Astrovirus Replication Is Inhibited by Nitazoxanide *In Vitro* and *In Vivo*

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Running Head: Nitazoxanide Inhibits Astrovirus Replication

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Abstract Word Count: 186

Text Word Count: 2749

Abstract

Astroviruses (AstV) are a leading cause of diarrhea especially in the very young, the elderly, and immunocompromised populations. Despite their significant impact on public health, no drug therapies for astrovirus have been identified. In this study we fill this gap in knowledge and demonstrate that the FDA-approved broad-spectrum anti-infective drug nitazoxanide (NTZ) blocks astrovirus replication *in vitro* with a 50% effective concentration (EC_{50}) of approximately 1.47μM. It can be administered up to 8 hours post-infection and is effective against multiple human astrovirus serotypes including clinical isolates. Most importantly, NTZ reduces viral shed and clinical disease (diarrhea) *in vivo*, exhibiting its potential as a future clinical therapeutic.

Importance

Human astroviruses (HAstV) are thought to cause between 2 and 9% of acute, non-bacterial diarrhea cases in children worldwide. HAstV infection can be especially problematic in immunocompromised people and infants where the virus has been associated with necrotizing enterocolitis, severe and persistent diarrhea, as well as systemic and often fatal disease. Yet no antivirals have been identified to treat astrovirus infection. Our study provides the first evidence that nitazoxanide may be an effective therapeutic strategy against astrovirus disease.

Introduction

Diarrheal disease is the second leading cause of death in children under 5 years of age, with nearly 1.7 billion cases and 525,000 deaths each year (1). Since their discovery in 1975, human astroviruses have consistently ranked among the leading causes of diarrhea worldwide (2). However, human astrovirus infections can range anywhere from asymptomatic to mild diarrhea and even fatal systemic disease (3, 4). Infections in immunocompetent individuals typically present as watery diarrhea that resolves within 1 to 3 days post-infection without the need for hospitalization (2). Astrovirus outbreaks of frequently occur in assisted living facilities, hospitals and child care centers, where the young, elderly, and immunocompromised

53 populations are at risk of persistent diarrhea leading to wasting (5), and extra-gastrointestinal
54 disease that requires medial intervention, including respiratory disease (6–10), and fatal
55 encephalitis, and meningitis (11).

56 Despite its high prevalence and the risk of severe disease, no vaccines or drug
57 treatments exist for astrovirus. Only oral or parenteral fluids and electrolytes available to prevent
58 and treat dehydration caused by astrovirus-induced diarrhea. In these studies, we provide the
59 first evidence that nitazoxanide (NTZ), an FDA-approved broad-spectrum anti-parasitic and anti-
60 viral drug, inhibits the replication of multiple strains of human astrovirus *in vitro* even when
61 administered up to 8 hours post-infection, and reduces viral shed and diarrhea *in vivo*. This work
62 highlights the potential use of NTZ as an effective therapeutic strategy against astrovirus
63 infection.

64

65 **Results**

66 **Nitazoxanide blocks astrovirus replication *in vitro* by inhibiting dsRNA production.** To
67 identify an effective antiviral drug against astrovirus, human colon carcinoma (Caco-2) cells
68 were infected with a laboratory strain of human astrovirus strain-1(HAstV-1) at a multiplicity of
69 infection (MOI) of 1 before increasing concentrations of foscarnet, ribavirin, acyclovir,
70 nitazoxanide or DMSO (vehicle control) were added 1 hour post-infection (hpi). Viral capsid
71 protein levels were quantitiated at 24 hpi by immunofluorescent microscopy as described (12).
72 Foscarnet, ribavirin, and acyclovir failed to inhibit HAstV-1 replication even at concentrations of
73 250 μM (Figure 1a). In contrast, NTZ inhibited HAstV-1 replication in a dose-dependent manner
74 with the 5 μM treatment completely blocking virus replication (Figure1a-b). The 50% effective
75 concentration was calculated as approximately 1.47 μM (Figure 1c). Concentrations of NTZ
76 above 5 μM were associated with decreased cell viability as compared to vehicle alone (DMSO)
77 (Figure 1d). Thus, subsequent studies were performed with NTZ at a concentration of 2.5 μM.

To determine the stage of the viral replication cycle blocked by NTZ, drug was added at 2, 4, 6, 8, and 12 hours after HAstV-1 infection and viral capsid expression quantitated (Figure 2a). Addition of NTZ up to 8hpi completely inhibited HAstV-1 replication (Figure 2b) suggesting it blocks an early stage of the viral life cycle. Indeed, NTZ reduced the formation of double-strand RNA that occurs when the astrovirus genome is generated via its RNA-dependent RNA polymerase (Figure 2c). This method serves as a proxy for early replication given our lack of antibodies to the HAstV-1 non-structural proteins. Since NTZ appeared to exert antiviral activity early in the astrovirus replication cycle, we asked if NTZ activated any innate antiviral pathways. Previous research has shown thiazolides, the active form of NTZ, up-regulate type I and II IFN (13), HAstV has already been shown to be sensitive to IFN treatment, where exogenous addition of IFNβ limited astrovirus replication in a dose-dependent manner (14). We treated Caco-2 cells with NTZ and looked for up-regulation of type I and III interferons (IFN). At 4 hours post NTZ treatment, the shortest amount of time in which NTZ still inhibits HAstV replication, IFNα, IFNβ, or IFNλ were not significantly up-regulated compared to non-treated cells (Figure 2f). This suggests that the induction of IFN is not the mechanism by which NTZ blocks HAstV replication.

Finally, we asked if NTZ was effective against multiple HAstV serotypes and clinical isolates. Briefly, Caco-2 cells were infected with four different lab-adapted HAstV serotypes (HAstV-1, 2, 6 and 8) and four clinical isolates SJ177.110 (HAstV-2), SJ60.212 (HAstV-8), SJ88123.E120 (HAstV-1), and SJ88027.E259 (HAstV-1) obtained from patient samples and treated with 2.5 μM NTZ or DMSO (vehicle control). NTZ completely inhibited all HAstV isolates suggesting it is broadly protective against multiple HAstV serotypes (Figure 3a). Within the past decade, novel HAstV more closely related to animal AstV and associated with severe extra-gastrointestinal symptoms have been discovered. To determine if NTZ could also inhibit these non-classical HAstV, we infected Caco-2 cells with VA1, treated the cells with NTZ, and at 24hpi stained for dsRNA. Again, NTZ completely inhibited VA1 replication (Figure 3b). Thus, NTZ

exhibits compelling potential as a treatment option across HAstV serotypes and possibly individuals experiencing extra-gastrointestinal symptoms of astrovirus infection.

NTZ reduces viral replication and clinical disease in *vivo*. To determine if NTZ reduced disease, we used the only small animal model exhibiting astrovirus-induced diarrheal disease, turkey poults (15). Briefly, 5 day old turkey poults were orally gavaged with 100 mg/kg NTZ once daily for 4 days prior to oral infection with intestinal filtrate containing between 10^{12}-10^{13} genome copy units turkey astrovirus (TAstV-2) in 500 µl PBS and 3 days post-infection (Fig 4a). Stool samples were scored daily by four blinded volunteers as previously described (16). The scoring scale ranged from 1 to 4 based on color and consistency, with scores of 3 and 4 being considered diarrhea. Stool was also collected to quantitate viral titers. Fewer NTZ-treated poults had clinical disease (Fig 4b) and these poults had significantly less virus in their stool throughout the course of infection (Fig 4c). The poults showed no adverse symptoms from receiving NTZ alone. Excitingly, this gives evidence NTZ may be an effective therapeutic for astrovirus-induced diarrheal disease in patients.

Discussion

Our study provides the first evidence of an effective antiviral for astrovirus infection. We showed NTZ is broadly protective against multiple HAstV serotypes and reduces the production of dsRNA during infection *in vitro* with an EC_{50} of approximately 1.47 µM. Additionally, we showed the potential NTZ has as a clinical therapeutic by its ability to reduce viral shed and clinical disease in our symptomatic turkey poult model.

Nitazoxanide, 2-acetyloxy-N-(5-nitro-2-thiazolyl) benzamide (Alinia; Romark Laboratories), is a thiazolide compound for treatment of both intestinal protozoal and helminthic infections specifically *Giardia lamblia* and *Cryptosporidium parvum* (17). Recently this compound has been shown to have antiviral properties as well. The use of NTZ in vitro has been reported as an antiviral against influenza virus (18), rotavirus (19), norovirus (20),

Japanese encephalitis virus (JEV) (21), rubella virus (22), Zika virus (23), hepatitis C virus (24), and hepatitis B virus (25). Successful clinical trials have demonstrated its effectiveness in treating influenza (26), norovirus and rotavirus (20, 27, 28), hepatitis B virus (29), and hepatitis C virus (24, 30). Its mechanism of action against protozoa is due to its interference with pyruvate:ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reactions (31). While its antiviral action is currently unknown, research suggests it may be through the induction of the interferon response via activation of protein kinase R, or disruption of the unfolded protein response (17). We show that NTZ disrupts astrovirus infection early in the replications cycle causing a significant decrease in the production of dsRNA. The inhibition by NTZ at an early stage of infection was also seen with JEV (21). Recent research has shown thiazolides up-regulate type I and II IFN (13), which modulate the immune system and could be how NTZ creates a broadly antiviral state. However, the rapid kinetics with which NTZ inhibits HAstV replication (Figure 2) suggests that the induction of IFN is not responsible.

Astroviruses are classified into genotypes, but within the classical human genotype, *Mamastrovirus 1 (MAstV1)*, strains are further divided into serotypes (HAstV-1-8) based on their antigenicity and genetic differences in the complete capsid sequence (2). These genetic differences between astrovirus serotypes can confer differences in replication kinetics and symptom severity (32). Thus, finding a compound that broadly inhibits astroviruses across genotypes and serotypes is crucial. We found that NTZ is broadly protective across multiple HAstV serotypes, including the dominant strain worldwide, HAstV-1 (33), and patient isolates. Excitingly, NTZ shows efficacy against at least one non-classical HAstV genotype, *MAstV9*, specifically the VA1 serotype (Figure 3b). Non-classical HAstV (*MAstV6, 8, and 9*) have been linked to severe extra-gastrointestinal symptoms. To date, the non-classical HAstV genotypes have been associated with eight cases of encephalitis or meningitis (11), with VA1 being identified in five of those cases. Since the 1980's, the incidence of classic HAstV has been declining (5), studies have shown seroprevalence of VA1 and MLB1 is 65% (34) and 86% (35),

156 respectively, and could account for the displacement of circulating classic HAstV. Testing the
157 susceptibility of primary astrovirus isolates as well as non-classical HAstV to NTZ increases our
158 confidence that this drug would be effective in a clinical setting against circulating strains of
159 HAstV.

160 Turkey poults exhibit age-dependent diarrhea similar to humans when infected with
161 TAstV making them the only clinically relevant small animal model for astrovirus identified to
162 date (15, 36). We found that NTZ reduced virus levels shed in stool. We found a significant
163 decrease in stool viral titers with NTZ-treated poults having nearly 2 logs less virus at 5 days
164 post-infection. We also showed that viral titers began to plateau in the NTZ-treated poults at 5
165 days post-infection while untreated poults still showed increasing titers. This suggests NTZ
166 treatment may lead to faster clearance of the virus, however additional studies taken out further
167 are needed to definitively prove this.

168 These studies were repeated, however, due to the seasonality of turkey breeding and
169 limited availability of poults, a different breed of turkey, royal palm, was used. With the royal
170 palm poults, we again saw a reduction of viral titer in the stool. Additionally, we tested the small
171 intestinal tissue to quantitate viral RNA. The reduction in stool titers was recapitulated in the
172 tissue, where NTZ-treated poults had 2 logs lower virus in the duodenum, and about 1 log lower
173 virus in both the jejunum and the ileum (data not shown). However, NTZ-treatment had no effect
174 on the reduction of clinical symptoms in the royal palm poults. The mechanism by which TAstV-
175 2 induces diarrhea could be why this reduction was not statistically significant throughout
176 infection. We know that administration of capsid alone is sufficient to induce diarrhea in turkey
177 poults (16). From our *in vitro* work we believe NTZ blocks replication around the point where the
178 AstV genome is copied. Therefore, NTZ treatment may not be able to fully inhibit AstV-induced
179 diarrhea. In addition to this point, we administered between 10^{12}-10^{13} genome copy units to
180 each poult. While there have been reports of virus shed in humans at this level (32), it is a large
181 viral dose that may not be representative of natural infection. This work provides the first

182 evidence that NTZ may be an effective antiviral option against a broad range of HAstV,
183 including both classical and non-classical genotypes and limits viral titers *in vivo*.
184
185 **Materials and Methods**
186 Cells and Virus Propagation
187 The human intestinal adenocarcinoma cell line Caco-2 was obtained from ATCC (HTB-37).
188 Cells were propagated in minimum essential medium (MEM; Corning) supplemented with 20%
189 fetal bovine serum (FBS; Benchmark), GlutaMax-I (Gibco), 1 mM sodium pyruvate (Gibco), and
190 penicillin-streptomycin (Gibco).
191 Lab adapted human astrovirus stocks (HAstV-1, HAstV-2, HAstV-6, HAstV-8) were propagated
192 in Caco-2 cells, and the titer of the viruses were determined on Caco-2 cells by the fluorescent-
193 focus assay (focus-forming units [FFU]) as previously described (12).
194 Clinical isolates (SJ177.110, SJ60.212, SJ88123.E120, and SJ88027.E259) were derived from
195 remnant fecal samples submitted for clinical diagnostic testing at St. Jude Children's Research
196 Hospital. All samples were de-identified before testing. The St. Jude Institutional Review Board
197 approved this study with a waiver of consent. All isolates were propagated in Caco-2 cells.
198 Briefly, a 10-20% dilution of stool extract, positive for HAstV by RT-PCR, was filtered through a
199 0.22μm filter. The extract was diluted 1:10 in MEM + 5μg/ml porcine trypsin before adsorption
200 onto Caco-2 cell monolayers. Following a 1 hour adsorption period at 37°C, the inoculum was
201 removed and replaced with MEM containing 10μg/ml porcine trypsin and 0.3% BSA. The titer of
202 the viruses were again determined on Caco-2 cells by the fluorescent-focus assay (12).
203 TAstV-2 stocks were prepared from intestines collected from infected turkey poults. Briefly,
204 pieces of intestine were suspended in 0.5 ml PBS in multiple tubes, homogenized using 2-mm
205 zirconium oxide beads (Next Advance) beads for 4 minutes on speed setting 4 (Next Advance
206 air cooling bullet blender), and pelleted by centrifugation at 12,000 rpm for 5 minutes. The

supernatants were pooled and filtered through a 0.2-μm filter (fecal filtrate), and viral copy number was quantified by real-time RT-PCR.

In vitro HAstV Infection

Briefly, 5×10^4 cells were seeded into 96-well tissue culture plates (Corning), and after 2 days, the cells were inoculated with virus (HAstV-1, clinical isolates, VA1) in serum-free MEM for 1 hour at 37°C, at which time the virus was replaced with MEM containing 0.3% BSA and infection was allowed to proceed until 24hpi unless otherwise stated.

NTZ treatment was carried out in serum-free MEM and added following the 1 hour virus adsorption period unless otherwise stated in the experimental design.

Immunofluorescent Staining

Cells were fixed with 100% ice-cold methanol for 15 minutes, and then blocked with 5% normal goat serum (NGS; Gibco) in PBS at room temperature. The cells were stained with HAstV mouse monoclonal antibody 8E7 (2 μg/ml DakoCytomation) for 1 hour at room temperature followed by anti-mouse IgG labeled with Alexa Fluor 488 (anti-mouse IgG-Alexa Fluor 488; Invitrogen) secondary antibodies and 4′,6′-diamidino-2-phenylindole (DAPI; Sigma) for 30 minutes at room temperature. Staining was imaged on EVOS® FL Cell Imaging System and analyzed using ImageJ 1.50i software.

MTT Cell Viability Assay

Cell viability was tested using an MTT Cell Proliferation assay kit (Abcam) according to the manufacturer protocol. Briefly, cells were treated with varying concentrations of nitazoxanide in serum free media for 24 hours. The nitazoxanide containing media was removed and replaced with a 50:50 mixture of MTT reagent and serum free media. The cells were incubated with the mixture at 37°C for 3 hours. Following incubation, an MTT solvent solution was added and the

plate was placed on an orbital shaker for 15 minutes. The absorbance was then measured at OD595. Cell viability was calculated as a percentage of non-treated cells.

Animals and NTZ treatment

Broad-breasted white turkey poults were obtained from a commercial hatchery. Five-day-old poults were randomly assigned to groups (n = 6 per group) and housed in individual, temperature-controlled Horsfall units with HEPA-filtered inlet and exhaust air valves, where they were given free access to water and routine turkey starter feed. Poults were orally inoculated with 500µl of TAstV-2 intestinal filtrate, containing approximately 10^{12}-10^{13} genome copies, or PBS alone. Stool from individual birds was scored from 1 to 4. Scoring was performed daily post-infection. Scores of 3 (liquid or loose stool with some undigested food or solid material) and 4 (watery stool with no solids present) were defined as diarrhea, in accordance with previously published work from Meliopoulos et al. (16).

For NTZ treatment, poults were orally administered 100mg/kg nitazoxanide in 500ul of ultra-pure water. Administration of NTZ was carried out 4 days prior to infection and 3 days post-infection.

Turkey Astrovirus qRT-PCR assay

TAstV-2 genome copies were determined as previously described (16). Briefly, viral RNA was isolated from 10% stool by the MagMAX-96 AI/ND Viral RNA Isolation Kit (Applied Biosystems) according to the manufacturer's protocol. PCR was performed on 3 µl of each sample using TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosciences) with 600nM forward primer 5′GACTGAAATAAGGTCTGCACAGGT, 600nM reverse primer 5′AACCTGCGAACCCTGCG, and 200nM probe 6-carboxyfluorescein (6FAM)-ATGGACCCCCTTTTTCGGCGG-BHQ1 (black hole quencher) under the following conditions: 50°C for 5 min, 95°C for 20 s, followed by 45 cycles, with one cycle consisting of 95°C for 3 s and 60°C for 30 s on a Bio-Rad CFX96 real-

time PCR detection system. The number of genome copies/µL of total RNA was determined using a standard curve generated from a synthesized TAstV-2 DNA from nucleotides 4001 to 4201 with a known copy number (calculated using Thermo Fisher Scientific DNA Copy Number and Dilution Calculator). Log_{10} dilutions of the synthesized TAstV-2 DNA were used for real-time RT-PCR as described above.

Acknowledgments

We thank Rebekah Honce, Pamela Freiden and Dr. Victoria Meliopoulos for their expert poop scoring abilities; Sean Offord and Sharon Lokey from the St. Jude Animal Resources Center for assistance with turkey studies; and Dr. David Wang for graciously providing VA1 for these studies.

These studies were funded by National Institute of Allergy and Infectious Diseases R21 AI135254-01, and ALSAC.

References

1. World Health Organization. 2017. Diarrhoeal Disease Fact Sheet.

2. Bosch A, Pintó RM, Guix S. 2014. Human Astroviruses. Clin Microbiol Rev 27:1048–1074.

3. Méndez-Toss M, Griffin DD, Calva J, Contreras JF, Puerto FI, Mota F, Guiscafré H, Cedillo R, Muñoz O, Herrera I, López S, Arias CF. 2004. Prevalence and Genetic Diversity of Human Astroviruses in Mexican Children with Symptomatic and Asymptomatic Infections. J Clin Microbiol 42:151–157.

4. Kurtz JB, Lee TW, Craig JW, Reed SE. 1979. Astrovirus infection in volunteers. J Med Virol 3:221–230.

5. Bosch A, Guix S, Pintó RM. 2013. Epidemiology of Human Astroviruses, p. 1–18. *In* Schultz-Cherry, S (ed.), Astrovirus Research: Essential Ideas, Everyday Impacts, Future Directions. Springer New York, New York, NY.

6. Lin H-C, Kao C-L, Chang L-Y, Hsieh Y-C, Shao P-L, Lee P-I, Lu C-Y, Lee C-Y, Huang L-M. 2008. Astrovirus gastroenteritis in children in Taipei. J Formos Med Assoc 107:295–303.

7. Tseng W-C, Wu F-T, Hsiung CA, Chang W-C, Wu H-S, Wu C-Y, Lin J-S, Yang S-C, Hwang K-P, Huang Y-C. 2012. Astrovirus gastroenteritis in hospitalized children of less than 5 years of age in Taiwan, 2009. J Microbiol Immunol Infect 45:311–317.

8. Amaral MSC, Estevam GK, Penatti M, Lafontaine R, Lima ICG, Spada PKP, Gabbay YB, Matos NB. 2015. The prevalence of norovirus, astrovirus and adenovirus infections among hospitalised children with acute gastroenteritis in Porto Velho, state of Rondônia, western Brazilian Amazon. Mem Inst Oswaldo Cruz 110:215–221.

9. Ueda Y, Nakaya S, Takagi M, Ushijima H. 1996. [Diagnosis and clinical manifestations of diarrheal virus infections in Maizuru area from 1991 to 1994--especially focused on small round structured viruses]. Kansenshogaku Zasshi 70:1092–1097.

10. Cordey S, Zanella M-C, Wagner N, Turin L, Kaiser L. 2018. Novel human astroviruses in pediatric respiratory samples: A one-year survey in a Swiss tertiary care hospital. J Med Virol 90:1775–1778.

11. Vu D-L, Cordey S, Brito F, Kaiser L. 2016. Novel human astroviruses: Novel human diseases? Journal of Clinical Virology 82:56–63.

12. Marvin S, Meliopoulos V, Schultz-Cherry S. 2014. Human Astrovirus Propagation, Purification and Quantification. BIO-PROTOCOL 4.

13. Trabattoni D, Gnudi F, Ibba SV, Saulle I, Agostini S, Masetti M, Biasin M, Rossignol J-F, Clerici M. 2016. Thiazolides Elicit Anti-Viral Innate Immunity and Reduce HIV Replication. Scientific Reports 6:27148.

14. Marvin SA, Huerta CT, Sharp B, Freiden P, Cline TD, Schultz-Cherry S. 2015. Type I Interferon Response Limits Astrovirus Replication and Protects against Increased Barrier Permeability In Vitro and In Vivo. J Virol 90:1988–1996.

15. Koci MD, Moser LA, Kelley LA, Larsen D, Brown CC, Schultz-Cherry S. 2003. Astrovirus induces diarrhea in the absence of inflammation and cell death. J Virol 77:11798–11808.

16. Meliopoulos VA, Marvin SA, Freiden P, Moser LA, Nighot P, Ali R, Blikslager A, Reddivari M, Heath RJ, Koci MD, Schultz-Cherry S. 2016. Oral Administration of Astrovirus Capsid Protein Is Sufficient To Induce Acute Diarrhea In Vivo. MBio 7.

17. Rossignol J-F. 2014. Nitazoxanide: A first-in-class broad-spectrum antiviral agent. Antiviral Research 110:94–103.

18. Rossignol JF, La Frazia S, Chiappa L, Ciucci A, Santoro MG. 2009. Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. J Biol Chem 284:29798–29808.

19. Rossignol J-F, Abu-Zekry M, Hussein A, Santoro MG. 2006. Effect of nitazoxanide for treatment of severe rotavirus diarrhoea: randomised double-blind placebo-controlled trial. Lancet 368:124–129.

20. Rossignol J-F, El-Gohary YM. 2006. Nitazoxanide in the treatment of viral gastroenteritis: a randomized double-blind placebo-controlled clinical trial. Alimentary Pharmacology & Therapeutics 24:1423–1430.

21. Shi Z, Wei J, Deng X, Li S, Qiu Y, Shao D, Li B, Zhang K, Xue F, Wang X, Ma Z. 2014. Nitazoxanide inhibits the replication of Japanese encephalitis virus in cultured cells and in a mouse model. Virol J 11:10.

22. Perelygina L, Hautala T, Seppänen M, Adebayo A, Sullivan KE, Icenogle J. 2017. Inhibition of rubella virus replication by the broad-spectrum drug nitazoxanide in cell culture and in a patient with a primary immune deficiency. Antiviral Res 147:58–66.

23. Li Z, Brecher M, Deng Y-Q, Zhang J, Sakamuru S, Liu B, Huang R, Koetzner CA, Allen CA, Jones SA, Chen H, Zhang N-N, Tian M, Gao F, Lin Q, Banavali N, Zhou J, Boles N, Xia M, Kramer LD, Qin C-F, Li H. 2017. Existing drugs as broad-spectrum and potent inhibitors for Zika virus by targeting NS2B-NS3 interaction. Cell Res 27:1046–1064.

24. Rossignol J, Elfert A, El–Gohary Y, Keeffe EB. 2009. Improved Virologic Response in Chronic Hepatitis C Genotype 4 Treated With Nitazoxanide, Peginterferon, and Ribavirin. Gastroenterology 136:856–862.

25. Korba BE, Montero AB, Farrar K, Gaye K, Mukerjee S, Ayers MS, Rossignol J-F. 2008. Nitazoxanide, tizoxanide and other thiazolides are potent inhibitors of hepatitis B virus and hepatitis C virus replication. Antiviral Res 77:56–63.

26. Haffizulla J, Hartman A, Hoppers M, Resnick H, Samudrala S, Ginocchio C, Bardin M, Rossignol J-F. 2014. Effect of nitazoxanide in adults and adolescents with acute uncomplicated influenza: a double-blind, randomised, placebo-controlled, phase 2b/3 trial. The Lancet Infectious Diseases 14:609–618.

343 27. Teran CG, Teran-Escalera CN, Villarroel P. 2009. Nitazoxanide vs. probiotics for the treatment of
344 acute rotavirus diarrhea in children: a randomized, single-blind, controlled trial in Bolivian children.
345 International Journal of Infectious Diseases 13:518–523.

346 28. Siddiq DM, Koo HL, Adachi JA, Viola GM. 2011. Norovirus gastroenteritis successfully treated with
347 nitazoxanide. Journal of Infection 63:394–397.

348 29. Rossignol J-F, Keeffe EB. 2008. Thiazolides: a new class of drugs for the treatment of chronic
349 hepatitis B and C. Future Microbiol 3:539–545.

350 30. Rossignol J-F, Elfert A, Keeffe EB. 2010. Treatment of chronic hepatitis C using a 4-week lead-in
351 with nitazoxanide before peginterferon plus nitazoxanide. Journal of Clinical Gastroenterology
352 44:504–509.

353 31. Hoffman PS, Sisson G, Croxen MA, Welch K, Harman WD, Cremades N, Morash MG. 2007.
354 Antiparasitic drug nitazoxanide inhibits the pyruvate oxidoreductases of Helicobacter pylori,
355 selected anaerobic bacteria and parasites, and Campylobacter jejuni. Antimicrob Agents
356 Chemother 51:868–876.

357 32. Caballero S, Guix S, El-Senousy WM, Calicó I, Pintó RM, Bosch A. 2003. Persistent gastroenteritis in
358 children infected with astrovirus: association with serotype-3 strains. J Med Virol 71:245–250.

359 33. Vu D-L, Bosch A, Pintó RM, Guix S. 2017. Epidemiology of Classic and Novel Human Astrovirus:
360 Gastroenteritis and Beyond. Viruses 9.

361 34. Burbelo PD, Ching KH, Esper F, Iadarola MJ, Delwart E, Lipkin WI, Kapoor A. 2011. Serological
362 studies confirm the novel astrovirus HMOAstV-C as a highly prevalent human infectious agent.
363 PLoS ONE 6:e22576.

35. Holtz LR, Bauer IK, Jiang H, Belshe R, Freiden P, Schultz-Cherry SL, Wang D. 2014. Seroepidemiology of astrovirus MLB1. Clin Vaccine Immunol 21:908–911.

36. Schultz-Cherry S, Kapczynski DR, Simmons VM, Koci MD, Brown C, Barnes HJ. 2000. Identifying agent(s) associated with poult enteritis mortality syndrome: importance of the thymus. Avian Dis 44:256–265.

Figure Legends

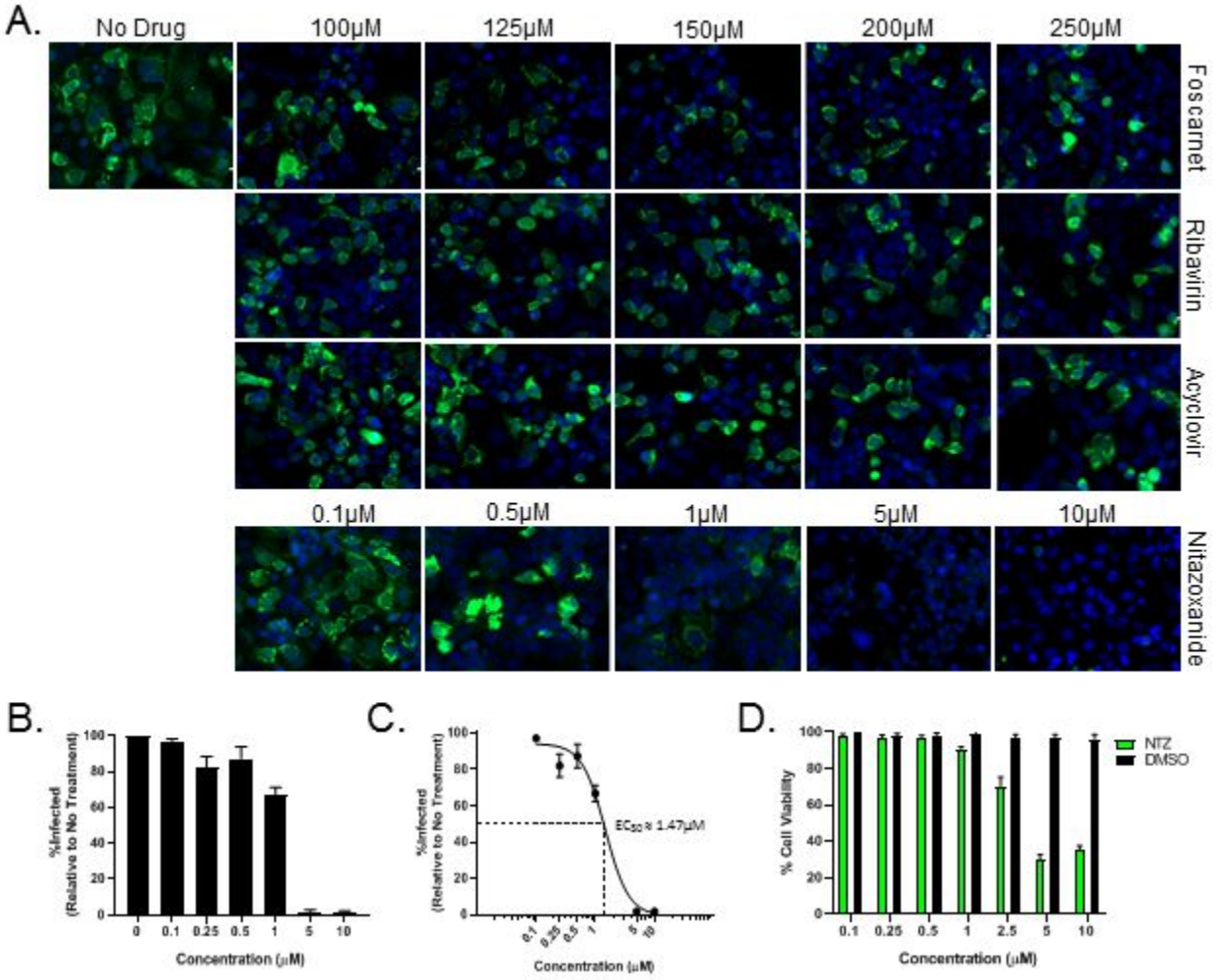
Figure 1 | Nitazoxanide inhibits HAstV-1 replication in Caco-2 cells. (A) Caco-2 cells were infected with HAstV-1 at an MOI of 1 and treated with a panel of antivirals (foscarnet, ribavirin, acyclovir, and nitazoxanide) at the indicated concentrations. At 24hpi, cells were fixed and stained with DAPI (blue) and for the presence of astrovirus capsid protein (green). (B) The percent of infected cells was calculated and compared to non-treated cells. (C) Non-linear regression analysis of percent infection data was used to determine the 50% effective concentration (EC_{50}). (D) Cell viability of Caco-2 cells following 24 hour treatment with NTZ (green bars) or vehicle alone (DMSO; black bars) at the indicated concentrations was determined by MTT assay. All error bars indicate standard error of the means.

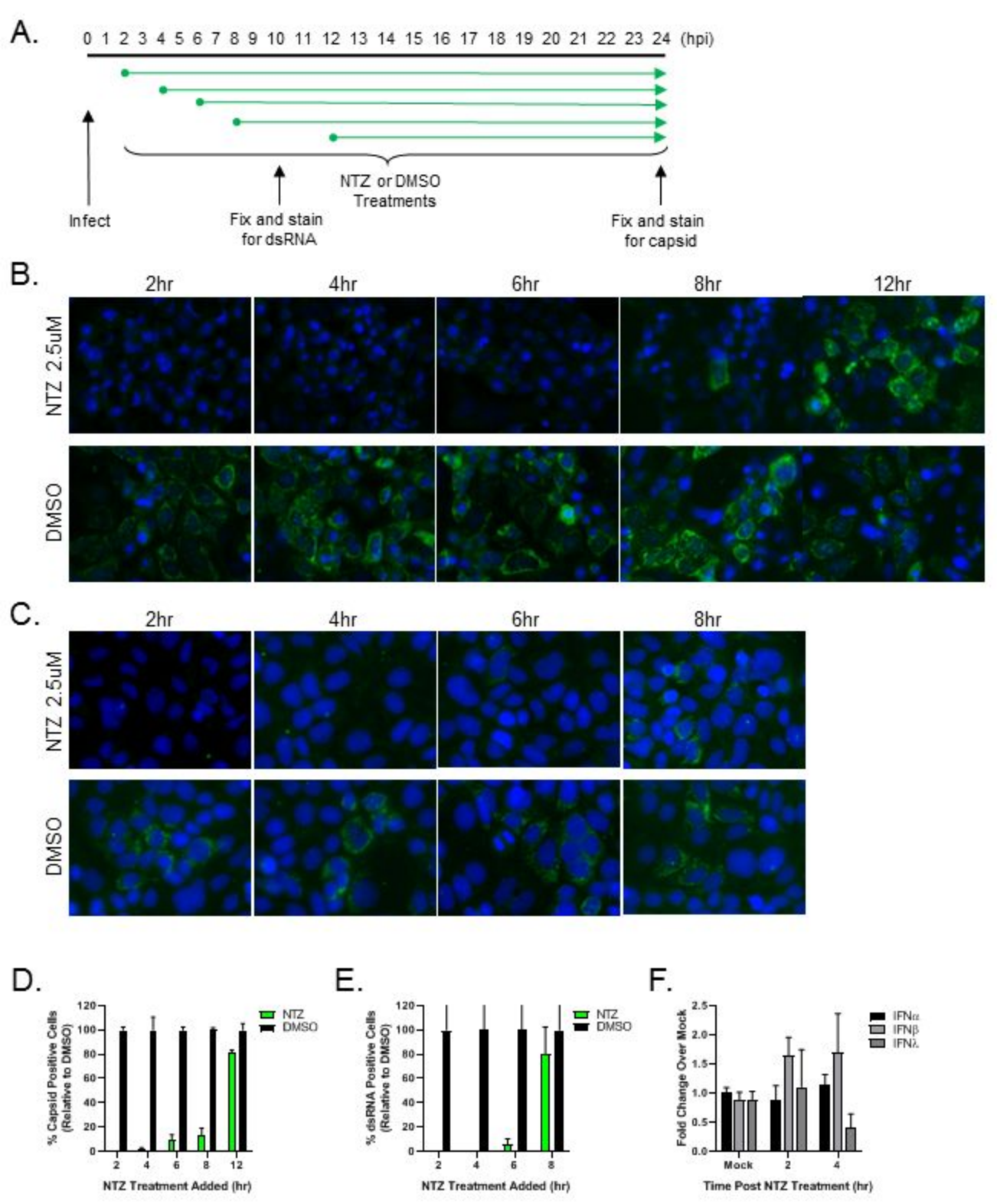
Figure 2 | Nitazoxanide inhibits HAstV-1 replication in vitro when added up to 8 hpi. Caco-2 cells were infected with HAstV-1 and at the various times post-infection 2.5μM NTZ or vehicle alone (DMSO) was added as indicated by the schematic in panel A. (B) At 24hpi, cells were fixed and stained with DAPI (blue) and for the presence of astrovirus capsid protein (green). (C) At 10hpi, cells were fixed and stained with DAPI (blue) and for the presence of dsRNA (green). (D) Quantification of the percent of cells with capsid staining from panel B. (E) Quantification of the percent of cells with dsRNA staining from panel C. (F) Real-time RT-PCR for IFNα, IFNβ,

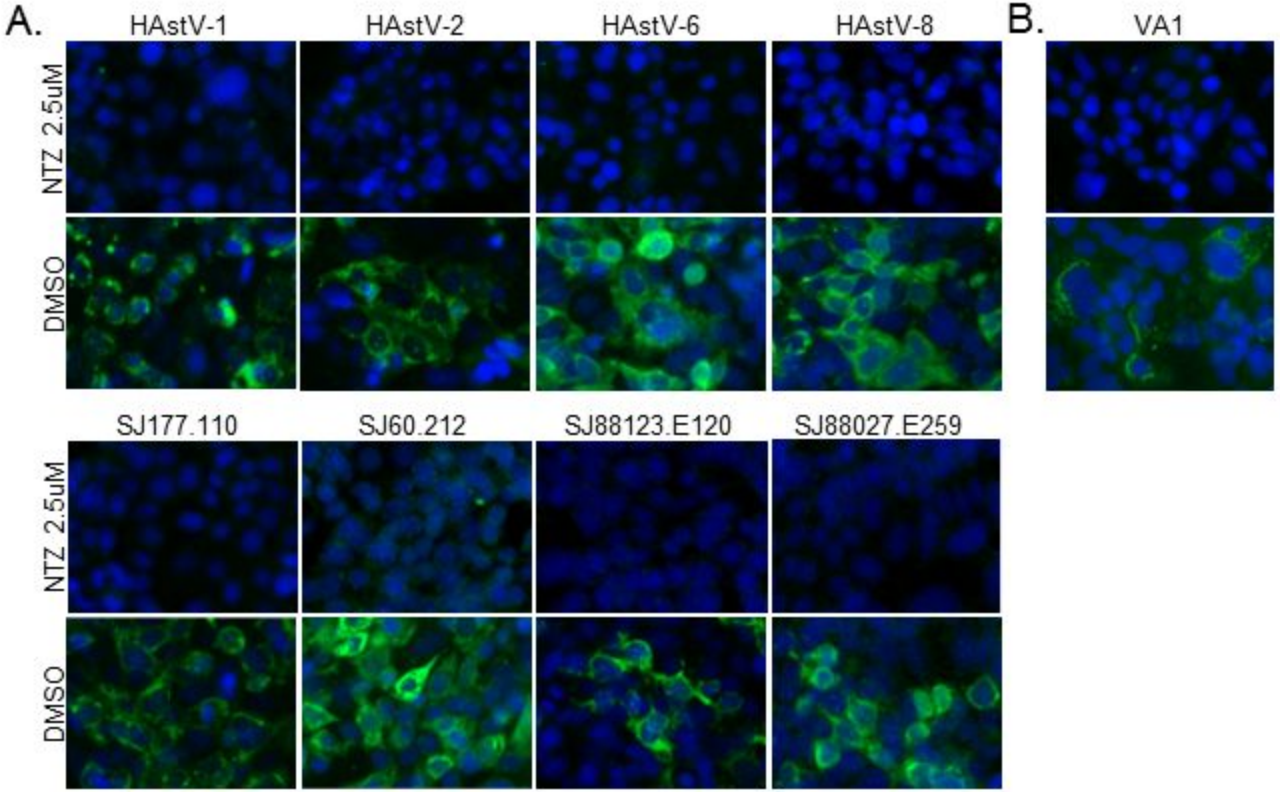
and IFNλ was performed on RNA was collected from Caco-2 cells treated with 2.5μM NTZ and normalized to GAPDH. Results are shown as fold increase over untreated cells and error bars indicate standard error of the means.

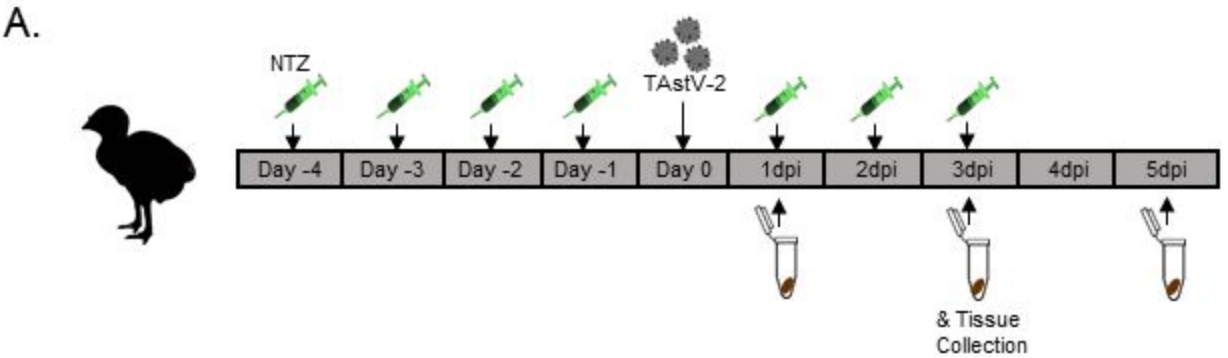
Figure 3 | Nitazoxanide inhibits the replication of multiple serotypes and clinical isolates of human astrovirus. (A) Caco-2 cells were infected with lab adapted virus serotypes (upper panels) or clinical isolates (lower panels) and treated with 2.5μM NTZ or vehicle alone (DMSO). At 24hpi, cells were fixed and stained with DAPI (blue) and for the presence of astrovirus capsid protein (green). (B) Caco-2 cells infected with VA1 were fixed at 24hpi and stained with DAPI (blue) and for the presence of dsRNA (green).

Figure 4 | Nitazoxanide reduces clinical symptoms and viral titers in turkey poults. (A) Turkey poults (n=6/group) were infected with turkey astrovirus (TAstV-2) from intestinal filtrate. Four days prior to infection and three days post-infection poults were treated with NTZ. Poults were monitored for clinical score daily and stool was collected to measure viral RNA titer every other day. (B) Percentage of poults with clinical scores of 3 or higher in the groups: NTZ treatment alone (green circle), no antiviral treatment (gray square), TAstV-2 infected without antiviral treatment (black triangle), and TAstV-2 infected with NTZ treatment (green triangle). (C) Viral RNA titer of stool collected from infected poults with NTZ treatment (green triangles) or no antiviral treatment (black triangles). All error bars indicate standard error of the means, and dashed line represents the limit of detection.


A.
No Drug
100μM
125μM
150μM
200μM
250μM
Foscarnet
Ribavirin
Acyclovir
0.1μM
0.5μM
1μM
5μM
10μM
Nitazoxanide
B.
%Infected (Relative to No Treatment)
Concentration (μM)
C.
%Infected (Relative to No Treatment)
EC50 ≈ 1.47μM
Concentration (μM)
D.
% Cell Viability
NTZ
DMSO
Concentration (μM)


A.
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 (hpi)
Infect
Fix and stain for dsRNA
NTZ or DMSO Treatments
Fix and stain for capsid
B.
2hr
4hr
6hr
8hr
12hr
NTZ 2.5uM
DMSO
C.
2hr
4hr
6hr
8hr
NTZ 2.5uM
DMSO
D.
% Capsid Positive Cells (Relative to DMSO)
NTZ
DMSO
NTZ Treatment Added (hr)
E.
% dsRNA Positive Cells (Relative to DMSO)
NTZ
DMSO
NTZ Treatment Added (hr)
F.
Fold Change Over Mock
IFNα
IFNβ
IFNλ
Mock
Time Post NTZ Treatment (hr)


A.
HAstV-1
HAstV-2
HAstV-6
HAstV-8
NTZ 2.5uM
DMSO
B.
VA1
SJ177.110
SJ60.212
SJ88123.E120
SJ88027.E259
NTZ 2.5uM
DMSO


A.
NTZ
TAstV-2
Day -4
Day -3
Day -2
Day -1
Day 0
1dpi
2dpi
3dpi
4dpi
5dpi
& Tissue Collection
B.
% with clinical score ≥3
100
80
60
40
20
0
0
1
2
3
4
5
Days Post-Infection
NTZ only
PBS only
NTZ+TAstV2
PBS+TAstV2
C.
Stool
Genome Copy Number
10^{14}
10^{12}
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
L.O.D.
0
1
3
5
Days Post-Infection
NTZ+TAstV2
PBS+TAstV2