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# A thiazepino[4,5-*a*]benzimidazole derivative hampers the RNA replication of Eurasian serotypes of foot-and-mouth disease virus

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ABSTRACT

The stamping-out policy for the control of foot-and-mouth disease virus (FMDV) in countries that are free from FMD without vaccination has a dramatic socio-economic impact, huge animal welfare issues and may result in the loss of farm animal genetic resources. As an alternative to pre-emptive culling or emergency vaccination we further explore the possibility to use antiviral drugs in the event of an FMD outbreak. In the present study, we tested the in vitro cytotoxicity and anti-FMDV activity of 1,2,4,5-tetrahydro-[1,4]thiazepino[4,5-*a*]benzimidazole. The molecule was shown to inhibit the replication of reference strains of the Eurasian FMDV serotypes O, A, C and Asia but not the FMDV serotypes from the South African Territories (SAT) neither a related picornavirus, i.e. swine vesicular disease virus. The molecule can be added until 2 h post inoculation in a 'single replication cycle experiment' without losing its antiviral activity. The genetic characterization of progressively selected resistant FMD viruses shows that the molecule presumably interacts with the non-structural 2C protein of FMDV. Further studies are required on the use of this molecule in vivo.



## 1. Introduction

Foot-and-mouth disease virus (FMDV) is an aphthovirus from the family *Picornaviridae* that causes a highly morbid, fast spreading disease in (domestic) cloven-hoofed animals. FMD is listed by the World Organization of Animal Health (OIE) and countries and zones are classified, not only depending on the presence or absence of the virus, but also on the use of vaccination to control the infection. Control measures of potential outbreaks in free-without vaccination countries include the culling on-the-spot of all susceptible animals of the infected holdings and those from epidemiologically linked holdings, which might result in socio-economically devastating consequences and animal welfare issues.

After the 2001 FMD epizootic in Western Europe, the European guidelines were amended to increase the support for emergency vaccination in case of a future outbreak of FMD [1]. However, the currently available emergency vaccines only confer complete clinical protection from 7 days post-vaccination onwards [2]. These emergency vaccines are serotype- and subtype-specific and their use requires specific diagnostic tests to differentiate between vaccinated and non-vaccinated animals [3]. Alternative strategies such as the use of small chemical molecules [4–6], interferons [7,8], immunostimulatory CpG oligonucleotides [9], poly IC [10], RNA or DNA(-like) interference [11–13] or a combination of these strategies [14] have the potential to induce immediate, serotype non-specific protection against FMDV replication under field circumstances. They could support the EU emergency vaccination policy or might even on itself be a valid alternative for pre-emptive culling of animals at risk to get infected with FMDV, in the EU or elsewhere.

The aim of the present study was to characterize the in vitro activity of 1,2,4,5-tetrahydro-[1,4]thiazepino[4,5-*a*]benzimidazole

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against reference strains of FMDV. This molecule is derived from a class of compounds of which the synthesis and structural features were originally described by Chimirri et al. [15]. We were first able to identify in vitro activity of this class of compounds against FMDV during a high-throughput screening of a library of drug-like small molecules [16].

## 2. Materials and methods

### 2.1. Compound

The molecule 1,2,4,5-tetrahydro-[1,4]thiazepino[4,5-*a*]benzimidazole, hereafter referred to as CHI-83, has a molecular weight of 204.29 g/mol. Its structural formula is shown in Fig. 1 and was originally described by Chimirri et al. [15].

### 2.2. Cells and viruses

The present in vitro antiviral studies were performed on SK-6 (swine kidney) cells. The SK-6-adapted FMDV reference strains O1/MAN/TUR/69 (O1 Manisa), A22/IRQ/24/64, A/IRN/11/96 (A Iran 11/96), C1/Noville/SWI/65, Asia1/Shamir/ISR/89, Asia1/CAM/9/80, SAT1/ZIM/25/89, SAT2/ZIM/3/97 and SAT3/ZIM/4/99 were used, as well as swine vesicular disease virus (SVDV) UKG/27/72. The latter is an enterovirus (family *Picornaviridae*) and is the most important differential diagnosis for FMDV in pigs. These FMD and SVD viruses were originally obtained from the World Reference Laboratory for FMDV and the European Community Reference Laboratory for SVDV at the Pirbright Institute, UK.

### 2.3. In vitro assays

The cytotoxicity of CHI-83 was determined as previously described by Goris et al. [5]. Briefly, after 48 h of incubation of 5-fold serial dilutions of CHI-83 on SK-6 cells, the cell viability was determined with the CellTiter 96®AQueous One Solution Cell Proliferation Assay (Promega, Leiden, The Netherlands) according to the manufacturer's instructions, and confirmed by light microscopic examination. The 50% cytotoxic concentration ($CC_{50}$) of CHI-83 was calculated as the concentration that reduced the cell viability by 50%, as described by Goris et al. [5].

The in vitro antiviral activity of CHI-83 was determined in 96-well plates as described by Goris et al. [5]. Briefly, the culture medium was removed from SK-6 cell monolayers with 80–90% confluency and 100 µl of 5-fold serial dilutions of CHI-83 was added per well, immediately followed by 100 µl containing 50 Tissue Culture Infectious $Dose_{50}$ ($TCID_{50}$) of FMDV or SVDV. At 48 h post inoculation (hpi), the inhibition of viral replication-induced cytopathic effect (CPE) was determined with the cell viability assay described above and confirmed by light microscopic examination. The 50% and 90% effective concentrations ($EC_{50}$ and $EC_{90}$) of CHI-83 were calculated as the concentrations which protected 50% and 90% of the SK-6 cells against viral replication-induced CPE, respectively, as previously described [5].

Similarly, the effect of CHI-83 on the viral RNA yield was determined by adding 2-fold serial dilutions of CHI-83 per well in 96-well plates, immediately followed by 50 $TCID_{50}$ of FMDV strain O1 Manisa. After a 30 min adsorption period, the inoculum was removed, the cells were washed twice and fresh culture medium containing 2-fold serial dilutions of CHI-83 was added. At 48 hpi, supernatant fluids and cells were collected. The nucleic acids were extracted with the Nucleospin® RNA virus columns (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions. One-step real-time RT-PCR for the FMDV RNA-dependant RNA-polymerase (FMDV 3D gene) was performed as adapted from the reference method from Callahan et al. [17].

So-called time-of-drug addition assays were performed by inoculating SK-6 cells in 96-well plates with 50 $TCID_{50}$ of FMDV strain O1 Manisa for 30 min [5]. After two washes, fresh medium was added. Every hour post inoculation until 6 hpi, CHI-83 was added to a final concentration of 60 µM, which is the lowest concentration that completely inhibited CPE formation and infectious virus yield in preliminary assays (data not shown). At 6 hpi (i.e. after one replication cycle [5]) supernatant fluids and cells of compound-treated cultures and control-infected non-treated cultures were collected and stored at −80 °C until use. The viral RNA levels of the collected fractions were determined by RT-PCR as described above [17].

To unravel the mode of action of CHI-83, CHI-83 resistant mutant FMDV was selected by culturing 50 $TCID_{50}$ of O1 Manisa and A Iran 11/96 in the presence of serial dilutions of CHI-83 ranging from 50 µM to 5 µM in 96-well plates. After 2 days of incubation, the content of 5–10 wells of the highest concentration of CHI-83 that showed CPE were serially passaged in 24-well plates with increasing but sub-optimal concentrations of CHI-83. This was repeated until full-blown CPE was observed at 100 µM of CHI-83. Infected, untreated cells (virus controls) were passaged in parallel.

### 2.4. Virus amplification and sequencing

To investigate the effect of continuous treatment with sub-optimal and increasing concentrations of CHI-83, the complete FMDV genome (except for the poly(C) region) of the resistant mutant virus, the virus control and the original virus was amplified, sequenced and analysed as described before [18,19]. Briefly, viral RNA was extracted and reverse-transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, CA, USA) and the UKFMD Rev 6 primer (5′-GGC GGC CGC TTT TTT TTT TTT TTT-3′). After cDNA purification, the Platinum® High Fidelity Taq (Invitrogen, CA, USA), 12 universal primer pairs for the non-structural protein (NSP) and 3′-poly(A) tail; and 12 lineage-specific primers to amplify the 5′-untranslated region (UTR) and capsid-coding region [18] were used to amplify the virus genome in 24 overlapping fragments. Cycle sequencing reactions, using the same primers pairs used for amplification and using the Big Dye-Terminator v3.1 Cycle Sequencing Reaction Kit, were analysed in an ABI 3730 DNA Analyzer (Applied Biosystems, USA). Sequences were assembled, proof-read, and edited with the Lasergene version 10.1 package (DNASTAR Inc, USA). Subsequent nucleotide and amino acid sequence alignment were performed as described by Valdazo-Gonzalez et al. [19].

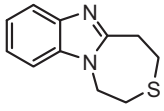


**Fig. 1.** Structural formula of CHI-83.

## 3. Results

The cytotoxicity and antiviral activity of CHI-83 were assessed with a colorimetric assay that measures the cell viability. The $CC_{50}$ of CHI-83 was 382.2 ± 18.5 µM. CHI-83 demonstrated antiviral activity against the 4 FMDV serotypes of the Eurasian continent (O, A, C and Asia). Antiviral activity could not be demonstrated for the SAT (South African Territories) serotypes or SVDV. This is shown in Table 1.

**Table 1**
Reduction of FMDV- and SVDV-induced CPE by treatment with CHI-83.

| Virus strain | $EC_{50}$ (μM)[a] | $EC_{90}$ (μM)[a] |
|---|---|---|
| FMDV O1/MAN/TUR/69 | 15.6 ± 8.6 | 44.2 ± 14.3 |
| FMDV A22/IRQ/24/64 | 18.4 ± 8.2 | 41.1 ± 4.3 |
| FMDV A/IRN/11/96 | 8.0 ± 1.5 | 28.5 ± 16.7 |
| FMDV C1/Noville/SWI/65 | 39.7 ± 27.8 | 58.8 ± 32.9 |
| FMDV Asia1/Shamir/ISR/89 | 51.4 ± 27.1 | 83.8 ± 39.6 |
| FMDV Asia1/CAM/9/80 | 26.6 ± 5.8 | 51.2 ± 11.6 |
| FMDV SAT1/ZIM/25/89 | >100 | >100 |
| FMDV SAT2/ZIM/3/97 | >100 | >100 |
| FMDV SAT3/ZIM/4/99 | >100 | >100 |
| SVDV UKG/27/72 | >100 | >100 |

[a] Data represent the mean ± S.D. from 3 independent experiments.

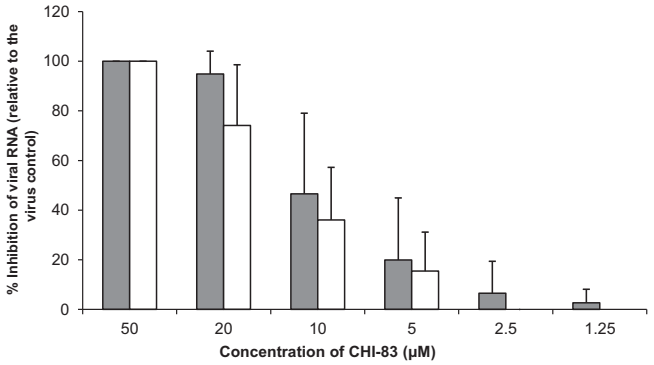


**Fig. 2.** Dose-activity curve for FMDV RNA reduction by CHI-83. Grey bars represent extracellular viral RNA and white bars represent intracellular viral RNA.

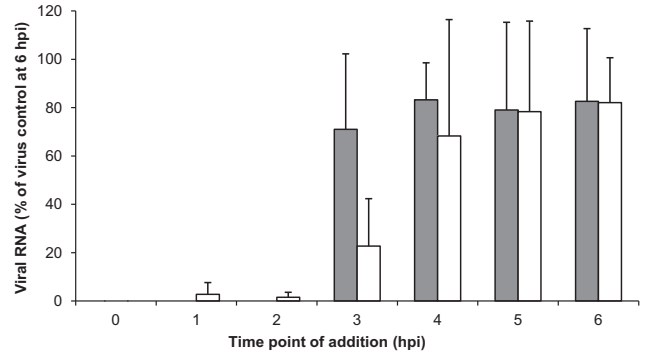


**Fig. 3.** Effect of delayed addition of CHI-83 on intra- and extracellular viral RNA levels. Grey bars represent extracellular viral RNA and white bars represent intracellular viral RNA.

The $EC_{50}$ and $EC_{90}$ values of CHI-83 for inhibition of viral RNA yield of the FMDV strain O1 Manisa were 11.3 ± 5.1 μM and 23.3 ± 7.7 μM for inhibition of the extracellular viral RNA yield and 13.6 ± 5.4 μM and 34.0 ± 13.7 μM for the inhibition of the intracellular RNA yield, respectively. Fig. 2 depicts the dose-activity curve and represents the mean ± S.D. from 3 independent experiments.

Time-of-drug addition assays were performed during one single replication cycle of the FMDV strain O1 Manisa [5]. When CHI-83 was added at a concentration of 60 μM during the first 2 hpi, the intracellular viral RNA synthesis was inhibited for 99% and extracellular viral RNA could not be observed. A gradual loss of the antiviral activity was observed when the compound was added between 3 and 6 hpi, as shown in Fig. 3. The data in Fig. 3 represent the mean ± S.D. from 3 independent experiments.

To determine the mode of action of CHI-83, FMDV O1 Manisa and A Iran 11/96 were serially passaged in sub-optimal and increasing concentrations of CHI-83. For both strains it was observed that after 4 passages 50 $TCID_{50}$ of FMDV induced full-blown CPE in the presence of 100 μM of CHI-83. Comparative consensus sequencing analysis revealed amino acid (aa) changes T1242I (Isoleucine instead of Threonine) for O1 Manisa and Q1371R (Arginine instead of Glutamine) for A Iran 11/96 respectively. Both aa changes were located in the non-structural 2C protein and were not observed in the wild type viruses or in the serially passaged control viruses. The present data confirm previous data in which similar aa changes were observed in the 2C protein of an O1 Manisa that was resistant to a precursor molecule of CHI-83 (data not shown).

## 4. Discussion

The European Council Directive 2003/85/EC has opened the door for animal friendly containment strategies for FMD. An alternative for emergency vaccination might be the prophylactic use of small chemical molecules that inhibit the viral replication cycle. This is not wishful thinking as recent modelling studies from Ribbens et al. [20] and Backer et al. [21] have for example shown that an outbreak of classical swine fever in a densely populated livestock area could be successfully and cost-effectively contained by the prophylactic use of the molecule 5-[(4-bromophenyl)methyl]-2-phenyl-5H-imidazo[4,5-c]pyridine or BPIP. Recently, our research consortium has described the activity of small chemical molecules against FMDV in laboratory animals, i.e. the ribonucleoside analogue 2′-*C*-methylcytidine (2′CMC) in severe combined immunodeficient mice [22] and the 3-oxo-3,4-dihyro-2-pyrazincarboxamide derivative (T-1105) in Guinea pigs [23]. However, the dose and quantity of 2′CMC or T-1105 to be used in farm animals may be too high to allow an economically viable FMD control strategy in large numbers of animals.

In the present manuscript, the in vitro activity of CHI-83 is demonstrated against the 4 Eurasian serotypes of FMDV. However, antiviral activity could not be demonstrated against the SAT serotypes of FMDV even not at a high concentration (100 μM). This is particularly an issue in the EU as FMDV SAT2 has spread northward in the recent years, crossing the historical physical barrier of the Sahara, and causing several independent outbreaks in North-Africa and the Middle East [24,25]. The ideal antiviral drug should be active against all 7 serotypes of FMDV.

Goris et al. previously observed the onset of the viral RNA synthesis between 2 and 3 hpi and extracellular viral RNA was observed from 5 hpi onwards [5]. This was confirmed in the present study (data not shown). The data of the present study thus suggest that CHI-83 interferes at a stage that coincides with the onset of the viral RNA replication, presumably by interacting with the viral 2C protein. The latter is a large membrane-associated RNA-binding protein with ATP-ase activity [26,27] that interacts with and modulates different host cell factors during the viral replication cycle [28,29]. To our knowledge, this is the first description of an antiviral compound that specifically targets the viral 2C protein of FMDV, although such inhibitors are described for other picornaviruses [30,31]. Although it has been described that single mutations from Threonine to Isoleucine [32] or from Glutamine to Arginine [33] can attenuate viral replication in vivo, we have no indications that the described aa mutations in the FMDV 2C protein may alter viral replication on SK-6 cells in vitro (data not shown). Further research might give a better insight into the functions of the FMDV 2C protein, particularly in natural hosts.

In conclusion, the present study further illustrates the potential of small molecule inhibitors to interfere with the replication cycle

of FMDV, which alludes to their prospective use for containing future outbreaks of FMD. Although practical applications are not yet commercially available, further research and development towards this goal is ongoing.

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