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Ribavirin has proven to be effective against several viruses in the clinical setting and a multitude of viruses in vitro. With up to five different proposed mechanisms of action, recent advances have begun to discern the hierarchy of antiviral effects at play depending on the virus and the host conditions under scrutiny. Studies reveal that for many viruses, antiviral mechanisms may differ depending on cell type in vitro and in vivo. Further analyses are thus required to accurately identify mechanisms to more optimally determine clinical treatments. In recent years, a growing number of ribavirin resistant and sensitive variants have been identified. These variants not only inform on the specific mechanisms by which ribavirin enfeebles the virus, but also can themselves be tools to identify new antiviral compounds.

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**Ribavirin and its broad spectrum of antiviral activities**
Several recent studies have continued to explore RBV as an antiviral and therapeutic treatment against different viruses. In recent years, these studies have focused on whether RBV is directly inhibiting the RdRp, mutating the genome, inhibiting IMPDH, or a combination of the three.

Inhibition of RNA synthesis is observed for the influenza virus polymerase in cells treated with either RBV or methotrexate, an inhibitor of purine synthesis that decreases intracellular concentrations of purines. In this case, loss of polymerase activity at low concentrations of nucleotide is considered to be the culprit [7]. For hepatitis E virus (HEV) replication in vitro, RBV has the same antiviral effect as two other IMPDH inhibitors, mycophenolic acid (MPA) and 5-ethyl-1-β-d-ribofuranosyl-1H-imidazole-4-carboxamide (EICAR) that can be neutralized by the addition of guanosine. HEV replication appears to be inhibited through the depletion of GTP pools and inhibition of IMPDH [8]. Similarly, RBV’s effect on HCV seemingly acts through the inhibition of IMPDH, since the addition of guanosine negates this effect and HCV RNA synthesis is suppressed in IMPDH-knockdown cells. No mutagenic activity was observed for HCV infection of Li23 hepatoma cells [9]. These results are in contradiction with those later obtained in the Huh-7.5 cell line, where mutagenic activity was indeed observed [10,11]. This mutagenesis...
is not mediated through the depletion of GTP levels, since the addition of guanosine does not significantly alter these effects. Depletion of GTP by MPA also has no mutagenic effect, suggesting that direct incorporation of RBV into the HCV genome is the underlying mechanism. Nevertheless, the study did uncover NTP imbalances resulting from RBV treatment that might contribute to the inhibition of RNA synthesis by mechanisms related to, or independent of, mutagenesis. In the case of arenaviruses, it appears that RBV has multiple effects. With lymphocytic choriomeningitis virus (LMCV), inhibition is likely mediated through a decrease in GTP levels caused by ribavirin-5'-monophosphate inhibition of IMPDH, while a mutagenic activity also occurs through incorporation of RBV into nascent RNA [12†]. Finally, a direct interaction with the viral polymerase was suggested in the case of Hantaan virus (HTNV). In Vero cells treated with RBV, a reduction in viral genomic RNA and infectious particles is observed that is not associated with a significant reduction in GTP levels, as would be expected if IMPDH inhibition were the primary antiviral effect. Rather, an observed increase in ribavirin-5'-triphosphate supports that direct interactions with the RdRp is the mechanism of inhibition [13†,14]. It is likely then, that for most viruses, ribavirin does indeed have pleiotropic effects that may differ in importance depending on concentration and cell type.

These studies highlight the possible contradiction of results due to choice of cell line, and question to what extent cell lines represent in vivo settings. Although the study of ribavirin as an antiviral drug has been justly focused its effects on the virus, an important set of recent studies examined how host cells may react to RBV exposure. One study revealed dramatic variations in the accumulation of RBV in seven different cell lines [15***]. The authors showed that while uptake of RBV by the equilibrative nucleoside transporters (ENT1 and 2) is similar for all cell types, the levels of intracellular RBV in seemingly resistant cells like BHK21, A549 and Vero are noticeably lower than those in BSRT7, HeLa, 4T1 or HEp2. Importantly, these profiles correlate with the lack of antiviral efficacy against VSV and Sendai virus. The authors concluded that the ability of a given cell type to metabolize RBV may determine which of RBV’s antiviral effects will be at play. In the case of the hCoV-EMC coronavirus, a lack of sensitivity to interferon-α2b and RBV is also observed in Vero cells, while LLC-MK2 cells are more responsive [16†]. Interestingly, RBV-responsive HuH7.5 cells passaged in the presence of ribavirin develop resistance phenotypes that correlate with reduced RBV uptake and/or processing. Furthermore, reduced uptake develops in PBMC of healthy donors treated for 1 week with RBV, and in the PBMC of HCV-infected patients receiving ribavirin-interferon combination therapy [17**].

**Ribavirin and its clinical use**

Although RBV has enough antiviral benefit against some viruses to warrant its clinical use despite its in vivo mechanisms remaining unclear, it is possible that this

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**Table 1**

| Family       | Genus      | Type species                                                                 |
|--------------|------------|------------------------------------------------------------------------------|
| Arenaviridae | Arenavirus | Junin virus, Lassa fever virus, Pichinde virus, Lymphocytic choriomeningitis virus |
| Bornaviridae | Bornavirus | Borna disease virus V and Hs80                                                |
| Bunyaviridae | Bunyavirus | San Angelo                                                                   |
|              | Hantavirus | Hantaan virus, Seoul virus                                                    |
|              | Nairovirus | Crimean Congo hemorrhagic fever virus                                         |
|              | Phlebovirus| Rift Valley fever virus, Sandy Fever Sicilian virus                           |
| Coronavirus  | Coronavirus| Severe Acute Respiratory Syndrome virus, Middle East Respiratory Syndrome virus, murine hepatitis virus |
|              |            |                                                                              |
|  Flaviviridae | Flavivirus | Dengue virus 1, 2, 4, Japanese encephalitis virus, Langat virus, Usutu virus, Wesselsbron virus, West Nile virus, Yellow Fever virus 17D and FNV, Zika virus |
| Hepeviridae  | Hepacivirus| GB virus B, Hepatitis C virus                                                  |
|              | Hepevirus  | Hepatitis E virus                                                             |
| Orthomyxoviridae | Influenzavirus | Influenza virus A, Influenza virus B                                         |
| Papillomaviridae | Papillomavirus | Human papillomavirus                                                         |
| Paramyxoviridae | Metapneumovirus | Human Metapneumovirus                                                        |
|              | Morbillivirus| Measles virus                                                                |
|              | Pneumovirus | Bovine respiratory syncitial virus, Human respiratory syncitial virus         |
| Picornaviridae | Respirovirus | Sendai virus                                                                 |
|              | Aphtovirus | Foot and mouth disease virus                                                   |
|              | Enterovirus| Poliovirus, Coxsackie virus                                                    |
|              |            | B3, Enterovirus 71, Rhinovirus                                                |
| Poxviridae   | Orthopoxvirus| Camelpox virus, Cowpox virus, Monkeypox virus, Vaccinia virus                |
| Retroviridae | Lentivirus  | Human immunodeficiency virus, Maedi Virusa virus                              |
| Rhadoviridae | Vesiculovirus| Vesicular stomatitis Indian virus                                              |
| Togaviridae  | Alphavirus | Chikungunya virus, Semliki Forest virus, Sindbis virus                        |
| Unassigned   | Deltavirus | Hepatitis delta virus                                                         |

Adapted and updated from [50]
lack of knowledge results in suboptimal targeting and efficacy.

The standard treatment against non-genotype 1 HCV, until the recent emergence of small molecule direct acting antivirals, has been a combination of pegylated interferon-α and RBV, although neither drug seems to have direct effects on the virus. In this regard, RBV enhances the pSTAT4 and IFN-γ response of natural killer cells to IFN-α stimulation [20]. By assessing RBV physiological levels in mono-infected and HIV co-infected patients, one study correlated lower response rates of HIV-infected patients with lower RBV bioavailability [21]. Interestingly, this combined therapy was surprisingly effective against disseminated human papillomavirus infection in a patient having previously undergone treatment for psoriasis [22], in HIV-HCV co-infected patients [23], as well as in a patient with acquired aplastic anemia [24].

In HCV infected patients undergoing RBV monotherapy, only a moderate and transient antiviral effect is observed. A lack of clear and direct inhibitory/mutagenic effects of RBV on HCV in vivo is thought to be a question of physiological concentrations of the drug or an issue of sampling the wrong compartment (blood versus liver). However, in recent studies, RBV was found to induce significantly more G-to-A and C-to-U transitions, a genetic signature that is indicative of ribavirin-induced mutagenesis [25**.26]. Whether the effect is sufficiently strong to be of benefit is not yet determined. For other clinical infections, RBV monotherapy remains the treatment of choice. For example, in transplant patients treated for hepatitis E infection, RBV therapy ensures a sustained virologic response in most patients as a first-line, or as a second and prolonged, therapy [27]. It is also a well-tolerated option to treat respiratory syncytial virus infections in immunocompromised patients [28]. As the only antiviral treatment against Crimean-Congo hemorrhagic fever, it appears to be beneficial when administered early, with decreased severity and fatality rates [29]. In a preliminary study, combination treatment was also shown to be effective against rhinovirus (RV) in patients with hypogammaglobulinemia, who are more susceptible to RV infections [30]. To explore an intervention strategy for the recently identified β coronavirus isolate hCoV-EMC/2012, treatment of rhesus macaques with RBV alone reduced virus replication. In combination with interferon-α2b, treatment was even more effective and permitted lower doses that are in line with clinical use [31]. Finally, RBV was shown to have protective potential against lethal hantavirus pulmonary syndrome after intranasal exposure of hamsters to Andes Virus, where the presence of live virus was reduced or abolished in serum [32].

Ribavirin and its isolated resistant variants

A number of RBV resistant variants have been either generated or isolated within the Picornaviridae family, all mapping to the RdRp and for many of them, resistance was accompanied by increased fidelity (Table 2). The first ribavirin-resistant poliovirus variant contained G64S in the RdRp [33], which led to the generation of four other RBV-resistant variants at this amino acid position (G64A, L,V and T) [34]. These variants resist the effects of several mutagens through increased replication fidelity. Mutations similar to those in poliovirus, G64R and G64T, were generated in human enterovirus 71 (HEV71) that

| Virus          | Variant | RBV phenotype — mechanism | Refs |
|---------------|---------|----------------------------|------|
| Poliovirus    | G64S/A/L/V/T | Resistant — higher fidelity | [33,34] |
| FMDV          | M296I   | Resistant — RBV-specific   | [44,45] |
|               | RB44H   | Resistant — higher fidelity | [36]  |
|               | A38V    | Resistant — RBV-specific   | [43]  |
| Coxsackie B3  | D5N:A38V:M194I:M296V (DAMM) | Resistant — RBV-specific | [43]  |
|               | A372V   | Resistant — higher fidelity | [37]  |
|               | P48K, S164P, A239G, L241I | Sensitive — lower fidelity | [46**] |
|               | I176V, I230F, F232Y, Y268W/H | Sensitive — lower fidelity | [46**] |
|               | S299T   | Sensitive — lower fidelity | [37]  |
| HEV71         | G64R/T  | Resistant — higher fidelity | [35]  |
|               | L129F   | Resistant — higher fidelity | [38]  |
| Chikungunya   | C483Y   | Resistant — higher fidelity | [39]  |
|               | C483M   | Resistant — higher fidelity | [47]  |
|               | C483A/G/W | Sensitive — lower fidelity | [47]  |
| Sindbis virus | C482A/G | Sensitive — lower fidelity | [47]  |
| HCV           | J6/JFH1 resistant populations | Resistant to RBV and 5-FU | [41,42] |
|               | G404S, E442G in NS5A protein | Resistant to RBV | [40] |
| Coronavirus   | MHV-ExoN | Sensitive — lower fidelity | [51]  |
|               | SARS-ExoN | Sensitive — lower fidelity | [48]  |
| Influenza A   | D27N in PB1 | Resistant to RBV | [7]    |
confers resistance to RBV and increased fidelity [35]. Recently, a FMDV mutagen-resistant RdRp variant was isolated using another mutagenic compound, 5-fluorouracil (5-FU). This R84H variant is also resistant to RBV and to a third mutagen, 5-azacytidine (AZC), as would be expected of high fidelity viruses [36]. A naturally occurring RdRp variant A372V of Coxsackie virus B3 is also resistant to these three mutagens through increased fidelity [37]. Yet another high fidelity HEV71 RdRp variant, L123F, was selected under RBV passage [38]. Within the Togaviridae family, the polymerase mutant C483Y of chikungunya virus was isolated using mutagenic passaging in RBV and 5-FU [39], which demonstrates higher replication fidelity. For HCV, ribavirin resistant mutations in the NSSA protein were first described using a replicon system [40]. More recently, resistant populations were isolated by serially passaging J6/JFH1 virus in RBV, which present dozens of mutations throughout the genome that could be responsible, including in the RdRp, but the precise determinant is not known [41]. In a later study, they confirmed that this population is also resistant to 5-FU, supporting increased fidelity as the mechanism behind its resistance [42].

In some cases, however, the precise mechanisms of resistance remain unclear and fidelity may or may not be involved. In the Orthomyxoviridae, an influenza A virus variant D27N, in the PB1 catalytic subunit of the viral replicase, was isolated that displays higher activity than wild type virus in the presence of RBV, as well as of an inhibitor of purine biosynthesis, methotrexate [7]. The authors suggest that this residue is involved in nucleotide recognition, but whether resistance results from increased fidelity or from increased processivity remains to be determined. Recently, another ribavirin-resistant FMDV polymerase variant A38V and a mutant carrying four RdRp amino acid substitutions called DAMM were isolated, but do not tolerate 5-FU or AZC treatment [43]. These variants have RBV-specific resistance profiles, similar to the first RBV-resistant FMDV polymerase variant M296L [44,45], suggesting that increased fidelity is not the basis of their resistance.

Whereas RBV resistant variants have been widely reported and are more readily isolated by mutagen screening, some RBV sensitive variants have also been recently isolated or generated. A dozen variants in different residue positions of Coxsackie virus B3 polymerase, and several variants of the same RdRp residue of chikungunya and Sindbis virus, are more sensitive to base analog mutagens because of their reduced polymerase fidelity, further confirming the mutagenic affect of RBV on RNA viruses [37,46,47]. In the case of coronaviruses (CoV) a hyper-sensitive ExoN-mutant has generated interesting data in recent years. Given that coronaviruses have the largest RNA genomes, they would expectedly be hyper-sensitive to RNA mutagens such as RBV; however, this did not seem to be the case. Following the discovery of a potential 3’-to-5’ exoribonuclease (ExoN) proofreading activity of the nsp14 [48], it was hypothesized that deletion of this activity would result in RBV sensitivity. This hypothesis was validated using murine hepatitis virus (MHV) and SARS-CoV ExoN-mutants, treated with RBV or 5-FU [49*].

Concluding remarks
In the last few years, in vitro studies on RBV have sought to identify which mechanisms are most contributing to the observed antiviral effect. It appears that in many cases, this pleiotropic compound is simply being true to its pleiotropic nature. A significant recent advance, and complication, toward this goal is the variability observed between cell types and tissues in RBV uptake and metabolism, that could impact the efficacy of a drug being administered with one mechanism in mind, while another mechanism is ultimately at work. Future research requires a better validation of these mechanisms depending on the cell system used, especially in the case of HCV. The most important goal is to determine how ribavirin treatment affects the virus in vivo, to more properly tailor treatments and identify alternative compounds with similar effects. Currently, the use of animal models to bridge laboratory and clinical studies are relatively scarce. Additionally, the recently isolated RBV-resistant and sensitive variants represent new tools to improve the efficacy of current mutagenic compounds and to identify new compounds with previously unknown antiviral mutagenic activity. For example, the isolation of the same Coxsackie virus B3 RdRp variant A372V in two independent screens for RNA mutagen and amiloride resistance, led to the discovery of a previously unknown, indirect mutagenic activity for the latter class of compounds [37]. This is especially relevant in this era of combination therapies, where drugs with different modes of action are needed whether they are inhibitors or mutagens.

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