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Inhibition of HEV Replication by FDA-Approved RdRp Inhibitors

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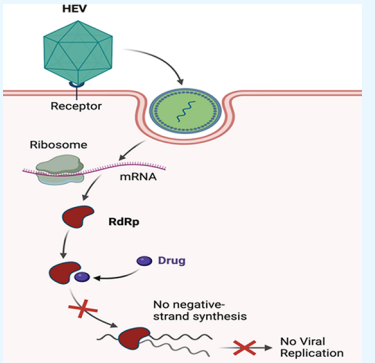
ABSTRACT: Hepatitis E virus (HEV) is primarily a hepatotropic virus that is responsible for acute hepatitis E in the general population and for chronic hepatitis in immunocompromised individuals. In the absence of a globally accessible vaccine, pegylated interferon-α and ribavirin are the only antiviral agents available for the treatment of chronic patients. As viral RNA-dependent RNA polymerases (RdRps) are indispensable for RNA replication, they are considered potential drug targets. In this study, we screened some well-known RdRp inhibitor molecules, notably, favipiravir, sofosbuvir, remdesivir, filibuvir, and tegobuvir. Of these, monotherapy with favipiravir and sofosbuvir inhibited the RdRp activity with an IC_{50} value of 10.2 ± 4.9 and 5.2 ± 2.9 μM, respectively, compared to the reference drug ribavirin (3.5 ± 1.6 μM). Further investigation of the combination therapy showed a reduction in viral RNA copy numbers by approximately 90%. Therefore, favipiravir has an additive effect when used with sofosbuvir. Therefore, we propose that favipiravir is a promising anti-HEV drug that can be used in combination with sofosbuvir.



1. INTRODUCTION

Hepatitis E virus (HEV) is a quasi-enveloped, positive-sense single-stranded RNA (+ssRNA) virus, which is mainly transmitted via contaminated water and undercooked pork. HEV primarily infects liver and causes acute hepatitis, which also has extrahepatic manifestations in some patients.[1] In immunocompromised clinical cases, HEV infection can lead to chronic hepatitis, wherein pegylated interferons and ribavirin are the only off-label treatment options.[2] However, pegIFN-α-associated nonresponse or side effects, as well as the emergence of ribavirin-resistant HEV mutants, further restrict their clinical use. Therefore, further research is necessary to develop safe and effective anti-HEV agents.

The RNA genome of HEV is ∼7.2 kb which encodes three open reading frames (ORF1−ORF3); however, an additional ORF4 has been recently reported in HEV genotype-1.[3] ORF1 encodes all nonstructural replicase proteins of HEV, whereas ORF2 codes for the capsid protein, and ORF3 encodes a small phosphoprotein required for virion egress.[4] HEV uses RNA-dependent RNA polymerase (RdRp), a nonstructural protein involved in RNA replication. In +ssRNA viruses, the RdRp enzyme is a vital component of the replication machinery as it catalyzes the synthesis of a complementary RNA strand from a template RNA in infected cells. Inhibition of RdRp has been shown to suppress or inhibit the replication of several RNA viruses, making it a promising antiviral target, and FDA-approved RdRp inhibitors are currently used for the treatment of coronaviruses, Hepatitis C virus, influenza virus, and Ebola virus infections[5−8] Sofosbuvir targets the RdRp gene of hepatitis C virus and is highly effective.[9] It targets the RdRp gene of SARS-CoV-2 and has been approved for emergency use.[10] Studies have shown that HEV RdRp is highly conserved among different strains (genotypes), suggesting it as a potential drug target.[11] Ribavirin, a nucleoside analogue, is the only treatment available for chronic hepatitis E; therefore, treatment failures due to significant mutations in HEV-RdRp remain a bottleneck in clinics.[12] Hence, there is an urgent need to develop novel, effective, and safe antiviral agents for the treatment of hepatitis E.

In this study, we expressed and purified the ∼56 kDa HEV-RdRp protein and studied its enzyme activity using a biochemical assay. Furthermore, the impact of some well-known anti-RdRp drugs, including sofosbuvir and favipiravir, on the enzyme activity of RdRp and ultimately on HEV replication was investigated. Ribavirin, sofosbuvir, and favipiravir effectively inhibited HEV replication, and the combination of sofosbuvir and favipiravir resulted in an additive antiviral effect. Favipiravir is used in the treatment of various viral diseases, but its effect on HEV has not yet been studied. RdRp plays a critical role in the replication and spread of the virus, and research on this enzyme continues to be an active area of study in the field of virology.

2. MATERIALS AND METHODS

2.1. Expression and Purification of HEV-RdRp. The HEV-RdRp gene was cloned in the pET-28a vector (kindly provided by Kavita Lole, Pune, India), and the construct was

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transformed into Lemo21 cells for protein expression. Various conditions were studied by varying the IPTG concentration, induction time, and temperature to optimize the protein expression. Lemo21 cells were grown in Terrific Broth (HiMedia) until the optical density (OD_{600}) reached 0.7, and the culture was induced with 0.5 mM IPTG at 26 °C for 5 h. The protein was solubilized as described previously.[13] Briefly, the protein was solubilized in 10 mM Tris−Cl (pH 7.5), 100 mM NaCl, and 0.5% *N*-lauryl sarcosine (NLS, Sigma-Aldrich), and the solubilized protein was purified using Ni-NTA chromatography and eluted in 10 mM Tris−Cl (pH 7.5), 100 mM NaCl, 0.1% NLS, and 250 mM imidazole. The eluents were pooled and loaded onto a HiLoad Superdex 6/200 pg column for further purification. The purified protein was finally eluted with 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.06% NLS.

2.2. Enzyme Activity of HEV-RdRp. The enzyme activity of the purified protein was determined in a reaction buffer containing 20 mM Tris−Cl (pH 7.5), 120 mM NaCl, 7.5% glycerol, 5 mM $MgCl_2$, and 7 mM β-ME. Therein, GTP was used as the substrate, while poly(C) was used as a template. The formation of dsRNA was quantified using a fluorescent dye, PicoGreen (Invitrogen), specific to the double-stranded structure.[14] The reactions were performed in black 96-well plates (Tarsons) in a total volume of 25 μL. A 5 μM amount of purified RdRp was incubated with 0.5 mM GTP and 1 μL of 1 mg/mL poly(C) in the reaction buffer. The reaction was incubated for 3−4 h at room temperature (RT) and subsequently stopped using 10 mM EDTA. The 200× PicoGreen dye (Invitrogen) was diluted with TE buffer, and 75 μL of 1× dye was added to the reaction mixture. Readings were taken using a microplate reader at standard wavelengths (excitation, 480 nm; emission, 520 nm).[15]

To check the effect of different RdRp inhibitor compounds (favipirvir, sofosbuvir, remdesivir, filibuvir, tegobuvir, and ribavirin), 5 μM purified RdRp was incubated with 1 μM of compounds in the reaction buffer for 30 min at RT. Subsequently, 0.5 mM GTP and 1 μL of 1 mg/mL poly(C) were added, and the reaction was incubated for 3−4 h at RT. The reaction was stopped with 10 mM EDTA, and 75 μL of 1× dye was added to quantify dsRNA formation. Readings were taken using a Tecan plate reader (Tecan) at standard wavelengths. For the 50% inhibitory concentration (IC_{50}) determination, the purified RdRp was incubated with different concentrations of favipiravir, sofosbuvir, and ribavirin. The IC_{50} values for the compounds were determined in triplicate, using GraphPad Prism software with log concentration vs normalized response.

2.3. Microscale Thermophoresis. The purified HEV-RdRp protein was labeled using a His-tag labeling kit (NanoTemper Technologies), and its interactions with the ligands favipiravir, sofosbuvir, and ribavirin were studied. While the RdRp protein was used at a concentration of 50 nM, the ligand concentration varied from 500 μM to 150 pM. Notably, for favipiravir, the concentration varied from 5 mM to 300 nM. The capillaries were loaded, and the data analysis was performed using Monolith NT.115 analysis software (NanoTemper Technologies). All experiments were performed as three independent experiments.

2.4. HEV Infectious Clones, In Vitro Transcription, and Transfection. For the full-length HEV genome, a cDNA clone of HEV genotype 3 (pSHEV-3; accession no. AY575859.1) was used. For the subgenomic HEV replicon, HEV-p6 luciferase (accession no. JQ679013) was used. For in vitro transcription, both full-length and subgenomic replicons were linearized using XbaI and MluI, respectively, and transcribed, as mentioned previously.[16] The transcript was transfected in Huh7 cells using lipofectamine (Lipofectamine, 2000, Invitrogen), as described earlier.[16,17] The transfection mixture was replaced with DMEM media containing different concentrations of favipiravir, sofosbuvir, and ribavirin. Post transfection, the cells were incubated for 4−5 days, and the anti-HEV effect of the inhibitors was evaluated using qPCR, IFA, and luciferase assay, as described below.

2.5. Cell Viability. Cell viability in the presence of inhibitors was assessed by using the MTT assay (HiMedia). Huh7 cells were seeded in a 96-well plate, and the next day, the cells were treated with different concentrations of inhibitors ranging from 0 to 5 mM. The cells were incubated with the inhibitors for 72 h, and the MTT assay was performed according to the manufacturer's protocol (HiMedia). Cell viability (%) was calculated in relation to the untreated cells. The normalization was performed by using blank.

2.6. Quantification of Viral RNA. For the quantification of viral RNA copy number, the untreated and treated cells were harvested 5−6 days post transfection. The cells were washed twice with PBS to ensure the removal of any bound viral RNA. Total cellular RNA was extracted from the cell pellet by using the TRIzol reagent (Invitrogen). The RNA was treated with DNase at 37 °C for 30 min, and the DNase-treated RNA was purified using a column purification method (Qiagen, Hilden, Germany). Random hexamer primers were used for the synthesis of cDNA (Verso cDNA synthesis kit) following the manufacturer's protocol. The synthesized cDNA was used for viral RNA quantification through qPCR, which was performed using HEV-ORF2-specific primers.[18] The viral RNA copy number was calculated using a standard equation described previously.[18] The normalization of viral RNA copies was performed by using the host GAPDH mRNA.

2.7. Immunofluorescence Assay. IFA was performed to confirm the effect of the inhibitors on HEV replication. Briefly, Huh7 cells were transfected using lipofectamine with in vitro-transcribed HEV RNA and seeded on glass coverslips in a complete DMEM (Gibco). The cells were then incubated at 37 °C in a CO_2 incubator. Next day, the medium was replaced with DMEM in the wells containing different inhibitor concentrations. After 4−5 days of transfection, the cells were washed using PBS and fixed using 4% PFA (paraformaldehyde) at RT for 10−20 min. The cells were then washed and permeabilized with PBS containing 0.1% triton-X and 3% BSA used for blocking. The cells were further incubated with anti-HEV-ORF2 primary antibody (1:400) for 1 h at 37 °C.[18] After washing with PBS-T (0.05% tween-20), the cells were incubated further with Alexa Fluor 488 antirabbit secondary antibody (1:600). The cells were further stained with DAPI and mounted on a slide using mounting media containing DAPI (Invitrogen). The images were analyzed by using a confocal microscope (Nikon). For fluorescence quantification, the 100 most fluorescing cells from three random different panels were selected, and the mean fluorescence of the selected cells was quantified using ImageJ software.

2.8. Additive Effect of Sofosbuvir and Favipiravir. Approximately 5−7 × 10^3 cells were seeded in a 96-well plate 24 h prior to transfection, and the cells were washed with 1×-PBS and Opti-MEM, as mentioned above. The cells were transfected with the in vitro-transcribed RNA of the HEV

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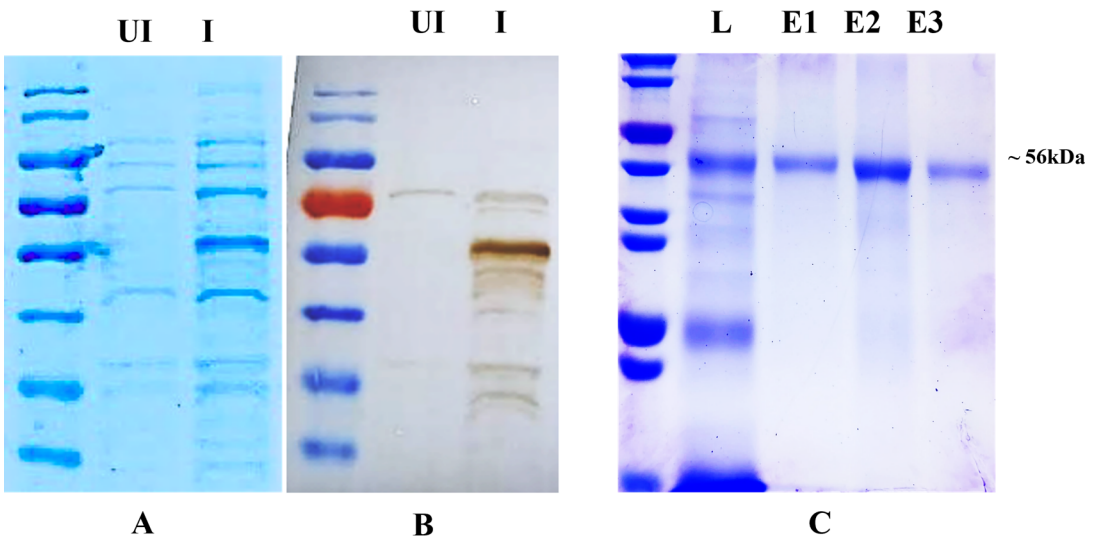

Figure 1. SDS-PAGE and Western blot analysis to check the expression and purification of HEV RdRp. (A,B) SDS-PAGE and Western blot confirming the expression of HEV-RdRp; M, marker; UI, uninduced; I, induced; Western blot was performed using HEV-RdRp epitope-specific antibodies.[18] (C) SDS-PAGE showing the purification of HEV-RdRp; L, load; E1, elution 1; E2, elution 2; E3, elution 3.

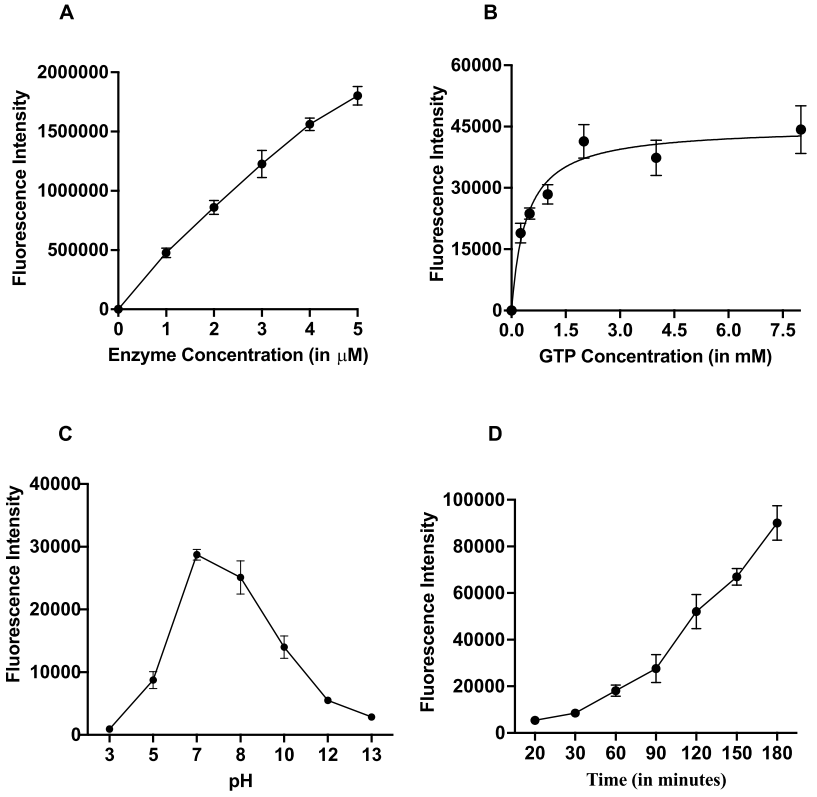

Figure 2. Enzyme activity of purified HEV-RdRp. (A) Upon varying enzyme concentrations. A linear increase in enzyme activity was observed upon increasing the concentration of enzyme from 0 to 5 μM. (B) Upon varying substrate concentration. The enzyme activity was evaluated by varying the concentration of GTP from 0 to 8 mM. The graph was fitted using the Michaelis−Menten equation, and the calculated *Km* was found to be 0.4 mM. (C) At varying pH. The enzyme activity was determined by varying the pH of the reaction buffer, and the maximal activity was reported at pH 7, and a loss of activity was reported under both acidic and alkaline conditions. (D) At various time points. The graph represents the enzyme activity w.r.t. time. All data points in the graph represent the mean or average value of three readings, and the error bars indicate the standard deviation.

replicon using lipofectamine, as mentioned above. After transfection, the cells were incubated for 6−7 h, and subsequently, different concentrations of sofosbuvir (0−50 μM) and favipiravir (0−500 μM) were added along with the complete DMEM by replacing the transfection mixture. Renilla luciferase assay was performed after 72 h, according to the manufacturer's protocol (Promega). The readings were normalized in terms of % HEV replication by assuming 100% replication in the no-compound control and 0% replication in the naïve cells. The SynergyFinder software

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(https://synergyfinder.fimm.fi) was used to determine the effect of the drug combination.[19]

2.9. Statistical Analysis. All the statistical analyses were performed using GraphPad Prism 9.0.0. The statistical significance of data was checked using Student's *t* test (unpaired), and the *p*-value <0.05 was considered significant.

3. RESULTS

3.1. Expression and Purification of HEV RdRp. The expression of HEV-RdRp was seen using SDS-PAGE and Western blot (Figure 1A,B) using the HEV-RdRp epitope-specific antibody.[18] The cells were harvested at 6000*g*, and the bacterial culture pellet was solubilized in the 10 mM Tris−Cl (pH 7.5) buffer, 100 mM NaCl, and 0.5% NLS. The solubilized fraction was filtered and loaded onto a His-Trap column for protein purification and finally eluted with 10 mM Tris−Cl (pH 7.5), 100 mM NaCl, 0.1% NLS, and 250 mM imidazole. The eluted fractions were pooled and concentrated and further loaded on a HiLoad Superdex 75 6/200 pg column, and peak fractions were collected in 10 mM Tris, 100 mM NaCl, and 0.08% NLS. The purified fractions were concentrated and loaded on an SDS-PAGE system, and a band of ∼56 kDa was seen (Figure 1C).

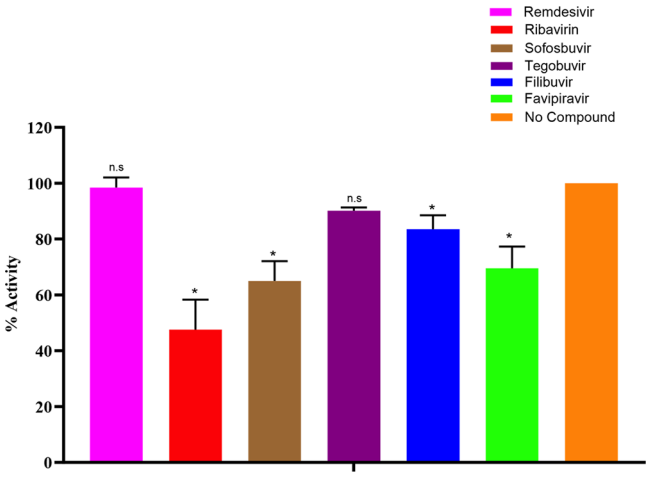

Figure 3. Effect of anti-RdRp drugs on the enzymatic activity of HEV-RdRp. It has been observed that ribavirin, sofosbuvir, favipiravir, and filibuvir significantly reduced the polymerase activity of HEV-RdRp. The graph was plotted in terms of % activity by assuming 100% activity in no compound control and 0% activity in no enzyme control. The significance of the data was checked using unpaired Student's *t* test, and a *p*-value <0.05 was considered statistically significant. *p-value <0.05; n.s. nonsignificant.

Table 1. Percentage Reduction of Viral RNA Copies in Treated Cells with Respect to Untreated Cells

treatment	CC_{50}	% reduction of viral RNA
untreated		0
ribavirin (100 μM)	<100 μM	∼72
sofosbuvir (125 μM)	<125 μM	∼68
favipiravir (500 μM)	<500 μM	∼60
ribavirin:sofosbuvir (1:10) (10: 100 μM)	>50%	∼78
ribavirin:favipiravir (1:10) (25: 250 μM)	>50%	∼60
sofosbuvir:favipiravir (1:10) (50: 500 μM)	>50%	∼85

3.2. Enzyme Activity of HEV-RdRp. Enzymatic kinetics was performed in 96-well black plates (Tarsons). Each reaction was performed in a 25 μL reaction volume, under the conditions mentioned in Materials and Methods. Poly(C) (1 μg) was used as a template, and GTP was used as the substrate to study the enzyme kinetics. The enzymatic activity of RdRp was first studied by varying the enzyme concentration from 0 to 5 μM to optimize the enzyme concentration to be used in studying the various parameters. Accordingly, the concen-

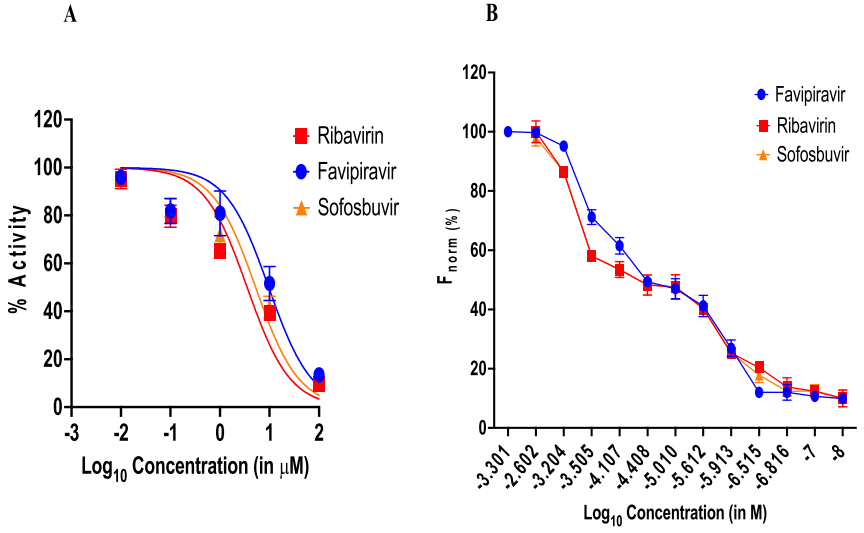

Figure 4. IC_{50} determination and MST. (A) IC_{50} determination of ribavirin, sofosbuvir, and favipiravir. It represents the mean or average values of triplicate measurements for IC_{50} determination of ribavirin, sofosbuvir, and favipiravir, respectively. IC_{50} was determined by fitting the curve using log[inhibitor] vs the normalized response. It has been observed that ribavirin, sofosbuvir, and favipiravir inhibit the HEV-RdRp activity, with IC_{50} values of 3.5 ± 1.6, 5.2 ± 2.9, and 10.2 ± 4.9 μM, respectively. (B) MST. It represents the dose−response curve for determining the dissociation constant (K_D) for ribavirin, sofosbuvir, and favipiravir with RdRp and was found to be 30 nM, 15 nM, and 39 μM, respectively. The graphs represent the mean values of three individual experiments, and the error bars represent the standard deviation.

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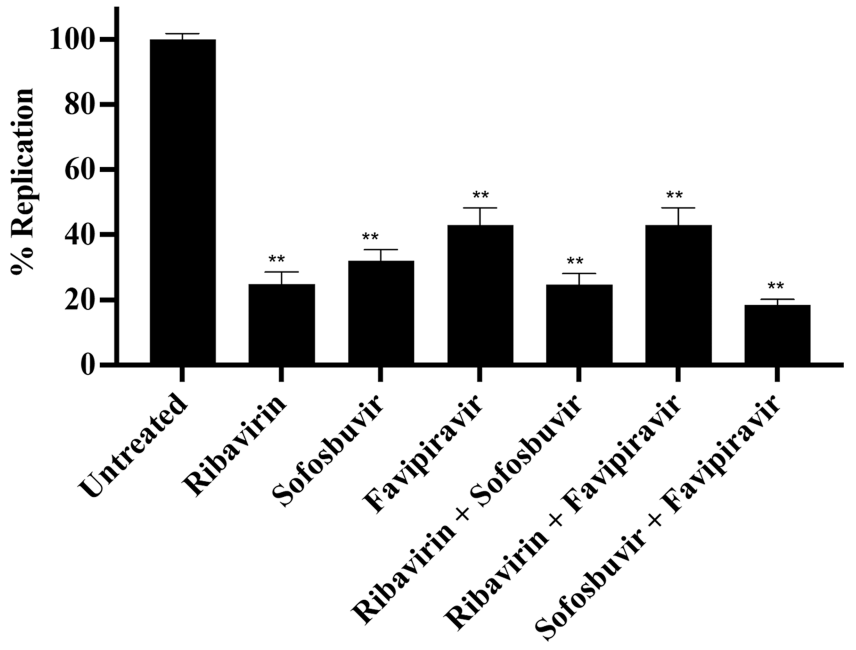

Figure 5. Percentage of viral RNA replication in treated cells with respect to the untreated cells (100% viral replication). The viral RNA copies determined using qPCR have been transformed to % replication by assuming 100% replication in untreated cells. The graph represents the mean value of the percentage replication of three independent experiments, and the error bar represents the standard deviation. Student's *t* test was used to check the statistical significance of the data, and a *p*-value <0.05 was considered statistically significant. ** *p*-value <0.01.

trations of the template and substrate were kept constant at 1 μg and 0.4 mM, respectively. A linear increase in the enzyme activity was observed with increasing enzyme concentration (Figure 2A). The effect of the substrate concentration (GTP) was also studied by varying its concentration from 0 to 8 mM, keeping the enzyme concentration at 5 μM. The K_m value of the equation was derived using the Michaelis−Menten equation, which was found to be 0.4 mM (Figure 2B). To determine the optimal pH of the rection, the enzyme activity was conducted under different pH conditions. The pH of the buffer was varied from 3 to 13, and the maximal activity was found to be at pH 7, leading to a loss of activity at more acidic or alkaline pH (Figure 2C). The enzyme activity was also studied for different times where the reaction was incubated for different time points from 20 to 180 min and subsequently stopped using 1 mM EDTA (Figure 2D). The enzyme conditions were optimized, and further all the reactions were performed using 5 μM of the purified enzyme and 0.4 mM GTP at pH 7.5 for 3−4 h. To further validate the RdRp activity, the activity was performed in the presence of actinomycin-D, which is an inhibitor of all DNA-dependent polymerases. 5 μM actinomycin-D was added to the reaction mixture, and no significant effect on the enzyme activity of RdRp was observed in the presence or absence of actinomycin-D (Supporting Information).

3.3. Effect of Compounds on RdRp Activity. RdRp has always been considered the most promising drug target since it is a vital component of viral RNA replication. To investigate the potential antiviral activity against HEV, we selected a few anti-RdRp compounds that are available in house. We evaluated ribavirin, sofosbuvir, remdesivir, favipiravir, filibuvir, and tegobuvir on the enzyme activity of purified RdRp. It has been observed that ribavirin, sofosbuvir, favipiravir, and filibuvir reduced the polymerase activity of HEV-RdRp (Figure 3). Based on the inhibition profile, we selected ribavirin, sofosbuvir, and favipiravir for further studies.

3.4. IC_{50} Determination and the Binding Affinity of Ribavirin, Sofosbuvir, and Favipiravir. Since sofosbuvir, favipiravir, and ribavirin effectively inhibited the activity of purified RdRp, they were further assessed for the determination of IC_{50} and dissociation constant (K_D) of these compounds with HEV-RdRp. To determine the IC_{50} value, their concentrations were varied from 10 to 100 μM, and they were incubated with 5 μM purified RdRp in the reaction buffer for 30−40 min. Subsequently, the template poly(C) and substrate (GTP) were added, and the reaction was incubated for 3−4 h. The quantification was performed, as described above. GraphPad Prism was used for the determination of the IC_{50} value by fitting the curve using log[inhibitor] versus normalized response, and it has been seen that ribavirin, sofosbuvir, and favipiravir inhibit the HEV-RdRp activity with the IC_{50} values of 3.5 ± 1.6, 5.2 ± 2.9, and 10.2 ± 4.9 μM respectively (Figure 4A).

Furthermore, the binding affinity of these compounds to RdRp was studied by using microscale thermophoresis (MST). The concentration of ribavirin and sofosbuvir was varied from 500 μM to 150 pM. For favipiravir, the drug concentration varied from 5 mM to 300 nM (Figure 4B), representing the dose−response curves for ribavirin, sofosbuvir, and favipiravir, respectively. The *Y*-axis of the graph represents the normalized fluorescence (%), and the *X*-axis represents the ligand concentration. The K_D values for ribavirin, sofosbuvir, and favipiravir were found to be approximately 30 nM, 15 nM, and 39 μM, respectively. The graph represents the mean value of three individual experiments, and the error bars represent the standard deviation (Figure 4B).

3.5. Effect of Ribavirin, Sofosbuvir, and Favipiravir on HEV Replication Using qPCR. Ribavirin, sofosbuvir, and favipiravir effectively inhibited the activity of purified RdRp; hence, they were used to study their effects on HEV RNA replication. Huh7 cells were transfected with whole HEV RNA and treated with ribavirin, sofosbuvir, and favipiravir alone and

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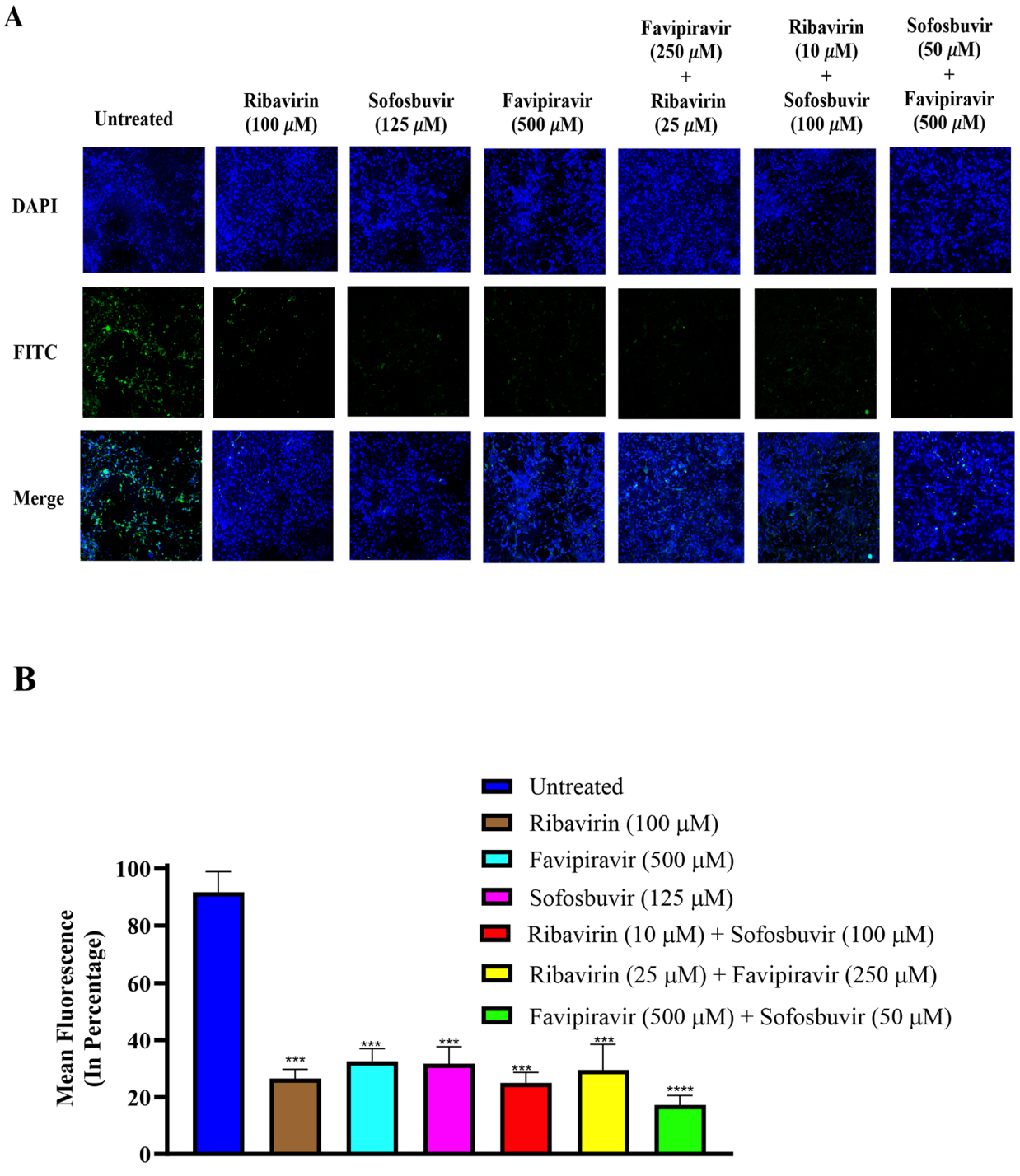

Figure 6. Immunofluorescence assay. (A) DAPI, FITC (ORF2), and merged panels of untreated cells and cells treated with ribavirin, sofosbuvir, and favipiravir and their combinations. (B) Graph represents the % mean fluorescence intensity of the treated cells with respect to the untreated cells. The maximum reduction in viral replication was observed in cells treated with sofosbuvir and favipiravir (1:10; 50:500 μM). Fluorescence was quantified from three different panels, and the error bars indicate the standard deviation. Student's t test was used to check the statistical significance of the data, and a p-value <0.05 was considered statistically significant. ***p-value <0.001 and ****p-value <0.0001.

in combination. The treatment was performed based on CC_{50} values, as mentioned in Table 1.

The cells were harvested 5 days post transfection, and the total cellular RNA was isolated. cDNA was synthesized from the isolated RNA and used for viral copy number quantification by qPCR. For IFA, the cells were grown on coverslips and processed further, as mentioned earlier. It was observed that there was a significant reduction in viral copies in treated cells compared to that in untreated cells. The maximum reduction was observed when sofosbuvir and favipiravir were used in combination (1,10; 50:500 μM). However, there was only a 60% reduction in the number of viral RNA copies when favipiravir (500 μM) was used for treatment. The % reduction of viral RNA copies upon various treatments has been mentioned in Table 1, and Figure 5 represents the % replication of viral RNA w.r.t. the untreated

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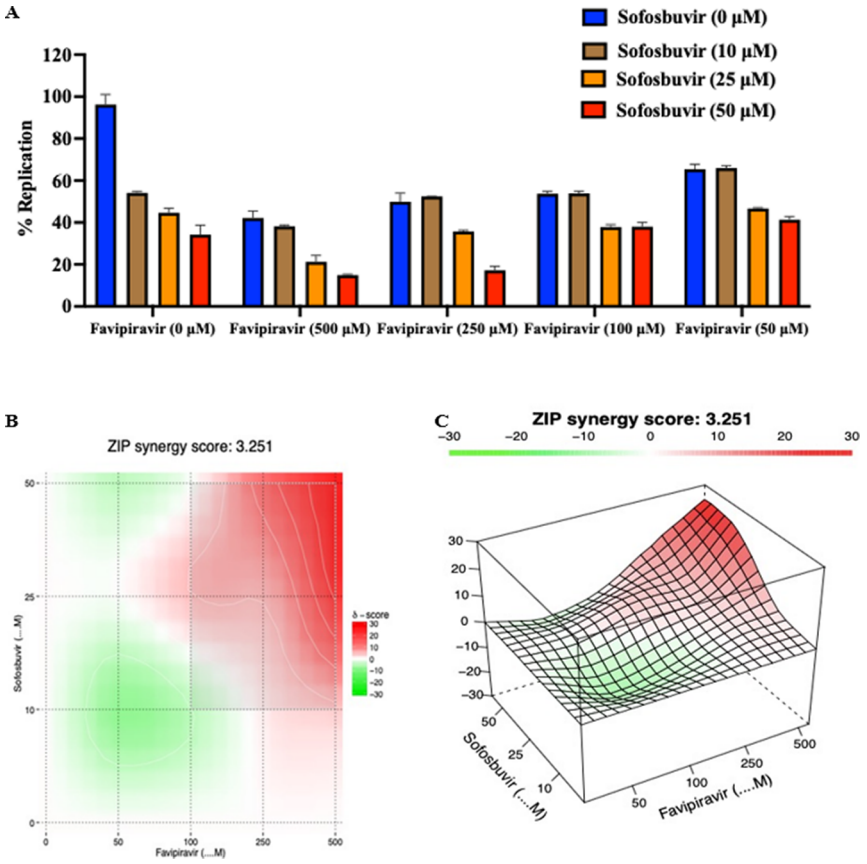

Figure 7. Combinatorial effects of sofosbuvir and favipiravir. (A) % replication of HEV replicons in treated cells was calculated with respect to the untreated cells. (B) Synergy plot describing the additive effect of sofosbuvir and favipiravir with a ZIP synergy score of 3.251. (C) Synergy plot represents the percentage of antiviral activity above or below the expected activity of sofosbuvir and favipiravir. It has been observed that the combination of sofosbuvir and favipiravir leads to an additive antiviral effect on HEV-RNA replication.

cells. The viral RNA copies determined by qPCR were transformed to % replication by assuming 100% replication in untreated cells.

3.6. Effect of Ribavirin, Sofosbuvir, and Favipiravir on HEV Replication Using Immunofluorescence. The anti-HEV-ORF2 epitope-specific antibodies were used to detect the expression of ORF2 (a marker of HEV replication) in untreated and treated cells. Figure 6 represents the ORF2 expression in untreated and treated cells. For quantification, 100 cells from different panels were selected, and the mean fluorescence intensity was measured. Figure 6B represents the percentage of the mean fluorescence intensity of treated cells with respect to untreated cells. The maximum reduction in viral replication was also observed in cells treated with sofosbuvir and favipiravir (1:10; 50:500 μM). Thus, while favipiravir significantly inhibited HEV replication, its effectiveness increased when used in combination with sofosbuvir.

3.7. Combinatorial Effect of Sofosbuvir and Favipiravir. The combination of sofosbuvir and favipiravir was further investigated by using transient HEV replication using in vitro transcribed HEV-p6 luciferase RNA. The transfected cells were treated with different combinations of sofosbuvir (0−50 μM) and favipiravir (0−500 μM). Renilla luciferase activity was further quantified 3 days post treatment, and the combinatorial effect of the drugs was calculated using Synergyfinder2.0. Figure 7A represents the % replication of HEV replicon in treated cells compared to the untreated cells. The ZIP synergy score was calculated using Synergyfinder2.0 (Figure 7B). Figure 7C shows the synergy plot indicating the antiviral activity (%) above or below the expected activity for sofosbuvir and favipiravir. It has been observed that the combination of sofosbuvir and favipiravir results in an additive anti-HEV effect with a ZIP synergy score of 3.251.

4. DISCUSSION

Little is known about the HEV-encoded replicase proteins, their enzyme activities underlying viral life cycle, and pathogenesis. Therefore, an in-depth understanding of viral pathobiology through the characterization of its enzyme could be helpful in the development of direct-acting anti-HEV drugs. The present study is an attempt to characterize the HEV-ORF1-encoded RdRp and identify its potential inhibitor molecules. We assessed the impact of several FDA-approved RdRp inhibitor compounds, including favipiravir,[20] remdesivir,[21] tegobuvir, filibuvir, sofosbuvir, and ribavirin known for their antiviral activities. We found that favipiravir, sofosbuvir, filibuvir, and ribavirin significantly inhibited the in vitro expressed and purified HEV-RdRp activity in the replicon cell culture model. However, remdesivir, recently approved as an emergency drug for the treatment of COVID-19, did not show any effect on HEV-RdRp activity. In addition to this, we also studied the effect of lamivudine, a nucleoside analogue that inhibits viral reverse-transcriptases, and is used for the treatment of hepatitis B virus and HIV.[22] In our assay, although lamivudine inhibited the in vitro enzyme activity of HEV-RdRp, it did not show any effect on viral replication.

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Finally, we selected three RdRp inhibitors, favipiravir, sofosbuvir, and ribavirin to further assess their effect on HEV RNA replication. Ample reports suggest that sofosbuvir and ribavirin effectively inhibit HEV replication, when used as combination therapy.[23] Notably, while sofosbuvir with or without ribavirin has also been undertaken to clinical trials,[24,25] the anti-HEV efficacy of favipiravir alone or in combination with ribavirin has not been studied so far. In a recent study, favipiravir has been shown to inhibit the replication of hepatitis A virus.[26] In previous studies, the role of RdRp inhibitor drugs, including the combination of NITD008 and GPC-N114 in HEV replication, has been reported.[27] In our study, favipiravir at 500 μM markedly inhibited HEV replication where an ∼60% decrease in viral RNA copies was observed. Notably, when used in combination with sofosbuvir, it further suppressed viral RNA copies by ∼85% Hence, to study the combinatorial effect of favipiravir and sofosbuvir, their different combination ratios were also evaluated, wherein an additive effect was observed with a ZIP score of 3.2. Previous studies have indicated that higher concentrations of favipiravir can exhibit antiviral effects by efficiently inhibiting viral replication.[28−30] Although the dosage of favipiravir used in our study was relatively higher, it reasonably aligned with these findings and supported its potential as an ant6i-HEV drug.

Thus, our study aimed to explore potential therapeutic drugs for treating HEV. Subsequently, future research could focus on conducting animal or preclinical trials to assess the effectiveness of favipiravir alone and in combination with sofosbuvir. These drugs exhibit promise as potential candidates to combat HEV infection. Additionally, gaining a deeper understanding of the role of HEV RdRp in viral replication may bring us closer to developing effective treatments that improve the outcomes for patients with hepatitis E.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05637.

RdRp activity in the presence of actinomycin-D (PDF)

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■ ABBREVIATIONS

HEV, hepatitis E virus; RdRp, RNA-dependent RNA polymerase; K_D, dissociation constant; K_m, Michaelis−Menten constant

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