Ribavirin Inhibits *In Vitro* Hepatitis E Virus Replication through Depletion of Cellular GTP Pools and Is Moderately Synergistic with Alpha Interferon

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Hepatitis E virus (HEV) is a common cause of acute hepatitis that results in high mortality in pregnant women and may establish chronic infections in immunocompromised patients. We demonstrate for the first time that alpha interferon (IFN- α) and ribavirin inhibit *in vitro* HEV replication in both a subgenomic replicon and an infectious culture system based on a genotype 3 strain. IFN- α showed a moderate but significant synergism with ribavirin. These findings corroborate the reported clinical effectiveness of both drugs. In addition, the antiviral activity of ribavirin against wild-type genotype 1, 2, and 3 strains was confirmed by immunofluorescence staining. Furthermore, the *in vitro* activity of ribavirin depends on depletion of intracellular GTP pools, which is evident from the facts that (i) other GTP-depleting agents (5-ethynyl-1- β -D-ribofuranosylimidazole-4-carboxamide [EICAR] and mycophenolic acid) inhibit viral replication, (ii) exogenously added guanosine reverses the antiviral effects, and (iii) a strong correlation ($R^2 = 0.9998$) exists between the antiviral activity and GTP depletion of ribavirin and other GTP-depleting agents.

Hepatitis E virus (HEV) is a positive-sense, single-stranded RNA virus and is classified as the sole member of the *Hepeviridae* family (1, 2). HEV is usually transmitted feco-orally and may cause self-limiting acute hepatitis. At least four genotypes are currently recognized: genotypes 1 and 2 seem to infect only humans and are endemic in developing regions, with an estimated 70,000 deaths annually (3), while genotypes 3 and 4 are zoonotic agents, with domestic pigs as their main reservoir (1, 2). The latter two genotypes cause sporadic autochthonous infections in both developing and industrialized parts of the world, e.g., through consumption of undercooked pig meat (1, 2). In addition, related viruses have been found in several animal species, including chickens (avian HEV) (4), rats (5), rabbits (6), ferrets (7), bats (8), and trout (cutthroat trout virus [CTV]) (9).

Infections with HEV are often severe in pregnant women infected with genotype 1, with up to 25% mortality (1, 10). In addition, genotype 3 can cause chronic infections, particularly in immunocompromised individuals (11). The first consideration in treating chronic hepatitis E, especially in transplant patients, is lowering immunosuppressive therapy, which induces clearance in over 30% of cases (12). An additional course of pegylated alpha interferon (IFN- α) for 3 months or longer proved successful in most cases described (13), but ribavirin (RBV) monotherapy is probably the most frequently used option for chronic hepatitis E (11), and it also seems effective in severe acute infections (14, 15).

Research on HEV has long been hampered by a lack of efficient cell culture models; however, *in vitro* cultures have been established in recent years (16, 17). By employing a replicon, an infectious virus yield assay, and immunofluorescence staining, we investigated the antiviral activities of both IFN- α and RBV against *in vitro* HEV replication. Depletion of intracellular GTP pools was found to be an important aspect of the mechanism of action of RBV.

MATERIALS AND METHODS

***In vitro* transcription and capping.** HEV Kernow-C1 p6 (GenBank accession number JQ679013) and p6/luc-encoding RNAs were transcribed *in vitro* from MluI-digested plasmid DNA (17) by use of the T7 RiboMAX Large Scale RNA production system (Promega, Madison, WI) and were capped with the ScriptCap m7G capping system (Cellscript, Madison, WI). To generate a transfection control for luminescence-based antiviral assays, the T7-IRES-FFLuc-YFsfRNA DNA fragment was PCR amplified from pT7-IRES-FFLuc-YFsfRNA with primers 5'-CATATGTCGACTAATACGACTCACTATAGGGATCCGCCCTCTCCC-3' and 5'-AGTGGTTTGTGTTTGTTCATCC-3' and with Kapa HiFi HotStart ReadyMix master mix (Kapa Biosystems, Woburn, MA). The resulting fragment was *in vitro* transcribed. The internal ribosome entry site (IRES) alleviates the need for capping, and the yellow fever virus-derived small flaviviral RNA (YFsfRNA) enhances the cellular stability of this firefly luciferase (FFLuc)-encoding RNA (18). The generation of pT7-IRES-FFLuc-YFsfRNA will be described elsewhere.

Cells and viruses. Huh7 cells (a kind gift from Ralf Bartenschlager, University of Heidelberg, Heidelberg, Germany) and HepG2/C3A cells (a kind gift from Luc Verschaeve, Scientific Institute of Public Health, Brussels, Belgium) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Integro, Leuvenheim, Netherlands) in a humidified 5% CO₂ incubator at 37°C.

To obtain infectious HEV stocks, HepG2/C3A cells seeded in a 6-well plate (BD Falcon, Franklin Lakes, NJ) were transfected with capped Kernow-C1 p6 RNA by use of DMRIE-C (Invitrogen, Carlsbad, CA) and were

Received 20 August 2013 Returned for modification 9 September 2013

Accepted 16 October 2013

Published ahead of print 21 October 2013

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doi:10.1128/AAC.01795-13

incubated at 35°C. Part of the culture medium was changed every 2 to 3 days to ensure long-term cell survival, and culture medium was harvested from the original plate after 34 days of incubation. The obtained virus stock was propagated once more in HepG2/C3A cells, with weekly changing of half of the culture medium. Culture medium was harvested after 22 days. Since the Kernow-C1 p6 strain has been adapted to growth in HepG2/C3A cells (19), culturing of HEV p6 virus stocks in these cells resulted in higher-titer stocks than those obtained by culture in Huh7 cells (results not shown).

HEV strain Sar 55 (GenBank accession number [M80581](#)), Akluj (accession number [AF107909](#)), LBPR-0379 (accession number [JN564006](#)), and Kernow-C1 (accession number [HQ389543](#)) inocula were obtained from human stool samples, while the human Mex 14 strain (accession number [M74506](#)) was passaged once in a rhesus macaque. The work with human isolates was carried out under approved protocols of the Carolinas Medical Center (IRB-10-0709B) and the Royal Cornwall Hospital Trust (IRB-06Q2101/61), with the informed consent of the patients. The housing, maintenance, and care of the rhesus macaque met or exceeded all requirements for primate husbandry as specified in the *Guide for the Care and Use of Laboratory Animals* (20).

Compounds. IFN- α 2a (Roferon-A) was purchased from Roche Pharmaceuticals (Basel, Switzerland), diluted to 3×10^5 international units (IU)/ml in phosphate-buffered saline (PBS; Lonza, Basel, Switzerland) supplemented with 10% glycerol and 0.1% bovine serum albumin, stored at -80°C , and kept at 4°C after thawing. RBV [1-(β -D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (Virazole)] was purchased from ICN Pharmaceuticals (Costa Mesa, CA). 5-Ethynyl-1- β -D-ribofuranosylimidazole-4-carboxamide (EICAR) was a gift from Akira Matsuda (Hokkaido University), and mycophenolic acid (MPA) was purchased from Santa Cruz Biotechnology (Dallas, TX). Guanosine was from Sigma-Aldrich (St. Louis, MO). Stock solutions of the compounds were made in dimethyl sulfoxide (DMSO) and stored at 4°C .

Luminescence-based antiviral assay. Huh7 cells were seeded in 96-well plates (BD Falcon) at 7.5×10^3 cells per well in 100 μl of DMEM with 10% FBS and were incubated at 37°C . After 24 h, cell layers were washed once with DMEM and transfected with IRES-FFLuc-YFsfRNA and capped p6/luc RNA (100 ng per well [each]) by use of DMR1E-C reagent (0.2 μl per well) according to the manufacturer's instructions. For cell control (CC) wells, viral RNA was omitted. Plates were incubated at 37°C for 4 h. Afterwards, the transfection medium was removed, cell layers were washed twice with PBS (100 μl per well), and 100 μl of compound diluted in DMEM with 10% FBS was added to each well. For virus control (VC) and CC wells, the compound was omitted. After incubation at 35°C for 3 days, 20 μl of the culture medium was transferred to a white 96-well CulturPlate (PerkinElmer, Waltham, MA), and luminescence produced by the secreted *Gaussia* luciferase (GLuc) was determined after addition of 50 μl of diluted *Renilla* luciferase assay substrate (Promega). A pilot study indicated that incubation for 3 days was sufficient to obtain a 100-fold increase in *Gaussia*-generated luminescence (data not shown), allowing for a fast and relatively sensitive readout. The remaining culture medium was removed, and 20 μl of passive lysis buffer (Promega) was added to each well. Next, the buffer with lysed cells was transferred to a white 96-well CulturPlate containing 100 μl of diluted FFLuc assay substrate (Promega) per well, and luminescence counts were obtained. GLuc luminescence values were normalized with the following formula: $\text{LucNorm}_{\text{compound}} = (\text{GLuc}_{\text{replicon+compound}} - \text{GLuc}_{\text{CC}}) / \text{FFLuc}_{\text{replicon+compound}}$. The percent antiviral activity was calculated as $100 - (\text{LucNorm}_{\text{compound}} / \text{LucNorm}_{\text{VC}} \times 100)$. The 50% effective concentration (EC_{50}) was defined as the concentration of compound that caused a 50% reduction in the LucNorm signal compared to that of VC and was calculated through logarithmic interpolation. For toxicity evaluation, plates were prepared in parallel with antiviral assay mixtures, but the transfection step was omitted. After incubation for 3 days at 35°C , medium was removed and replaced with 100 μl of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazinemethosulfate (MTS/PMS; Promega) solution,

as described previously (21). After an incubation period of 2 h at 37°C , the optical density (OD) at 498 nm was determined for each well. The percent cell viability was calculated as follows: $\% \text{ viability} = \text{OD}_{\text{compound}} / \text{OD}_{\text{CC}} \times 100$.

For combination studies, a checkerboard with 3-fold dilutions of RBV and 4-fold dilutions of IFN- α was used. Data were analyzed with the MacSynergy II template at the 95% confidence level (22). Volumes of synergy or antagonism below 25 $\mu\text{M}^2\%$ are considered insignificant, while values between 25 and 50 $\mu\text{M}^2\%$ are minor but significant. Volumes between 50 and 100 $\mu\text{M}^2\%$ indicate moderate synergy or antagonism (22–24).

Infectious virus yield assay. Huh7 cells were seeded in 6-well plates at 5×10^5 cells per well in 2 ml of DMEM with 10% FBS, penicillin (100 units/ml; Gibco), streptomycin (100 $\mu\text{g}/\text{ml}$; Gibco), and amphotericin B (Fungizone) (2.5 $\mu\text{g}/\text{ml}$; Gibco) and were incubated at 37°C . After 24 h, culture medium was removed and cell layers were inoculated with infectious p6 virus at 2.1×10^5 RNA copies/ml in 1 ml of medium with DMSO (0.5%) or compound and then incubated at 35°C . After 5 h, the inoculum was removed, cell layers were washed 3 times with 2 ml of PBS, and 3 ml of medium with DMSO or compound was added to each well. After 1 h, a 150- μl sample was taken and stored at -80°C . Since HEV replication *in vitro* is rather poor, regular changing of the culture medium was required to allow cell survival over the full duration of the assay. Consequently, 750 μl was removed from each well every 2 to 3 days and stored at -80°C , and 1 ml of fresh medium with DMSO or compound was added. The viral loads in samples from 1 h, 12 or 13 days, and 20 days postinfection were determined by reverse transcription-quantitative PCR (RT-qPCR). Statistical significance was calculated with the Mann-Whitney U test. On day 20 postinfection, all medium was removed from the plate, cell layers were washed with 2 ml of PBS, and 1 ml of MTS/PMS solution was added for evaluation of compound cytotoxicity. Cultures were incubated for 2 h at 37°C , and subsequently, the OD at 498 nm was determined for 100 μl from each well. Cell viability was expressed as a percentage of the DMSO control level.

Immunofluorescence analysis and focus-forming assay. HEV strains Sar 55, Akluj, Mex 14, LBPR-0379, Kernow-C1, and Kernow-C1 p6 were inoculated onto HepG2/C3A cells seeded in 8-well Lab-Tek II CC2 chamber slides (Nunc, Penfield, NY). Cells in triplicate wells were incubated with medium with or without RBV at 200 μM . After 3 days, cells were fixed and stained for capsid protein as described before (19). Each well was manually scanned, and fluorescent cells or foci were counted. Cell viability of HepG2/C3A cells treated with RBV at 200 μM for 3 days was assessed with the MTS/PMS method. RBV was used at a higher concentration than that in Huh7 cells because HepG2 cells appear to require higher concentrations to induce intracellular GTP depletion (e.g., see reference 25).

Quantification of intracellular GTP concentrations. RBV, MPA, or EICAR was added to 1-day-old cultures of Huh7 cells in DMEM with 10% FBS in 25-cm² flasks. For RBV, concentrations of 2.5, 10, 25, and 100 μM were tested. For MPA and EICAR, concentrations of 0.1, 0.25, 1, and 2.5 μM were used. Untreated CC cultures were included as well. Flasks were incubated at 35°C for 3 days, and subsequently, cells were detached using trypsin-EDTA solution (Gibco) and collected by centrifugation. Nucleotides were extracted from the cell pellet, and GTP levels were quantified by high-performance liquid chromatography as described previously (26). The 50% inhibitory concentration (IC_{50}) was defined as the concentration of compound required to reduce the GTP levels to 50% of the levels in untreated control cultures.

RT-qPCR. Viral RNA was extracted from culture medium by use of a NucleoSpin RNA virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Primer and probe sequences for TaqMan-based quantification of HEV RNA were published previously (27). The forward primer 5'-GGTGGTTTCTGGGGTGAC-3' and the reverse primer 5'-AGGGGTTGGTTGGATGAA-3' were used. The probe was labeled with 6-carboxyfluorescein (FAM) at the 5' end and with a minor groove binder (MGB) at the 3' end (5'-FAM-TGATTCTCAGCCCTTCGC-MGBNFQ-3') (28). Reactions were performed with One-Step qRT-PCR mix (Eurogentec, Seraing, Belgium) in a final volume of 25 μl

FIG 1 Inhibitory activities of IFN- α and RBV against HEV replication. Huh7 cells were transfected with capped p6/luc RNA and treated with RBV (A) or IFN- α (B) for 3 days. (C) Antiviral activities below or above those expected for RBV-IFN- α combinations. (D) Antiviral activities were also assessed in an infectious virus yield assay with RT-qPCR detection of viral RNA. *, $P < 0.05$; **, $P < 0.01$. GE, genome equivalents. (E) RBV (200 μ M) inhibited formation of foci in HepG2/C3A cells infected with strains Sar 55, Akluj, Mex 14, LBPR-0379, Kernow-C1, and Kernow-C1 p6. (F) Cell viability of Huh7 cells used in the infectious virus yield assay after 20 days in the presence of IFN- α or ribavirin, as assessed by the MTS/PMS method. (G) Cell viability of HepG2/C3A cells treated with RBV at 200 μ M for 3 days was assessed by the same method. Values represent means $\pm$ standard deviations (SD) for at least 3 independent experiments (A to D, F, and G) or 3 replicates (E).

containing 250 nM of each primer, 100 nM of probe, and 5 μ l of RNA sample. PCR was performed using an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA) under the following conditions: 30 min at 48°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed with ABI Prism 7500 SDS software (version 1.3.1; Applied Biosystems). For absolute quantification, standard curves were generated using 10-fold dilutions of the cloned target cDNA.

RESULTS

We used a transient GLuc-expressing genotype 3 replicon (Kernow-C1 p6/luc) (17) in Huh7 hepatoma cells to assess the potential anti-HEV activity of RBV and IFN- α , two drugs whose clinical efficacy has been reported in several case studies of HEV-infected patients (11, 13). Both RBV and IFN- α efficiently inhibited HEV replication, with EC₅₀s of 3 ± 2 μ M (0.7 ± 0.5 μ g/ml) and $1.3 \pm$

0.5 IU/ml, respectively (Fig. 1A and B). The combination of both inhibitors resulted in a moderately synergistic antiviral effect, with a synergy volume of 72 $\mu\text{M}^2\%$ (Fig. 1C). The combined antiviral activity was maximally 22% above the expected value (at about 4 IU/ml IFN- α and 0.4 μM RBV).

Next, the potential antiviral activity was assessed in Huh7 cells infected with the Kernow-C1 p6 virus (17). Viral replication was quantified by means of RT-qPCR detection of viral RNA in the culture medium. Both IFN- α and RBV resulted in significant reductions of viral titers at 12 and 20 days postinfection (Fig. 1D), without decreasing cell viability (RBV concentrations of up to 100 μM for 20 days) (Fig. 1F). A dose-dependent inhibition of viral replication was observed with RBV, with a 4.1- $\log_{10}$ reduction in viral titer after 20 days at a concentration of 100 μM . Some differences in the antiviral potency of RBV observed between the replicon and virus yield assays, though limited, may be explained by the different setups (luminescence versus quantification of released viral RNA) and the respective time windows (3 versus 20 days) of the assays.

Since RBV was reported to be effective in the treatment of acute genotype 1 infections (14, 15), the antiviral activity of RBV against genotypes 1 and 2 was assessed. To this end, HepG2/C3A cells were infected with wild-type isolate Sar 55, Akluj (both genotype 1), or Mex 14 (genotype 2) and stained for capsid protein. These strains do not spread in cell culture but are able to replicate intracellularly. RBV treatment (200 μM) reduced the number of fluorescent foci to almost zero (Fig. 1E). Comparable results were obtained for the wild-type genotype 3 strains LBPR-0379 and Kernow-C1 and for the cell culture-adapted Kernow-C1 p6 strain. No cytotoxicity was observed for RBV at 200 μM for 3 days (Fig. 1G). In addition, treatment of Sar 55 Gluc-containing replicon cells with RBV resulted in a pronounced reduction of viral replication, even when addition of RBV was delayed until 4 days posttransfection and activity was assessed at 15 days posttransfection (data not shown).

One of the proposed mechanisms of action for the broad-spectrum antiviral activity of RBV is that inhibition of IMP dehydrogenase (IMPDH) by RBV 5'-monophosphate results in depletion of the intracellular GTP pools, thus impeding RNA virus replication (29, 30). To determine whether GTP depletion is involved in inhibition of *in vitro* HEV replication, we assessed the antiviral activities of two known IMPDH inhibitors: MPA (an immunosuppressive agent) and EICAR, a 5-ethynylimidazole analogue of RBV (31, 32). Marked inhibition of HEV replication was observed in the replicon assay, with EC_{50} s of 0.20 ± 0.04 μM and 0.115 ± 0.007 μM for MPA and EICAR, respectively (Fig. 2A and B).

It has been shown for several viruses that are sensitive to RBV that replenishing of intracellular GTP pools through addition of exogenous guanosine restores virus replication (29, 33). Concordantly, the anti-HEV activities of RBV, MPA, and EICAR were reversed following addition of guanosine to the cell culture medium (Fig. 3A to C). Next, we quantified the intracellular GTP pools of Huh7 cells and the effects of different concentrations of the three compounds. Normal GTP concentrations were calculated to be around 350 μM , and RBV, MPA, and EICAR effectively depleted GTP pools, with IC_{50} s of 18 ± 9 μM , 0.5 ± 0.1 μM , and 0.3 ± 0.2 μM , respectively. A strong correlation between both the IC_{50} s for GTP depletion and the respective antiviral EC_{50} s was calculated ($R^2 = 0.9998$) (Fig. 3D).

FIG 2 Dependence of the antiviral activity of RBV on the depletion of intracellular GTP pools. MPA (A) and EICAR (B) are known inhibitors of IMPDH and are potent inhibitors of HEV replication in the replicon assay. Values represent means $\pm$ SD for at least 3 independent experiments.

DISCUSSION

Pegylated IFN- α and RBV are the only drugs that are currently available to treat acute and chronic hepatitis E (5, 7, 9). Treatment duration is at least 12 weeks for chronic infections (13) and a minimum of 3 weeks for acute infections (14, 15, 34) (compare with the current standard of care for hepatitis C, i.e., pegylated IFN- α plus RBV for up to 48 weeks, combined with a protease inhibitor for genotype 1 infections [35]). The scientific evidence for these hepatitis E treatments is limited to a number of case series and the *in vitro* activity against CTV, a HEV surrogate (36). None of these interventions or the superiority of one over the other has been validated in controlled trials yet. Here we demonstrate the antiviral activity of IFN- α and RBV against HEV *in vitro*, thus providing additional evidence for the clinical use of these drugs. In addition, a moderate but statistically significant synergy was calculated for the combination of IFN- α and RBV (Fig. 1C). The clinical relevance of this observation is unclear, however, since antiviral activity was maximally 22% above that expected. Nevertheless, since no antagonistic effects were observed, it might be an option to treat patients with a combination of IFN- α and RBV, as is the case in the management of infections with hepatitis C virus (HCV). Lower doses of both drugs may possibly be sufficient in a combination regimen, thus reducing the frequency and severity of adverse effects. In fact, successful combination therapy has already been reported for a chronically HEV-infected HIV patient (37).

FIG 3 Replenishing GTP pools salvages HEV replication. Addition of exogenous guanosine to the culture medium (40 μM) salvages HEV replication and abolishes the antiviral effects of RBV (A), MPA (B), and EICAR (C) in the replicon assay. (D) Relationship between the IC₅₀s for GTP depletion and EC₅₀s for inhibition of viral replication. Values represent means ± SD for at least 3 independent experiments.

When the *in vitro* inhibitory concentrations of IFN-α and RBV are compared to the serum concentrations typically obtained in (HCV-infected) patients, they seem to be in a roughly similar concentration range. For instance, mean pegylated IFN-α serum concentrations of around 80 IU/ml have been reported (38), which is considerably higher than the EC₅₀ for inhibition of the HEV replicon, although this is lower than the 300 IU/ml that resulted in a 0.9-log₁₀ reduction in the virus yield assay. For RBV, serum concentrations between 8 and 13 μM have been reported (38, 39), and hepatic accumulation of ribavirin has been claimed, with a steady-state liver concentration of 250 μM (40). We calculated an EC₅₀ of 3 μM for RBV and observed strong antiviral effects in the virus yield assays for concentrations above 10 μM. Although the relevance of such comparisons may be questionable, the fact that both IFN-α and RBV are active *in vitro* is in agreement with the observed clinical efficacy of both in chronically HEV-infected patients.

In a recent study by Dong and colleagues, the HEV ORF3 protein was shown to inhibit IFN-α signaling (41). In line with this finding, high concentrations of IFN-α were required to reduce release of HEV RNA into the culture medium after infection (e.g., 50% reduction at concentrations as high as 1,000 units/ml). A similarly high concentration of 300 IU/ml was needed in our infectious HEV system to achieve reductions of viral RNA levels in

the virus yield assay of 98% and 87% at 12 and 20 days postinfection, respectively (Fig. 1D). In the replicon assay, on the other hand, we observed strong inhibition by IFN-α at concentrations as low as 10 IU/ml (Fig. 1B). Slight differences obtained in the infectious HEV system may be explained by the use of different cell lines (A549 versus Huh7) and virus isolates and/or the fact that the HEV infection was already established before the start of IFN-α treatment in the study of Dong et al., while IFN-α was included in the inoculum during initial infection in our virus yield system. However, the pronounced potency of IFN-α against the subgenomic HEV replicon system can readily be explained by the lack of an interferon-antagonizing activity in p6/luc, where expression of ORF3 is ablated by replacement with a reporter cassette, making the replicon especially susceptible to inhibition by IFN-α. This is fully in line with the findings of Dong et al. (41).

The data presented here indicate that depletion of cellular GTP pools is the predominant mechanism by which ribavirin inhibits *in vitro* HEV replication. It is unclear, however, to what extent hepatic GTP levels can be decreased by RBV *in vivo* (30). Other proposed mechanisms of action of RBV include immunomodulatory effects, modulation of IFN-stimulated gene expression, lethal mutagenesis forcing the virus into an error catastrophe, interference with viral methyltransferase activity, direct inhibition of the viral polymerase, and inhibition of eukaryotic initiation factor

4E interfering with cap-dependent translation (30). Although one or several of these mechanisms could be involved in the antiviral activity of RBV against HEV, the results presented here suggest that GTP depletion is an important mechanism contributing to its antiviral activity.

Interestingly, the use of mycophenolate mofetil, a prodrug of MPA, for immunosuppressive therapy in transplant patients with chronic hepatitis E was significantly associated with HEV clearance (42). This observation is in accordance with the strong antiviral activity observed in our replicon assay (Fig. 2A). However, it is questionable whether the antiviral effect outweighs the immunosuppression that initially allowed HEV to establish a chronic infection.

The replicon-based antiviral assay as described here is rather labor-intensive, but to our knowledge, it is the first reported system that allows testing of potential antiviral molecules targeting HEV replication. In addition, the results of the infectious virus yield indicate that it is feasible to study the impact of potential antiviral compounds on the replication of full-length replication-competent HEV. Although the isolation of the Kernow-C1 p6 strain was a major step forward, the replication kinetics are (still) rather slow, thus requiring long incubation times to obtain sufficiently high HEV titers for detection by RT-qPCR. Further adaptation to cell culture may be a possible solution; this strategy proved successful, for instance, for hepatitis A virus and allowed for the development of relatively fast virus yield assays (43).

In conclusion, the observed *in vitro* antiviral activities provide additional support for the clinical use of both RBV and (pegylated) IFN- α for the treatment of severe cases of hepatitis E. However, both therapies require long treatment periods and can have severe adverse effects. RBV dose reductions because of anemia have resulted in treatment failure and death in some cases (15). Consequently, the assays presented here may serve as a starting point for developing convenient systems for high-throughput screening. This should allow the development of more potent anti-HEV drugs with a better safety profile. Ideally, these drugs should be safe during pregnancy. Since HEV appears to be emerging and its impact on public health has been underestimated, it would be wise to invest in research toward such antivirals.

ACKNOWLEDGMENTS

We thank Ralf Bartenschlager (University of Heidelberg, Germany) for Huh7 cells, Luc Verschaeve (Scientific Institute of Public Health, Brussels, Belgium) for HepG2/C3A cells, and Akira Matsuda (Hokkaido University, Japan) for providing EICAR. We are grateful to Hendrik Thibaut, Pieter Leyssen, Jan Paeshuyse, and Suzanne Kaptein for helpful discussions and to Ria Van Berwaer for excellent technical assistance.

Y.D. is a fellow of the Research Foundation-Flanders (FWO). Q.P. is supported by a Sheila Sherlock Fellowship from the European Association for the Study of the Liver (EASL). This work was supported by KU Leuven Geconcerteerde Onderzoeksacties (GOA/10/014) and by EU FP7 project SILVER (260644) and in part by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases (S.U.E.).

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