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Inhibition of human norovirus by a viral polymerase inhibitor in the B cell culture system and in the mouse model

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Abstract

The recently developed human norovirus (HuNoV) B cell culture and mouse models hold promise for drug discovery and development but their suitability for antiviral studies has not been assessed. We demonstrate the inhibitory effect of the nucleoside analogue 2'-C-methylcytidine (2CMC) on HuNoV replication in the human B cell BJAB cell line and in Balb/c Rag/gamma chain-deficient (Rag- γ c^{-/-}) mice. These data suggest the applicability of both models for future study and development of antiviral drugs for the treatment of HuNoV infections.

Keywords

human norovirus; polymerase inhibitor; positive-sense RNA virus; enteric virus; antiviral

Human noroviruses (HuNoVs) are the leading cause of viral gastroenteritis and food-borne disease worldwide constituting a global economic problem (Bartsch *et al.*, 2016; Havelaar *et al.*, 2015; Kirk *et al.*, 2015; Koo *et al.*, 2010; Patel *et al.*, 2008; Payne *et al.*, 2013). No vaccines or antiviral drugs are currently approved for clinical use. However, potent and safe antiviral therapy is urgently needed to reduce the burden of norovirus disease, particularly in vulnerable populations – young children, the elderly and immunocompromised – where prolonged and severe disease is linked to morbidity and mortality (Bok & Green, 2012; Hall *et al.*, 2012; Moreno-Espinosa *et al.*, 2004). Research into HuNoVs has been hampered by difficulties in culturing HuNoVs in cells (Duizer *et al.*, 2004; Herbst-Kralovetz *et al.*, 2013; Takanashi *et al.*, 2014). The cultivable genogroup V murine norovirus (MuNoV) is therefore a widely used surrogate for HuNoV (Karst & Wobus, 2015; Karst *et al.*, 2014; Wobus *et al.*,

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2006). However, the genetic diversity between these viruses represents one challenge for HuNoV drug development. Thus, the recently reported successes in propagation of HuNoV in immunodeficient mice (Taube *et al.*, 2013) and in a human B cell line (Jones *et al.*, 2015; Jones *et al.*, 2014) provide exciting tools for the evaluation of potential antiviral strategies and vaccine candidates directed against HuNoV. A number of anti-NoV strategies, including targeting essential viral proteins, have been described in recent years (for a recent review see (Thorne *et al.*, 2016)). In particular, the nucleoside analogue 2'-C-methylcytidine (2CMC) has been well characterized for its antiviral efficacy against MuNoV *in vitro* and in a mouse model as well as against the human Norwalk virus in a replicon system (Costantini *et al.*, 2012; Rocha-Pereira *et al.*, 2012; Rocha-Pereira *et al.*, 2013; Rocha-Pereira *et al.*, 2015). Therefore, the goal of these studies was to perform proof-of-concept studies to validate the recently developed HuNoV culture systems for antiviral studies using 2CMC as a tool compound.

A previous study (Jones *et al.*, 2014) and accompanying protocols article (Jones *et al.*, 2015) demonstrate infection of genogroup II genotype 4 (GII.4) HuNoVs *in vitro* in the B cell line BJAB in the presence and absence of synthetic H type histo-blood group antigen or *Enterobacter cloacae*. This infection can be prevented by UV treatment of the virus stock prior to infection. We established this culture system in our laboratory to test the applicability of this new HuNoV culture system for antiviral efficacy testing (Fig. 1). We first determined the nontoxic concentrations of 2CMC in BJAB cells using the WST-1 assay (Roche, San Francisco, CA, USA). BJAB cells were exposed to increasing concentrations of 2CMC for 3 days, the length of the viral infection assay (Fig. 1A, line graph). The 50% cytotoxic concentration (CC₅₀) was $75.5 \pm 0.6 \mu\text{M}$. Next, the viral inoculum with a given genome copy number was identified that resulted in the greatest increase over input in the BJAB system over a 3 day infection period (Fig. 1B). BJAB cells were infected with unfiltered HuNoV-positive stool containing the indicated genome copy numbers of HuNoV GII.4 Sydney as described (Jones *et al.*, 2015; Jones *et al.*, 2014) without extra addition of synthetic H type histo-blood group antigen or *Enterobacter cloacae*. After removing the unbound virus inoculum, cells were resuspended in fresh media. The day 0 sample was taken immediately to quantify the amount of viral genome bound to cells. The remaining cells were incubated at 37°C for 3 days to allow the infection to proceed. The combined content of each well, including cells and supernatant, was then harvested, and RNA was extracted from a 500 μl aliquot per sample collected on days 0 and 3 postinfection (p.i.). HuNoV genomes were measured by qRT-PCR as described (Taube *et al.*, 2013). The fold increase in genome titers over day 0 (i.e., bound genomes) was calculated and graphed. Similar to previous work (Jones *et al.*, 2015; Jones *et al.*, 2014), an input genome copy number of ~3500 genome copies of GII.4 Sydney resulted in the greatest fold increase over input. To determine the background signal of the assay, the virus stock was UV treated prior to infection or left untreated (Fig. 1C). Based on these data, a genome increase over input of 3 fold or greater is considered to provide an indication of viral replication in this assay.

To determine the antiviral efficacy of 2CMC on GII.4 HuNoV, BJAB cells were infected with unfiltered stool containing ~3500 genome copies as described above. After 1 h of infection and removal of the unbound virus inoculum, cells were resuspended in fresh media containing a dilution series of 2CMC (0.16 – 40 μM) or PBS as vehicle control and were

incubated for 3 days. In vehicle-treated cells, a maximal difference of approximately 3 PCR cycle thresholds (3 Cts) between 0 (mean Ct 23) and 3 (mean Ct 20) days p.i. was observed (not shown). This is equivalent to a log₁₀ difference in copy number in line with a previous report (Brown *et al.*, 2016). 2CMC significantly reduced HuNoV genome titers in BJAB cells in a concentration-dependent manner (Fig. 1A, bar graph). The half maximal 50% effective concentration (EC₅₀) was $0.3 \pm 0.02 \mu\text{M}$ and the therapeutic index (CC₅₀/EC₅₀) ~300. Thus, 2CMC exerted an antiviral effect at non-toxic concentrations. This antiviral potency of 2CMC against HuNoV was more pronounced than the *in vitro* activity reported against MuNoV (EC₅₀ = ~2 μM) and in the replicon system (EC₅₀ = ~18 μM) (Rocha-Pereira *et al.*, 2012; Rocha-Pereira *et al.*, 2013). The latter may be due to one or more differences in cell type (BJAB vs. Huh-7), virus strains (GI.1 vs. GII.4), or assay (replicon vs. infectious cycle). Importantly, these data demonstrate that the BJAB culture system is well suited to assess activity of potential inhibitors of HuNoV replication. Furthermore, this confirms that the RNA-dependent RNA polymerase (RdRp) is an excellent target to inhibit the replication of NoVs from multiple genogroups.

We also recently described the first mouse model of HuNoV infection, wherein Balb/c Rag- $\gamma\text{c}^{-/-}$ infected intraperitoneally supported replication of a HuNoV (Taube *et al.*, 2013). Therefore, the antiviral efficacy of 2CMC was next determined in this new animal model. Animal studies were performed according to the local and federal guidelines and protocols were approved by the University of Michigan Committee on Use and Care of Animals (UCUCA Number: PRO 00004534). Six to eight week old mice were administered 2CMC (100 mg/kg/day, divided into 2 daily treatments) or PBS vehicle control for 48 h intraperitoneally. One hour after the first drug treatment, mice were infected with HuNoV-positive stool containing 5×10^3 genome copies of GII.4 HuNoVs. Tissues were harvested 48 h p.i., and viral titers were determined by qRT-PCR as described (Taube *et al.*, 2013). 2CMC reduced HuNoV genome loads by ~1.0 – 1.5 log₁₀ in the colon, mesentery, liver and spleen, whereas no antiviral effect was observed in the jejunum, ileum, kidney and the bone marrow (Fig. 2A, data not shown). No viral genomes were detected in the stomach, duodenum, brain, heart and lung of 2CMC-treated or vehicle-treated animals (data not shown), indicating these sites were not infected. We next calculated the total viral load in 2CMC-treated or vehicle-treated animals and compared it to the inoculum titer. Analysis of the fold increase in HuNoV genome titers over input demonstrated significant decreases (~1 log₁₀) in genome loads in the presence of 2CMC compared to vehicle controls (Fig. 2B). This decrease provided independent confirmation of productive HuNoV replication in this mouse model. Importantly, these data demonstrated that 2CMC significantly inhibits the replication of HuNoV infection *in vivo*, suggesting infection of Balb/c Rag- $\gamma\text{c}^{-/-}$ can be used as an infection model for pre-clinical testing of antivirals against HuNoV.

Taken together, these data open up the exciting possibility of using both models for future study and development of antiviral drugs for the treatment of HuNoV infections, although both models in their current form have some caveats. For example, HuNoV replication and consequent increase in viral genome copies over input in BJAB cells and in Balb/c Rag- $\gamma\text{c}^{-/-}$ mice is not as robust as what was observed using MuNoV infection of RAW 264.7 cells (~5 log₁₀) and Balb/c Rag- $\gamma\text{c}^{-/-}$ (~2 log₁₀) (data not shown). This suggests that the number of HuNoV replication cycles is limited, for example by transformation of B cells or

species-specific restrictions of HuNoV infection in mice. Thus, HuNoV adaptation and/or identification of host restriction factors may be required in both models to increase overall viral yields in both systems in the future. Current efforts to optimize both models by trying to adapt HuNoV to replicate more efficiently in BJAB cells and Balb/c Rag- γ c^{-/-} mice are ongoing in our laboratories. In addition, further improvements in the mouse model to more closely mimic aspects of HuNoV infection, especially the natural route of entry, would also help with testing orally delivered antiviral treatments. These efforts will contribute to the development of next generation propagation systems that broaden the applicability to the large number of existing HuNoV genotypes, to increase the dynamic range of the assays, and to make the cell culture system more suitable for automation. Ultimately, this will allow the testing of larger libraries of antiviral molecules and provide complementary platforms to currently available technologies (e.g., Norwalk virus replicon system (Chang *et al.*, 2006)) in assessing the activity of clinical drug candidates.

In summary, our findings demonstrate that 2CMC inhibits infection of HuNoV in cell culture and in a mouse model. Specifically, our data demonstrate that both the new *in vitro* HuNoV propagation assay in BJAB cells and the HuNoV infection model in Balb/c Rag- γ c^{-/-} mice allow the evaluation of the anti-HuNoV activities of small molecule inhibitors of viral infection. Thus, these two models will be important tools in the future development of antiviral strategies for the treatment and prophylaxis of HuNoV infections.

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Highlights

- the polymerase inhibitor 2'-C-methylcytidine inhibits HuNoV replication in the recently established B cell culture system
- the nucleoside analogue 2'-C-methylcytidine inhibits HuNoV replication in a mouse model of HuNoV infection.
- both models show promise for future study and development of antiviral drugs for the treatment of HuNoV infections

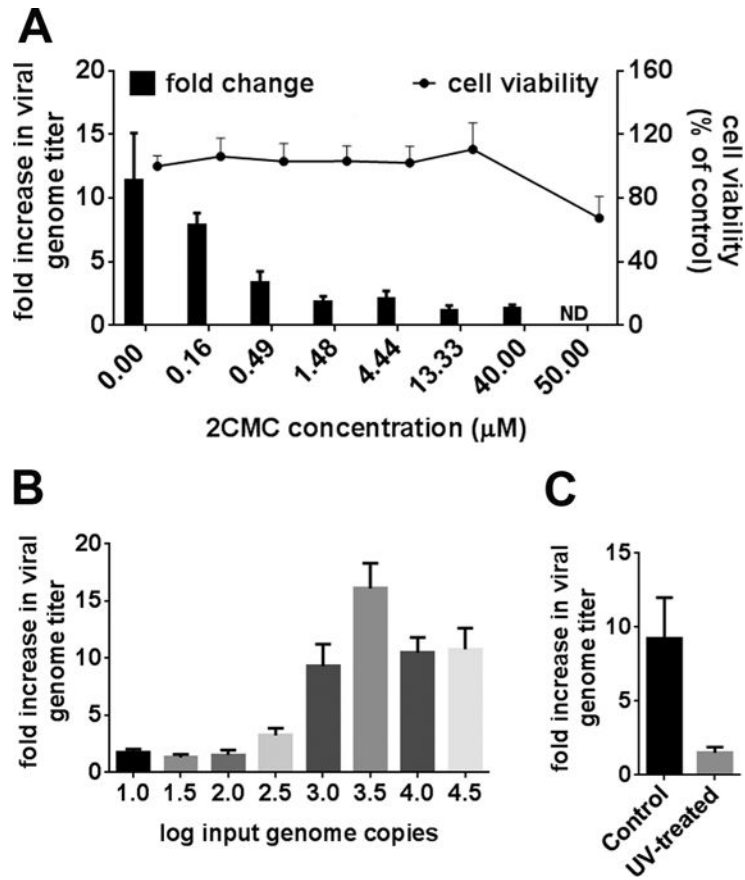
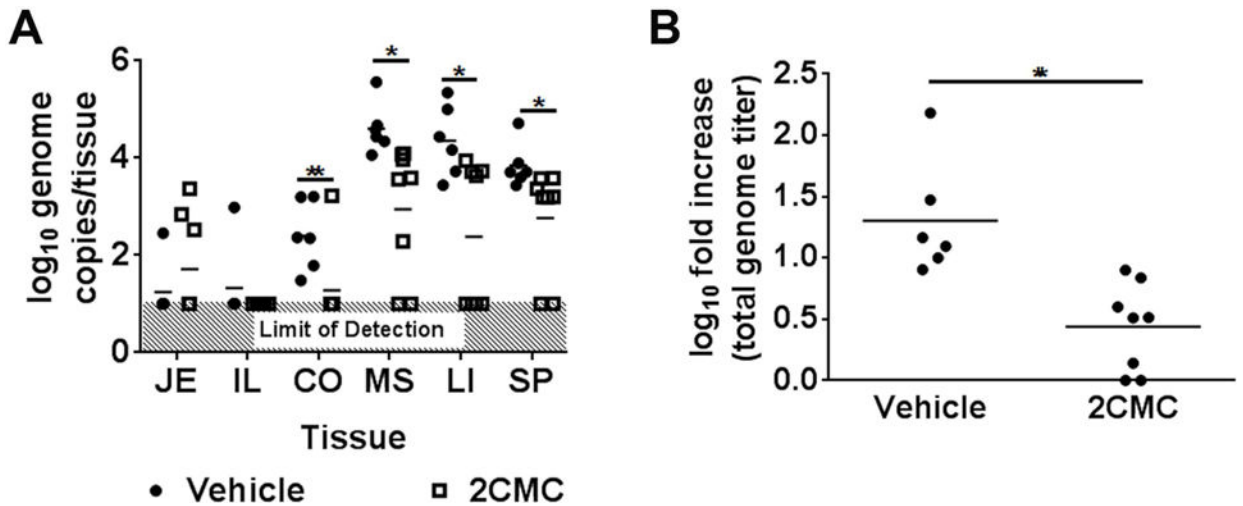


Fig. 1. Anti HuNoV efficacy of 2CMC *in vitro*. A) BJAB cells were treated for 3 days with the indicated concentrations of 2CMC and cell viability was determined by WST-1 assay (Roche) (line graph). BJAB cells were infected with unfiltered HuNoV-positive stool for 3 days in the presence of the indicated concentrations of 2CMC. HuNoV genomes were measured by qRT-PCR. Genome titers at 3 days post infection were compared to inoculum titers to determine the fold increase in viral titer over inoculum (bar graph). B) BJAB cells were infected with unfiltered HuNoV-positive stool containing the indicated genome copy numbers. HuNoV genomes were measured by qRT-PCR 3 days postinfection. Genome titers were compared to inoculum titers to determine the fold increase in viral titer over inoculum. C) BJAB cells were infected with the unfiltered HuNoV-positive stool inactivated with UV light for 30 seconds or left untreated for 3 days. All data are presented as means \pm SE of at least two independent experiments.

**Fig. 2.**

2CMC inhibits HuNoV replication in Balb/c Rag- $\gamma c^{-/-}$ mice. Mice were intraperitoneally administered either 2CMC (open square) or vehicle control (closed circle) and infected with HuNoV-positive stool 1 hour later. (A) HuNoV genomes were measured by qRT-PCR in jejunum (JE), ileum (IL), colon (CO), mesentery (MS), liver (LI), and spleen (SP) from mice sacrificed 48 hours post infection (hpi). (B) Total genome titers recovered from each mouse at the time of harvest were compared to inoculum titers to determine the log fold increase in viral titer over inoculum. Data are from two independent experiments, and each symbol represents individual mouse. Data were analyzed using the Mann-Whitney U test. **, P < 0.01