Extinction of Hepatitis C Virus by Ribavirin in Hepatoma Cells Involves Lethal Mutagenesis

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Abstract

Lethal mutagenesis, or virus extinction produced by enhanced mutation rates, is under investigation as an antiviral strategy that aims at counteracting the adaptive capacity of viral quasispecies, and avoiding selection of antiviral-escape mutants. To explore lethal mutagenesis of hepatitis C virus (HCV), it is important to establish whether ribavirin, the purine nucleoside analogue used in anti-HCV therapy, acts as a mutagenic agent during virus replication in cell culture. Here we report the effect of ribavirin during serial passages of HCV in human hepatoma HuH-7.5 cells, regarding viral progeny production and complexity of mutant spectra. Ribavirin produced an increase of mutant spectrum complexity and of the transition types associated with ribavirin mutagenesis, resulting in HCV extinction. Ribavirin-mediated depletion of intracellular GTP was not the major contributory factor to mutagenesis since mycophenolic acid evoked a similar decrease in GTP without an increase in mutant spectrum complexity. The intracellular concentration of the other nucleoside-triphosphates was elevated as a result of ribavirin treatment. Mycophenolic acid extinguished HCV without an intervening mutagenic activity. Ribavirin-mediated, but not mycophenolic acid-mediated, extinction of HCV occurred via a decrease of specific infectivity, a feature typical of lethal mutagenesis. We discuss some possibilities to explain disparate results on ribavirin mutagenesis of HCV.

Introduction

Hepatitis C virus (HCV) infections affect about 180 million people worldwide, and an estimated 75% of newly infected patients progress towards a chronic infection, which constitutes a risk for severe liver diseases such as cirrhosis and hepatocarcinoma [1–4]. HCV is an hepacivirus of the Flaviviridae family that displays the error-prone replication and quasispecies dynamics typical of RNA viruses [3,5–7]. No vaccine is available to prevent HCV infections, and the current standard of care (SOC) treatment consists of the combination of pegylated interferon-α (IFN-α) and the purine nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1-H-1,2,4-triazole-3-carboxamide) (Rib) (IFN-α+Rib). However, on average only about 60% of the chronically infected patients show a sustained virological response that results of ribavirin treatment. Mycophenolic acid extinguished HCV without an intervening mutagenic activity. Ribavirin-mediated, but not mycophenolic acid-mediated, extinction of HCV occurred via a decrease of specific infectivity, a feature typical of lethal mutagenesis. We discuss some possibilities to explain disparate results on ribavirin mutagenesis of HCV.
Currently used in the investigations on lethal mutagenesis because it has been licensed for human use for several decades, and proven to be mutagenic first for poliovirus (PV) [37,53,56], and then for several other RNA viruses [35,40,48,49,51,57–62], but not for others [63–65]. It is not clear whether Rib exerts its anti-HCV activity through mutagenesis or other mechanisms [58,66–77]. Alternative antiviral mechanisms of Rib are: (i) immunomodulation and enhancement of the Th1 antiviral immune response [78,79]; (ii) up-regulation of genes involved in IFN signaling [80,81]; (iii) inhibition of viral RNA-dependent RNA polymerases (RdRps) [82–87]; (iv) depletion of intracellular GTP levels [88]; (v) inhibition of mRNA cap formation [89].

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While some studies in cell culture have suggested a mutagenic activity of Rib on HCV [58,73,76,90–92], other investigations have failed to provide evidence of Rib mutagenesis [93,94]. Elucidation of the anti-HCV mechanism of Rib is highly relevant because it may influence the design of antiviral protocols. Recent model studies with different virus-host systems have indicated that when a mutagenic agent participates in therapy, a sequential administration of an inhibitor first, followed by a mutagenic agent may be more effective than the corresponding combination therapy to achieve virus extinction [49,95–97]. The advantage of a sequential administration depends on the concentrations of mutagen and inhibitor used, and it requires that one of the drugs used for therapy acts as a mutagenic agent [95–97]. Thus, with the advent of DAAs, it is important to clarify the mechanism of Rib activity against HCV to compare the effectiveness of alternative antiviral protocols first in cell culture and then in animal models and clinical trials.

The development of systems which allow infection of cultured cells with HCV particles containing the entire viral genome [98–100] permits addressing HCV evolution over extended periods of time (serial infections) in the presence of antiviral agents, without possible confounding effects derived from host cell evolution [12]. Here we study the effect of exposure of HCV in cell culture [12,101] to several drugs or drug combinations in a multiple passage design, as depicted in Fig. 1. The results indicate that Rib is mutagenic for HCVp0, and that Rib-mediated, but not mycophenolic acid-mediated, HCV extinction displays the attributes of lethal mutagenesis.

**Results**

**Cytotoxicity and inhibitory activity of ribavirin and mycophenolic acid**

To determine cytotoxicity of the drugs (concentration that reduces cell viability by 50%, CC50) used in the present study, Huh-7.5 reporter cells were incubated with increasing concentrations of Rib or mycophenolic acid and cell viability determined after 72 h of treatment. To quantify the inhibition of infectious HCV progeny production (drug concentration that produces a 50% decrease in progeny production, IC50), Huh-7.5 cells were infected with HCVp0 at a multiplicity of infection (MOI) of 0.2–0.5 TCID50/cell in the presence of increasing concentrations of the drugs and infectious progeny production measured. From the data, CC50 and IC50 values were calculated (Fig. 2), that yielded a therapeutic index (TI) (CC50/IC50) of 12.8, and >212.8 for Rib and mycophenolic acid, respectively. These values served as guide for the choice of drug concentrations in the experiments.

**Influence of infection conditions on the response of HCV to ribavirin treatment during replication in human hepatoma cells**

The parental HCVp0 virus [12,101] was prepared as described in Materials and Methods, and used to investigate the response of HCV to Rib. HCVp0 was subjected to 10 serial passages in Huh-7.5 cells using either high (1–2 TCID50/cell) or low (0.1–0.2 TCID50/cell) initial MOI, in the absence or presence of different Rib concentrations. The results show a consistent decay of progeny infectivity and of intracellular HCV RNA as a result of Rib treatment, while a sustained viral replication was observed in the absence of Rib (Fig. 3). An exception was the decreased but continued replication of HCV in the presence of 50 μM Rib in the infection at high MOI (Fig. 3a). In all other cases, infectivity and viral RNA decreased to levels below the corresponding limits of detection by passages 3 to 9 (Fig. 3a, b). Decay of infectivity in the cell culture supernatant always preceded decay of intracellular viral RNA, an observation previously made during mutagenesis-based extinction of lymphocytic choriomeningitis virus (LCMV) [42].

In previous studies on lethal mutagenesis of foot-and-mouth disease virus (FMDV) and LCMV we defined a double criterion to consider the virus extinct: (i) absence of infectivity following three
blind passages of the undiluted cell culture supernatant in the host cells in the absence of any drug, and (ii) absence of intracellular viral RNA using a highly sensitive RT-PCR amplification protocol [47,52,57]. When these criteria were applied to the Rib-treated HCV populations, no infectivity could be rescued when supernatants devoid of detectable infectivity were further passaged in the absence of Rib. However, some residual HCV RNA persisted as judged by an amplification band (above the background level of the negative control) even when the treatment was extended to 15 passages (Fig. 3b).

Mutant spectrum complexity in hepatitis C virus populations passaged in the absence or presence of ribavirin

To examine whether the decrease of infectivity upon HCV replication in the presence of Rib was accompanied with a mutagenic activity of Rib, the mutant spectrum complexity at the E2-, NS5A- and NS5B-coding regions (chosen because they encode structural and non-structural proteins displaying different degrees of conservation) of several HCV populations was evaluated (Table 1). NS5A and NS5B of populations passaged three times at low MOI in the presence of Rib underwent a significant increase of mutation frequency (1.4- to 3.0-fold with 50 μM Rib, and 1.6- to 4.8-fold with 100 μM Rib), relative to the population passaged in absence of Rib (p<0.05 to p<0.0005; χ² test). Statistically significant increases of nucleotide diversity were also observed for increasing Rib concentrations (NS5A p<0.004; NS5B p<0.04 and p<0.005; Permutation test) (Table 1). Ribavirin induces preferentially G→A and C→U transitions in HCV and other RNA viruses [33,56,57,77,102]. While the percentage of transitions relative to the total number of mutations did not vary as a result of Rib treatment (79.1%, 75.6% and 79.4% in the absence and presence of 50 and 100 μM Rib, respectively), the corresponding percentage of [(G→A)+(C→U)] transitions increased from 32.5% in the absence of Rib to 51.7% and 57.7% in the presence of 50 and 100 μM Rib, respectively, reflected also in an increase of the [(G→A)+(C→U)]/[(A→G)+(U→C)] ratio (Fig. 4a). The mutational bias reinforces the conclusion that Rib acts as a HCV mutagen. [The amino acid substitutions and their score according to the PAM 250 substitution matrix [103] – a measure of their probability of occurrence – are listed in Tables S1, S2 and S3.]

To further investigate the mutagenic activity of Rib, mutant spectrum complexity of the NS5A-coding region in the populations passaged at a MOI of 1 to 2 TCID₅₀/cell was investigated by molecular cloning and Sanger sequencing, and ultra-deep pyrosequencing. The analysis of molecular clones (Table 2) indicates a significant increase of mutation frequency (2.5-fold with 75 μM Rib, and 2.8-fold with 100 μM Rib), relative to the population passaged in the absence of Rib (p<0.05 and p<0.005, respectively; χ² test). A significant increase of nucleotide diversity was observed in the NS5A-coding region for populations treated with increasing concentrations of Rib (passage 4 populations, p<0.0017; Permutation test). Concerning the distribution of mutation types, again while the percentage of transitions relative to the total number of mutations did not vary as a result of Rib treatment (82.1%, 76.6% and 88.0% in the absence and presence of 75 and 100 μM Rib, respectively), the corresponding percentage of [(G→A)+(C→U)] transitions increased significantly [from 14.3% in the absence of Rib to 59.5% and 68.0% in the presence of 75 and 100 μM Rib, respectively (p<0.05 and p<0.01, respectively; χ² test).
The effect of ribavirin on infectious HCV progeny production and intracellular HCV RNA. (a) Huh-7.5 reporter cells were infected with HCVp0 at a MOI of 1–2 TCID50/cell (105 Huh-7.5 reporter cells infected with 1×105 to 2×105 TCID50 of HCVp0), in the absence or presence of Rib. Infections indicated in the upper box. Infections with HCV GNN were carried out in parallel (negative control). The progeny from each infection was used to infect fresh cells, as described in Materials and Methods. Virus infectivity was determined in the cell culture supernatant (left panel), and intracellular viral RNA measured by quantitative RT-PCR (right panel). The discontinuous line parallel to the abscissa indicates the limit of detection of infectivity and viral RNA. Below, RT-PCR amplification bands using a highly sensitive HCV-specific amplification protocol that yields a 539 bp fragment, using as template total intracellular RNA from the infection series and passage numbers indicated at the top of the corresponding lanes; +, +++, ++, +++, +++++ and +++++++ indicated in the absence or presence of amplification band; the position of DNA size markers is indicated on the left. (b) Same as in (a) but with infections carried out at an initial MOI of 0.1–0.2 TCID50/cell, using two different protocols: 4×105 Huh-7.5 reporter cells infected with 4×105 to 8×105 TCID50 of HCVp0 (duplicate assays, namely 1 and 2, left panels), and 4×105 Huh-7.5 reporter cells infected with 4×104 to 8×104 TCID50 of HCVp0 (duplicate assays, namely 1 and 2, right panels). Symbols and RT-PCR amplification bands shown at the bottom were obtained as is explained in part (a). Procedures are described in Materials and Methods. doi:10.1371/journal.pone.0071039.g003

The effect of guanosine on the inhibitory and mutagenic activity of ribavirin. Alteration of nucleotide pools

Ribavirin is metabolized by cellular enzymes to produce the mono- di- and tri-phosphate nucleotide derivatives (RMP, RDP, RTP, respectively) [88,104]. RMP is a competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), resulting in reduced intracellular GTP levels [88,105,106]. Guanosine can reverse the inhibitory effect of RMP on IMPDH [35,60,104,107]. Therefore, we examined the effect of guanosine alone and guanosine plus Rib on HCV multiplication. Replication of HCVp0 in the presence of 200 μM guanosine resulted in a 10- to 100-fold decrease in HCV infectious progeny production and intracellular viral RNA, and the decrease diminished with passage number (Fig. 5a). Guanosine partially compensated the inhibition exerted by 50 μM Rib, but only minimally the inhibition by 100 μM Rib. In all cases the presence of guanosine delayed the Rib-mediated extinction of HCV. Only a minor reduction of mutant spectrum complexity (that did not reach statistical significance) was observed in the presence of guanosine at the two Rib concentrations tested (Table 4). Likewise, the Rib-mediated increase of nucleotide diversity and [(G→A)+(C→U)]/[(A→G)+(U→C)] ratio diminished only modestly in the presence of guanosine (Table 4 and Fig. 4d; the amino acid substitutions are listed in Table S6). To determine the effect of Rib treatment on the GTP level, the intracellular amount of GTP and RTP was measured in Huh-7.5 reporter cells at 8 h and 72 h after treatment with 50 μM and 100 μM Rib. The results (Fig. 5b) show a 3.0- to 8.5-fold reduction in the intracellular concentration of GTP. RTP reached maximum levels of 0.07 fmol/cell at 72 h of treatment with 100 μM Rib. In contrast to GTP, all other nucleotides increased their concentration as a result of Rib treatment, with maximum increases for the pyrimidine nucleotides (4.2-fold for UTP and 3.4-fold for CTP); the increase was partially reversed by the presence of guanosine (Fig. 5c). Thus, addition of guanosine to Huh-7.5 reporter cells did not restore the GTP depletion resulting from Rib addition. In view of these results, it was interesting to examine the effect of mycophenolic acid on HCV progeny production and mutagenesis.

The effect of mycophenolic acid on hepatitis C virus progeny production and nucleotide pools

Mycophenolic acid is an inhibitor of IMPDH, which contrary to RTP or GTP cannot be incorporated into RNA [105,108–110]. To explore whether depletion of GTP by itself (in the absence of an added mutagenic agent) could jeopardize infectious HCV progeny production, serial passages were carried out in the presence of mycophenolic acid in the absence or presence of guanosine. Mycophenolic acid produced a dramatic inhibition of HCV production that resulted in HCV extinction (Fig. 6a). Significantly, in the transition towards extinction, loss of infectivity preceded loss of intracellular viral RNA. The inhibitory effects of mycophenolic acid was almost fully reversed by guanosine, a reversal that was not observed previously with other hepatoma cells or HCV replicons [93,111]. Depletion of GTP by mycophenolic acid was comparable to that produced by Rib, and also not reversed by guanosine addition, whereas the increase in the concentration of the other standard nucleotides was 1.5- to 4-fold lower (compare Figs. 5c and 6b). Passages in the presence of mycophenolic acid did not result in any significant increase of mutant spectrum complexity or mutational bias (Fig. 4d and Tables 5 and S7). Mycophenolic acid in the 2.5–5 μM range drove viral infectivity and RNA below the limit of detection. Furthermore, no HCV RNA could be amplified from the cell culture supernatant, using the sensitive RT-PCR protocol to diagnose extinction (Fig. 6a). The results suggest that depletion of GTP contributes to inhibition of HCV progeny production, without involving a mutagenic activity [see Discussion]. The inhibitory effect of mycophenolic acid can drive HCV to extinction.

Probing features of lethal mutagenesis in the extinction of hepatitis C virus by ribavirin and mycophenolic acid

Mutagenesis-driven extinction of FMDV and LCMV occurred with 105- to 107-fold decreases of specific infectivity (the ratio between viral infectivity and the amount of genomic viral RNA) and without any detectable variation in the consensus genomic nucleotide sequence [42,112,113]. These features are a hallmark of virus extinction by lethal mutagenesis [reviewed in [114,115]]. Extinction by Rib occurred with a 5-fold to 23-fold decrease of specific infectivity, as quantified by infectivity and viral RNA in samples of the cell culture supernatants (Fig. 7a). In contrast, no variation in specific infectivity was observed during mycophenolic acid-mediated extinction (Fig. 7b). In both cases the consensus sequence remained invariant. Thus, Rib-mediated, but not
### Lethal Mutagenesis of Hepatitis C Virus

| Drug | No drug | Rib 50 μM | Rib 100 μM |
|------|---------|-----------|------------|
|      |         |           |            |
| ![Table Cells](image1.png) | ![Table Cells](image2.png) | ![Table Cells](image3.png) | ![Table Cells](image4.png) |

**Panel (a):**
- No drug
- Rib 50 μM
- Rib 100 μM

**Panel (b):**
- No drug
- Rib 75 μM
- Rib 100 μM

**Panel (c):**
- No drug
- Rib 75 μM

**Panel (d):**
- No drug
- Rib 50 μM
- Rib 100 μM
- MPA 5 μM

**Gua 200 μM**
- Rib 50 μM + Gua 200 μM
- Rib 100 μM + Gua 200 μM
- MPA 5 μM + Gua 200 μM

**Note:** The values in the table represent the ratio of specific nucleotide changes ([G-A](C-U))/([A-G](U-C)).
mycophenolic acid-mediated, HCV extinction displayed the hallmarks of lethal mutagenesis. In conclusion, we have provided evidence that Rib alters the mutant spectrum composition of HCV quasispecies replicating in hepatoma cells in a manner compatible with a mutagenic activity of Rib.

Discussion

Lethal mutagenesis is gradually finding its way as a potential antiviral therapy with reduced probability of selection of escape mutants [54,116,117], and with prospects of applying the same principles to anti-cancer therapy [118–120]. The mechanism by which Rib exerts its antiviral activity is still a debated issue since multiple, unrelated modes of action have been observed with different viruses [59,106,121], and more than one mechanism may be operating on a given virus. Recent studies of antiviral designs based on lethal mutagenesis render important a clarification of whether mutagenesis of the viral genome occurs in the course of an antiviral treatment. Indeed, a potential advantage of a sequential inhibitor-mutagen administration over the corresponding combination antiviral treatment. Indeed, a potential advantage of a sequential inhibitor-mutagen administration over the corresponding combination antiviral treatment would lead to a mutational bias in favor of G→A and C→U transitions. In the NS5A-coding region, UDPS identified a total of 38 and 127 different mutations in the populations passaged in the absence and presence of Rib, respectively. The corresponding numbers identified by molecular cloning and Sanger sequencing were 28 and 46, respectively. Of the total number of different mutations scored, only 5 and 12, in the absence and presence of Rib, respectively, were common to Sanger sequencing and UDPS (Tables S4 and S5). Thus, despite sampling different sequences, both procedures provide evidence of a mutagenic activity of Rib on HCV. Other activities of Rib in Huh-7.5 cells may contribute to the anti-HCV action of Rib. It must be indicated, however, that solely a change in mutant spectrum composition does not provide evidence of a mutagenic activity, since a drug may alter the relative fitness of viral subpopulations. This might cause a variation in the broadness of the mutant spectrum. In our case, however, it seems unlikely that such mutant spectrum reorganization would lead to a mutational bias in favor of G→A and C→U.

Table 1. Quasispecies analysis of HCVp0 populations passaged in the absence or presence of ribavirina.

| Genomic region | Ribavirin conc. (μM)a | Number of nt analyzed (clones/haplotypes)c | Mutation frequencyd | Nucleotide diversity * \( \times 10^3 \) (95% CI) |
|---------------|-----------------------|------------------------------------------|---------------------|---------------------------------------------|
| E2 (nt 1490–2590) | 0                     | 15,122 (15/9)                           | 9.3 \times 10^{-4}  | 1.11 (0.73–1.58)                          |
|               | 50                    | 18,860 (17/14)                          | 1.3 \times 10^{-3}  | 1.49 (1.15–2.01)                          |
|               | 100                   | 20,657 (21/16)                          | 1.5 \times 10^{-3}  | 1.74 (1.33–2.15)                          |
| N5SA (nt 6269–7666) | 0                     | 29,704 (22/18)                          | 7.7 \times 10^{-4}  | 1.16 (0.93–1.51)                          |
|               | 50                    | 23,766 (17/17)                          | 1.4 \times 10^{-3}  | 1.54 (1.16–2.11)                          |
|               | 100                   | 21,396 (20/20)                          | 2.1 \times 10^{-3}  | 2.43 (1.99–3.44)                          |
|               | 0                     | 22,691 (18, 10/5, 3)                    | 2.5 \times 10^{-4}  | 0.74 (0.53–1.03)                          |
| N5SB (nt 7667–8442/8443–9442) | 50         | 37,862 (24, 22/17, 9)                  | 7.6 \times 10^{-4}  | 1.60 (1.22–2.20)                          |
|               | 100                   | 16,676 (12, 11/7, 8)                    | 1.2 \times 10^{-3}  | 1.25 (0.79–1.79)                          |

The population analyzed correspond to passage 3 of the infections at an initial MOI of 0.1 to 0.2 TCID50/cell described in Fig. 3b.

aThe HCV genome residue numbering corresponds to the JFH-1 genome (accession number #AB047639). The N5SB-coding region was covered by two overlapping amplifications.

bThe parenthesis indicates the number of clones analyzed, followed by the number of haplotypes (number of different RNA sequences); some clones did not contain the full length sequence; when the alignment of the sequenced region was correct such clones were entered in the calculation.

cAverage number of mutations per nucleotide relative to the corresponding consensus sequence. Mutation types are summarized in Fig. 4a and their position in the HCV genome and deduced amino acid substitutions are given in Tables S1, S2 and S3.

dThe calculation of nucleotide diversity (\( \pi \)) and 95% confidence interval (CI) is explained in Materials and Methods.

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transitions in the absence of a mutagenic activity associated with Rib treatment.

Depletion of intracellular GTP levels does not account for the majority of Rib-mediated mutagenesis, since no mutagenesis was observed under the same depletion as a result of treatment with mycophenolic acid. This immunosuppressant and some of its prodrugs have proven effective as inhibitors of HCV multiplication in cell culture and in vivo [93,111,122,123]. Significantly, extinction by the non-mutagenic mycophenolic acid did not alter the specific infectivity of HCV and, with our quantification procedures infectivity was still detectable when extracellular HCV RNA was not. This is in contrast to the decrease in specific infectivity associated with Rib-mediated extinction, a hallmark of lethal mutagenesis [35,42,57,62,97,113] (Fig. 7).

It is not clear whether the inhibition of HCV progeny production by Rib is directly linked to its mutagenic activity or to some other effect on the virus or the host cell, independent of its mutagenic activity. The difference in compensatory activity of guanosine on the inhibition produced by 50 μM and 100 μM Rib does not appear to be related to variations in intracellular GTP. Rather, Rib produced a considerable increase in intracellular ATP, CTP and UTP that was only partially compensated by guanosine addition. Concentrations of mycophenolic acid that depleted GTP to a similar extent than Rib led to a less accentuated increase of ATP, CTP and UTP. Thus, imbalances in the levels of the standard nucleotides might contribute to the inhibition produced by Rib. Modifications of hepatocyte gene transcription may affect completion of the virus life cycle [124]. ATP – which underwent a 2-fold higher increase upon treatment with Rib than with mycophenolic acid (Figs. 5 and 6) – is recruited in HCV-infected hepatocytes where it co-localizes with non-structural HCV proteins in cytoplasmic structures; the ATP concentration is about 5-fold higher at these sites than elsewhere in the cell [125]. ATP levels may modify the activity of the ATP-dependent

### Table 2. Quasispecies analysis of NSSA genomic residues of HCVp0 populations passaged in the absence or presence of ribavirin.

| Ribavirin conc. (μM)a | Passage number | Number of nt analyzed (clones/haplotypes)b | Mutation frequencyc | Nucleotide diversityd | × 10^(-5) (95% CI) |
|-----------------------|----------------|-------------------------------------------|---------------------|----------------------|-------------------|
| 0                     | 4              | 23,766 (17/13)                            | 6.7 × 10^(-4)       | 0.89 (0.72–1.19)     |                   |
| 0                     | 5              | 19,572 (14/8)                             | 6.1 × 10^(-4)       | 0.69 (0.42–1.12)     |                   |
| 75                    | 4              | 16,766 (12/12)                            | 1.4 × 10^(-3)       | 1.37 (0.96–2.05)     |                   |
| 75                    | 5              | 11,899 (9/8)                              | 1.9 × 10^(-3)       | 2.34 (1.76–3.06)     |                   |
| 100                   | 4              | 13,980 (10/10)                            | 1.8 × 10^(-3)       | 2.07 (1.75–2.44)     |                   |

aThe populations analyzed correspond to the infections at an initial MOI of 1 to 2 TCID50/cell described in Fig. 3a. Passage 5 in the infections with 100 μM Rib did not yield sufficient HCV RNA for analysis. The genomic residues analyzed are 6269 to 7666 of the NS5A-coding region. The HCV genome residue numbering corresponds to the JFH-1 genome (accession number AB047639).
bThe parenthesis indicates the number of clones analyzed, followed by the number of haplotypes (number of different RNA sequences); some clones did not contain the full length sequence; when the alignment of the sequenced region was correct such clones were entered in the calculation.
cMutation frequency and nucleotide diversity are defined in Table 1 legend and Materials and Methods. Mutation types are summarized in Fig. 4b and their position in the HCV genome and deduced amino acid substitutions are given in Table S4.
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### Table 3. Ultra-deep pyrosequencing analysis of hepatitis C virus populations passaged in the absence or presence of ribavirina.

| Parameter | Ribavirin conc. (μM)a | NSSA ampliconb |
|-----------|-----------------------|----------------|
|           | A1 (6152–6454)        | A2 (6446–6767) |
|           | A3 (6737–6954)        | A4 (6910–7252) |
|           | A5 (7224–7550)        | A6 (7432–7725) |
| Number of different mutations | 0 | 3 | 7 | 5 | 15 | 4 | 7 |
| Number of polymorphic sites | 75 | 18 | 25 | 15 | 35 | 22 | 24 |
| Number of haplotypesc | 0 | 4 (3/0) | 8 (7/0) | 6 (5/0) | 16 (12/3) | 4 (2/1) | 8 (6/1) |
| [(G→A)4(U→C)]d | 0 | 2 (2/1) | 0 (0/6) | 0.25 (1/4) | 0.50 (4/8) | 1 (1/1) | 0.33 (1/3) |
| [(A→G)4(U→C)]  | 75 | 3.5 (28/6) | 2.83 (17/6) | 6.5 (13/2) | 1.45 (16/11) | 13 (13/1) | 3.75 (15/4) |

aThe populations analyzed correspond to the infections at an initial MOI of 1 to 2 TCID50/cell described in Fig. 3a.
bThe HCV genome residue numbering corresponds to the JFH-1 genome (accession number AB047639). Amplicon (A) length ranged between 216 and 339 nucleotides. The number of nucleotides sequenced was 2.1 × 10^5 to 3.3 × 10^6, and the number of reads on which the parameters were calculated was 10,000 for each amplicon. Procedures are described in Materials and Methods.
cIn parenthesis the number of haplotypes with one and two mutations is given.
dIn parenthesis the two terms of the ratio are given. Mutation types are summarized in Fig. 4c and their position in the HCV genome and deduced amino acid substitutions are given in Table S5.
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Figure 5. The effect of ribavirin (Rib) and guanosine (Gua) on HCV infectious progeny production and intracellular viral RNA and nucleotide pools. (a) Huh-7.5 reporter cells were infected with HCVp0 at a MOI of 0.1–0.2 TCID₅₀/cell, in the absence or presence of the Rib and Gua concentrations indicated in the upper box. Infections with HCV GNN were carried out in parallel (negative control). The progeny form each infection was used to infect fresh cells, as described in Materials and Methods. Viral infectivity was determined in the cell culture supernatant (upper panels), and viral RNA quantitative RT-PCR in an extract of infected cells (lower panels). The discontinuous lines parallel to the abscissa indicates the maximum decrease possible is 100%. Procedures are described in Materials and Methods.

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Table 4. Quasispecies analysis of HCVp0 populations passaged in the absence or presence of ribavirin and guanosine.

| Ribavirin conc. (µM) | Guanosine conc. (µM) | Number of nt analyzed (clones/haplotypes) | Mutation frequency | Nucleotide diversity π, 10⁻⁶ (95% CI) |
|----------------------|----------------------|------------------------------------------|-------------------|----------------------------------|
| 0                    | 0                    | 29,704 (22/18)                           | 7.7 × 10⁻⁴         | 1.16 (0.93–1.51)                 |
| 0                    | 200                  | 30,493 (24/17)                           | 8.5 × 10⁻⁴         | 1.36 (1.09–1.68)                 |
| 50                   | 0                    | 23,766 (17/17)                           | 1.4 × 10⁻³         | 1.54 (1.16–2.11)                 |
| 50                   | 200                  | 35,979 (27/17)                           | 9.2 × 10⁻⁴         | 1.39 (1.17–1.72)                 |
| 100                  | 0                    | 21,396 (20/20)                           | 2.0 × 10⁻³         | 2.43 (1.99–3.44)                 |
| 100                  | 200                  | 38,125 (28/26)                           | 1.7 × 10⁻³         | 2.36 (2.04–2.79)                 |

*The populations analyzed correspond to the infections at an initial MOI of 0.1 to 0.2 TCID₅₀/cell described in Fig. 5a. About 25% to 50% of clones analyzed did not contain the full length sequence expected from the primers used; when the alignment of the sequenced region was correct such clones were entered in the calculation. The HCV genome residue numbering corresponds to the JFH-1 genome (accession number AB047639). The NSSA-coding region (nucleotides 6269 to 7666) was analyzed.

[a] The parenthesis indicates the number of nt analyzed, followed by the number of haplotypes/number of different RNA sequences; some clones did not contain the full length sequence; when the alignment of the sequenced region was correct such clones were entered in the calculation.

[b] Mutation frequency and nucleotide diversity are defined in Table 1 legend and Materials and Methods. Mutation types are summarized in Fig. 4d and their position in the HCV genome and deduced amino acid substitutions are given in Table S6.

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sample. Both, molecular cloning and sequencing and deep-seqencing methods are only sampling methods to approach the complex reality of a viral mutant spectrum. Low viral loads such as those produced by Rib will tend to underestimate the mutant spectrum complexity unless the amounts of template are normalized prior to RT-PCR amplification. (iv) In combination therapies, the interplay between an inhibitor and a mutagenic agent may affect the timing in which an increase in mutant spectrum complexity can be observed [95,96]. It may be significant that evidence of a mutagenic activity in vivo has been obtained during Rib monotherapy [77]. These possible sources of bias apply to determinations of mutant spectrum complexity both in cell culture and in vivo, and should be evaluated in comparing results from different studies.

An analysis of the molecular events that underlie the transition towards HCV extinction has indicated an apparently stochastic persistence of HCV RNA despite loss of HCV infectivity. There have been clinical descriptions of presence of HCV RNA in patients displaying a sustained response to treatment [139–141]. The nature of this persistent HCV RNA in hepatocytes has not been studied, but its presence cannot be dismissed because it might correspond to defective HCV RNAs. A class of defective viral genomes termed defectors participate in lethal mutagenesis and result in loss of infectivity preceding loss of viral RNA [42,114]. The possible involvement of lethal defectors in HCV extinction and the biological significance of HCV RNA remnants following an effective treatment deserve further investigation.

Materials and Methods

Cells and viruses

The origin of Huh 7.5, Huh-7-Lanet, Huh-7.5 reporter cell lines and procedures for cell growth in Dulbecco’s modification of Eagle’s medium (DMEM), have been previously described [142,143]. Infected and uninfected cells were cultured at 37°C and 5% CO2. The viruses used in the experiments are HCVcc [Jc1FLAG2p7-nsGluc2A] (a chimera of J6 and JFH-1 from genotype 2a) and GNN [GNNFLAG2p7-nsGluc2A] (carrying a mutation in the NS5B RNA-dependent RNA polymerase rendering it replication-defective) [144]. To control for the absence of contamination the supernatants of mock-infected cells maintained in parallel with the infected cultures were titrated; no infectivity in the mock-infected cultures was detected in any of the experiments.

Production of viral progeny and titration of infectivity

The procedures used to prepare the initial virus stock HCVp0 and for serial infections of the hepatoma Huh-7.5 cells have been previously described [12]. Briefly, Huh-7-Lanet cells were electroporated with 10 μg of the infectious transcript of HCVcc (Jc1 or the negative control GNN) (260 volts, 950 μF). Electroporated cells were then passaged every 3–4 days without allowing the cells to become confluent, passages were continued until 30 days post-electroporation, and the cell culture supernatants were pooled. The virus was then concentrated 20 times using 10,000 MWCO spin columns (Millipore) as instructed by the manufacturer, and stored in aliquots (at −70°C). To increase virus infectivity, Huh-7.5 reporter cells were infected with concentrated virus stocks at a MOI of 0.5 TCID50/cell, and the cells were passaged to obtain the working viral stock HCVp0 [12]. For titration of HCV infectivity, serially diluted cell culture supernatants were applied to Huh-7.5 cells and 3 days post-infection the cells were washed with PBS, fixed with ice-cold methanol, and stained using anti-NS5A monoclonal antibody 9E10, as previously described [12,145].

| Mycophenolic acid conc. (μM) | Guanosine conc. (μM) | Number of nt analyzed (clones/haplotypes) | Mutation frequency | Nucleotide diversity
|-------------------------------|-------------------|---------------------------------|-------------------|-------------------|
| 0 | 0 | 29,704 (22/18) | 7.7 × 10^{-4} | 1.16 (0.93–1.51) |
| 0 | 200 | 30,493 (24/17) | 8.5 × 10^{-4} | 1.36 (1.09–1.68) |
| 5 | 0 | 27,214 (23/15) | 9.6 × 10^{-4} | 1.11 (0.81–1.67) |
| 5 | 200 | 23,766 (17/12) | 1.1 × 10^{-3} | 1.57 (0.90–2.45) |

*The populations analyzed correspond to passage 3 (except MPA for 5 μM which is passage 2 because at passage 3 the amount of HCV RNA template was insufficient for the analysis) of infections at an initial MOI of 0.1–0.2 TCID50/cell described in Fig. 6a. The analyses for the control passages in the absence of mycophenolic acid are the same reported in Table 4. They are included here to facilitate comparisons. The HCV genome residue numbering corresponds to the JFH-1 genome (accession number #AB047639). The NS5A-coding region (nucleotides 6269 to 7066) was analyzed.

**The parenthesis indicates the number of clones analyzed, followed by the number of haplotypes (number of different RNA sequences); some clones did not contain the full length sequence; when the alignment of the sequenced region was correct such clones were entered in the calculation.

| Mutation frequency | Nucleotide diversity
|-------------------|-------------------|
| p. 10^{-6} (95% CI) | p. 10^{-5} (95% CI) |
| 1.16 (0.93–1.51) | 1.36 (1.09–1.68) |
| 1.11 (0.81–1.67) | 1.57 (0.90–2.45) |

*Mutation frequency and nucleotide diversity are defined in Table 1 legend and Materials and Methods. Mutation types are summarized in Fig. 4d and their position in the HCV genome and deduced amino acid substitutions are given in Table 57.

Table 5. Quasispecies analysis of HCVp0 populations passaged in the absence or presence of mycophenolic acid and guanosine.

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Treatment with ribavirin, guanosine and mycophenolic acid

Solutions of Rib (Sigma), guanosine (Sigma) and mycophenolic acid (Sigma) were prepared at concentrations of 100 mM, 200 mM and 50 mg/ml in PBS, DMEM and methanol, respectively. They were sterilized by filtration, and stored at –70°C. Prior to use, the stock solutions were diluted in DMEM to reach the desired concentration. Huh-7.5 reporter cells were pretreated with the appropriate drug concentrations during 16 h prior to infection. Then, 4x10⁶ cells were infected with 8x10⁵ TCID₅₀ of HCVp0; the adsorption time was 5 h, and the infection continued for 72 to 96 h in the presence of Rib. For successive viral passages, 4x10⁶ Huh-7.5 reporter cells were infected with 2.5 ml of the supernatant from the previous infection; the MOI ranged between 0.1 and 10⁻⁴ TCID₅₀/cell; each MOI can be calculated from the infectivity values given for each experiment. In some cases the initial MOI was 1 to 2 TCID₅₀/cell, as detailed in the corresponding experiments.

Toxicity test

The CC₅₀ of Rib, and mycophenolic acid was measured by seeding 96-well plates with Huh-7.5 cells to 70% confluence and exposing the cells to up to 250 μM Rib and 100 μM mycophenolic acid for 72 hours. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-}

Figure 7. Effect of ribavirin (Rib) and mycophenolic acid (MPA) on the specific infectivity of HCV. (a) Huh-7.5 reporter cells were infected with HCVp0 at an initial MOI of 0.1–0.2 TCID₅₀/cell, in the absence or presence of the Rib concentrations indicated in the upper box; infection with GNN was used as negative control. The experiment is the one shown in Fig. 3(b). At different passages, extracellular viral RNA was measured by quantitative RT-PCR (left panel). Specific infectivities (right panel) were calculated with the infectivities given in Fig. 3b and the RNA concentrations indicated in the left panel. The discontinuous horizontal line indicates the limit of detection. Statistically significant differences are indicated by one (p<0.001) or two (p<0.0001) asterisks (one way analysis of variance as detailed in Materials Methods). (b) Infections as in (a), in the absence or presence of MPA concentrations indicated in the boxes above the panels. The experiment is the one shown in Fig. 5(a). At different passages, extracellular viral RNA was measured by quantitative RT-PCR (left panel). Specific infectivities (right panel) were calculated with the infectivities given in Fig. 5(a) and the RNA concentrations indicated in the left panel. The discontinuous horizontal line indicates the limit of detection. Differences were not statistically significant. Procedures are described in Materials and Methods.

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diphenyltetrazolium bromide] was added to each well at a final concentration of 500 µg/ml; 4 h later crystals were dissolved in 100 µl of DMSO and the O.D. measured at 550 nm; 50% cytotoxicity was calculated from four different determinations as previously described [144].

Inhibitory concentration
The IC50 of Rib, and mycophenolic acid was calculated relative to the untreated controls (defined as 100% infectivity), as described previously [57]; determinations were carried out in triplicate.

Nucleotide pool analysis
The procedure used has been previously described [146,147]. Huh-7.5 cells (2 x 10⁶ cells) either untreated or treated with the drugs indicated for each experiment were washed with PBS and incubated on ice for 10 min with 900 µl of 0.6 M trichloroacetic acid. A precooled mixture of 720 µl of Uvasol® (1,1,2-trichlorotrifluoroethane, Sigma) and 180 µl of Tri-n-octylamine (Sigma) was added to 900 µl of the supernatant, vortexed for 10 s and centrifuged 30 s at 12,000 g at 4°C and stored at −80°C until further analysis. Nucleotides were separated in a Partisol 10 SAX analytical column (4.6 mm x 50 mm) (Whatman) with a Partisol 10 SAX guard cartridge column (4.6 x 30 mm) (Capital HPLC). Each sample (100 µl) was injected into an Alliance 2695 HPLC system connected to a 2996 photodiode array detector (Waters); the eluent flow rate was 0.8 ml/min, and the nucleotides were detected at a wavelength of 254 nm, except Rib that was detected at 220 nm. Prior to injections, the column was equilibrated with 60 ml of 7 mM NH₄H₂PO₄, pH 3.8 (buffer A). The separation program started with 22.5 min of an isocratic period with buffer A followed by a linear gradient of 112.5 min to the high concentration buffer 250 mM NH₄H₂PO₄, 500 mM KCl, pH 4.5 (buffer B) and a final isocratic period of 37.5 min with buffer B. Prior to sample analysis, 50 µl of 20 pmol/µl UTP, CTP, ATP and GTP, and 80 pmol/µl Ribavirin (Jena Bioscience), were separated to create a processing method using the Waters Empower™ Chromatography Data Software. Determinations were carried out with two independent biological samples, each one in triplicate. The amount of each nucleotide in cell extracts was normalised relative to the number of cells.

RNA extraction, cDNA synthesis, PCR amplification and nucleotide sequencing
Intracellular viral RNA was extracted from infected cells using the Qiagen RNeasy kit according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA). RT-PCR amplification was carried out using AccuScript (Agilent), as specified by the manufacturers. Several genomic regions were amplified using specific oligonucleotides as primers (Table S8). Nucleotide sequences of genomic HCV RNA were determined using the 23 ABI 3730XLs sequencer. To evaluate the complexity of mutant spectra, HCV RNA was extracted as described above and subjected to RT-PCR to amplify the E2-, NS3A- and NS5B-coding regions as previously described [12]. Amplification products were analyzed by agarose gel electrophoresis using HindIII-digested Φ-29 DNA as molar mass standards. Negative controls (amplifications in the absence of RNA) were included in parallel to ascertain absence of contamination with undesired templates. RT-PCR amplifications were performed in triplicate and mixed equimolarly prior to the analysis. Then, PCR products were purified (Ampure Beads), quantified (Pico Green Assay), and analyzed for quality (Bioanalyzer) prior to the UDPS procedure. Negative controls (without template RNA) were run in parallel to ascertain absence of contamination with undesired templates. Data analysis was based on the fasta files obtained from the 454 GS system software which includes quality controls to guarantee the integrity of the amplicons. Data processing was done on the open source R environment [149], using Bioconductor [150] and the Biostrings library [151] for pattern matching, sequence alignment and functions developed for this purpose. Two levels of errors were considered: a background level of noise that affected both strands, and at a higher level which was neighborhood and structure-dependent, and therefore strand-specific. For reads data processing a demultiplexing step where the reads were assigned to samples and amplicons was followed by a quality filter and repair step, and by the intersection of haplotypes in the forward and reverse strands, and an abundance filter for these consensus haplotypes above 0.25% [152]. Accuracy validations were performed using published procedures [150,153–155]; the same conclusions on Rib mutagenesis were obtained with a cut-off value of 1.0%.

Population complexity
Population complexity was measured by determining mutation frequency, number of polymorphic sites and haplotypes as described in the corresponding tables. Nucleotide diversity (π) (average number of nucleotide differences per site between any two RNA sequences of the same mutant spectrum and genomic region) was calculated according to the formula \( \pi = n (n-1)/2 \sum_{i<j} \pi_{ij} \) where \( n \) is the number of clones, and \( \pi_{ij} \) is the number of different nucleotides between the pair of sequences \( ij \) divided by the sequence length in nucleotides [156]. Confidence intervals for nucleotide diversities were calculated using bias-corrected bootstrap resampling method.

Statistical analyses
The statistical significance of differences between mutation frequencies or repertoires of mutation types was evaluated by the chi-square test (χ² test). To determine the significance of increases of nucleotide diversity associated with drug treatments, permutation tests were carried out on the regression slope of nucleotide diversity as a function of drug concentration, with 10,000 permutations per test. Briefly, sequences were randomly assigned to one of the possible groups, keeping the group size as in the sample under analysis. For each permutation, a linear regression of the nucleotide diversity versus drug concentration was obtained, and the value of the slope was stored. In this manner, the distribution of the regression slope was obtained for the case in which differences in nucleotide diversity were due solely to random sampling. Then, a p-value for the observed slope was...
calculated as the number of times that an equal or higher slope than the observed was found in the null distribution divided by the number of permutations. Both, p-values from permutations tests and bias-corrected bootstrap confidence intervals were obtained using ad hoc scripts developed for MATLAB® (code available upon request).

To probe statistical significance of the differences in specific infectivity, one-way analysis of the variance was performed with statistical package SPSS 13.0 (SPSS, Inc.). For multiple comparisons, Bonferroni’s correction was applied. The data are presented as mean values and standard deviations.

Quantification of HCV RNA

Real time quantitative RT-PCR was carried out using the Light Cycler RNA Master SYBR Green I kit (Roche), according to the manufacturer’s instructions, as previously described [157]. The 5′-UTR non-coding region of the HCV genome was amplified using as primers oligonucleotide HCV-5UTR-F2 (5′- TGAGGAAC-TACTGTGCCTGACGGCACAGAAG; sense orientation; the 5′ nucleotide corresponds to genomic residue 47), and oligonucleotide HCV-5UTR-R2 (5′- TGCTCATGGTGCGACGGTGACGAG; antisense orientation; the 5′ nucleotide corresponds to genomic residue 347). Quantification was relative to a standard curve obtained with known amounts of HCV RNA, synthesized by in vitro transcription of plasmid GNN DNA. The specificity of the reaction was monitored by determining the denaturation curve of the amplified DNAs. Negative controls (without template RNA and RNA from mock-infected cells) were run in parallel with each amplification reaction, to ascertain absence of contamination with undesired templates.

Assessment of HCV extinction

We have taken as criteria to consider HCV extinct those previously described for lethal mutagenesis of FMDV [48,49]. HCV was considered extinct when no virus infectivity was detected and no viral RNA was amplified using a sensitive RT-PCR amplification protocol, either from the supernatant of the cell culture that contains the putatively extinguished virus, or following 3 blind passages of the cell culture supernatants using Huh-7.5 reporter cells in the absence of any drug. The highly sensitive RT-PCR consists in the amplification using the primers JC1-NS5A F1 and JC1-NS5A R1 (Table S8). It should be noted that infectivity below the level of detection did not necessarily imply extinction according to these criteria, and this is indicated in the corresponding results.

Supporting Information

Table S1 Mutations, corresponding amino acid and point accepted mutation (PAM) of the E2-coding region in the mutant spectra HCV p3 passaged in the absence or presence of ribavirin (Rib).

Table S2 Mutations, corresponding amino acid and point accepted mutation (PAM) of the NS5A-coding region in the mutant spectra HCV p3 passaged in the absence or presence of ribavirin (Rib).

Table S3 Mutations, corresponding amino acid and point accepted mutation (PAM) of the NS5B-coding region in the mutant spectra HCV p3 passaged in the absence or presence of ribavirin (Rib).

Table S4 Mutations, corresponding amino acid and point accepted mutation (PAM) of the NS5A-coding region in the mutant spectra HCV p4 and p5 passaged in the absence or presence of ribavirin (Rib).

Table S5 Mutations, corresponding amino acid and point accepted mutation (PAM) of the NS5A-coding region in the mutant spectra HCV p4 passaged in the absence or presence of ribavirin (Rib) analyzed by ultra deep sequencing.

Table S6 Mutations, corresponding amino acid and point accepted mutation (PAM) of the NS5A-coding region in the mutant spectra HCV p3 passaged in the absence or presence of ribavirin (Rib) and guanosine (Gua).

Table S7 Mutations, corresponding amino acid and point accepted mutation (PAM) of the NS5A-coding region in the mutant spectra HCV p3 passaged in the absence or presence of guanosine (Gua) and/or myco-phenolic acid (MPA).

Table S8 Oligonucleotides used to amplify and sequence the HCV genomes.

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Author Contributions

Conceived and designed the experiments: ED AMO-P CP. Performed the experiments: AMO-P JS CP. Analyzed the data: AMO-P HT AG-P JQ JIE CP. Contributed reagents/materials/analysis tools: ED AG-P JQ. Wrote the paper: ED CP.

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