error threshold”) is unknown, the ribavirin mutagenesis data alone do not provide an answer to whether the increase in mutation rate would affect HCV RNA function. In contrast, the colony formation assay measures the ability of the HCV replicon RNA to de novo generate viable G418-resistant colonies in transfected Huh-7 cells, thereby linking ribavirin treatment to the functionality of the progeny HCV RNA. Third, in these experiments the effect of ribavirin on replicon CFE was dose-dependent while in the binary system there was no dose response of induced mutation frequencies (Contreras et al., 2002). Finally, while the studies with the binary HCV replication system focused on the mutagenic activity of ribavirin, the current studies underscore the importance of intracellular GTP pools in regulating the ribavirin anti-HCV replicon activity.

Recently Lanford et al., (2003) have described observations similar to ours in their hepatitis C subgenomic replicon system. The reductions in replicon quantity from ribavirin were comparable to the results reported above (e.g., 2- to 15-fold reduction using 100–160 μM in this study vs a 2- to 19-fold reduction using 100–200 μM). Lanford and colleagues also indicated complete abrogation of colony formation following 400 μM treatment for 72 h (while in this study no colonies were observed following 500 μM treatment after 10 days). One notable difference between the two studies was the apparent lack of cytotoxicity in the Lanford et al. report (i.e., no toxicity at 400 μM, 9 days whereas cellular toxicity was demonstrated in this study even at 100 μM, 10 days). The use of confluent replicon cultures by Lanford et al. to evaluate the cytotoxicity of ribavirin greatly reduces the apparent toxicity, something observed in the course of these studies as well (data not shown).

The recent demonstration that ribavirin induces “error catastrophe” of poliovirus by Crotty et al. (2001) has drawn extensive interest among investigators. In support of this theory, ribavirin has been shown by in vitro biochemical assays to be a pseudo-substrate of the HCV RdRp and to pair equally with either UMP or CMP (Maag et al., 2001). Subsequently, Contreras et al. reported that ribavirin increases the overall mutation rate of the HCV RNA in the binary HCV replication system (Contreras et al., 2002). These reports have led to a hypothesis that ribavirin may be incorporated into the HCV genome, mutagenize the progeny RNA, and induce “error catastrophe” in HCV. This report presents evidence for ribavirin-induced loss of colony-forming efficiency in HCV replicon. Does it provide direct support for the theory of “error catastrophe” in HCV ex vivo? Unfortunately, even though treatment with very high doses of ribavirin (≥ 500 μM) did result in complete loss of colony formation in one “infection”/transfection cycle (data not shown), it is not possible to determine whether this effect was replicon-based or due largely to cellular toxicity. The multiple rounds of “infection” needed to demonstrate “viral eradication” are not practical with replicon transfection, as it takes more than 1 month for each round of transfection/colony formation and expansion. Moreover, unlike the viral infection assay, the CFE experiment is limited by the relatively narrow linear range of input replicon RNA, making it difficult to use recovered RNA from high-dose ribavirin treatments in the assay, as the replicon RNA yield would be too low.

In these studies a 10-day treatment of replicon cells with 100 μM ribavirin reduced the intracellular viral transcript level only by ~40% (Fig. 2). Likewise ribavirin/MPA or ribavirin/VX-497 combination treatment reduced replicon copy number only 50 to 70% even though the replicon CFE was dropped 20- to 25-fold (Figs. 5, 6). In poliovirus studies, while the virus plaque-forming unit (PFU) (infectivity) was decreased 18- to 140-fold by ribavirin, the amount of viral RNA was reduced by only 6- to 16-fold (Crotty et al., 2001). The reason for the differential impact of ribavirin on the quantity and quality of the genetic material of poliovirus and HCV replicon is unclear. It would be of interest to determine whether true HCV infectivity following ribavirin treatment is changed in vivo, when a robust HCV culture system becomes available.

The nucleoside analog-induced loss of viral infectivity has been shown to parallel moderate increases in mutation frequency in several studies (Loeb et al., 1999; Sierra et al., 2000; Pariente et al., 2001; Crotty et al., 2001). The NS5A and neo genes of the replicon showed different susceptibility to the mutagenic effects of ribavirin, with and without IMPDH inhibitors. Although the NS5A gene mutation frequency seemed to increase slightly by these treatments, the differences were not statistically significant except for that from the 300 μM ribavirin treatment (Table 2). In contrast, the neo gene was mutated to a greater extent, with the combination treatments yielding statistically significantly increases in the error rate (~2-fold, P < 0.05) (Table 3). Since there was no G418 (neomycin) selection pressure during the treatment, the differential impact of the drugs on the mutation rates of the NS5A and neo genes may have been due to the total absence of selection pressure on the
antibiotic resistance gene; that is, replicon RNA bearing a defective NS5A gene may have been selected against during treatment whereas replicon RNA with a mutated neo fragment may have had no deleterious effect. Alternatively, as Grande-Perez et al. have recently reported in lymphocytic choriomeningitis virus (LCMV) infection system, the mutagenesis extinction by 5-fluorouracil does not correlate linearly with the increase in the viral genome mutation frequency; and the mutations were not evenly distributed throughout the virus genome (Grande-Perez et al., 2002). Therefore the detailed molecular mechanism for nucleoside-driven virus “error catastrophe” may be complex. Further sequencing analysis, targeting other replicon regions, is needed to determine whether the reduction in replicon CFE correlates more directly with the increase in the mutation rate of a particular region of the HCV replicon.

A second possible explanation for the lack of simple correlation between observed mutation frequency and drop in infectivity is that the replicon error rate is already so high that it may mask small increases induced by ribavirin (compare the mutation rate of the untreated replicon genomes maintained in the absence of G418 for 10 days (4–6 × 10⁻³) (Tables 2, 3) with that of the binary HCV system (9 × 10⁻³) (Contreras et al., 2002) or the VP1 gene of the poliovirus (1.5 × 10⁻⁴) (Crotty et al., 2001). The reason for this high apparent mutation frequency of the replicon genome is unclear. Comparable amplification/cloning/sequencing strategies employed by others (Contreras et al., 2002, i.e., amplification of 1–2 µg of cellular RNA, 35–40 cycles, followed by cloning and DNA template preparation for sequencing) have yielded significantly lower mutation frequencies (see above), suggesting that this high error rate is an authentic property of the replicon system. Another possibility is that the low level of ribavirin misincorporation, although not significantly increasing the overall mutation of the HCV genome, can dramatically impact the function of viral genomes by either changing certain RNA secondary and tertiary structures important for HCV function (such as stem-loops in the 3’ and 5’ UTRs and/or the IRES) and/or by hampering the HCV translational process by disrupting codon–anticodon recognition (Cameron and Castro, 2001). More studies are needed to fully address these possibilities.

The role of IMPDH inhibition in the ribavirin antiviral effect was also examined. The current studies suggest that exogenous guanosine suppressed the ribavirin effect on CFE and potent IMPDH inhibitors such as MPA and VX-497 enhanced this effect (Figs. 5, 6). The logical inference is that IMPDH inhibition contributes to ribavirin’s anti-HCV activity by reducing the competition with intracellular GTP for incorporation into nascent replicon genomes. The fact that MPA or VX–497 alone had only minimal effect on both the quantity and the quality of the HCV replicon suggests that IMPDH inhibition alone may not be the primary mechanism by which ribavirin inhibits HCV in vivo.

Whether the ability of ribavirin to reduce the replicon CFE plays a role in the clinical treatment of HCV is not clear. The peak concentration of ribavirin in patients’ serum is ~3 to 17 µM and higher dosing is usually limited by hemolysis and decreased red blood cell count (Glue, 1999; Preston et al., 1999), whereas in these studies, reduction in replicon CFE was not obvious below 50 µM. Ribavirin at clinically relevant doses (e.g., 10 µM) did not appear to affect either the copy number or the CFE of the replicon, agreeing with clinical data that ribavirin monotherapy is not effective in reducing HCV viral load in serum (Dusheiko et al., 1996). Although some accumulation of ribavirin may occur in the liver (Glue, 1999), it is unclear if the achievable intracellular drug concentration is sufficient to exert a major mutagenic effect on HCV in patients. As the ribavirin/IMPDH inhibitor combination did not incur additional cytotoxicity to Huh-7 cells (data not shown), these studies suggest that combination treatment may have potential clinical value, by eliciting enhanced anti-HCV effect at clinically tolerable ribavirin doses. Further clinical studies are needed to address the therapeutic relevance of this approach.

Materials and methods

Compounds

Ribavirin (1-ß-D-ribofuranosyl-1,2,4-triazole-3-carboximide) was obtained from Schering-Plough Research Institute (SPRI) and was stored frozen as a 20 mM stock solution in phosphate-buffered saline (PBS). Mycophenolic acid (MPA) and guanosine were both purchased from Sigma (Catalog Nos. M3536 and G6264, respectively) and were stored frozen in dimethyl sulfoxide (DMSO) as 20 mM stock solutions. VX-497 was synthesized at SPRI as described (Markland et al., 2000) and was stored frozen in DMSO as 20 mM stock solutions.

Cell cultures

Human hepatoma cell line Huh-7 cells were grown in Dulbecco’s modified minimal essential medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum, 4 mM l-glutamine, nonessential amino acids, 10 mM Heps, 0.075% sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM sodium pyruvate. HCV subgenomic replicon (containing NS5A S1179I adaptive mutation) was constructed at SPRI in a manner similar to that previously reported (Lohmann et al., 1999) and was identical in sequence to the “S1179I” replicon reported by Blight et al., (2000) The replicon-bearing Huh-7 cells were maintained in the Huh-7 medium (described above) supplemented with 1 mg/ml G418 (Geneticin, Gibco-BRL).
RNA quantification by real-time RT-PCR (“Taqman”) assays

Replicon cells were seeded at 3000 cells per well in 96-well Bio-coated plates (Becton Dickinson) and allowed to adhere overnight. The culture medium was replaced with 5% serum medium containing the experimental compound the next day. Seventy-two hours later the plates were harvested by aspirating medium, washing twice with PBS, and adding in 30 μl cell lysis buffer (Ambion, Catalog No. 8721). The plates were then heated at 75°C for 5 min followed by freezing at −70°C for 10 min. The cell lysate was assayed for HCV replicon copies by one-step real-time RT-PCR (“Taqman”) assays using the ABI 7700 instrument. More specifically, replicon RNA in the cell lysate was reverse-transcribed into cDNA, which then served as a template for the real-time PCRs. The amount of cDNA template (which correlates with the replicon copy number in the original cell lysate) was determined by the PCR cycle number (“Ct”) at which a threshold level of PCR product is generated. The primers used to monitor the HCV NS5B gene were (forward) ATGAGACGGCAGCCTGTA and (reverse) TTGATGGGCAGCTTGGTTTC. The double-fluorescence-labeled probe (CACGCCATGCGCTGCGG) was purchased from Applied Biosystems (Part No. P6448-6464). Cellular GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA from same cell lysate was used as an internal control for cell number and metabolic status (primers/probes purchased from Perkin–Elmer, Part No. 4310859). Every assay was carried out in triplicate and each study was independently performed at least two times. The results were graphed using mean values from a representative study.

Cytotoxicity determination

Cytotoxicity of a given drug or combination of drugs was determined by two methods: MTS assay using the compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (CellTiter 96 Aqueous One Solution cell proliferation assay, Promega, Part No. TB245) following manufacturer’s instructions and by quantification of cellular GAPDH RNA using quantitative real-time RT-PCR as described above.

Replicon RNA colony formation efficiency assay

Replicon cells were treated with compound(s) for 10 days in complete DMEM (without G418), with the medium/compound being changed every 2–3 days. Total cytoplasmic RNA was extracted using the RNA Easy Kit from Qiagen (Catalog No. 74104) and the RNA concentration determined by absorbance at 260 nm. HCV replicon copy number in each RNA sample was determined by real-time RT-PCR using a replicon RNA-standard curve. Eight microgram of total RNA suspended in 50 μl PBS was mixed with 50 μl cell suspension (2 × 10⁶ Huh-7 cells in PBS in a 0.1-cm electroporation cuvette; Bio-Rad, Catalog No. 165-2089). After two pulses at 960 μF and 350 V using the Gene Pulser System (Bio-Rad), cells were immediately transferred to 10 ml of complete DMEM medium (without G418) and seeded onto 10-cm culture dishes. After 24 h, medium was replaced by complete DMEM with 0.5 mg/ml G418. The G418-containing medium was changed every 2–3 days. Three weeks later, colonies were counted following staining with crystal violet (0.5 g/liter in 30% methanol, 3.2% formaldehyde). Representative results of multiple (≥2) independent transfections are shown.

Cloning and sequencing of replicon RNA

Total cytoplasmic RNA was isolated from compound-treated replicon cells and used as a template for amplification of NS5A region (positions 5077 to 6417 of the HCV 1b genome). Reverse transcription (RT) was carried out with 0.2–1 μg of RNA, 200 U superscript II reverse transcriptase (Invitrogen, Catalog No. 18064-014), and 100 ng random hexamer primers (Invitrogen, Catalog No. 48190-011) at 42°C for 50 min. cDNAs were then amplified with Pfu Turbo DNA polymerase (Stratagene, Catalog No. 600252) by 40 PCR cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 2 min. The primers for NS5A were (forward) ATGTCCGGCTCTGTTGCTAAAGAG and (reverse) GCAGCAGACGCAGTCCTCAC. The neo gene primers were (forward) TCAAGACGGACCTGTCCTG and (reverse) CTTTGACCTGGGCAACAGTTCCGC. PCR products were then cloned into the pCR-Blunt II-TOPO vector (Invitrogen, Catalog No. K2800-20) and plasmid DNA from independent colonies for each treatment (or no drug control) was sequenced in both directions. Sequencing data were analyzed with the Vector NTI program.

Acknowledgments

We are grateful to Dr. Michael Cable and Dr. Xiao Tong for helpful discussions. We also thank Paul Ingravallo, Sony Agrawal, Chuan-Kui Jiang, and Rong Kong for their technical assistance and Dr. Musaddeq Hussain and Linda Hamilton for quantification of the HCV subgenomic replicon RNA.

References


Holland, J.J., Domingo, E., de la Torre, J.C., Steinhauser, D.A., 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, 3960–3962.


