2′-C-Methylcytidine, a potent and selective inhibitor of the replication of the foot-and-mouth disease virus

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Abstract:

Introduction: The foot-and-mouth disease virus (FMDV) is the causal agent of one of the most contagious and devastating animal diseases. With the adoption of Council Directive 2003/85/EC greater emphasis has been put on emergency vaccination to control a future outbreak within the European Community. However, FMD vaccines are serotype- (and subtype) specific and confer complete clinical against challenge only 7 days post vaccination. Possible alternatives would be the use of antiviral agents that inhibit FMDV replication. We examined the potential of the nucleoside analogue, 2′-C-hydroxymethylcytidine (2′-C-MetCyt), for this purpose and compared its antiviral activity to that of ribavirin, a known FMDV inhibitor.

Materials and Methods: Anti-FMDV assays and cytotoxicity assays were performed to assess the in vitro antiviral activity. Effect on viral RNA yield was investigated using a quantitative real-time RT-PCR. Virus yield and plaque reduction assays were undertaking, as well as time of drug addition studies to obtain initial information on the mode of action of 2′-C-MetCyt.

Results: The EC50 and EC90 for inhibition of FMDV-induced cytopathic effect were 6.4 ± 3.8 µM and 10.8 ± 5.4 µM, making it 100- to 140-fold more potent than ribavirin. Comparable EC50 values for inhibition of viral RNA synthesis were observed. Treatment of FMDV-infected BHK-21 wells with 77 µM 2′-C-MetCyt resulted in a 1.6 10³ to 3.2 10³-fold reduction of infectious virus yield. Time of drug addition assays suggest that 2′-C-MetCyt interacts with viral replication at a time point that coincides with the onset of viral replication.

Discussion: In contrast to emergency vaccination, a potent and selective antiviral agent, such as 2′-C-MetCyt, may provide almost immediate (prophylactic/therapeutic) protection against infection and, thus, constitute an important alternative/supplementary option to contain outbreak such as those caused by FMDV.

Introduction:

The foot-and-mouth disease virus (FMDV) is the prototype member of the Aphthovirus genus within the Picornaviridae. The virus is antigenically variable and seven distinct serotypes of the virus have been defined [O, A, C, Asia1 and the SAT types 1, 2 and 3] as well as multiple subtypes. The non-enveloped, single-stranded (ss), positive-sense RNA virus affects all domesticated cloven-hoofed animals, including cattle, swine, sheep and goats as well as a variety of wild animal species. The ability of FMDV to spread rapidly in susceptible animal herds as well as its trans-boundary character, make foot-and-mouth disease (FMD) a disease listed by the World Organisation for Animal Health (OIE) and recognised as a major constraint to international trade (Leforban, 1999). The swine vesicular disease virus (SVDV) is a porcine picornavirus that causes clinical signs, which are (symptomatically) indistinguishable from FMD. Recently, swine vesicular disease (SVD) caused unexpected problems in Portugal, where outbreaks were reported in 2003 (D.J. Paton, Report of the annual meeting of EU national swine vesicular disease laboratories, Brussels, 17 November 2004, p. 3-4). Although it has been successfully eradicated since then, SVDV remains endemic in the southern regions of Italy (Reid et al., 2004) and, thus, continues to pose a risk to other European countries.

Because of the severe socioeconomic consequences related to an FMD incursion, the main concern of FMD-free countries is to prevent the introduction of the virus and/or to rapidly eradicate it in case of an outbreak. Until recently, emergency vaccination had a minor role in the European control policy, but the 2001 outbreaks have prompted the European Commission and the Member States to revise the legislation, placing greater emphasis on the use of emergency vaccination.
(Council Directive 2003/85/EC). However, since FMD vaccines are serotype- and, to a lesser extent, subtype-specific, an effective control strategy is dependent upon vigilant epidemiological monitoring to identify relevant circulating strains for inclusion in antigen banks (Paton et al., 2005). Moreover, even the best currently available, chemically inactivated vaccines only confer complete clinical protection against homologous challenge 7 days after vaccination and partial protection in 4 days (Golde et al., 2005). The delay in protection implies that during the initial – critical – stages of an outbreak the livestock population remains highly susceptible to infection. Therefore, the use of current FMD vaccines to induce early protection is limited and alternative/supplementary methods to rapidly reduce the spread of FMDV in outbreak situations are needed.

One possible alternative would be the use of antiviral agents that inhibit FMDV replication. 5'-Fluorouracil and 5-azacytidine have been reported to reduce viral progeny by 50 to 100-fold in vitro (Sierra et al., 2000), whereas the nucleoside analogue ribavirin \([1\beta-D\text{-}ribofuranosyl\text{-}1H\text{-}1,2,4\text{-}triazole\text{-}3\text{-}carboxamide}\) was found to eliminate FMDV from acute and persistently infected BHK-21 cell cultures (De la Torre et al., 1987). The oral prodrug NM283 (Valopicitabine) of a novel ribonucleoside analogue, 2’-C-methylysidine (2’-C-MetCy), has recently been reported as an inhibitor of the replication of hepatitis C virus (HCV) (Pierra et al., 2005). We studied whether 2’-C-MetCy also elicits antiviral activity against FMDV and SVDV, akin to HCV positive-sense ssRNA viruses.

**Materials and Methods:**

1. **Compounds**

Ribavirin was purchased from ICN Pharmaceuticals and 2’-C-MetCy was synthesized according to standard methods (Eldrup et al., 2004; Clark et al., 2005) (Fig. 1).

2. **Cells and viruses**

Baby hamster kidney (BHK-21) cells were grown in Glasgow MEM BHK-21 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 7% v/v tryptose-phosphate (MEM-FBS-A). Swine kidney (SK6) cells were grown in Leibovitz medium (LM) supplemented with 3% heat-inactivated FBS, 80 IU/ml gentamycin and 1 µg/ml fungizone antimitotic (LM-FBS-A).

The FMDV strains O1 Manisa, A22 Iraq 24/64, A Iran 1996, C4 Noville, Asia1 Shamir, SAT1 ZIM 25/89, SAT2 NAM 1/92/2 and SAT3 ZIM 4/81 and the SVDV strain UKG 27/72 were obtained from the Institute for Animal Health, Pirbright, United Kingdom.

3. **Anti-FMDV assay**

BHK-21 cells were seeded in 96-well plates at a density of 1.5 x 10^5 cells per well in MEM-FBS-A. Following a 24h incubation at 37°C and 5% CO2, medium was removed, cells were washed three times with phosphate-buffered saline (PBS, pH 7.4), and 100 µl of FMDV inoculum was added (approximately 50 CCID50/100 µl, in which CCID50 is defined as the concentration of virus capable of causing CPE in 50% of the cell culture). After a 30-minute adsorption period at 37°C and 5% CO2, the FMDV suspension was removed and cells were washed three times with PBS. Fivefold serial dilutions of test compound (ribavirin or 2’-C-MetCy) were added to the infected cell culture in a total volume of 100 µl MEM-A supplemented with 2% FBS (MEM-2%FBS-A) and cell cultures were subsequently incubated at 37°C and 5% CO2. Non-infected, untreated cells (i.e. cell controls, CC) and infected, untreated cells (i.e. virus controls, VC) were included in each assay. When at least 90% destruction of the cell monolayer was observed microscopically for the VC wells (between 24h to 48h after infection), 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine ethosulfate (MTS/PES) solution was added to each well to induce a colorimetric reaction. Subsequently, following a 2-hour incubation period at 37°C, the optical density (OD) of each well was read at 490 nm in a microplate reader. The percent CPE reduction was calculated as follows:

\[
\text{%CPE reduction} = \frac{([\text{OD}_{\text{treated}}]_{\text{FMDV}} - \text{OD}_{\text{CC}})}{\text{OD}_{\text{CC}} - \text{OD}_{\text{VC}}} \times 100
\]

in which (OD_{treated})_{FMDV} is the OD_{490 nm} of cells infected with a defined FMDV strain and treated with a certain dilution of compound. The 50% and 90% effective concentrations (EC50 and EC90) were defined as the compound concentrations that protect respectively 50% and 90% of the cells against virus-induced CPE. Both the EC50 and EC90 were calculated using logarithmic interpolation.

4. **Anti-SVDV assay**

The EC50 and EC90 values of both ribavirin and 2’-C-MetCy were determined for SVDV strain UKG 27/72 in a manner identical to the one described for FMDV, apart from the SK6 cell line and the LM-FBS-A medium that were used.

5. **Cytotoxicity assay**
In parallel with the anti-FMDV and anti-SVDV assays, BHK-21 and SK6 cells were seeded in 96-well plates at a density of 1.5 x 10^5 cells per well in MEM-FBS-A and LM-FBS-A, respectively. Following 24h of incubation at 37°C and 5% CO₂, medium was removed and cells were washed three times with PBS. Fivefold serial dilutions of a test compound (ribavirin and 2'-C-MetCyt) in a total volume of 100 µl MEM-2%FBS-A or LM-2%FBS-A were added. Plates were subsequently incubated at 37°C and 5% CO₂ for an additional 24h to 48h. Untreated CC were included in each assay. Subsequently, 20 µl of a MTS/PES solution was added to each well and plates were placed at 37°C in the dark for a 2-hour period. The OD_{490 nm} in each well was read and the percentage of viable cells was calculated as follows:

\[
\%\text{viable cells} = \left( \frac{\text{OD}_{490\ nm}}{\text{OD}_{490\ nm}^{\text{treated}}} \right) \times 100
\]

in which OD_{treated} is the OD_{490 nm} of cells treated with a certain dilution of compound. The 50% toxic concentration (TC_{50}) was defined as the compound concentrations that reduce the number of viable cells by 50% and was calculated using logarithmic interpolation.

6. RNA extraction and quantitative real-time RT-PCR for FMDV

The effect of 2'-C-MetCyt on FMD viral RNA yields for strains O Manisa and A Iran 1996 was studied using a serotype-independent two-step quantitative real-time RT-PCR (qRT-PCR) system. The experimental design was identical to that of the anti-FMDV assay described above. However, instead of adding an MTS/PES solution, cell culture supernatant and cells were collected and stored at -80°C until use. RNA was extracted from the collected fractions using commercially available RNeasy silica-based spin-columns according to the manufacturer's instructions. In a separate reverse transcription (RT) step, 9.625 µl of 1:10 diluted, extracted RNA was added to a 1x Multiscribe RT Buffer supplemented with 62.5 pmol random hexamer primers, 12.5 nmol dNTP's, 137.5 nmol MgCl₂, 10 IU RNase inhibitor and 31.25 IU Multiscribe reverse transcriptase in a total volume of 25 µl. RT conditions were as follows: 10 min at 25°C, followed by 30 min at 48°C and 5 min at 95°C.

Subsequently, a modified version of the real-time PCR described by Reid and colleagues (2003) was performed. Briefly, 10 µl template cDNA was added to a 2x universal TaqMan PCR master mix without AmpErase® UNG supplemented with 22.5 pmol forward and reverse primers and 5 pmol probe (for primer and probe sequences, refer to Reid et al., 2003) in a total volume of 25 µl. The mixture was placed in an ABI 7900HT thermocycler to perform 45 cycles of 15 sec at 95°C and 60 sec at 60°C, preceded by 10 min at 95°C. A tenfold dilution series of a known FMDV RNA positive sample and a negative water sample were incorporated as internal controls in both the RT and PCR steps. All samples were analysed in three replicate reactions.

7. Virus yield reduction assay for FMDV

Confluent monolayers of BHK-21 cells in 24-well plates were inoculated with 300 µl of a viral suspension of approximately 50 CCID₅₀/100 µl (FMDV strain O Manisa or A Iran 1996). Following a 30-minute adsorption period at 37°C and 5% CO₂, the inoculum was removed and the cells were washed three times with PBS. Subsequently, either 1 ml of a 77 µM solution of 2'-C-MetCyt in MEM-2%FBS-A or 1 ml MEM-2%FBS-A was added to the wells and plates were incubated at 37°C and 5% CO₂ for 24h. CC and non-infected, treated cells (product controls, PC) were incorporated in each assay. Following the incubation period, plates were read microscopically for CPE and cytotoxicity. In the absence of the latter, plates were stored at -80°C until further use. After thawing, the well content of infected, treated and VC cells was collected and centrifuged (4°C, 3000 x g, 15 minutes) to eliminate cell debris. The resulting supernatant was used in a backtitration experiment with BHK-21 cells in suspension (OIE, 2004). Virus-induced CPE was recorded microscopically after a 48h incubation period at 37°C and 5% CO₂. Virus titres were expressed as CCID₅₀/ml according to the method described by Reed and Muench (1938) to determine the efficacy of 2'-C-MetCyt in reducing infectious FMD viral yields.

8. Plaque reduction assay for FMDV

24-well plates were seeded with BHK-21 cells at a density of 3 x 10⁵ cells per well in MEM-FBS-A medium. The plates were incubated at 37°C and 5% CO₂ until the cell monolayer obtained 95% confluence. Medium was removed, cells were washed with PBS and 300 µl of a tenfold dilution series of either FMDV strain (O Manisa or A Iran 1996) in MEM-2%FBS-A was added. Following a 30-minute incubation period at 37°C and 5% CO₂, the viral suspension was removed and cells were washed three times with PBS. Subsequently, either 1 ml of a defined concentration of 2'-C-MetCyt in MEM-2%FBS-A (77 µM) or 1 ml MEM-2%FBS-A was added. Plates were incubated for an additional 7h30 at 37°C and 5% CO₂. Supernatant was removed and the cell monolayer was overlaid with 1 ml MEM-2%FBS-A supplemented with 0.6 % w/v agar and incubated for 48h at 37°C and 5% CO₂. Afterwards, 300 µl of 30% formaldehyde was deposited in each well and left to diffuse for 30 minutes. The agar overlay was carefully removed and 100 µl of a 1% crystal violet in 20% ethanol was added to each well to stain the cells. Following a 5-minute coloration period, the
solution was removed and each well was washed three times with PBS. CC and PC were incorporated in each assay as internal controls. Plates were read microscopically for cytopathic efficiency.

9. Time-of-drug addition assay for FMDV
Briefly, confluent BHK-21 cells in 96-well plates were infected with approximately 3 CCID_{50}/cell of virus (FMDV strain O, Manisa or A Iran 1996). Following a 30-minute incubation period at 37°C and 5% CO₂, the inoculum was removed, cells were washed three times with PBS and 100 µl of MEM-2%FBS-A was added. Every hour post-inoculation during a timespan of 6 hours, supernatant and cell pellets of these infected cultures were harvested and stored at -80°C until further use. In parallel, a final concentration of 77 µM of 2'-C-MetCyt was added every hour post-inoculation during a timespan of 6 hours to the supernatant. The compound-treated cell cultures were incubated until 6h post inoculation, at which time cell pellets were collected and stored at -80°C until further use. The RNA level of the collected fractions was quantified by the real-time PCR method for FMDV as described above. Additionally, the supernatant of the untreated cell culture was used to assess infectious viral yields in a titration assay with BHK-21 cells in suspension (OIE, 2004).

Results:

1. Antiviral activity of 2'-C-MetCyt
The ability of the nucleoside analogue 2'-C-MetCyt to inhibit FMDV and SVDV-induced CPE formation was assessed in a BHK-21 and SK6 cell multicycle growth assay, respectively. The antiviral activity was compared to that of ribavirin, a broad-spectrum antiviral agent, earlier reported to inhibit acute FMDV replication in vitro (De la Torre et al., 1987). The EC_{50} and EC_{90} were determined by a CPE reduction assay using the MTS method. Both compounds exerted antiviral activity against all FMDV strains tested (Table 1) and inhibited virus-induced CPE formation in a dose-dependent manner (R² > 0.9 for each compound) (data not shown). However, differences in antiviral efficiency among FMDV serotypes were observed. On average, the EC_{50} and EC_{90} values of 2'-C-MetCyt for inhibition of FMDV replication were respectively 6.4 ± 3.8 µM and 10.8 ± 5.4 µM, making it 100- to 140-fold more potent than ribavirin in inhibiting FMDV replication (p<0.001). Moreover, and in contrast to ribavirin, 2'-C-MetCyt also exerted antiviral activity against SVDV [EC_{50} = 45.2 ± 0.5 µM and EC_{90} = 71.0 ± 0.1 µM (n = 3)]. The mean 50% cytotoxic concentrations of 2'-C-MetCyt and ribavirin were 762 ± 130 µM and greater than 2047 µM, respectively.

From here on, only FMDV strains O, Manisa and A Iran 1996 were used, because of their importance to Europe.

2. Effect of 2'-C-MetCyt on viral RNA yield
To further validate the antiviral activity of 2'-C-MetCyt against FMDV O, Manisa and A Iran 1996, the effect of the compound on viral RNA yield was determined by qRT-PCR (Fig. 2). The EC_{50} for inhibition of extracellular viral RNA yield were 2.2 ± 0.4 µM and 2.0 ± 0.3 µM, respectively, whereas the EC_{50} for inhibition of intracellular viral RNA yield for both strains were slightly higher (respectively, 5.0 ± 1.8 µM and 6.4 ± 0.9 µM).

3. Virus yield and plaque reduction
To assess the effect of the compound on infectious FMDV yields and FMDV plaque formation efficiency, 2'-C-MetCyt was added to infected BHK-21 cell monolayer at a concentration of 77µM. Addition of 2'-C-MetCyt resulted in a reduction of 1.6 10^{3} to 3.2 10^{3} CCID_{50}/ml of infectious virus yield for FMDV strains O, Manisa and A Iran 1996 as compared to untreated controls and a 1.5 10^{3} to 5 10^{3} reduction in plaque formation efficiency (Fig. 3).

4 Time-of-drug addition studies
Time-of-drug addition assays for FMDV strains O, Manisa and A Iran 1996 were set up to obtain initial information on the mode of action of 2'-C-MetCyt in inhibiting the FMDV replication. The kinetics of one replication cycle of FMDV was first determined by means of qRT-PCR and virus yield assay. In untreated infected cells, release of viral RNA was first detected at 5 h post inoculation (Fig. 4), whereas infectious viral particles were detected 1 h later post inoculation (10^{2} CCID_{50}/ml). A single replication cycle of FMDV, thus, takes approximately 5 to 6 h, which is in line with earlier published data (Grubman and Baxt, 2004). Onset of intracellular RNA synthesis was first observed at 3h post inoculation (Fig. 4). When 2'-C-MetCyt was added (at 77 µM) during the first 2 h post inoculation, it resulted in a significant decrease of intracellular viral RNA levels as compared to the 6 h post inoculation untreated control (Fig. 5). A complete loss of activity was noted when the
compound was added at a time point later than 2 h post inoculation. Data for FMDV strain A Iran 1996 were similar, but not shown.

Discussion:

Various 2′-modified ribonucleoside analogues have recently been reported to be selective inhibitors of the replication of HCV (Carroll et al., 2003). Of these, the oral prodrug of 2′-C-MetCyt (Valopicitabine) is currently being evaluated clinical trials in patients with chronic HCV infections (http://www.idenix.com). We therefore hypothesised that 2′-C-MetCyt may elicit antiviral activity against animal picornaviruses, such as FMDV and SVDV, and compared its antiviral activity to that of ribavirin.

We found that both ribavirin and 2′-C-MetCyt exhibited antiviral activity against all seven FMDV serotypes and subtypes tested, but only 2′-C-MetCyt proved active against SVDV, a virus causing a clinically indistinguishable disease in pigs. However, 2′-C-MetCyt was not active against vesicular stomatitis virus, a member of the Rhabdoviridae that also causes comparable clinical signs in a number of ruminants (data not shown). The therapeutic index (i.e. ratio of TC50/EC50) of 2′-C-MetCyt against FMDV was ~119. Overall 2′-C-MetCyt was at least 100- to 140-fold more potent in inhibiting FMDV-induced CPE than ribavirin, even if De la Torre and colleagues (1987) reported earlier that ribavirin inhibited FMDV yield by 50%, in case of a cytoplytic infection, at a concentration of 5- to 6-fold lower than the EC50 concentration reported here. In addition to inhibiting FMD virus-induced CPE, we report that 2′-C-MetCyt also causes a comparable reduction of extracellular and intracellular RNA yields. Furthermore, 2′-C-MetCyt proved, compared to data of earlier studies, approximately 30- to 50-fold more effective in reducing viral progeny than 5-fluorouracil and 5-azacytidine (Sierra et al., 2000).

From the information derived from the time-of-drug addition studies with FMDV, it can be postulated that 2′-C-MetCyt interferes with FMDV replication at a step that coincides with the onset of intracellular viral RNA synthesis. The 5′-triphosphorylated metabolites of the ribonucleoside analogues are the active entity and inhibit the catalytic activity of HCV RNA polymerase (Carroll et al., 2003) and may thus be expected to inhibit the polymerase of picornaviruses. Hence, the 5-fold higher sensitivity to 2′-C-MetCyt of the FMDV SAT types as compared to the Eurasian FMDV strains, might be explained by genomic variations in the regions encoding for non-structural proteins (NSP) involved in virus replication, among which the RNA-dependent RNA-polymerase 3D and the NSP 3A (Grubman and Baxt, 2004). Even though genetic variation in the NSP regions is tolerated to a lesser extent than in regions encoding for the structural proteins, marked differences are observed between the 3A coding regions for the SAT isolates and other FMDV strains (Heat et al., 2001).

Although further studies are required to study viral resistance, the mechanism of antiviral activity, the in vivo toxicology and pharmacokinetics of 2′-C-MetCyt, this or other compounds with anti-FMDV activity may have the potential to serve as an important additional strategy in the current FMD control policy. An effective antiviral could protect the animals from infection during the 4 to 7-day-window period after vaccination, i.e. before the vaccine induces a protective immune response. Moreover, antiviral agents that result in an early protection against FMDV infection may have the potential to protect small animal herds against infection without the need for vaccination and subsequent culling (i.e. "small herd problem", Paton et al., 2004).

In conclusion, we have demonstrated in vitro antiviral activity of the ribonucleoside analogue 2′-C-MetCyt, against FMDV and SVDV. It may be important to have potent and broad-spectrum inhibitors of FMDV replication at hand to help containing outbreaks. Indeed an effective "druggable" antiviral could be used in a prophylactic scenario (in small herds and in farms surrounding an infected herd) and should almost immediately protect against infection (in contrast to emergency vaccination). In addition, during the asymptomatic incubation period in infected animals, an antiviral should be able to cause rapid reduction in viremia and thus further spread of infection, especially by FMDV infected pigs that are known to shed huge amounts of FMDV. Other possible uses of antiviral drugs would be to prophylactically treat valuable animals in zoological collections and expensive, rare animals in scientific or breeding programmes.

Conclusions:

- 2′-C-MetCyt inhibits the replication of FMDV
- Antiviral agents could constitute important alternative/supplementary options to control FMDV outbreaks (e.g. small herd problem)
- Antiviral agents could induce almost immediate protection and are not FMDV serotype specific
Recommendations:

- Further research is needed to study in vivo feasibility and drugeability of 2′-C-MetCyt
- Mode of action of 2′-C-MetCyt needs to be investigated thoroughly

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References:


### Table 1. The effect of ribavirin and 2'-'C-methylcytidine on FMDV and SVDV cytopathic effect formation in a multicyle growth assay

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Ribavirin EC(_{50}) (µM)</th>
<th>2'-'C-MetCyt EC(_{50}) (µM)</th>
<th>Ribavirin EC(_{90}) (µM)</th>
<th>2'-'C-MetCyt EC(_{90}) (µM)</th>
</tr>
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<tbody>
<tr>
<td>FMDV O(_{1}) Manisa</td>
<td>350 ± 30</td>
<td>8.7 ± 0.4</td>
<td>940 ± 48</td>
<td>15 ± 0.9</td>
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<tr>
<td>FMDV A(_{22}) Iraq 24/64</td>
<td>970 ± 30</td>
<td>10 ± 1.6</td>
<td>1697 ± 125</td>
<td>15 ± 0.5</td>
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<tr>
<td>FMDV A Iran 1996</td>
<td>560 ± 95</td>
<td>9.9 ± 0.2</td>
<td>1412 ± 269</td>
<td>15 ± 0.6</td>
</tr>
<tr>
<td>FMDV C(_{1}) Noville</td>
<td>1867 ± 256</td>
<td>5.3 ± 3.9</td>
<td>&gt; 2047</td>
<td>13 ± 0.8</td>
</tr>
<tr>
<td>FMDV Asia1 Shamir</td>
<td>757 ± 28</td>
<td>10 ± 1.1</td>
<td>1830 ± 58</td>
<td>14 ± 0.4</td>
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<tr>
<td>FMDV SAT1 ZIM 25/89</td>
<td>526 ± 47</td>
<td>3.7 ± 3.3</td>
<td>984 ± 38</td>
<td>7.8 ± 5.5</td>
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<tr>
<td>FMDV SAT2 NAM 1/92/2</td>
<td>NT</td>
<td>1.4 ± 0.2</td>
<td>NT</td>
<td>2.9 ± 0.0</td>
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<tr>
<td>FMDV SAT3 ZIM 4/81</td>
<td>1095 ± 65</td>
<td>1.8 ± 0.0</td>
<td>&gt; 2047</td>
<td>2.8 ± 0.0</td>
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<tr>
<td>SVDV UKG 27/72</td>
<td>&gt; 2047</td>
<td>45.2 ± 0.5</td>
<td>&gt; 2047</td>
<td>71.0 ± 0.1</td>
</tr>
</tbody>
</table>

NT = not tested
Data are mean ± SD from 3 independent experiments

**Figure 1. Structural formulae of ribavirin and 2'-'C-methylcytidine**

A

![Structural formulae of ribavirin and 2'-'C-methylcytidine](image)
Figure 2. Effect of 2'-C-methylcytidine on extracellular and intracellular viral RNA yields for FMDV strains O₁ Manisa (A) and A Iran 1996 (B) at 48 h post infection

The insets represent extracellular (white bars) and intracellular (grey bars) viral RNA levels on a more detailed scale. Data are mean values ± SD for 3 independent experiments.
Figure 3. Effect of 77 μM 2’-C-methylcytidine on FMDV-induced cytopathic efficiency for strains O₁ Manisa (left) and A Iran 1996 (right)

CC = non-infected, untreated cells
PC = non-infected, treated cells.

Figure 4. Levels of extracellular (filled diamonds) and intracellular (open diamonds) viral RNA during a single replication cycle of FMDV strain O₁ Manisa on BHK-21 cells inoculated with 3 CCID₅₀/cell

Viral RNA levels were monitored at various time points post inoculation during a timespan of 6 h by quantitative real-time RT-PCR.
Intracellular RNA levels were monitored by quantitative real-time RT-PCR at 6 h post inoculation in cells that had been treated with 77 μM 2'-C-MetCyt added at different time points post inoculation. Data are expressed as percentage of the untreated infected cells (VC). Data are mean ± SD from 3 independent experiments.