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Antiviral activity of ribavirin and favipiravir against human astroviruses

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Background

Long considered to be pathogens exclusive to the gastrointestinal tract, astroviruses have been the focus of renewed interest due to the recognition of invasive infection in humans and other mammals, including central nervous system (CNS) infection and viremia[1–4]. While gastrointestinal astrovirus infection is a self-limited disease, six out of ten cases of human meningoencephalitis caused by astroviruses have resulted in death [5–12]. Currently, there are no drugs approved by the US or European regulatory agencies for treatment of astrovirus infections, and there are no published treatment trials of astrovirus infections. It is unclear what role broad-spectrum antivirals may against astroviruses because they have not been empirically tested *in vitro* or *in vivo.* In one case of survival from astrovirus encephalitis, the patient received intravenous immunoglobulin, steroids, ribavirin, and pegylated interferon-α2b[5]. In a fatal case, the patient received intravenous immunoglobulin and ribavirin prior to death[6]. Flavonoids and immunomodulatory drugs like interferon-α/β have also demonstrated efficacy in reducing astrovirus replication in cell culture[13–16].

Ribavirin is the prototypical broad-spectrum antiviral as it has multiple mechanisms of action against many RNA viruses, including direct inhibition of the RNA-dependent RNA polymerase (RdRp)[17]. Ribavirin has *in vitro* activity against noroviruses (10 μM caused 64% reduction in human norovirus replication) and enteroviruses (EC_{50} range of 106 to >1100μM), members of two viral families that share phylogenetic relationships to the

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Credit author statement

Andrew Janowski: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft, Visualization. Holly Dudley: Investigation, Formal analysis. David Wang: Conceptualization, Resources, Writing- Review and Editing, Supervision.

Conflict of Interest Declaration

None to declare

astrovirus RdRp, but the activity of ribavirin against astroviruses has not been quantified[18–20]. Favipiravir also has *in vitro* antiviral activity against many RNA viruses, including enteroviruses and noroviruses (EC_{50} range 31–248μM); however, the efficacy of favipiravir against astroviruses has also not been determined[21].

Objectives

We quantified the effect of both ribavirin and favipiravir on the *in vitro* replication of two astrovirus genotypes, astrovirus VA1 (VA1) and human astrovirus 4 (HAstV4), two strains that have been associated with central nervous system disease in humans[2].

Study Design

Ribavirin (Sigma-Aldrich) was suspended in Dulbecco's Modified Eagle Media (DMEM, Gibco) with serial dilutions performed to generate dosages over a range of 0.1–1000μM. Due to poor water solubility, favipiravir (Biovision) was suspended in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 5mM with serial dilutions made into DMEM over a range of 0.1–1000μM. All cells treated with favipiravir had 1% DMSO (v/v) added to each well to normalize to the DMSO concentration present in 1000μM of favipiravir.

Caco-2 cells were grown to confluence in 96-well plates. Both VA1 and HAstV4 stocks were originally isolated from stool specimens and propagated in Caco-2 cells [13, 22]. HAstV4 was pretreated with 100μg/mL of trypsin for 30 minutes as HAstV4 but not VA1 requires trypsin for propagation in cell culture[13, 23]. Cells were infected with a MOI 0.01 of VA1, HAstV4, or Influenza A WSN (IAV) in the presence of ribavirin or favipiravir for one hour, the cells were then washed once, and growth media added to the cells containing ribavirin or favipiravir[22, 24]. IAV was used to confirm the efficacy of both drugs. For VA1, the cells were maintained in growth media containing DMEM with 10% fetal bovine serum (FBS), For HAstV4 and IAV infections, the cells were maintained in DMEM with 3.3μg/mL of trypsin and no FBS. At 72 hours post-inoculation, the supernatant was removed, TRIzol (ThermoFisher) was added to the cells, and RNA extracted using Direct-zol 96-well RNA extraction plates (Zymo Research). VA1, HAstV4, and IAV RNA were quantified using previously published qRT-PCR assays[13, 22, 25]. Viral RNA cycle threshold (CT) values were normalized to the CT values for the housekeeping gene, Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0), using the delta CT method and then normalized to no drug treatment using the delta-delta CT method[26]. Geometric means of the change in RNA relative to no treatment was graphed in Prism 8.1.0 (GraphPad) and compared using one-way or Brown-Forsythe ANOVA tests with post-hoc analysis by Dunnett's with or without T3 multiple comparisons test. The 50% effective concentration (EC_{50}) values were calculated using Prism. Cells treated with favipiravir or ribavirin in the absence of virus using the aforementioned media conditions for 72 hours were also analyzed by the CellTiter-Glo assay (Promega) for quantification of toxicity. Mean relative luminescence units (RLU) were plotted in GraphPad. Because we did not detect >50% cytotoxicity over the concentrations of the drugs tested, the lower limit 50% cytotoxicity concentration (CC_{50}) value was set at 1000μM for calculation of selectivity indices (SI; CC_{50} value divided by

EC_{50} value). For all analyses, two independent experiments were conducted with 3 replicates per experiment.

Results and discussion

Ribavirin treatment of Caco-2 cells infected with VA1 or HAstV4 caused a 10–100-fold reduction of viral RNA with greater effect on VA1 replication (Figure 1). The EC_{50} value was also lower for VA1 (154μM) compared to HAstV4 (268μM) (Table 1). Only mild toxicity of ribavirin was observed at 1000μM as a 17–31% decrease in luminescence in the CellTiter-Glo assay was observed (Figure 2; $P \leq 0.004$). Favipiravir treatment resulted in a 10-fold reduction of VA1 replication, but in contrast, only a 44% reduction of HAstV4 replication was observed at 1000μM (Figure 1). No significant reduction of cell viability was observed at any concentration of favipiravir (Figure 2). We confirmed the activity of our formulations of ribavirin and favipiravir as both significantly inhibited IAV with EC_{50} values consistent with previously published values (Table 1)[21, 27].

The recent recognition of fatal astrovirus infections in humans has highlighted a greater need effective anti-astrovirus therapies. Previously, there was no *in vitro* data regarding the inhibitory effect of broad-spectrum antivirals on astroviruses. Our results demonstrate that both ribavirin and favipiravir inhibit replication of VA1 and ribavirin inhibits HAstV4 in cell culture. The *in vitro* EC_{50} values identified for both drugs have similar activities for other enteric viruses like enteroviruses and noroviruses[18, 19, 21].

Interestingly, very modest inhibition of HAstV4 with favipiravir was observed, despite the drug demonstrating greater activity against VA1 and IAV. The cause of this differential effect of favipiravir could be specific to differences in the RdRp of HAstV4 (AZB52200.1), which shares 52.3% amino acid identity with VA1 (YP_003090286.1). Resistance to favipiravir has been described in other viruses with amino acid substitutions in the RdRp[28]. Alternatively, HAstV4 may utilize unique host factors for replication that are not inhibited by favipiravir.

Currently, there are no animal models of human astrovirus infection, precluding our ability perform *in vivo* efficacy studies. The ribavirin and favipiravir EC_{50} concentrations for VA1 are comparable to the EC_{50} concentrations identified for yellow fever virus (YFV), and both drugs were found to be protective in a hepatitis model of fatal YFV infection[29]. Nonetheless, effective treatment of astrovirus infection of the central nervous system may require penetration of the blood-brain barrier (BBB) by the candidate antiviral. There is conflicting data whether ribavirin is able to penetrate the human BBB while the penetration of favipiravir into the CNS has not been described[30].

We did not observe significant cytopathic effect with our drug concentrations of ribavirin or favipiravir, with or without trypsin/FBS, consistent with other previously published CC_{50} values that were also >1000μM [18, 19, 21]. We did observe a small increase in cellular adenosine triphosphate (ATP) in the CellTiter-Glo assay in ribavirin treated cells at 10–200μM (Figure 2), consistent with a previous publication[31]. Because of the limits of solubility and the need to maintain sufficient volume of cell culture media, we did not achieve 50% cytopathic effect with the drug concentrations that were tested, so our

estimation of the SI is an underestimate. Nonetheless, the calculated SIs indicate the likelihood of a narrow therapeutic window for these drugs *in vivo* and it could be difficult to achieve serum concentrations for sufficient inhibition of astrovirus replication. In future studies, the possibility of additive or synergistic effects of combination therapy of ribavirin and favipiravir can be evaluated, and the addition of recombinant interferon may also enhance the activity of either drug. Future studies may also lead to identification of novel antivirals that are astrovirus specific.

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References

[1]. Bosch A, Pinto RM, Guix S. Human astroviruses. Clinical microbiology reviews. 2014;27:1048–74. [PubMed: 25278582]

[2]. Reuter G, Pankovics P, Boros A. Nonsuppurative (Aseptic) Meningoencephalomyelitis Associated with Neurovirulent Astrovirus Infections in Humans and Animals. Clinical microbiology reviews. 2018;31.

[3]. Selimovic-Hamza S, Boujon CL, Hilbe M, Oevermann A, Seuberlich T. Frequency and Pathological Phenotype of Bovine Astrovirus CH13/NeuroS1 Infection in Neurologically-Diseased Cattle: Towards Assessment of Causality. Viruses. 2017;9.

[4]. Matias Ferreyra FS, Bradner LK, Burrough ER, Cooper VL, Derscheid RJ, Gauger PC, et al. Polioencephalomyelitis in Domestic Swine Associated With Porcine Astrovirus Type 3. Vet Pathol. 2019:300985819875741.

[5]. Fremond ML, Perot P, Muth E, Cros G, Dumarest M, Mahlaoui N, Seilhean D, Desguerre I, Hebert C, Corre-Catelin N, Neven B, Lecuit M, Blanche S, Picard C, Eloit M Next-Generation Sequencing for Diagnosis and Tailored Therapy: A Case Report of Astrovirus-Associated Progressive Encephalitis. Journal of the Pediatric Infecctious Diseases Society 2015:1–5.

[6]. Naccache SN, Peggs KS, Mattes FM, Phadke R, Garson JA, Grant P, et al. Diagnosis of neuroinvasive astrovirus infection in an immunocompromised adult with encephalitis by unbiased next-generation sequencing. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2015;60:919–23.

[7]. Quan PL, Wagner TA, Briese T, Torgerson TR, Hornig M, Tashmukhamedova A, et al. Astrovirus encephalitis in boy with X-linked agammaglobulinemia. Emerging infectious diseases. 2010;16:918–25. [PubMed: 20507741]

[8]. Lum SH, Turner A, Guiver M, Bonney D, Martland T, Davies E, et al. An emerging opportunistic infection: fatal astrovirus (VA1/HMO-C) encephalitis in a pediatric stem cell transplant recipient. Transplant infectious disease : an official journal of the Transplantation Society. 2016.

[9]. Brown JR, Morfopoulou S, Hubb J, Emmett WA, Ip W, Shah D, et al. Astrovirus VA1/HMO-C: an increasingly recognized neurotropic pathogen in immunocompromised patients. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2015;60:881–8. [PubMed: 25572899]

[10]. Sato M, Kuroda M, Kasai M, Matsui H, Fukuyama T, Katano H, et al. Acute encephalopathy in an immunocompromised boy with astrovirus-MLB1 infection detected by next generation sequencing. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology. 2016;78:66–70. [PubMed: 26991054]

[11]. Cordey S, Vu DL, Schibler M, L'Huillier AG, Brito F, Docquier M, et al. Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection. Emerging infectious diseases. 2016;22:846–53. [PubMed: 27088842]

[12]. Wilson MR, Sample HA, Zorn KC, Arevalo S, Yu G, Neuhaus J, et al. Clinical Metagenomic Sequencing for Diagnosis of Meningitis and Encephalitis. The New England journal of medicine. 2019;380:2327–40. [PubMed: 31189036]

[13]. Janowski AB, Bauer IK, Holtz LR, Wang D. Propagation of astrovirus VA1, a neurotropic human astrovirus, in cell culture. Journal of virology. 2017.

[14]. Guix S, Perez-Bosque A, Miro L, Moreto M, Bosch A, Pinto RM. Type I interferon response is delayed in human astrovirus infections. PloS one. 2015;10:e0123087. [PubMed: 25837699]

[15]. Marvin SA, Huerta CT, Sharp B, Freiden P, Cline TD, Schultz-Cherry S. Type I Interferon Response Limits Astrovirus Replication and Protects against Increased Barrier Permeability In Vitro and In Vivo. Journal of virology. 2016;90:1988–96. [PubMed: 26656701]

[16]. Superti F, Seganti L, Orsi N, Desideri N, Stein ML, Tinari A, et al. In vitro effect of synthetic flavanoids on astrovirus infection. Antiviral Res. 1990;13:201–8. [PubMed: 2116755]

[17]. Graci JD, Cameron CE. Mechanisms of action of ribavirin against distinct viruses. Reviews in medical virology. 2006;16:37–48. [PubMed: 16287208]

[18]. Dang W, Yin Y, Wang Y, Wang W, Su J, Sprengers D, et al. Inhibition of Calcineurin or IMP Dehydrogenase Exerts Moderate to Potent Antiviral Activity against Norovirus Replication. Antimicrobial agents and chemotherapy. 2017;61.

[19]. Smee DF, Evans WJ, Nicolaou KC, Tarbet EB, Day CW. Susceptibilities of enterovirus D68, enterovirus 71, and rhinovirus 87 strains to various antiviral compounds. Antiviral Res. 2016;131:61–5. [PubMed: 27063860]

[20]. Wolf YI, Kazlauskas D, Iranzo J, Lucia-Sanz A, Kuhn JH, Krupovic M, et al. Origins and Evolution of the Global RNA Virome. mBio. 2018;9.

[21]. Furuta Y, Komeno T, Nakamura T. Favipiravir (T-705), a broad spectrum inhibitor of viral RNA polymerase. Proc Jpn Acad Ser B Phys Biol Sci. 2017;93:449–63.

[22]. Janowski AB, Klein RS, Wang D. Differential In Vitro Infection of Neural Cells by Astroviruses. mBio. 2019;10.

[23]. Lee TW, Kurtz JB. Serial propagation of astrovirus in tissue culture with the aid of trypsin. The Journal of general virology. 1981;57:421–4. [PubMed: 6798168]

[24]. Janowski AB, Wang D. Infection and Propagation of Astrovirus VA1 in Cell Culture. Curr Protoc Microbiol. 2018:e73. [PubMed: 30444308]

[25]. Kawakami E, Watanabe T, Fujii K, Goto H, Watanabe S, Noda T, et al. Strand-specific real-time RT-PCR for distinguishing influenza vRNA, cRNA, and mRNA. Journal of virological methods. 2011;173:1–6. [PubMed: 21185869]

[26]. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402–8. [PubMed: 11846609]

[27]. Sidwell RW, Bailey KW, Wong MH, Barnard DL, Smee DF. In vitro and in vivo influenza virus-inhibitory effects of viramidine. Antiviral Res. 2005;68:10–7. [PubMed: 16087250]

[28]. Delang L, Segura Guerrero N, Tas A, Querat G, Pastorino B, Froeyen M, et al. Mutations in the chikungunya virus non-structural proteins cause resistance to favipiravir (T-705), a broad-spectrum antiviral. The Journal of antimicrobial chemotherapy. 2014;69:2770–84. [PubMed: 24951535]

[29]. Julander JG, Shafer K, Smee DF, Morrey JD, Furuta Y. Activity of T-705 in a hamster model of yellow fever virus infection in comparison with that of a chemically related compound, T-1106. Antimicrobial agents and chemotherapy. 2009;53:202–9. [PubMed: 18955536]

[30]. Rhoades RE, Tabor-Godwin JM, Tsueng G, Feuer R. Enterovirus infections of the central nervous system. Virology. 2011;411:288–305. [PubMed: 21251690]

[31]. Shah NR, Sunderland A, Grdzelishvili VZ. Cell type mediated resistance of vesicular stomatitis virus and Sendai virus to ribavirin. PloS one. 2010;5:e11265. [PubMed: 20582319]

Highlights

- Ribavirin inhibits replication of astrovirus VA1 and classic human astrovirus 4.
- Replication of astrovirus VA1 is reduced by favipiravir
- No significant cytotoxicity was detected for favipiravir or ribavirin

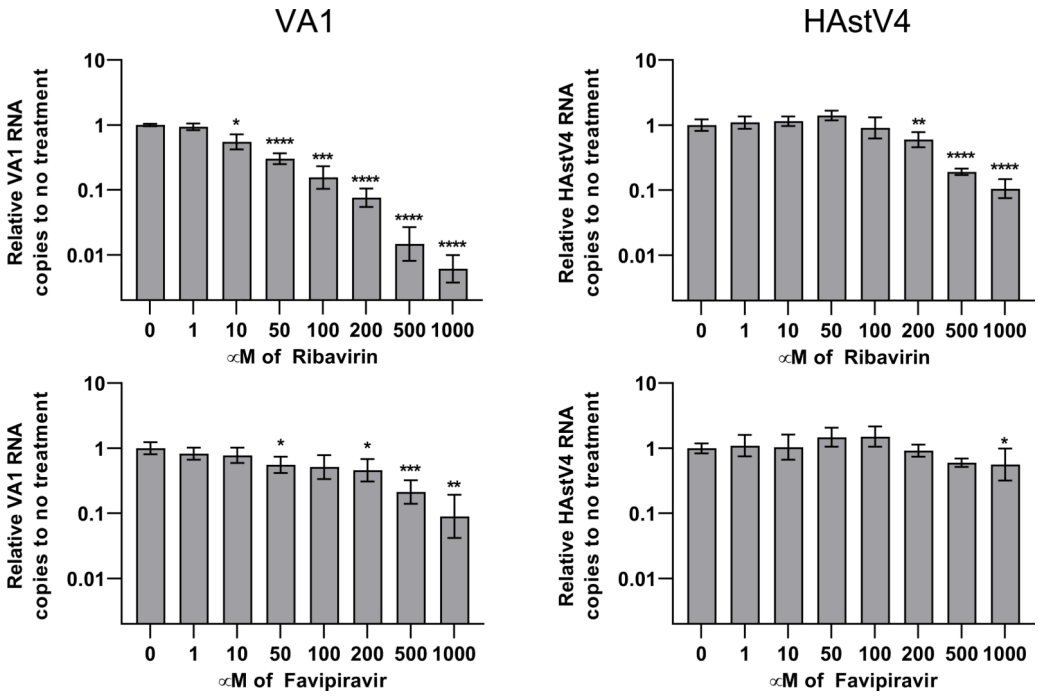

Figure 1:

Quantification of VA1 or HAstV4 RNA upon treatment with ribavirin or favipiravir, normalized to no drug treatment. Error bars represent one geometric standard deviation. *, P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001.

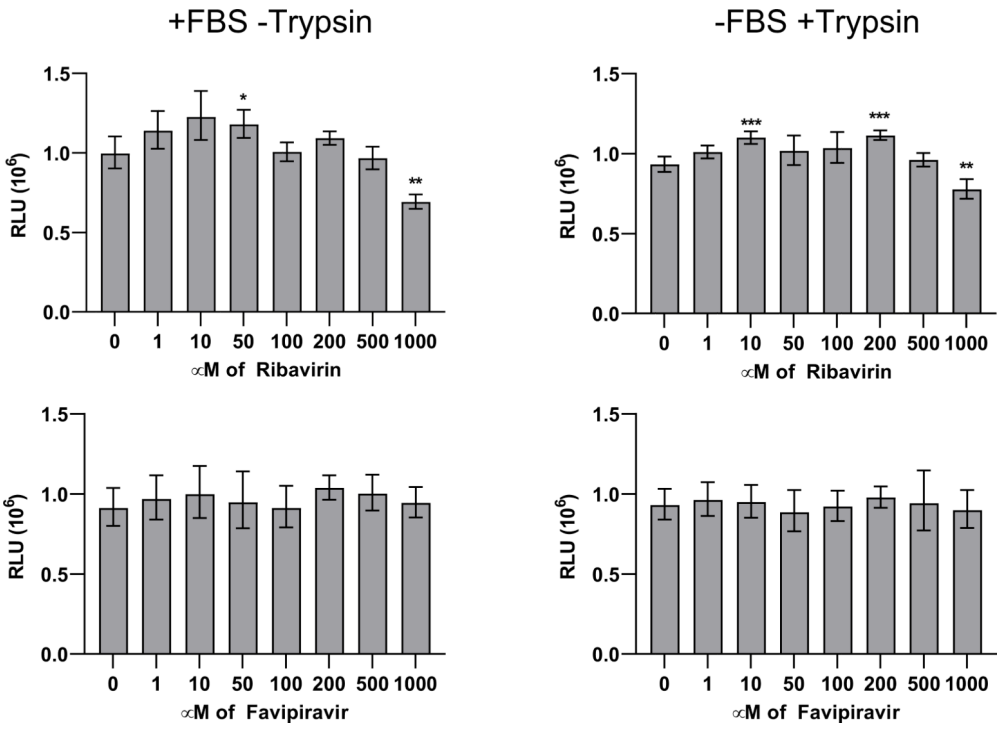

Figure 2:

Measurement of luminescence (RLU, relative luminescence units) for each antiviral drug concentration, reflecting the number of viable cells in each treatment group. Error bars represent one geometric standard deviation. *, P≤0.05; **, P≤0.01; ***, P≤0.001.

Table 1:

Calculated EC_{50} concentrations in μM of each virus-antiviral treatment with ±95% confidence intervals and selectivity indices (SI).

	VA1		HAstV4		IAV	
	EC_{50}	**SI**	**EC_{50}**	**SI**	**EC_{50}**	**SI**
Ribavirin	154 μM ± 21	>6.5	268 μM ± 39.5	>3.7	4.54 μM ± 0.97	>220
Favipiravir	246 μM ± 76.4	>4.1	>1000 μM	1	4.73 μM ± 1.01	>211