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Repeated exposure to 5D9, an inhibitor of 3D polymerase, effectively limits the replication of foot-and-mouth disease virus in host cells

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Abstract

Foot-and-mouth disease (FMD) is a highly contagious disease of livestock caused by a highly variable RNA virus (FMDV) that has seven serotypes and more than sixty subtypes. Both prophylactic and post-infection means of controlling the disease outbreak, including universally applicable vaccines and emergency response measures such as therapeutic treatments, are on high demand. In this study, we analyzed the long-term exposure outcome to a previously identified inhibitor of 3D polymerase (FMDV 3Dpol) for controlling FMDV infection and for the selection of resistance mutants. The results showed that no escape mutant viruses were isolated from FMDV A24 Cruzeiro infections in cell culture treated with gradually increasing concentrations of the antiviral compound 5D9 (4-chloro-N'-thieno, [2,3-d]pyrimidin-4-ylbenzenesulfonohydrazide) over ten passages. Biochemical and plaque assays revealed that when 5D9 was used at concentrations within a non-toxic range in cells, it drove the virus to undetectable levels at passage eight to ten. This is in contrast with observations made on parallel control (untreated) passages exhibiting fully viable and stable virus progenies. Collectively, the results demonstrated that under the experimental conditions, treatment with 5D9 does not confer a resistant phenotype and the virus is

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Disclosure statement

The authors declare no competing interests.

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unable to evade the antiviral effect of the inhibitor. Further efforts using quantitative structure-property relationship (QSPR) based modifications of the 5D9 compound may result in the successful development of an effective in vivo antiviral drug targeting FMDV.

Keywords

foot-and-mouth disease virus (FMDV); 5D9 compound; 3D polymerase inhibitor; FMDV therapeutic treatment; antiviral

Foot-and-mouth disease virus (FMDV) is the etiologic agent of a highly contagious vesicular disease that affects cattle, sheep, goats, and other cloven-hoofed animals. While mortality rates are low in infected animals, morbidity can reach extremely high levels leading to loss of productivity, culling of infected and susceptible animals, and great personal and financial detriments due to both the loss of livestock and the international trade capabilities (Mort et al., 2008; Paarlberg, Lee, and Seitzinger, 2002). Recent outbreaks in South Korea, Japan, Egypt, and the UK have brought to the forefront the importance of controlling this disease, as well as the devastating local and global effects both during and post-outbreak (Ghoneim et al., 2010; Knowles et al., 2012; Reid et al., 2009).

FMDV belongs to the Picornaviridae family of the genus Aphthovirus. It has seven distinct serotypes (A, C, O, Asia 1, Sat 1, Sat 2, and Sat 3) (Bachrach, 1968; Grubman and Baxt, 2004) and more than 60 subtypes. Due to such a high level of diversity the development of a universal vaccine against FMDV has been a challenging task (Paton et al., 2005). The current most-effective vaccines are serotype-specific and consist of chemically inactivated whole-virus FMDV, offering protection only after seven days of vaccination (Grubman, 2005). The combined efforts including both vaccination and antivirals have been proposed as one strategy to more effectively treat FMD-infected animals and contain the spread of disease.

The FMDV genome consists of an 8.5 kb long single-stranded, positive sense RNA genome that is translated into a single polyprotein, which is processed into four structural and ten non-structural proteins (Grubman and Baxt, 2004). The non-structural RNA-dependent RNA polymerase (RdRp) protein also known as 3Dpol is coded within the 3' end of the FMDV genome. It is essential for the synthesis of viral RNA and pivotal to the virus lifecycle. A number of crystal structures of apo enzyme, enzyme complexes containing template-primer (Ferrer-Orta et al., 2004), Vpg (Ferrer-Orta et al., 2006), RNA template-primer and incoming NTP or mutagenic nucleotides ribavirin triphosphate (RTP) and 5-fluorouridine triphosphate (FUTP) (Ferrer-Orta et al., 2007; Ferrer-Orta et al., 2010) and biochemical studies (Arias et al., 2008; Belsham, 1992; Bentham et al., 2012; Ferrer-Orta et al., 2004; Ferrer-Orta et al., 2007; Nayak et al., 2006) have provided significant insights into the RNA replication mechanism of 3Dpol over the years. Although a number of approaches have been pursued to develop anti-FMD therapies (Airaksinen et al., 2003; Dias et al., 2011; Dias et al., 2012; Uddowla et al., 2012; Vagnozzi et al., 2007) no clinically approved antiviral compounds are available for treatment of FMDV infection. This is in contrast with other viral diseases for which there are approved antiviral drugs that specifically target their

polymerases (Airaksinen et al., 2003; Crotty et al., 2000; De Clercq, 2005; Parniak and Sluis-Cremer, 2000; Sarafianos et al., 2009).

In a previous study we identified seven compounds that inhibit 3Dpol at low micromolar concentrations. One of these inhibitors, 5D9 inhibited both FMDV 3Dpol enzyme and virus with comparable IC₅₀ for the enzyme and the EC₅₀ for the virus in cell-based assay suggesting that 5D9 acts by a single mechanism of action, inhibition of 3Dpol. The crystal structure of FMDV 3Dpol containing RNA template-primer, UTP and leaving PPi (after AMP incorporation) (PDB entry 2E9Z) as well as apo enzyme (PDB entry 1U09) was used to dock 5D9 in a cavity close to but distinct from the NTP binding site (Durk et al., 2010). This pocket consists of amino acid residues V55, I56, S58, K59, R168, G176, K177, T178, R179 and I180. The binding of 5D9 in this pocket was confirmed by site-directed mutagenesis of K59 and K177, which are located at two sides of the binding pocket (Durk et al., 2010). K59 is located near the junction of fingers (index finger, Ferrer-Orta, 2004, JBC) and palm subdomains of 3Dpol, whereas K177 and R168 belongs to motif F in the fingers subdomain (Bruenn, 2003; Ferrer-Orta et al., 2004; Poch et al., 1989). None of these two residues bind to either RNA template-primer or NTP. In fact, only one residue of the inhibitor binding pocket (R168) has additional interaction with substrate (incoming NTP). In the ternary complex of FMDV 3Dpol, NH₂ atom of R168 interacts with β -phosphate (distance NH₂ – β PO₄ = 3.2 Å) of incoming UTP (Ferrer-Orta et al., 2007; PDB entry 2E9Z). R168 is also neighbor to P169, which associated to fidelity changes in fmdv and Coxsacivirus 3Dpol (Gnadig et al., 2012). It is possible that the interaction of 5D9 with R168 (due to its proximity to residue P169) may have an impact on the fidelity of FMDV 3Dpol (Agudo et al., 2010).

The goal of this study was to examine the outcome of long-term exposure of FMDV to 5D9, which had exhibited the highest level of reduction in FMDV replication in our earlier work. The antiviral potency of 5D9 in terms of therapeutic index compares favorable (~80 fold higher) over ribavirin (EC₅₀ = 970 μ M, EC₉₀ = 1697 μ M), and comparable to 2'-C-methylcytidine (EC₅₀ = 10 μ M, EC₉₀ = 15 μ M) (Durk et al., 2010; Goris et al., 2007). Here, we serially passed the A24 Cruzeiro parental virus in the presence of a gradually increasing dosage of 5D9 and this led to a reduction of viral titers to undetectable levels after eight passages, while control passages in the absence of treatment grew similar to parental levels. Western blot analysis indicated a dose-dependent decrease in the expression of 3Dpol when 5D9 was present.

First we determined the cytotoxicity of 5D9. To this end, 1 $\times$ 10⁴ BHK-21 cells/well were seeded in a 96-well plate in BHK growth medium (BME containing 10% fetal calf serum, L-glutamine, sodium-pyruvate and 1X antibiotic, antimycotic). 5D9 was added to confluent cell monolayers at concentrations of 0–200 μ M and incubated at 37°C for 24 hours in a total volume of 100 μ L in the presence of 4% DMSO (Fig. 1). After 24 hours cell viability/proliferation was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay method (American Type Culture Collection, ATCC) according to the manufacturer's instructions. Values were compared with those obtained from untreated control cells. 80% cell viability was set as cut off for the

determination of cytotoxicity of the compound. As shown in Fig. 1 the inhibitor did not cause any significant change in the cell viability at these concentrations.

In order to assess the antiviral activity of 5D9 on FMDV infection under different compound dose and multiplicity of infection (MOI, ratio of number of infectious virus particles in plaque forming units per cell) conditions, a confluent monolayer of BHK-21 cells pretreated with concentrations of 5D9 within the 0–25 μ M range (Fig. 2A) was infected with FMDV A24 Cruzeiro at 0.01 and 0.5 MOI and incubated at 37°C. One hour post infection (hpi), unadsorbed viruses were inactivated by two acidic washes [25 mM MES (2-morpholinoethanesulfonic acid) pH 5.5; 145 mM NaCl] and then neutralized with virus growth medium, VGM [(Eagle's basal medium (BME) (Life Technologies, Gaithersburg, MD), 1% antibiotic/antimycotic]. The compound was re-administered in a total volume of 500 μ l VGM containing 4% DMSO. Twenty-four hours post infection the plates were frozen, then freeze-thawed and viral plaque assays were performed for the quantification of infectious virus in treated and untreated samples as described (Rieder et al., 1993). Fig. 2A shows the effects of 5D9 on the replication of FMDV A24 Cruzeiro. The compound inhibited virus replication both at 0.01 and 0.5 MOIs to similar extent (~50% reduction in viral titer at 10 μ M concentration). The specific effect of 5D9 on FMDV replication in the cells was also demonstrated by a reduction in 3Dpol expression in western blot analysis when BHK cells were infected with A24 Cruzeiro in the presence of the compounds at an MOI of ten. (Fig. 2B).

Reduction in 3Dpol expression also correlated with lower expression of the viral structural protein VP1, which suggests that the decrease in viral protein expression observed is indeed due to the inhibition of viral replication and not to the misfolding or degradation of 3Dpol. For western blot analysis, first the cell lysates were electrophoresed on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using a 12% Nu-PAGE® pre-cast gel system (Invitrogen) to separate the proteins. Subsequently, the separated proteins were electro-blotted onto a nitrocellulose membrane (Sigma). After blocking with 5% milk in PBS, FMDV 3Dpol and VP1 were probed with mouse-monoclonal anti-FMDV 3Dpol antibody (a gift from A. Clavijo at the National Centre for Foreign Animal Disease, Winnipeg, Manitoba, Canada) and rabbit polyclonal anti-VP1 sera (a gift from M. Grubman, ARS, USDA, USA) at 1:500 and 1:1000 dilutions, respectively in 1% milk and PBST (Phosphate Buffered Saline with Tween 20). Primary antibodies reacted with alkaline phosphatase (ALKP)-conjugated secondary antibodies and the blot was developed with SIGMAFASTTMPCIP®/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium). Cellular tubulin, employed as an internal loading control protein, was detected with anti-tubulin- α (Sigma). The 5D9 dose-dependent disappearance of 3Dpol corroborated the dose-dependent inhibitory effect of this compound observed in plaque assays (Fig. 2B). Tubulin controls were included to ensure equal loading in each of the test wells.

On the basis of similar antiviral effects of 5D9 at the two different MOIs observed in Fig. 2 and the role of 3Dpol in virus replication, we next pursued an experimental design to evaluate viral fitness in the presence of 5D9 and the selection of resistant mutants. To this end A24 Cruzeiro was used to infect BHK-21 cells at an MOI of 0.01 and exposed to a low (5 μ M) compound concentration in the first passage. Further passages were performed using

a fraction (1/5) of total volume of freeze-thawed cell lysates at MOIs calculated within a range of 2–5 (passages 2–7). Compound treatment was performed as described earlier and the plates containing virus were freeze-thawed for further passages or analysis. The MOI was calculated by dividing plaque forming units (PFUs) by the cell number (4.5×10^5). The passage scheme presented in Fig 3A shows that each virus passage was conducted 10 times in parallel quadruplicates. At passages 4, 6, and 8, samples were processed for sequencing and quantitative analysis. For sequencing the viral genome and RT-PCR of viral RNA, the RNA extraction was carried out using the RNeasy Mini Kit column method (Qiagen, Catalog Number 74014, Valencia, CA). Post-RNA extraction, cDNA was produced using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Catalog Number 18080-051) and PCR was subsequently performed using the Phusion High-Fidelity PCR KIT (New England Biolabs, Catalog Number E0553L, Ipswich, MA). Sequencing was performed on an ABI sequencer (Applied Biosystems, Bedford, MA, USA. Fig. 3B shows that the compound treatment reduced the virus titer gradually from 107 PFU/ml at passage four to 103 PFU/ml at passage 8. In contrast, no significant changes in viral titers were observed in mock-treated virus passages, except for a slight increase (less than 1 log) after 10 passages (data not shown). This suggests that ten passages of A24 Cruzeiro in the absence of compound was not detrimental to the virus. Virus titration (Figs. 3B) and RT-PCR results (Fig. 4) show that no detectable virus is present at and after passage nine.

To determine the sequence of the viruses passaged in the presence or absence of 5D9, samples at passages 4 and 8 were examined by RT-PCR and sequenced as previously described (Rieder et al., 2005). In addition, individual clones from infected cell culture monolayers at passage 5 were isolated under low melting point agarose (SeaPlaque) overlay followed by RT-PCR and sequencing. Analysis of whole viral population showed that no predominant mutations were fixed in the region coding for 3Dpol within the 5D9-treated viruses (at passage 4 and passage 8). Interestingly, one out of 12 randomly picked clones at 5D9-treated passage 5 displayed a P169Q change in 3Dpol and another clone exhibited multiple mutations within the 3Dpol sequence [(amino acid substitutions P44R, E259V, S335F and L376F) and one silent mutation (C>T) at nucleotide position 7585]. Based on the fact that under the experimental conditions no mutant viruses became predominant in subsequent parallel passages, it can be suggested that none of these mutation were able to confer a selective advantage to the virus against the antiviral compound. Although these residues are not part of the validated 5D9 binding pocket (Durk et al., 2010), evidence suggest that P44 and P169 are important determinants of RdRp fidelity whereas S335 is important for RdRp function (Castro, Arnold, and Cameron, 2005; Gnadig et al., 2012; Gong and Peersen, 2010; Korneeva and Cameron, 2007; Verdaguer and Ferrer-Orta, 2012; Weeks et al., 2012). In particular, Agudo et al reported changes at P44 and P169 in response to ribavirin treatment for FMDV 3Dpol affecting the enzyme fidelity (Agudo et al., 2010). However, failure of these mutants to adapt to 5D9 compound challenge could possibly be explained in two ways: (I) If the mutations increase the fidelity, the virus quasispecies may be critically reduced in size leading to its extinction; (II) The decrease in fidelity could lead the virus towards another extreme, where virus will accumulate too many errors in its genome and will eventually be unable to survive. It should be noted that the isolation/emergence of resistant mutants is an important step in the determination of target specificity

for an antiviral drugs and deciding the antiviral therapy (Aloia, Locarnini, and Beard, 2012; Koev and Kati, 2008); (Wainberg and Friedland, 1998). In addition to intrinsic error rate of viral RdRPs (10⁻³-10⁻⁵/copy for a 10 Kb genome), a number of other factors including the host environment contribute to the virus evolution (Domingo, 1989; Domingo, 1997; Domingo et al., 1996; Elena et al., 2006; Elena and Sanjuan, 2005; Furio, Moya, and Sanjuan, 2005; Sanjuan et al., 2007; Sanjuan et al., 2010). In this context, we cannot absolutely exclude the possibility that using higher virus inputs and/or different conditions resistance mutants to 5D9 may emerge.

In conclusion, the results demonstrated that compound 5D9 is a very efficient inhibitor of FMDV replication, and long-term exposure of FMDV A24 Cruzeiro to this compound did not result in the selection of resistant mutants in cell culture. The fact that the drug-binding pocket in FMDV 3Dpol targeted by 5D9 (via a single mechanism of action, see Durk et al., 2010) is conserved among different serotypes (Fig. 4C) suggests that this compound could potentially be developed as a broad-spectrum inhibitor of FMDV. Clinically effective inhibitors acting either alone or in conjunction with current vaccines may be very helpful as an emergency response measure in controlling the spread of disease in the outbreaks.

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Abbreviations

FMD	Foot- and- Mouth Disease
FMDV	Foot –and- Mouth Disease Virus
3Dpol	3D polymerase
5D9	4-chloro-N'-thieno, [2,3-d]pyrimidin-4-ylbenzenesulfonohydrazide
QSPR	Quantitative Structure-Property Relationship
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid
NTP	Nucleoside triphosphate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
BME	Eagle's Medium Basal
ATCC	American Type Culture Collection
BHK	Baby Hamster Kidney
MOI	Multiplicity of Infection

hpi	Hours Post Infection
PBST	Phosphate Buffered Saline with TWEEN-20
DMSO	Dimethylsulphoxide
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation assay

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We report an antiviral compound that binds in a novel pocket in FMDV 3D^{pol}.
The virus became undetectable after prolong exposure to 5D9.
No resistance mutants were selected following treatment up to ten passages.
The low micromolar IC₅₀ of 5D9 compound is far superior to ribavirin

Figure 1.

Determination of cytotoxicity of 5D9: 10×10^4 baby hamster kidney (BHK) cells were seeded in BHK growth medium in the presence of either 4% DMSO only (vehicle control) or 5–200 μM 5D9. 24 hr later MTT assay was conducted to determine the cellular viability. The numbers on X-axis represent the 5D9 concentration and Y-axis values represent % viability of the cells in comparison to 4% DMSO treated cells.

Figure 2.

Antiviral effect of 5D9: (A) Virus titer reduced upon treatment with 5D9. 0–25 μM 5D9 was administered in the presence on 4% DMSO to a confluent monolayer of BHK-21 cells prior to infection with FMDV. After 1 hr cells were infected with A24 Cruzeiro strain of FMDV at 0.01 and 0.5 MOI. After 1 hpi the unadsorbed viruses were washed and the compound was re-administered and incubated for another 24 hours at 37°C and 5% CO₂. Following this incubation, samples were taken and plaque assays were performed. Results are reported as percent inhibition compared to a sample without inhibitor, and demonstrate a dose-dependent inhibition of virus replication. At the highest concentrations of 5D9 (25 μM) there was a greater than 90% inhibition of virus replication. Error bars represent standard deviation for two independent experiments. (B) 5D9 caused dose-dependent reduction in the expression of FMDV 3Dpol and VP1. Compound treatment and virus infections were performed as described as above. The numbers below each lane represent μM concentration of 5D9. The FMDV A24 Cruzeiro strain was used at a MOI of 10 for the infection. After 5 hours of treatment cells were harvested, washed with PBS, pH 7.4 supplemented with complete protease inhibitor cocktail, lysed with RIPA buffer and viral genomic RNA was degraded using benzonase nuclease; equal protein was loaded in each lane as reflected by

tubulin expression. The details of antibody concentrations and protocol for western blot analysis are described in the main text.

Figure 3.

Continued treatment with 5D9 drove virus to undetectable levels. (A) Scheme of virus passage. FMDV A24 Cruzeiro strain was passed in the presence of gradually increasing concentrations of 5D9 in quadruplicates (right panel shown with filled diamonds as arrowhead), 4% DMSO used as to dissolve 5D9 was run as control (in quadruplicate) throughout passage (right panel shown with filled circles as arrowhead). (B) Effect of 5D9 on the virus titer over the passage. Zero PFU/ml was set the detection limit. At passage 10, the virus in the compound treated group was not detectable by plaque assay.

Figure 4.

Reverse transcriptase-polymerase chain reaction (RT-PCR) detection of viral RNA. (A) Viral RNA was not detectable at and after passage 9 in the presence of 5D9. C2 and C10 represent control (vehicle only) treated viral RNA from passage 2 and 10, respectively. The numbers after (–) represent sample replicate. Similarly, P1-P10 represent 5D9 treated viral RNA from passage numbers, P1-P10, respectively. PC is positive control for the PCR reaction with HeLa RNA template and negative control (NC) represents the PCR reaction without RNA. Lane M is marker lane showing 1Kb DNA ladder. (B) In order to recover (if any present) viral RNA from passage 10 of 5D9 treatment group, the lysates from were further passaged for three generations in the absence of 5D9. As the gel shows there was no detection of viral RNA. The numbering and denotations are similar to figure A. (C) Alignment of 3Dpol sequences from seven FMDV serotypes. Sequence alignment shows absolute conservation (highlighted with red bar) of K59, R168 and K177 residues critical to 5D9 binding (Durk et al., 2010). The text starting each line indicates the serotype, the numbers at left and right hand side of each sequence denote the position of sequence in 3Dpol protein.