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Inhibition of norovirus replication by the nucleoside analogue 2′-*C*-methylcytidine

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ABSTRACT

We here report on the activity of 2′-*C*-methylcytidine (2CMC) [a nucleoside polymerase inhibitor of the hepatitis C virus (HCV)] on the *in vitro* replication of (murine) norovirus (MNV). 2CMC inhibits (i) virus-induced CPE formation, (ii) viral RNA synthesis and (iii) infectious progeny formation with EC_{50} values of ~2 μM. 2CMC acts at a time-point that coincides with the onset of viral RNA synthesis. Even following 30 passages of selective pressure no MNV-resistant virus was selected, which is in line with the high barrier to resistance of the nucleoside analogue for HCV. When combined with the broad-spectrum RNA virus inhibitor ribavirin, a marked antagonistic activity was observed indicating that these molecules should not be combined for the treatment of norovirus infections. Our results suggest that 2′-*C*-methyl nucleoside analogues should be further explored for the treatment and prophylaxis of norovirus infections.

1. Introduction

Noroviruses are today recognized as the leading cause of food-borne outbreaks of gastroenteritis worldwide, affecting millions of individuals and resulting in heavy health and economic burden every year [1,2]. However, no vaccine or specific antiviral therapy is available today for treatment or prevention of norovirus illness.

Noroviruses are positive-sense single stranded (ss) RNA viruses belonging to the genus *Norovirus*, family of the *Caliciviridae* [3]. The six/seven nonstructural proteins of norovirus include a viral protease [4], a NTPase/helicase [5] and a RNA-dependent RNA polymerase (RdRp) [6,7], responsible for the synthesis and amplification of the genomic RNA. The crystal structure of the Norwalk virus RdRp has been resolved and overall showed catalytic and structural elements highly conserved among RdRps of other (+)ssRNA viruses [8,9]. Therefore, the norovirus RdRp is a critical enzyme for viral replication and an important antiviral target [3,10].

Insights into the norovirus life cycle and thus potential antiviral targets have long been hampered by the lack of efficient cell culture systems, given human noroviruses are not cultivable [11]. Significant progress has been made with surrogate viruses (murine norovirus (MNV) and others) and a Norwalk virus replicon-bearing cell line [9,12–14].

There are very few reports of antiviral activity of small molecule inhibitors of noroviruses [15–17]. However, a substantial number of selective inhibitors of other ssRNA(+) viruses, such as picornaviruses, have been described [18]. Given the similarities of the replication strategies between noroviruses and such (+)ssRNA viruses, compounds with activity against these viruses could serve as scaffolds for the development of antivirals for norovirus. We therefore wanted to evaluate the potential anti-norovirus activity of a selection of such molecules. To that end a rapid *in vitro* antiviral assay was elaborated using the infectious MNV as a surrogate for human norovirus. MNV is considered today the best surrogate since many molecular features and fundamental mechanisms of replication are conserved [13]. From the reference antiviral molecules screened using this assay, 2′-*C*-methylcytidine (2CMC) was identified as an inhibitor of MNV replication. We report on the particular characteristics of the anti-norovirus activity of this nucleoside analogue.

2. Materials and methods

2.1. Compounds

Ribavirin, 1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (Virazole; RBV) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Simvastatin and dextran sulfate 5000 and 10,000 were purchased from Sigma–Aldrich (Bornem, Belgium). All other molecules were synthesized as described before [19–22].

2.2. Cells and viruses

MNV (strain MNV-1.CW1) was propagated in RAW 264.7 cells grown in DMEM (Life Technologies, Gent, Belgium) supplemented with 10% or 2%FBS, 2 mM L-glutamine, 20 mM HEPES, 0.075 g/L

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sodium bicarbonate, 1 mM sodium pyruvate, 100U penicillin/mL,100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

For the plaque reduction assay, double concentrated E-MEM 10% FBS supplemented as above plus 0.2 mM non-essential amino acids, without phenol red mixed in equal parts with 1% agarose was used as overlay media. A second overlay media containing neutral red (0.6%) was used to visualize PFU (plaque forming units).

2.3. Antiviral assay

The antiviral activity of the selected compounds was initially determined using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based cytopathic effect (CPE) reduction assay. RAW 264.7 cells (1×10^4 cells/well) were seeded in a 96-well plate and infected with MNV (MOI of 0.001) in the presence (or absence) of a dilution series of compounds (0.023–50 or 100 μg/mL). Cells were incubated for 3 days, i.e. until complete CPE was observed in infected untreated cells. Then, a MTS-phenazinemethosulfate (MTS/PMS) stock solution [(2 mg/mL MTS (Promega, Leiden, The Netherlands) and 46 g/mL PMS (Sigma–Aldrich, Bornem, Belgium) in PBS at pH 6–6.5)] was diluted 1/20 in MEM (Life Technologies, Gent, Belgium) and 75 μL were added to each well. After 2 h, the optical density (OD) was read at 498 nm. The %CPE reduction was calculated as $[(OD_{treated})_{MNV} - OD_{VC}]/[OD_{CC} - OD_{VC}] \times 100$, where OD_{CC} represents the OD of the uninfected untreated cells, whereas OD_{VC} and $(OD_{treated})_{MNV}$ represent the OD of infected untreated cells and virus-infected cells treated with a compound concentration, respectively. The 50% effective concentration (EC_{50}) was defined as the compound concentration that protected 50% of the cells from virus-induced CPE.

2.4. Cytotoxicity assay

The cytotoxicity of the compounds was evaluated by the MTS-method, by exposing uninfected cells to the same concentrations of compounds for 3 days. The %cell viability was calculated as $(OD_{treated}/OD_{CC}) \times 100$, where OD_{CC} is the OD of uninfected untreated cells and $OD_{treated}$ are uninfected cells treated with compound. The CC_{50} was defined as the compound concentration that reduces the number of viable cells by 50%. The selectivity index (SI) was calculated as CC_{50}/EC_{50}.

2.5. MOI dependence assay

To evaluate the effect of the multiplicity of infection (MOI) on the anti-norovirus activity of 2CMC, RAW 264.7 cells (1×10^4 cells/well) were seeded in a 96-well plate and infected with MNV at a MOI between 0.002 and 2 in the presence of a dilution series of compound (0.023–50 μg/mL). The antiviral activity was evaluated by the MTS-method and the EC_{50} was calculated. A back-titration was performed in all the virus suspensions for which virus-induced CPE was recorded microscopically after 3 days of incubation. Virus titers were expressed as $CCID_{50}$ according to the Reed and Muench formula.

2.6. Plaque reduction assay

The method was essentially as reported earlier [16]. Briefly, confluent RAW cells (5×10^5 cell/mL seeded one day earlier in 6-well plates) were infected with MNV (20–80 PFUs/well) for 1 h at 37 °C and exposed to serial concentrations of compound (0.1–10 μg/mL). After MNV-infection, a first overlay containing the same concentrations of compound was added. Plaques were counted after 48 h incubation, in the presence of neutral red (0.6%). The %PFU reduction was calculated as $(PFU_{treated}/PFU_{VC}) \times 100$, in which PFU_{VC} is the number of PFU of untreated infected cell cultures and $PFU_{treated}$ are infected cells treated with compound. The EC_{50} was defined as the compound concentration that reduced the number of PFU by 50%.

2.7. Virus yield assay

RAW 264.7 cells (2×10^5 cells/mL) were infected with MNV at an MOI of 0.001. After 1 h at 4 °C, cells were washed with cold (4 °C) DMEM and seeded in 96-well plates, in the presence of a dilution series of 2CMC (0.023–50 μg/mL). Following 3 days of incubation, cell culture supernatants were collected for quantification of viral RNA load by quantitative RT-PCR (qRT-PCR).

2.8. RNA isolation and quantitative RT-PCR

Extracellular RNA was isolated from cell culture supernatant (150 μL) using the NucleoSpin RNA Virus Kit (Macherey–Nagel, Germany), intracellular RNA was extracted from cells using the RNeasy minikit (Qiagen, Netherlands) according to the manufacturer's protocol. Forward (5′-CAC GCC ACC GAT CTG TTC TG -3′ position 4972–4991) and reverse (5′-GCG CTG CGC CAT CAC TC-3′ position 5080–5064) primers were designed for the ORF1/2 junction, as described elsewhere [23]. A 6-FAM-MGB probe was used (5′-CGC TTT GGA ACA ATG-3′ position 5001–5015).

One-step qRT-PCR was performed in a 25 μL reaction mixture containing 6.25 μL One-Step Reverse Transcriptase qPCR Master Mix Plus Low ROX (Eurogentec, Belgium), 900 nM of each primer, 200 nM of probe, 0.0625 μL of RT-PCR enzyme mix and 3 μL of template MNV RNA. Cycling conditions were: reverse transcription at 48 °C for 30 min, initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min (ABI 7500 Fast Real-Time PCR System, Applied Biosystems, USA). For absolute quantification, standard curves were generated using 10-fold dilutions of MNV template DNA of known concentration.

2.9. Time-of-drug-addition assay

RAW 264.7 cells (2×10^5 cells/mL) were infected with MNV at a MOI of 0.1. After 1 h at 4 °C, cells were washed with cold (4 °C) DMEM and incubated at 37 °C. To study the replication kinetics of MNV in untreated cultures, supernatant and cells were harvested every 2 h until 24 h post-infection (pi) and viral RNA was quantified by qRT-PCR. In parallel, another set of infected cultures were treated with 2CMC (at 2 μg/mL). The molecule was added at different time points after infection (with 2 h intervals) and cultures were further incubated until 24 h pi, at which time supernatant and cells were collected separately for determination of the viral RNA by qRT-PCR.

2.10. Selection of drug-resistant variants

Drug-resistant virus was tentatively generated by culturing wild type MNV in RAW 264.7 cells in the presence of increasing concentrations of 2CMC (up to 5 μg/mL) in 96-well plates. After 4–5 days supernatants were collected from cultures which exhibited full CPE with the highest concentration of compound. The collected viruses were used for a successive round of infection in which the procedure was repeated and virus growing at higher concentrations of 2CMC was generated. Three independent serial passages with each 30 rounds of infection were completed.

2.11. Sequencing of MNV cultured in the presence of 2CMC

cDNA fragments that cover the entire MNV genome were generated by RT-PCR after extraction from infected cell culture supernatants. RT-PCR was performed in a 50 μL reaction mixture containing 1 μL of sample, 34 μL of H_2O, 10 μL of 5× Qiagen One Step RT-PCR Buffer, 2 μL of dNTPs, 0.6 μM of each primer and 2 μL of RT-PCR enzyme mix. Cycling conditions were: reverse transcription at 50 °C for 30 min, activation Hotstar Taq at 95 °C for 15 min followed by 40 cycles of initial denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. After gel purification, PCR fragments of both DNA strands were analyzed using the cycle sequencing method (ABI Prism BigDye terminator cycle sequencing ready reaction kit). Sequencing data were obtained with an ABI373 automated sequence analyzer (Applied Biosystems), and sequences were analyzed with the 4Peaks software (MacOSX application).

2.12. Combination studies of 2CMC and ribavirin

The combined antiviral activity of 2CMC and ribavirin was evaluated by the MTS-method. Three treatment conditions were performed: (i) cells were infected with MNV and treated with 2CMC (0.08–10 μg/mL); (ii) cells were infected with MNV, treated with 2CMC (0.08–10 μg/mL) and 100 μM ribavirin; (iii) cells were pre-treated with 100 μM ribavirin for 6 h prior to viral infection and treatment with 2CMC (0.08–10 μg/mL).

3. Results

3.1. Screening of reference antiviral compounds for anti-norovirus activity

A panel of molecules with known antiviral activity on (+)ssRNA viruses acting on different targets of the viral life cycle were screened for antiviral activity against norovirus by an MTS-based CPE reduction assay. None of these molecules, except for 2′-*C*-methylcytidine (2CMC) inhibited the replication of the virus (Supplementary data, Table 1).

3.2. Anti-norovirus activity of 2CMC

The protective effect of 2CMC on virus-induced CPE formation was confirmed by quantifying the effect of the molecule on viral RNA synthesis and virus-induced plaque formation. Norovirus-induced CPE and viral RNA synthesis (Fig. 1) was inhibited with EC_{50} values of 0.52 ± 0.27 μg/mL [2.0 ± 1.0 μM] and 0.41 ± 0.21 μg/mL [1.6 ± 0.8 μM], respectively and plaque formation with an EC_{50} of 0.35 ± 0.05 μg/mL [1.4 ± 0.3 μM] (Table 1). A CC_{50} value of 4.2 ± 0.5 μg/mL [16.4 ± 2.1 μM] was determined, resulting in a selectivity index of 8.1. The anti-norovirus activity of 2CMC appeared largely independent of the MOI. EC_{50} values varied between 0.55 and 1.70 μg/mL when cells had been infected with MOI ranging from 0.002 to 2 (Fig. 2).

3.3. Time-of-drug-addition assay

Time-of-(drug) addition experiments were carried out to estimate the moment when 2CMC interferes with the replication cycle of norovirus. To that end we first determined the kinetics of a single replication cycle of MNV. Extracellular and intracellular viral RNA was quantified by means of qRT-PCR for the first 24 h pi.

Levels of viral RNA in the culture supernatant of untreated infected cultures became detectable (above background) as of 18 h pi (data not shown). Thus under these experimental conditions one replication cycle of norovirus takes approximately 18 h. The onset of intracellular RNA synthesis was detected at 6 h pi and was followed by an exponential increase in intracellular levels of viral RNA (inset graph Fig. 3). When 2CMC was added during the first 6 h pi, it resulted in the complete inhibition of the formation of both extracellular and intracellular viral RNA synthesis (Fig. 3). When the compound was first added at a time-point later than 6 h pi, a gradual increase of both extra- and intracellular viral RNA was observed, reflecting the loss of the antiviral effect of 2CMC.

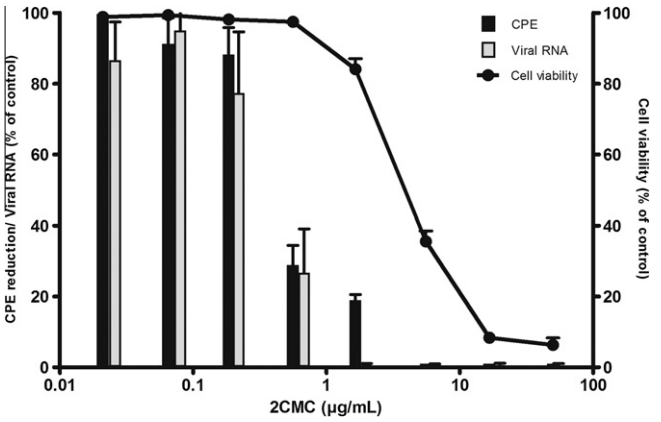

Fig. 1. *In vitro* anti-norovirus activity of 2CMC as quantified by means of (i) a MTS-based virus-induced CPE reduction assay and (ii) qRT-PCR assay.

3.4. Selection and sequencing of "2CMC-resistant" strains

We next tried to generate, by selective pressure with increasing concentrations, MNV variants resistant to 2CMC. Three cultures were independently passaged in the presence of the drug for a total of 30 passages each but the susceptibility of the resulting pools to 2CMC did not markedly change (maximum 4-fold increase in EC_{50}). Pools associated with higher EC_{50} values were sequenced but no mutations were detected in the genomes of these pools.

3.5. 2CMC combined with ribavirin results in antagonistic antiviral activity

The combined *in vitro* antiviral effect of ribavirin and 2CMC was studied. Ribavirin did not result in relevant antiviral activity in standard anti-norovirus assays. When cells were pre-treated with ribavirin for 6 h prior to infection, a maximum inhibitory effect of 30% was achieved with 100 μM (data not shown). Concentrations >100 μM were not considered for these experiments as they resulted in toxicity on the RAW cells.

When 100 μM ribavirin was added to 2CMC-treated infected cultures, a marked antagonistic activity was observed (Fig. 4), in particular at 2CMC concentrations <2.5 μg/mL. Indeed, whereas 2CMC alone results in virtual complete protection against virus-induced CPE, when combined with ribavirin, an antiviral effect was no longer detected. Concentrations of 2CMC >2.5 μg/mL combined with 100 μM ribavirin, still resulted in some antiviral activity but the protective efficacy was markedly lower than when 2CMC was used alone. When cells were pre-treated with ribavirin prior to MNV-infection, the antiviral effect of 2CMC disappeared completely even at the highest concentration tested (10 μg/mL).

The effect of a pre-treatment of cells with 2CMC before infection was also evaluated but no influence was observed in the antiviral activity of the compound (data not shown).

Table 1
In vitro anti-norovirus activity of 2CMC.

	CPE inhibition EC_{50}, μg/mL [μM]	Plaque reduction assay EC_{50}, μg/mL [μM]	Inhibition of viral RNA synthesis EC_{50}, μg/mL [μM]
2'-*C*-methylcytidine	0.52 ± 0.27 [2.0 ± 1.0]	0.35 ± 0.05 [1.4 ± 0.3][a]	0.41 ± 0.21 [1.6 ± 0.8]

Inhibitory effect of 2CMC on MNV replication as quantified by: (i) virus-induced CPE reduction using the MTS-method, (ii) reduction of plaque formation by plaque reduction assay and (iii) reduction of viral RNA levels by qRT-PCR. Results are means ± SEM of 6 independent experiments.
[a] Results are means ± SEM of 2 independent experiments.

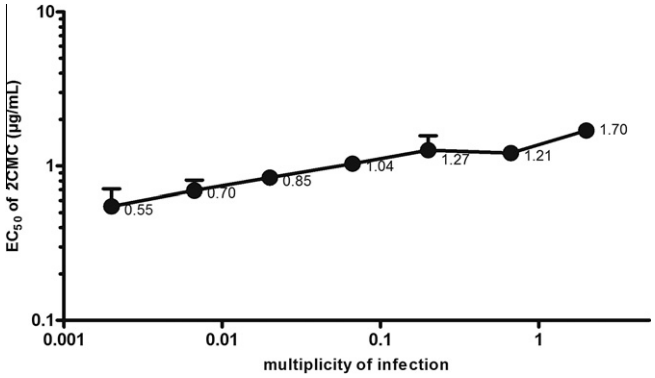

Fig. 2. Influence of MOI on the antiviral activity of 2CMC, evaluated by infecting cells with MNV at increasing MOIs (0.002–2). Results are means of 2 independent experiments ± SEM.

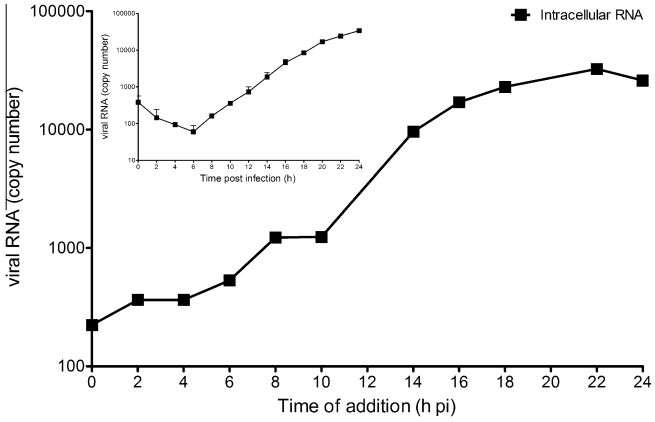

Fig. 3. Time-of-drug-addition studies of 2CMC. The effect of time-of-(drug) addition on the antiviral activity of 2CMC was evaluated by quantification of intracellular viral RNA by qRT-PCR at 24 h pi (in cells treated with 2 μg/mL 2CMC, starting at different times post-infection) and comparison with untreated infected cells. The inset presents yields of intracellular (squares) viral RNA during a single replication cycle of MNV in RAW cells. Viral RNA levels were monitored at various times post-infection by qRT-PCR.

4. Discussion

We identified the hepatitis C virus (HCV) nucleoside polymerase inhibitor 2′-*C*-methylcytidine as an inhibitor of the *in vitro* replication of MNV, a surrogate for the non-cultivable human norovirus.

2CMC inhibited viral replication (as quantified by three methods) with an EC_{50} value of ~2 μM, only slightly higher than the EC_{50} obtained for inhibition of *in vitro* HCV replication [22]. Equally important is the fact that 2CMC is able to completely inhibit viral replication at non-toxic concentrations, which further points to the selectivity of the antiviral effect. Of note is that the murine macrophage cell line used (RAW 264.7) proved to be much more sensitive to 2CMC than the hepatoma, epithelial or fibroblast cell

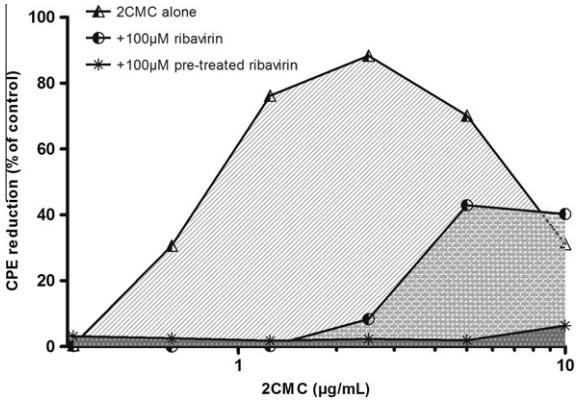

Fig. 4. Combined antiviral activity of 2CMC and ribavirin. Graph shows effect on the reduction of virus-induced CPE of MNV-infected RAW cells under three different conditions (i) cells were infected with MNV and treated with 2CMC (0.08–10 μg/mL); (ii) cells were infected with MNV and treated with 2CMC (0.08–10 μg/mL) and 100 μM ribavirin; (iii) cells were pre-treated with 100 μM ribavirin for 6 h prior to viral infection and treatment with 2CMC (0.08–10 μg/mL). Results are expressed as %CPE reduction and are means 2 independent experiments.

lines used to assess the antiviral activities of this molecule against picornaviruses and HCV (where CC_{50} was almost 10-fold higher) [20,24,25].

The 3′-valyl ester oral prodrug of 2′-*C*-methylcytidine i.e. valopicitabine was developed as an inhibitor of HCV replication. The nucleoside analogue inhibits, through its 5′-triphosphate metabolite, the HCV RdRp [24,26]. Valopicitabine resulted in antiviral efficacy in chronically HCV-infected patients [27,28] but development was halted because of adverse effects. During the submission process of the current paper another team also reported on the *in vitro* anti-norovirus activity of 2CMC and its closely related analogue 2′-F-2′-*C*-methylcytidine [29], with more or less comparable activity of 2CMC. Of note, the adenosine and the 7-deaza-adenosine [30] of 2CMC (active against HCV) exerted very little or no antiviral activity against MNV. Since 2CMC is a known polymerase inhibitor, the molecule may also be expected to inhibit the norovirus polymerase. In time-of-drug addition assays we demonstrated that 2CMC acts at a time-point that coincides with the onset of viral RNA synthesis, which is in line with the viral polymerase as the antiviral target. 2CMC-resistant HCV viruses carry a S282T mutation in their polymerase [31] while *in vitro* resistance of HCV to 2CMC is not readily selected. Indeed, the barrier to resistance is high for this and most HCV nucleoside analogues [32]. In an attempt to generate 2CMC-resistant MNV, we passaged the virus 30 times in the presence of the molecule. No clear drug-resistant variants were observed and no mutations in the viral genome were noted that could be associated with a drug resistant antiviral phenotype which is in line with the high barrier to resistance against for HCV [32].

Ribavirin, a drug used for the treatment of respiratory syncytial virus, Lassa virus and HCV infections (in combination with pegylated interferon) has been reported to inhibit the replication of norovirus in the Norwalk replicon model [15]. We therefore wanted to study the combined antiviral effect of ribavirin with 2CMC.

Although Costantini and colleagues [29] reported a slight synergistic activity of this combination on norovirus replication, we observed a marked antagonistic effect. This is in line with an antagonistic activity of ribavirin on the anti-HCV and anti-enterovirus activity of 2CMC [33,34]. Ribavirin may result in increased intracellular levels of cytosine 5′-triphosphate (CTP). The 5′-triphosphate form of 2CMC competes directly with CTP for binding to the RdRp active site hence explaining the antagonistic activity. Our findings thus argue against the potential combined use of ribavirin and 2′-*C*-methylpyrimidine analogues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.003.

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