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

Goris Nesya [a,*], De Palma Armando [b], Toussaint Jean-François [a], Musch Ina [a], Neyts Johan [b], De Clercq Kris [a]

[a] *Department of Virology, Epizootic Diseases Section, Veterinary and Agrochemical Research Centre, Groeselenberg 99, 1180 Brussels, Belgium*
[b] *Rega Institute for Medical Research, Department of Microbiology and Immunology, Katholieke Universiteit Leuven, Minderbroederstraat 10, 3000 Leuven, Belgium*

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Abstract

We report on the potent and selective in vitro antiviral activity of 2′-*C*-methylcytidine (2′-*C*-MetCyt) against foot-and-mouth disease virus (FMDV). FMDV belongs to the *Picornaviridae* and has the potential to cause devastating epidemics in livestock. The 50% and 90% effective concentrations (EC_{50} and EC_{90}) for inhibition of the FMDV-induced cytopathic effect (CPE) formation were 6.4 ± 3.8 and 10.8 ± 5.4 μM. Comparable EC_{50} values for inhibition of viral RNA synthesis were observed. Treatment of FMDV-infected BHK-21 cells with 77 μM 2′-*C*-MetCyt resulted in a $(1.6–3.2) \times 10^3$-fold reduction of infectious virus yield. Time-of-drug addition experiments suggest that 2′-*C*-MetCyt interacts with viral replication at a time point that coincides with the onset of intracellular viral RNA synthesis. In contrast to emergency vaccination, a potent and selective antiviral agent may provide almost immediate (prophylactic/therapeutic) protection against infection and thus constitute an important alternative/supplementary option to contain outbreaks such as those caused by FMDV.

Keywords: Foot-and-mouth disease; Swine vesicular disease; 2′-*C*-Methylcytidine; Ribavirin; Disease control

1. Introduction

The foot-and-mouth disease virus (FMDV) is the prototype member of the genus *Aphthovirus* within the family *Picornaviridae* (Rodrigo and Dopazo, 1995). The virus is antigenically variable and seven distinct serotypes of the virus have been defined [O, A, C, Asia1 and the South African Territories (SAT) types 1, 2 and 3] as well as multiple subtypes (Belsham, 1993). FMDV is the causal agent of one of the most contagious and, notably in areas with highly productive and unvaccinated susceptible livestock, devastating animal diseases. 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 (Donaldson, 2004). 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), also included in the study, is a porcine picornavirus that causes clinical signs, which are (symptomatically) indistinguishable from FMD (Kitching, 2002). Recently, swine vesicular disease (SVD) caused unexpected problems in Portugal, where outbreaks were reported in 2003 (Paton, 2003). 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.

In the past, FMD outbreaks have had catastrophic consequences for the agricultural sector owing to death in young animals (mortality rate may reach 50% or more because of virus-induced myocarditis) (Bachrach, 1985), weight loss and a decrease in milk production resulting in an economic loss in productivity. Moreover, there are direct costs related to mass slaughter of infected animals, pre-emptive culling of suspect animals (“stamping out”) (Rweyemamu and Leforban, 1999)

* Corresponding author at: CODA-CERVA-VAR, Department of Virology, Section Development of Diagnostic Tools for Epizootic Diseases, Groeselenberg 99, 1180 Brussels, Belgium. Tel.: +32 23790400; fax: +32 23790666.
E-mail addresses: negor@var.fgov.be (N. Goris), armando.depalma@rega.kuleuven.be (A. De Palma), jetou@var.fgov.be (J.-F. Toussaint), inmus@var.fgov.be (I. Musch), johan.neyts@rega.kuleuven.be (J. Neyts), krdec@var.fgov.be (K. De Clercq).

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and due to imposed trade restrictions during an outbreak and in the aftermath of it (Brooksby, 1982). Often, however, the indirect economic losses in sectors such as tourism are even higher, as exemplified by the 2001 epidemic in the United Kingdom (Thompson et al., 2002).

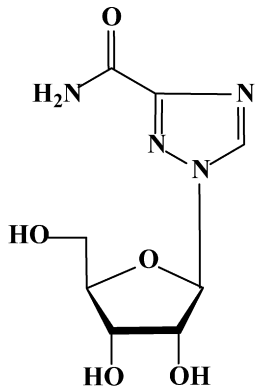
Because of the severe socioeconomic consequences related to an FMD incursion, the main concern of FMD-free countries is to prevent the introduction of the virus and/or to rapidly eradicate it in case of an outbreak. Until recently, emergency vaccination had a minor role in the European control policy, but the 2001 outbreaks of FMD in the United Kingdom, Ireland, France and The Netherlands 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 50–100-fold in vitro (Sierra et al., 2000), whereas the nucleoside analogue ribavirin [1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide] was found to eliminate FMDV from acute and persistently infected BHK-21 cell cultures (De la Torre et al., 1987). Others have shown that a replication-defective human adenovirus-mediated type I interferon completely protects pigs from FMDV infection (Chinsangaram et al., 2003) and delays and reduces disease symptoms in cattle (Wu et al., 2003), when challenged with FMDV 1 day post inoculation. The oral prodrug NM283 (valopicitabine) of a novel ribonucleoside analogue, 2′-*C*-methylcytidine (2′-*C*-MetCyt), has recently been reported as an inhibitor of the replication of hepatitis C virus (HCV) (Pierra et al., 2005). We studied whether 2′-*C*-MetCyt also elicits antiviral activity against FMDV and SVDV, akin to HCV positive-sense ssRNA viruses.

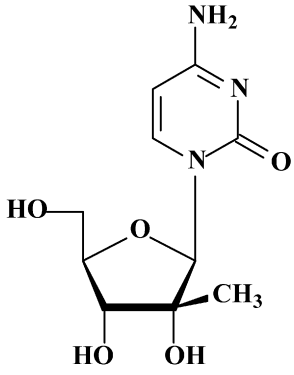
2. Materials and methods

2.1. Compounds

Ribavirin was purchased from ICN Pharmaceuticals (Costa Mesa, California, USA) and 2′-*C*-MetCyt was synthesized according to standard methods (Eldrup et al., 2004; Clark et al., 2005) (Fig. 1).



Ribavirin



2'-*C*-methylcytidine

Fig. 1. Structural formulae of ribavirin and 2′-*C*-methylcytidine.

2.2. Cells and viruses

Baby hamster kidney (BHK-21) cells (Ahl, 1974) were grown in Glasgow MEM BHK-21 medium (Gibco, Merelbeke, Belgium) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Cambrex Bio Sciences, Verviers, Belgium), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 7% (v/v) tryptose-phosphate (Gibco) (MEM-FBS-A). Swine kidney (SK6) cells (Kasza et al., 1972) were grown in Leibovitz medium (LM) supplemented with 3% heat-inactivated FBS, 80 IU/ml gentamycin (Vetoquinol, Aartselaar, Belgium) and 1 μg/ml fungizone antimycotic (Gibco) (LM-FBS-A).

The FMDV strains O_1 Manisa, A_{22} Iraq 24/64, A Iran 1996, C_1 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.

2.3. Anti-FMDV assay

BHK-21 cells were seeded in 96-well cell culture plates at a density of 1.5×10^5 cells per well in MEM-FBS-A. Following a 24 h incubation at 37 °C and 5% CO_2, medium was removed,

cells were washed three times with phosphate-buffered saline (PBS, pH 7.4, Gibco), and 100 μl of FMDV inoculum (each strain mentioned above) was added (approximately 50 $CCID_{50}$/100 μl, in which $CCID_{50}$ is defined as the concentration of virus capable of causing CPE formation in 50% of the cell culture). After a 30-min adsorption period at 37 °C and 5% CO_2, the FMDV suspension was removed and cells were washed three times with PBS. Five-fold serial dilutions of test compound (ribavirin or 2′-*C*-MetCyt) 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% CO_2. Non-infected, untreated cells (i.e. cell controls, abbreviated as CC) and infected, untreated cells (i.e. virus controls, abbreviated as VC) were included in each assay. When at least 90% destruction of the cell monolayer was observed microscopically for the VC wells (between 24 and 48 h after infection), 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazine ethosulfate (MTS/PES) solution (Promega, Leiden, The Netherlands) was added to each well to induce a colorimetric reaction. Subsequently, following a 2-h 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: % CPE reduction = $[(OD_{treated})_{FMDV} - OD_{VC}]/[OD_{CC} - OD_{VC}] \times 100$, 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 (EC_{50} and EC_{90}) were defined as the compound concentrations that protect, respectively 50% and 90% of the cells against virus-induced CPE. Both the EC_{50} and EC_{90} were calculated using logarithmic interpolation.

2.4. Anti-SVDV assay

The EC_{50} and EC_{90} values of both ribavirin and 2′-*C*-MetCyt 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.

2.5. Cytotoxicity assay

In parallel with the anti-FMDV and anti-SVDV assays, BHK-21 and SK6 cells were seeded in 96-well cell culture plates at a density of 1.5×10^5 cells per well in MEM-FBS-A and LM-FBS-A, respectively. Following 24 h of incubation at 37 °C and 5% CO_2, medium was removed and cells were washed three times with PBS. Five-fold serial dilutions of test compound (ribavirin or 2′-*C*-MetCyt) in a total volume of 100 μl MEM-2%FBS-A or LM-2%FBS-A were added. Cell culture plates were subsequently incubated at 37 °C and 5% CO_2 for an additional 24–48 h. Untreated CC were included in each assay. Subsequently, 20 μl of a MTS/PES solution was added to each well and culture plates were placed at 37 °C in the dark for a 2-h period. The $OD_{490\,nm}$ in each well was read and the percentage of viable cells was calculated as follows: % viable cells = $(OD_{treated}/OD_{CC}) \times 100$, 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 reduces the number of viable cells by 50% and was calculated using logarithmic interpolation.

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

The effect of 2′-*C*-MetCyt on FMD viral RNA yields for strains O_1 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 (Qiagen Benelux, Venlo, The Netherlands). In a separate reverse transcription (RT) step, 9.625 μl of 1:10 diluted, extracted RNA was added to a 1× Multiscribe RT Buffer (TaqMan® Reverse Transcription Reagents, Applied Biosystems, Nieuwerkerk, The Netherlands) supplemented with 62.5 pmol random hexamer primers (Applied Biosystems), 12.5 nmol dNTP's, 137.5 nmol $MgCl_2$, 10 IU RNase inhibitor and 31.25 IU Multiscribe reverse transcriptase (Applied Biosystems) 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 et al. (2003) was performed. Briefly, 10 μl template cDNA was added to a 2× universal TaqMan PCR master mix without AmpErase® UNG (Applied Biosystems) 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 s at 95 °C and 60 s at 60 °C, preceded by 10 min at 95 °C. A 10-fold 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.

2.7. Virus yield reduction assay for FMDV

Confluent monolayers of BHK-21 cells in 24-well cell culture plates were inoculated with 300 μl of a viral suspension of approximately 50 $CCID_{50}$/100 μl (FMDV strain O_1 Manisa or A Iran 1996). Following a 30-min adsorption period at 37 °C and 5% CO_2, 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 culture plates were incubated at 37 °C and 5% CO_2 for 24 h. 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, culture plates were stored at −80 °C until further use. After thawing, the well content of infected, treated and infected, non-treated cells

Table 1
The effect of ribavirin and 2′-*C*-methylcytidine on FMDV and SVDV cytopathic effect formation in a multicycle growth assay

Virus strain	EC_{50} (μM)		EC_{90} (μM)	
	Ribavirin	2′-*C*-MetCyt	Ribavirin	2′-*C*-MetCyt
FMDV O_1 Manisa	350 ± 30	8.7 ± 0.4	940 ± 48	15 ± 0.9
FMDV A_{22} Iraq 24/64	970 ± 30	10 ± 1.6	1697 ± 125	15 ± 0.5
FMDV A Iran 1996	560 ± 95	9.9 ± 0.2	1412 ± 269	15 ± 0.6
FMDV C_1 Noville	1867 ± 256	5.3 ± 3.9	>2047	13 ± 0.8
FMDV Asia1 Shamir	757 ± 28	10 ± 1.1	1830 ± 58	14 ± 0.4
FMDV SAT1 ZIM 25/89	526 ± 47	3.7 ± 3.3	984 ± 38	7.8 ± 5.5
FMDV SAT2 NAM 1/92/2	NT	1.4 ± 0.2	NT	2.9 ± 0.0
FMDV SAT3 ZIM 4/81	1095 ± 65	1.8 ± 0.0	>2047	2.8 ± 0.0
SVDV UKG 27/72	>2047	45.2 ± 0.5	>2047	71.0 ± 0.1

NT: not tested, data are mean ± S.D. from three independent experiments.

(VC) was collected and centrifuged (4 °C, 3000 × *g*, 15 min) to eliminate cell debris. The resulting supernatant was used in a back-titration 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% CO_2. Virus titres were expressed as $CCID_{50}$/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.

2.8. Plaque reduction assay for FMDV

Twenty four-well cell culture plates were seeded with BHK-21 cells at a density of 3×10^5 cells per well in MEM-FBS-A medium. The plates were incubated at 37 °C and 5% CO_2 until the cell monolayer obtained 95% confluence. Medium was removed, cells were washed with PBS and 300 μl of a 10-fold dilution series of either FMDV strain (O_1 Manisa or A Iran 1996) in MEM-2%FBS-A was added. Following a 30-min incubation period at 37 °C and 5% CO_2, 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. Culture plates were incubated for an additional 7 h 30 min at 37 °C and 5% CO_2. 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 48 h at 37 °C and 5% CO_2. Afterwards, 300 μl of 30% formaldehyde was deposited in each well and left to diffuse for 30 min. 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-min 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.

2.9. Time-of-drug addition assay for FMDV

Briefly, confluent BHK-21 cells in 96-well cell culture plates were infected with approximately 3 $CCID_{50}$/cell of virus (FMDV strain O_1 Manisa or A Iran 1996). Following a 30-min incubation period at 37 °C and 5% CO_2, 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 h, 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 h to the supernatant. The compound-treated cell cultures were incubated until 6 h 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).

3. Results

3.1. 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 ($R^2 > 0.9$ 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 and 10.8 ± 5.4 μM, making it 100–140-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. As an example, Fig. 2 depicts the effect of 2′-*C*-MetCyt on viral replication for FMDV strain C1 Noville and the BHK-21 cell viability. 92 ± 3.9% of the treated, non-infected BHK-21 cells were still viable at 77 μM 2′-*C*-MetCyt, whereas at that

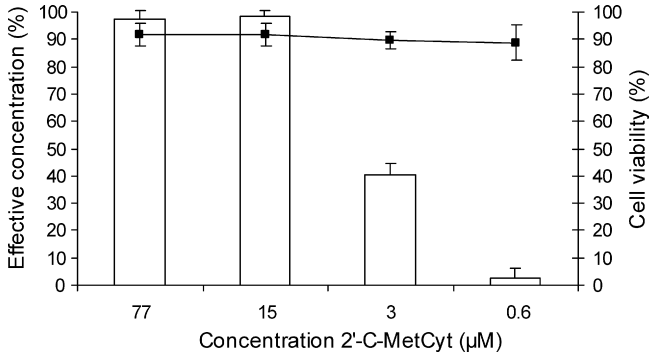


Fig. 2. Effect of 2′-*C*-methylcytidine on viral replication (white bars) and cell viability (filled squares) as determined by the MTS method for compound treated BHK-21 cells infected with 50 $CCID_{50}$/100 μl FMDV type C_1 Noville. Data are mean ± S.D. from three independent experiments.

compound concentration 2′-*C*-MetCyt almost completely prevented infection of BHK-21 cells with FMDV strain C1 Noville. Concentrations of 387 and 968 μM 2′-*C*-MetCyt led to a respective decrease of cell viability of 25% and 68% compared to 77 μM2′-*C*-MetCyt.

From here on, only FMDV strains O_1 Manisa and A Iran 1996 were used, because of their importance to Europe.

3.2. Effect of 2′-C-MetCyt on viral RNA yield

To further validate the antiviral activity of 2′-*C*-MetCyt against FMDV O_1 Manisa and A Iran 1996, the effect of the compound on viral RNA yield was determined by qRT-PCR (Fig. 3). The EC_{50} for inhibition of extracellular viral RNA yield were 2.2 ± 0.4 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 and 6.4 ± 0.9 μM).

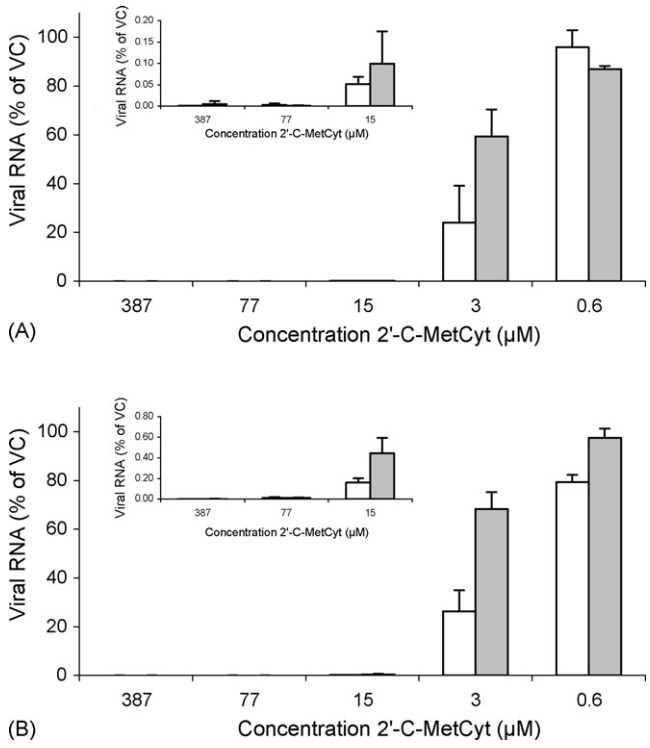


Fig. 3. Effect of 2′-*C*-methylcytidine on extracellular (white bars) and intracellular (grey bars) viral RNA yields for FMDV strains O1 Manisa (A) and A Iran 1996 (B) at 48 h post infection (the insets represent extracellular and intracellular viral RNA levels on a more detailed scale). Data are mean values ± S.D. for three independent experiments.

3.3. 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

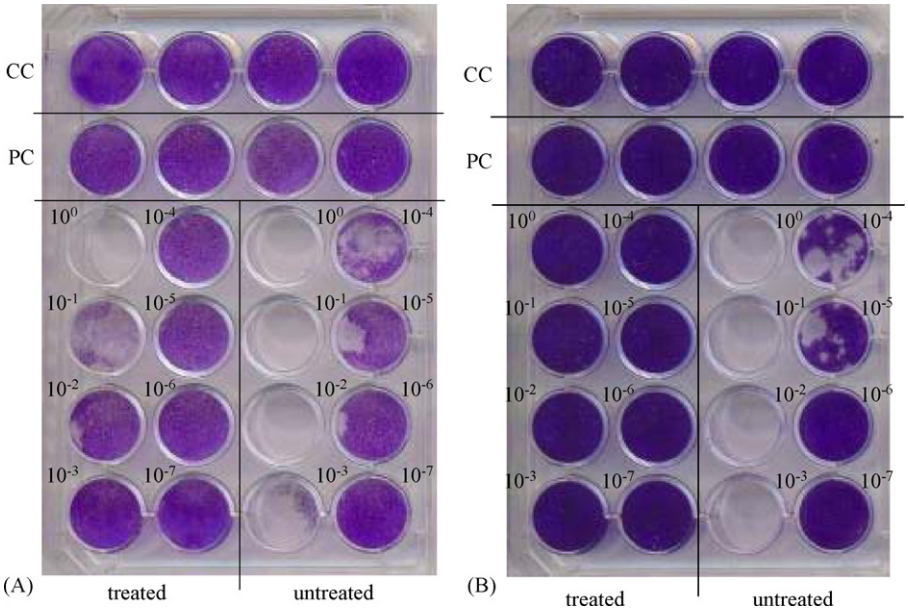


Fig. 4. Effect of 77 μM 2′-*C*-methylcytidine on FMDV-induced cytopathic efficiency [strains O_1 Manisa (A) and A Iran 1996 (B)]. CC: non-infected, untreated cells. PC: non-infected, treated cells.

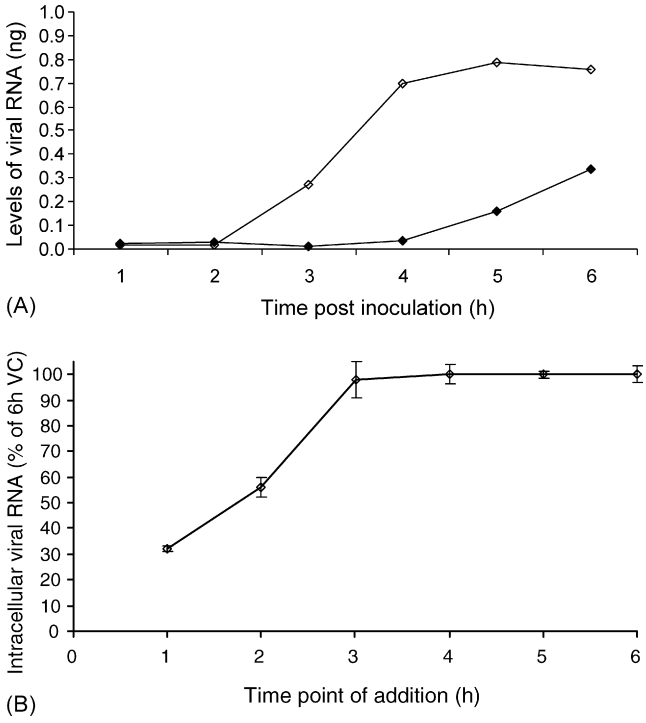


Fig. 5. (A) Levels of extracellular (filled diamonds) and intracellular (open diamonds) viral RNA during a single replication cycle of FMDV strain O_1 Manisa on BHK-21 cells inoculated with 3 $CCID_{50}$/cell. Viral RNA levels were monitored at various time points post inoculation during a timespan of 6 h by quantitative real-time RT-PCR. (B) Effect of delayed addition of 2′-*C*-MetCyt on the replication of FMDV strain O_1 Manisa. 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 ± S.D. from three independent experiments.

of 77 μM. Addition of 2′-*C*-MetCyt resulted in a reduction of (1.6–3.2) × 10^3 $CCID_{50}$/ml of infectious virus yield for FMDV strains O_1 Manisa and A Iran 1996 as compared to untreated controls and a (1.5–5) × 10^3 reduction in plaque formation efficiency (Fig. 4).

3.4. Time-of-drug addition studies

Time-of-drug addition assays for FMDV strains O_1 Manisa and A Iran 1996 were set up to obtain initial information about 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. 5A), 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 5–6 h, which is in line with earlier published data (Grubman and Baxt, 2004). Onset of intracellular RNA synthesis was first observed at 3 h post inoculation (Fig. 5A). 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. 5B). 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.

4. 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 in clinical trials in patients with chronic HCV infections (http://www.idenix.com). In addition, the 7-deaza-adenosine analogue, i.e. 7-deaza-2′-*C*-methyladenosine, was shown to inhibit in cell culture the replication of two picornaviruses, i.e. the poliovirus and the human rhinovirus (Olsen et al., 2004). 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 TC_{50}/EC_{50}) of 2′-*C*-MetCyt against FMDV was ∼119. Overall 2′-*C*-MetCyt was at least 100–140-fold more potent in inhibiting FMDV-induced CPE formation than ribavirin, even if De la Torre et al. (1987) reported earlier that ribavirin inhibited FMDV yield by 50%, in case of a cytolytic infection, at a concentration of five to six-fold lower than the EC_{50} concentration reported here. In addition to inhibiting FMD virus-induced CPE formation, 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 30–50-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 five-fold higher sensitivity to 2′-*C*-MetCyt of the FMDV SAT types as compared to the Eurasiatic 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). In addition, knowledge about the structure of the 3D polymerase of different FMDV strains would be a valuable tool to interpret the anti-FMDV activity

shown by nucleoside analogues such as 2′-*C*-MetCyt (Ferrer-Orta et al., 2004).

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. This control strategy is based upon emergency vaccination in conjunction with diagnostic tests that discriminate unequivocally between infected and vaccinated animals (Council Directive 2003/85/EC). The diagnostic sensitivity and specificity of currently available discriminatory diagnostic assays, however, do not allow detection of a single FMDV infected animal in vaccinated herds of less than 30 ruminants with 95% confidence and 5% prevalence (i.e. the "small herd problem") (Paton et al., 2004). Proposed strategies to circumvent the small herd problem include using a "vaccination-to-kill" policy in small herds (the vaccinated animals would be destroyed once the outbreak is brought under control) and avoiding vaccinating such herds in the first place (Paton et al., 2004).

An effective antiviral, however, could protect the animals from infection during the 4–7-days-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.

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 "drugeable" 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 FMD virus. 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.

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