Treatment with a Nucleoside Polymerase Inhibitor Reduces Shedding of Murine Norovirus in Stool to Undetectable Levels without Emergence of Drug-Resistant Variants

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Prolonged norovirus shedding may occur in certain patients, such as organ transplant recipients. We established a mouse model for persistent norovirus infection (using the mouse norovirus MNV.CR6 strain). The nucleoside viral polymerase inhibitor 2′-C-methylcytidine (2CMC), but not favipiravir (T-705), reduced viral shedding to undetectable levels. Viral rebound was observed after stopping treatment, which was again effectively controlled by treatment with 2CMC. No drug-resistant variants emerged.

Noroviruses are an important cause of chronic gastroenteritis in immunocompromised patients (1). Although most norovirus infections are acute and self-limiting, the infection can become chronic in (i) solid-organ and hematopoietic stem cell transplant recipients (2–4), (ii) patients undergoing chemotherapy (3, 5, 6), and patients with immunodeficiencies, including those caused by HIV infection (7–10). Prolonged norovirus infection can also be observed in young children (11) and the elderly (12, 13). Reduction of immunosuppressive therapy, when feasible, is the strategy of choice to control the infection in transplant recipients. Specific antiviral therapy to treat (chronic) norovirus gastroenteritis is not available. The effect of drugs such as ribavirin and nitazoxanide has been evaluated in a small number of patients, but no clear beneficial clinical effect was observed (10, 14, 15).

We wanted to establish a persistent mouse norovirus (MNV) infection model that can be used to assess the efficacy of norovirus inhibitors on such an infection. To that end, the MNV.CR6 strain (herein CR6) was employed. MNV is a genogroup V norovirus that has been widely used as a surrogate for human noroviruses (16, 17); CR6 is an a virulent MNV strain that can replicate for weeks to months in wild-type mice and (to higher titers) in innate immune-deficient mice (18, 19). The MNV.CR6 strain was shown to replicate at higher titers in the proximal colon but was also detected in the mesenteric lymph nodes, spleen, brain, liver, and lungs of mice 3 days after infection (18, 20). The site of persistence is reported to be the intestine, where CR6 is present for many weeks after infection (20). It was recently shown that both wild-type and IFNAR−/−-positive mice were cured of an ongoing MNV.CR6 infection following treatment with gamma interferon (IFN-α) (21).

Interferon receptor knockout mice (AG129) were infected orally with the CR6 strain (22), and the effects of two small-molecule inhibitors of in vitro norovirus replication (2′-C-methylcytidine [2CMC] and favipiravir [T-705]) on viral shedding in stool were assessed (23, 24). We demonstrated earlier that 2CMC is effective in the treatment and prophylaxis of acute MNV infection in AG129 mice (25).

2CMC was synthesized as described previously (26) and dissolved in sterile saline. Favipiravir (T-705) was purchased from BOC Sciences (NY, USA) and formulated in 0.4% carboxymethylcellulose (Sigma-Aldrich, Bornem, Belgium). The MNV.CR6 strain (kindly provided by H. W. Virgin, Washington University, St. Louis, MO, USA) was propagated in RAW 264.7 as described before (24).

AG129 mice (129/Sv mice) originally from BK Universal, United Kingdom, were bred and housed at the Rega Institute under specific-pathogen-free conditions. All experiments were performed under the guidelines and authorization of the Ethical Committee of the University of Leuven (P101/2012). For all experiments, age- and sex-matched mice 8 to 12 weeks of age were infected by oral gavage with 10⁶ CCID₅₀ (50% cell culture infective doses) of CR6. At 7 days postinfection (p.i.), mice were left untreated (n = 9) or were treated with 100 mg/kg daily of 2CMC subcutaneously for 5 (n = 4), 7 (n = 4), or 11 (n = 4) days. Two more rounds of a 14-day treatment (with a 4-week interval in between) with 2CMC (n = 10) or favipiravir (200 mg/kg daily by oral gavage [n = 5]) were given. On each day after infection, the general condition and weight of treated and untreated mice were assessed, individual stool samples were collected (whenever possible during one daily period of observation), and levels of MNV RNA were quantified by reverse transcriptase quantitative PCR (RT-qPCR). RNA was extracted from stool samples using the RNeasy minikit (Qiagen, Belgium) and assessed using the Thermo Scientific (Waltham, MA, USA) iTaq Universal SYBR Green One-Step RT-qPCR kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s protocol. For the MNV RT-qPCR, the primers and probe were used as described before, with minor modifications (24). The iTaq Universal SYBR Green One-Step RT-qPCR kit

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Universal Probes reaction mix (Bio-Rad, Belgium) was used, and cycling conditions included reverse transcription at 50°C for 10 min and initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 60°C for 30 s (Roche LightCycler 96; Roche Diagnostics, Belgium). Statistical analysis was performed using Prism 5 software (Graph-Pad Software, San Diego, CA). P values were determined with the nonparametric Mann-Whitney test.

Following oral infection with CR6, all AG129 mice remained healthy and did not show symptoms (e.g., ruffled fur, reduced activity, squinted eyes, diarrhea, or weight loss) that are typically observed in mice infected with MNV-1, a strain that causes acute infections (Fig. 1A). The consistency of the stool of infected animals remained normal throughout the experiment (data not shown), but viral RNA was detected from day 2 or 3 p.i. in some of the animals (Fig. 1B). From day 6 p.i. on, viral RNA was consistently detected at high titers in the stool of all infected mice (at >4 log_{10} RNA copies/g of stool in most mice). A parallel quantification of infectious virus particles was performed by endpoint titration (CCID_{50} determination) in stool samples collected in parallel and rendered comparable results and limits of detection (2.58 log_{10} CCID_{50}/g stool versus 1.81 log_{10} RNA copies/g stool) as shown in Fig. S1 in the supplemental material. Hence, the RT-qPCR has an adequate sensitivity to detect (reduced) MNV levels in infected mice.

Day 7 p.i. was selected as the start of treatment with 2CMC for 5, 7, or 11 consecutive days (Fig. 2A). Shedding of virus in the stool was quantified to monitor whether the inhibitor(s) can (i) reduce or even stop viral shedding and (ii) completely cure the animal of the infection. Treatment with 2CMC resulted in a significant reduction in virus shedding in the stool as early as 1 day after the start of treatment (day 8 p.i.) (reduction of 1.7 log_{10} RNA copies/g of stool in 2CMC-treated mice versus untreated mice) (Fig. 2). At day 14 p.i., i.e., after 7 days of treatment, CR6 RNA had become undetectable in the stool of all 2CMC-treated mice. Viral RNA remained undetectable in the stool of the 2CMC-treated animals until the end of treatment at day 17 p.i. (end of the 11-day treatment) (Fig. 2A). However, 3 days after the 11-day 2CMC-treatment period, viral RNA was again detectable in the stool of mice at levels comparable to those of the untreated controls (Fig. 2E). Hence, 2CMC treatment for ≤11 days proved insufficient in completely eliminating CR6 from the infected mice. Also, soon after cessation of the 5- or 7-day treatment period, rebound was observed in all mice.

Next, the effect of a treatment scheme consisting of two rounds of 14 consecutive days of 2CMC treatment (with a pause of 4 weeks in between) was evaluated; in parallel, the effect of favipiravir (for which we have demonstrated in vitro anti-MNV activity, [24]) was assessed (Fig. 3). In 6 of 10 2CMC-treated mice, CR6 RNA was undetectable in stool following another 14 days of treatment (day 21 p.i.). Similar to the experiment presented in Fig. 2, a rebound in viral RNA was noted following cessation of the treatment. Favipiravir did not reduce viral shedding (Fig. 3A). The dose selected for the study was determined on the basis of the solubility and stability of the formulation; this dose had also been shown to be effective against infection with yellow fever and West Nile virus in small models (27, 28). The in vitro activity of favipiravir against these flaviviruses is comparable to the activity of the compound against the murine norovirus (23, 29). In a recent study, 8 weeks of treatment with 600 mg/kg daily of favipiravir resulted in undetectable levels of MNV-3 RNA in the stool of some of the infected mice (30). At day 55 p.i. (i.e., about 4 weeks after stopping the first round of treatment), both of the groups treated earlier were again treated with either compound for 14 consecutive days (Fig. 3B). The second treatment round with favipiravir did not result in a reduction in viral shedding (Fig. 3B). The second round of 14-day treatment with 2CMC again proved to be effective in reducing the shedding of virus in the stool to undetectable levels (Fig. 3B), but following cessation of treatment, viral rebound was observed. Despite the fact that viral rebound was observed after the first 14-day 2CMC treatment period, the infection was still susceptible to the drug, suggesting that no drug-resistant variants had emerged. We tried but failed to sequence viral RNA isolated from stool samples at days 69 and 71 p.i. (which followed the end of treatment). We next attempted to sequence samples from day 16.
Although 2CMC is a relatively weak *in vitro* inhibitor of MNV replication, the compound effectively reduces virus shedding in the feces of mice chronically infected with MNV. Also, no drug resistance developed. Favipiravir, despite inhibiting *in vitro* MNV replication, was not effective in the mouse model. The activity of 2CMC in this chronic mouse infection model suggests that a (more) potent inhibitor of norovirus replication would be able to at least reduce viral replication in patients with chronic norovirus infections. In such patients, it is very important that drug-resistant variants not develop. Hence, antinorovirus drugs (e.g., 2CMC) should not readily select for drug-resistant variants; alternatively, a combination of drugs may also prevent the development of resistance.

In conclusion, we demonstrated for the first time that an inhibitor of norovirus replication can reduce viral shedding to undetectable levels in chronically infected animals. This validates this model for the evaluation of the potential *in vivo* efficacy of novel norovirus inhibitors.

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**FIG 2** (A) Scheme of CR6 infection and treatment with 2CMC at 100 mg/kg daily for 5, 7, or 11 days starting on day 7 p.i. (B) Viral RNA loads in stool samples of MNV.CR6-infected AG129 mice that were untreated (*n* = 9) or treated with 2CMC at 100 mg/kg daily starting on day 7 p.i. for a period of 5 (*n* = 4), 7 (*n* = 4), or 11 (*n* = 4) days. Data are presented only for mice that received treatment; values in brackets are the numbers of treated mice at particular time points. (C, D, E) Follow-up period after the end of 5, 7, or 11 days of treatment, respectively. Dotted line, limit of detection. *P* values were determined with the nonparametric Mann-Whitney test: ***, *P* < 0.001; **, *P* < 0.01; ns, *P* ≥ 0.05.
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FIG 3 Viral RNA loads in stool samples of MNV.CR6-infected AG129 mice that were untreated (n = 5) or treated with 2CMC at 100 mg/kg daily (n = 10) or T-705 at 200 mg/kg daily (n = 5) starting on day 7 p.i. for a first (A) or second (B) period of 14 consecutive days (with a 4-week drug-free interval). Dotted line, limit of detection. P values were determined with the nonparametric Mann-Whitney test: ***, P < 0.001; ***, P < 0.01; *, P < 0.05; ns, P ≥ 0.05.


