Broad-spectrum inhibition of common respiratory RNA viruses by a pyrimidine synthesis inhibitor with involvement of the host antiviral response

Nam Nam Cheung,1 Kin Kui Lai,1 Jun Dai,1 Jun Hang Kok,1 Honglin Chen,1,2 Kwok-Hung Chan,1 Kwok-Yung Yuen1,2,* and Richard Yi Tsun Kao1,2,*

Abstract

Our previous screening of 50,240 structurally diverse compounds led to the identification of 39 influenza A virus infection inhibitors (Kao R.Y., Yang D., Lau L.S., Tsui W.H., Hu L. et al. Nat Biotechnol 2010;28:600–605). Further screening of these compounds against common respiratory viruses led to the discovery of compound FA-613. This inhibitor exhibited low micromolar antiviral activity against various influenza A and B virus strains, including the highly pathogenic influenza A strains H5N1 and H7N9, enterovirus A71, respiratory syncytial virus, human rhinovirus A, SARS- and MERS-coronavirus. No significant cellular toxicity was observed at the effective concentrations. Animal studies showed an improved survival rate in BALB/c mice that received intranasal FA-613 treatments against a lethal dose infection of A/HK/415742Md/2009 (H1N1). Further cell-based assays indicated that FA-613 interfer with the de novo pyrimidine biosynthesis pathway by targeting the dihydroorotate dehydrogenase. Surprisingly, FA-613 lost its antiviral potency in the interferon-deficient Vero cell line, while maintaining its inhibitory activity in an interferon-competent cell line which showed elevated expression of host antiviral genes when infected in the presence of FA-613. Further investigation of the specific connection between pyrimidine synthesis inhibition and the induction of host innate immunity might aid clinical development of this type of drug in antiviral therapies. Therefore, in acute cases of respiratory tract infections, when rapid diagnostics of the causative agent are not readily available, an antiviral drug with properties like FA-613 could prove to be very valuable.

INTRODUCTION

Respiratory tract infections (RTIs) pose a major burden to public health in terms of mortality and morbidity [1]. In fact, acute RTI is estimated to cause 3.9 million deaths annually, making it one of the top five causes of mortality in both developing and developed countries [2]. Young children, the elderly and immunocompromised individuals are especially susceptible to severe RTIs. The World Health Organization (WHO) estimated that, from 2001–2005, only six causes were accountable for over 70% of the 10 million annual deaths worldwide in children under the age of five. Pneumonia and diarrhoea have been consistently identified as the top two causes of death in all WHO regions [3]. Additionally, RTI-related morbidity is associated with productivity loss and medical expenditure, leading to a significant economic burden [4–8].

Common viruses that cause the majority of RTIs include the influenza A virus (IAV), the influenza B virus (IBV), coronaviruses (CoV), respiratory syncytial virus (RSV), human rhinovirus (HRV) and some enteroviruses, such as the enterovirus A71 (EV-A71) [9, 10]. The latter is also the causative agent of hand, foot and mouth disease, with the potential of progressing to severe complications affecting the central nervous system [11–13]. Currently available rapid multiplex molecular assays to determine the causative agent of infection are costly and generally too time-consuming to have significant usefulness in the acute clinical setting. Even when the viral cause is determined, only supportive and symptomatic treatment are given, as specific therapeutic options are lacking for most respiratory viruses [14–16]. We therefore hypothesized that a broad-spectrum inhibitor of
Influenza A/WSN/33 (H1N1) virus infection was inhibited and thus the toxicity of FA-613 was evaluated in different ques. Multiple cell lines were used for the antiviral assays viruses that were less suitable for the clear formation of plaques. Antiviral activity was determined through MTT assays for initial assays were found against RSV, HRV and EV-A71. As shown in Table 1, inhibitory effects in the virus families. We expanded our field outside of the family of Orthomyxoviridae by testing FA-613 against other common respiratory viruses in vitro and partial protection against lethal doses of IAV in vivo. Our study into the mechanism of action of this compound found that FA-613 interferes with the cellular de novo pyrimidine biosynthesis pathway. Broad-spectrum antiviral activity of pyrimidine synthesis inhibitors has been identified previously and their antiviral effect has been mainly attributed to the depletion of the nucleosides necessary for replication of the viral genome [18–20]. Surprisingly, however, we found a connection between the antiviral activity of FA-613 and the use of cells that lack a functional interferon pathway. Furthermore, the addition of FA-613 to virus-infected cells induced an elevated expression of antiviral genes. This would support the findings recently reported by Lucas-Houran et al., which showed that inhibition of pyrimidine synthesis led to an enhanced expression of interferon-stimulated genes (ISGs) [21].

Here, we describe FA-613, a single compound that inhibits multiple RNA viruses that are commonly known to cause RTIs, with in vitro and partial in vivo activities. Its mechanism of action relies on interference with the de novo pyrimidine synthesis pathway, and involves induction of the host innate immunity.

RESULTS

FA-613 has broad-spectrum antiviral activity against respiratory viruses in vitro and antiviral activity against influenza A/H1N1 in vivo

Influenza A/WSN/33 (H1N1) virus infection was inhibited at an EC50 of 3.8±0.2 µM for FA-613 (Fig. 1a). Similar low micromolar activity was found in other IAV strains of the H3N2, H5N1 and H7N9 subtypes, while an EC50 of 0.2 ±0.2 µM was found in IBV, as determined by plaque reduction assay (PRA) (Fig. 1b). Immunoﬂuorescence assays were conducted using Madin–Darby canine kidney (MDCK) cells infected with WSN/33 at an m.o.i. of 10 in the presence of DMSO or 20 µM FA-613. As shown in Fig. 1 (c), diminished protein expression of the viral nucleoprotein (NP) was found in virus-infected cells treated with FA-613 at 8 h post-infection (h.p.i.).

We expanded our field outside of the family of Orthomyxoviridae by testing FA-613 against other common respiratory virus families. As shown in Table 1, inhibitory effects in the initial assays were found against RSV, HRV and EV-A71. Antiviral activity was determined through MTT assays for viruses that were less suitable for the clear formation of plaques. Multiple cell lines were used for the antiviral assays and thus the toxicity of FA-613 was evaluated in different cell lines. No significant toxic effect was found at the effective concentrations.

In vivo efficacy of FA-613 was explored by infecting BALB/c mice intranasally at three LD50 (100 PFU per mouse) of influenza A/HK/415742Md/2009 (H1N1). Treatment with FA-613 (2 mg kg−1 per day), zanamivir (2 mg kg−1 per day) or DMSO in PBS (negative control) was given by the intranasal route twice per day for 3 days, starting 10 min prior to infection. Mouse survival was monitored daily for 14 days to determine mortality. Two independent experiments were performed, consisting of six and seven mice per group, respectively. As expected, the group of mice treated with zanamivir showed complete survival. Partially prolonged survival was found for the compound FA-613-treated group when compared to the vehicle control group (Fig. 1d).

FA-613 interferes with influenza virus genome transcription and replication

To elucidate the mechanism of action of this compound, a time-of-addition assay was performed. As shown in Fig. 2 (a), more than 50% inhibition of viral yield was achieved when the virus was exposed to compound FA-613 in the time interval between 4 and 6 h.p.i. For typical influenza A infections of cells, virus genome transcription and replication processes take place during this period of time and, thus, this result could indicate that the compound’s target plays an essential role in this phase of the virus life cycle. A luciferase activity-based minigenome reporter assay was performed to investigate this further. Human embryonal kidney (HEK) 293 T cells were co-transfected with the individual components of the IAV polymerase, vRNP (PB2, PB1, PA and NP), a luciferase reporter driven by the viral polymerase (pLuc-Firefly) and a Renilla vector (pCMV-Renilla-luciferase). A serial dilution of FA-613 or DMSO was added to the cells 2 h post-transfection. Activity of the reconstituted viral polymerase complex was determined by measuring the Firefly luciferase activity the following day, and Renilla luciferase activity was used for normalization. Our results indicate that FA-613 interferes with vRNP function at an IC50 of 12.6±0.18 µM (Fig. 2b).

FA-613 interferes with pyrimidine metabolism

Several broad-spectrum antivirals were reported to be pyrimidine synthesis inhibitors through inhibition of dihydroorotate dehydrogenase (DHODH) [18–20], the fourth enzyme of the de novo pyrimidine biosynthesis pathway (Fig. 3a), and it has been speculated that the inhibition activity of these compounds is a direct consequence of the deprivation of the nucleosides necessary for virus replication. To test whether FA-613 harbours a similar mechanism of action, different nucleosides and bases were added to the antiviral assays. As shown in Fig. 3(b), the addition of uracil completely reversed the antiviral effect of FA-613. Furthermore, addition of orotic acid, the enzymatic product of DHODH, showed dose-dependent protection of the virus against FA-613, indicating that the compound is targeting DHODH (Fig. 3c).
**FA-613 antiviral activity is cell-dependent and involves the elevated expression of antiviral genes**

Despite broad-spectrum antiviral activity and an indication that FA-613 seems to target the host pyrimidine synthesis pathway through DHODH, no antiviral activity was found against SARS- and MERS-CoV. We hypothesized that this could be due to the use of different cell lines. PRAs for the coronaviruses were conducted using Vero cells, kidney epithelial cells derived from the African green monkey (*Chlorocebus sabaeus*), which are known to be interferon-deficient [22]. Consequently, it is conceivable that if a compound were to exert its antiviral effect through the involvement of host innate immunity, then the drug will not work in cells without an intact interferon pathway. To test this, we conducted simultaneous PRAs for WSN/33 using both Vero and MDCK cell lines. We found that, in fact, FA-613 lost its antiviral activity even against WSN/33 when we replaced MDCK cells with Vero cells (Fig. 4a). Furthermore, when we conducted antiviral activity assays against the SARS- and MERS-CoV using the FRHK4 and Huh-7 cell lines, respectively, the compound regained its activity (Fig. 4b).

To further investigate the effect of FA-613 on host innate immunity, A549 cells were either mock-infected or infected with delNS1-M-A14U [23] at an m.o.i. of 0.01, in the presence of DMSO or 20 µM FA-613. Cells on coverslips were collected at 8 h.p.i. and processed for immunofluorescence assay and staining of NP. Two individual experiments were performed, and a representative result is shown. (d) BALB/c mice were infected intranasally with three LD₅₀ of influenza A/HK/415742Md/2009 (H1N1). Treatment consisted of 2 kg mg⁻¹ per day FA-613, 2 kg mg⁻¹ per day zanamivir or vehicle control for 3 days in two doses per day for 3 days. Mouse survival was evaluated daily. Survival rate data are the average of two independent experiments, analysed using the Kaplan and Meier method (Graphpad Prism). *P<0.05.

**DISCUSSION**

We identified a broad-spectrum inhibitor of common respiratory viruses, compound FA-613. The range of viruses against which the compound has antiviral activity includes...
When we used the cell lines FRHK4 and Huh-7, FA-613 and other antiviral compounds showed antiviral activity against SARS- and MERS-CoV, while comparison of the DHODH genes in these cell lines, respectively, at a similar EC₅₀ showed antiviral activity against SARS- and MERS-CoV, when antiviral assays were conducted using the Vero cell line. Surprisingly, despite broad-spectrum antiviral activity of FA-613 on the nuclear export of NP. When the pathway of pyrimidine synthesis is perturbed, the pool of reserve pyrimidines is insufficient to facilitate efficient transcription and replication, causing a halt in infection.

Surprisingly, despite broad-spectrum antiviral activity of FA-613 in the aforementioned viruses, the compound was initially unable to inhibit SARS- and MERS-CoV infection when antiviral assays were conducted using the Vero cell line, while comparison of the DHODH genes in Homo sapiens and Chlorocebus sabaeus showed 98% homology. When we used the cell lines FRHK4 and Huh-7, FA-613 showed antiviral activity against SARS- and MERS-CoV, respectively, at a similar EC₅₀ as against the other viruses.

To test whether the antiviral activity of FA-613 is cell type-dependent, we conducted simultaneous PRAs using MDCK or Vero cells infected with WSN/33 (Fig. 4a). Interestingly, FA-613 completely lost its inhibitory effect on the virus in the interferon-deficient Vero cell line. We then speculated that the host innate immunity could be involved in the mechanism of action of this compound (Fig. 5). Although the expression of antiviral genes did not differ significantly between uninfected cells treated with DMSO or FA-613, the addition of FA-613 resulted in a marked increase in the expression of IFNB1 and other antiviral genes such as CXCL10 and ISG15 when compared to DMSO in A549 cells infected with delNS1-M-A14U. Similar results were previously reported by Lucas-Hourani et al., who linked pyrimidine biosynthesis to innate immunity by showing that inhibition of pyrimidine synthesis led to an enhanced expression of interferon-stimulated genes (ISGs) [21]. Our finding that elevated expression of antiviral genes under the influence of FA-613, in combination with the finding that FA-613 is not effective in Vero cells, could indicate that the antiviral activity of pyrimidine synthesis inhibitors depends on an intact innate immune system. The interaction between the host de novo pyrimidine synthesis pathway and host innate immunity is still unclear at present. A3, a compound with a distinct molecular structure from FA-613, was previously reported to similarly target the pyrimidine biosynthesis pathway through targeting of DHODH [19]. In contrast to FA-613, A3 did not show cell-type dependency and was able to inhibit infection in Vero cell lines effectively. The authors further mentioned that no induction of IFNB1 and other antiviral genes was found upon treatment of infected cells with A3 [19]. Another recent report describes a pyrimidine synthesis inhibitor that maintains its activity in Vero cells, and elicits an interferon-independent antiviral state [24]. The combination of both parallel and contrasting findings from different individual research groups led to the postulation that, although multiple different pyrimidine synthesis inhibitors have been reported, explicit underlying mechanisms of action could be at play for different classes of inhibitors, with or without the involvement of interferon at different levels of the pathway. In addition, it is plausible that adenoviruses, and possible involvement of interferon at different levels of the pathway.

**Table 1.** FA-613 is a broad-spectrum inhibitor of different RNA viruses that are the causative agents of RTIs without significant toxicity at the effective concentrations

<table>
<thead>
<tr>
<th>Virus strain or host cell</th>
<th>Group</th>
<th>Family</th>
<th>Method</th>
<th>EC₅₀ (µM)</th>
<th>TC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory Syncytial virus</td>
<td>(−) ssRNA</td>
<td>Paramyxoviridae</td>
<td>MTT</td>
<td>10.1±1.0</td>
<td>–</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>(+) ssRNA</td>
<td>Picornaviridae</td>
<td>PRA</td>
<td>9.7±0.3</td>
<td>–</td>
</tr>
<tr>
<td>Enterovirus 71</td>
<td>(+) ssRNA</td>
<td>Picornaviridae</td>
<td>PRA</td>
<td>8.6±1.2</td>
<td>–</td>
</tr>
<tr>
<td>Adenovirus (hAd5)</td>
<td>dsDNA</td>
<td>Adenoviridae</td>
<td>MTT</td>
<td>&gt;50</td>
<td>–</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>(+) ssRNA</td>
<td>Coronaviridae</td>
<td>PRA</td>
<td>&gt;30*</td>
<td>–</td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>(+) ssRNA</td>
<td>Coronaviridae</td>
<td>PRA</td>
<td>&gt;30*</td>
<td>–</td>
</tr>
<tr>
<td>MDCCK</td>
<td>–</td>
<td>–</td>
<td>MTT</td>
<td>–</td>
<td>&gt;100</td>
</tr>
<tr>
<td>RD</td>
<td>–</td>
<td>–</td>
<td>MTT</td>
<td>–</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HEp-2</td>
<td>–</td>
<td>–</td>
<td>MTT</td>
<td>–</td>
<td>30.4±1.2</td>
</tr>
<tr>
<td>Huh-7</td>
<td>–</td>
<td>–</td>
<td>MTT</td>
<td>–</td>
<td>&gt;100</td>
</tr>
<tr>
<td>FRHK4</td>
<td>–</td>
<td>–</td>
<td>MTT</td>
<td>–</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Vero</td>
<td>–</td>
<td>–</td>
<td>MTT</td>
<td>–</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Antiviral activity as determined in the Vero cell line; please refer to Fig. 4 for the EC₅₀ of FA-613 in SARS- and MERS-CoV as determined in the FRHK4 and Huh-7 cell lines, respectively.

–, Not applicable.

IAV, IBV, RSV, HRV, SARS- and MERS-CoV (Fig. 1, Table 1). The compound’s mechanism of action seems to specifically affect the transcription and replication of the virus. The finding that the addition of excess uracil or orotic acid is capable of relieving the virus from the inhibitory effect of FA-613 suggests that the cellular de novo pyrimidine synthesis pathway and of its key enzymes, DHODH, in particular are involved. The viral protein NP was located predominantly in the nucleus in influenza-infected cells treated with compound FA-613 (Fig. 1c). This is most likely due to the fact that general infection is delayed in the presence of this compound, rather than a direct effect of compound FA-613. This finding that elevated expression of antiviral genes under the influence of FA-613, in combination with the finding that FA-613 is not effective in Vero cells, could indicate that the antiviral activity of pyrimidine synthesis inhibitors depends on an intact innate immune system. The interaction between the host de novo pyrimidine synthesis pathway and host innate immunity is still unclear at present.
other DNA viruses, have developed an escape mechanism from the host innate immune response that is elicited by pyrimidine synthesis inhibition, rendering FA-613 unable to effectively inhibit adenovirus infection. Nonetheless, modulation of host pathways for enhancement of the innate immune response through chemicals could be a viable strategy for the development of antiviral therapy, and has been of growing interest recently [25, 26]. It can be expected that such immunomodulators are effective against a range of different viral pathogens through the many facets of the host immune response.

Despite promising in vitro antiviral activity, the use of pyrimidine synthesis inhibitors in animal challenge models has not been successful [27–29]. This could be due to the specific uptake and metabolism of the compound, as well as to a reservoir of uridine in living organisms. Intranasal treatment of influenza virus infection in mice could be preferable for compounds that lack activity when administered through the oral or intraperitoneal route. However, enhanced virus replication and lung pathology was previously found in mice that received intranasal treatments versus other routes of treatment [30]. Despite promising in vitro antiviral activity, the use of pyrimidine synthesis inhibitors in animal challenge models has not been successful [27–29]. This could be due to the specific uptake and metabolism of the compound, as well as to a reservoir of uridine in living organisms. Intranasal treatment of influenza virus infection in mice could be preferable for compounds that lack activity when administered through the oral or intraperitoneal route. However, enhanced virus replication and lung pathology was previously found in mice that received intranasal treatments versus other routes of treatment [30]. An adjusted low virus challenge dose is therefore recommended when testing the effectiveness of intranasal administration of a compound [30]. In this study, we opted for treatment by the intranasal route twice per day for 3 days, starting 10 min before infection of the mice with 3 LD_{50} of A/HK/415742Md/2009 (H1N1). Treatment for longer than 3 days led to excessive weight loss in all groups, including the positive and negative control, and was not sustainable. The direct topical (intranasal) regimen of FA-613 in combination with the prophylaxis treatment of 10 min prior to infection resulted in modest protection of the mice in terms of survival rate. This indicates that, despite several unsuccessful attempts in the past, pyrimidine synthesis inhibitors could potentially inhibit virus infections in vivo when given under appropriate conditions. Our work therefore provides novel insights in the clinical development of pyrimidine synthesis inhibitors as broad-spectrum antiviral drugs.

To conclude, we have demonstrated that FA-613 is a broad-spectrum inhibitor of respiratory RNA viruses in vitro through interference with the de novo pyrimidine synthesis pathway, possibly by targeting DHODH. Mice infected with lethal doses of IAV (H1N1) showed prolonged survival when treated with FA-613 in comparison with the vehicle control. In addition, our data indicate that addition of FA-613 to the infected cell leads to induction of the host innate immune response. Further research into the specific pathways in play could potentially lead to a new direction in the usage of pyrimidine synthesis inhibitors in the treatment of systemic viral infections.

Fig. 2. FA-613 inhibits replication and transcription in influenza virus A infection. (a) Results of time-of-addition assay. Compound and virus were pre-mixed and added to MDCK cells on ice to exclusively allow binding for synchronization of the infection process (−1 h). At 0 h, cells were washed, replenished with fresh MEM and heated to 37°C to start endocytosis. Cells were then exposed to FA-613 treatment for 2 h periods by adding and removing the drug at the indicated time-points. For each experiment, FA-613 or DMSO were used in parallel plates, with viral yield in the DMSO treatment group representing 100%. Averages±SEM shown represent the data collected from three independent experiments performed in triplicate. (b) Results of the luciferase activity-based minigenome reporter assay. HEK 293 T cells were co-transfected with different plasmids expressing the viral proteins constituting the IAV vRNP, pLuc-Firefly and pCMV-Renilla luciferase. Compounds were added 2 h post-transfection. The relative luciferase activity was determined on the following day to evaluate the activity of the reconstituted viral polymerase complex. Averages±SEM shown represent the data collected from three independent experiments performed in triplicate.
METHODS

Chemicals and reagents
All compounds were purchased from Chembridge and dissolved in DMSO for in vitro assays. For in vivo activity assays, compound stocks in DMSO were further diluted in PBS.

Cell lines and viruses
MDCK, HEK 293T, human lung carcinoma (A549) and Vero cell lines (purchased from ATCC) were maintained in MEM or DMEM supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen). Influenza virus A and B strains were propagated in MDCK cell cultures. RSV was propagated

Fig. 3. FA-613 antiviral activity in IAV is exclusively reversed by excess uracil and orotic acid. (a) Simplified representation of the de novo pyrimidine biosynthesis and salvage pathways. A series of enzymes are necessary for the production of pyrimidines. DHODH (in red) is the fourth enzyme and produces orotate. Alternatively, pyrimidines can be salvaged from uracil. Results of PRAs in MDCK cells infected with WSN/33 were performed using DMSO, 20 µM FA-613 alone, 20 µM FA-613 in combination with 400 µM of the indicated bases (b) or 20 µM FA-613 in combination with a serial dilution of orotate (c). Averages ± SEM shown represent the data collected from three independent experiments performed in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Plaque assay (PRA) Cell monolayers in 24-well plates were infected with ~50 PFU/well of the indicated virus premixed with DMSO or FA-613 in a serial dilution of concentrations. Viral inoculum was removed after 1.5 h of absorption at 37°C by aspiration and the cells were overlaid with 0.75 % low-melting agarose in MEM containing 0.5 % HI-FBS, 1 µg/ml TPCK-treated trypsin and the appropriate amount of DMSO or FA-613. Cells were fixed with 10 % formaldehyde at 72 h after infection and stained with 0.7 % crystal violet to evaluate plaque formation. In the nucleotide addition assays, indicated nucleotides were added during infection as well as in HEp-2 cells. RD cells were used for the propagation of rhinovirus and enterovirus 71. SARS- and MERS-CoV were grown in Vero and Huh-7 cells, respectively. Viruses were grown in either plain MEM or DMEM supplemented with 0.2 % FBS. Experiments involving live A/Vietnam/1194/2004 (H5N1), A/Anhui/1/2013 (H7N9), SARS- and MERS coronaviruses followed the standard operating procedures of the approved Biosafety Level 3 facility [31].

**Figure 4.** Cell-dependent activity of FA-613. (a) MDCK and Vero cells were individually and simultaneously infected with WSN/33 in the presence of DMSO or a serial dilution of FA-613. Viral yield was determined by PRA, where 100 % of viral yield represents the DMSO-treated group in each cell line. Averages±SEM shown represent the data collected from two independent experiments performed in triplicate. *P<0.05, **P<0.01. (b) Antiviral assays for SARS- and MERS-CoV were conducted in MTT assays using Vero cells or Huh-7 and FRHK4 cells. EC50 values from two independent experiments performed in triplicate were calculated using Graphpad Prism.

**Figure 5.** FA-613-treated cells elicit enhanced interferon-beta expression when infected with delNS1-WSN/33. A549 cells were either mock-infected or infected with delNS1-M-A14U at an m.o.i. of 0.01, in the presence of DMSO or compound. Cell lysate was collected at 16 h.p.i. for RNA extraction. Expression of the indicated genes was determined by qPCR. Averages±SEM shown represent the data collected from two independent experiments performed in triplicate.
in the agarose overlay. The percentage of viral yield relative to the vehicle control (DMSO) was determined for each compound concentration, and the 50% effective concentration (EC$_{50}$) was calculated using Graphpad Prism.

**Animal studies**

FA-613 was dissolved in DMSO and diluted to 1 mg ml$^{-1}$ using PBS. Eight-week-old BALB/c mice were anesthetized by intraperitoneal injection of ketamine-xylazine (50/5 mg kg$^{-1}$) before intranasal inoculation with three 50% lethal dose (LD$_{50}$) of influenza A/HK/415742Md/2009 (H1N1), in a volume of 20 μl on day 0 (100 PFU/mouse). FA-613 (treatment group; 1 mg ml$^{-1}$), zanamivir (positive control; 1 mg ml$^{-1}$) or DMSO in PBS (negative control) was administered intranasally in volumes of 20 μl twice per day for 3 days. The first dose was given 10 min prior to infection on day 0. Body weight and survival was evaluated daily for 14 days to observe mouse morbidity and mortality, respectively. Survival curve analysis was performed using the Kaplan and Meier method in Graphpad Prism.

**MTT assay**

The 50% toxic concentration (TC$_{50}$) of the compound in various cell lines and compound antiviral activity in the indicated viruses was determined by MTT assay. Cells were seeded one day prior to the assay in 96-well plates, and treated with serially diluted compounds or DMSO. Viruses were pre-mixed with DMSO or FA-613 prior to addition to the cells for the antiviral assays. After 72 h of incubation at 37°C, 5 mg ml$^{-1}$ of MTT solution was added followed by 4 h incubation at 37°C. Cells were lysed with 10% SDS with 0.01M HCl at 37°C overnight before determination of OD$_{570}$ with reference to OD$_{640}$. The relative MTT reading to the control was determined by dividing the reading of the tested compound concentration by the reading of the DMSO control in order to calculate the EC$_{50}$/TC$_{50}$.

**Mini-genome assay**

HEK 293 T cells seeded in 96-well plates were transfected using Lipofectamine LTX according to the manufacturer’s instructions with 80 ng of each of the following plasmids: PHW2000-PB1, -PB2, -PA, -NP, pLuc-Firefly and pCMV- Renilla-luciferase [32]. Serial dilutions of the compounds were added 2 h post-transfection. Activity of the reconstituted polymerase complex was determined the following day using the Dual Luciferase Assay kit (Promega) according to the manufacturer’s instructions.

**RNA extraction and quantitative RT-PCR**

The DELNS1-M-A14U-WSN [23] was a gift kindly provided by Professor Honglin Chen and was used to infect A549 cells at an m.o.i. of 0.01. RNA was isolated from infected cell cultures at 16 h.p.i. using the Qiagen RNeasy mini kit, according to the manufacturer’s instructions. One microgram total RNA was reverse transcribed using an oligo d(T) primer and the Transcriptor First Strand cDNA Synthesis Kit (Roche). The synthesized cDNA served as template for the amplification of genes of interest and the housekeeping gene by real-time PCR using the FastStart Universal SYBR Green Master (Roche), and the ABI-PRISM 7900 sequence detection system. Primer sequences used for the detection of target genes: IFNB1 (forward TGGAGGCTTGAATA, reverse GCCAGAGTTTC TCAAAATAG), CXCL10 (forward CCATTCTGATTTCG TGCCCTAT, reverse TTTTCCTGCTAATGCTTCTTACG TA), ISG15 (forward GAGAGGAGCGGAATCTCATCT, reverse GCCCTGTATTCTCTACCA), CCL5 (forward GCACTGGCTCCCCATATT, reverse AGCACTTGCCAC TGGTGTA), ACTB (forward CACTCTTCCAGGTTCTTC TTCCCTC, reverse GTACAGGTCTTTCGCGATGT). Values were normalized to the gene expression of ACTB (beta-actin) and relative to the values in mock-treated samples, after normalization to gene expression.

**Indirect immunofluorescence assay**

MDCK cells seeded to 80% confluence on coverslips were infected with WSN/33 at an m.o.i. 10 in the presence of DMSO or FA-613. Cells were fixed for 10 min using 3.7% formaldehyde in PBS at 8 h.p.i. and washed thrice with PBS before incubation with a primary mouse anti-NP antibody (Millipore) for 1 h at 37°C. After washing, cells were incubated with an Alexa Fluor 488-conjugated secondary antimouse antibody against mouse for 30 min at 37°C. Mounting oil containing DAPI were used to mount the coverslips. Fluorescent images were obtained using a Carl Zeiss LSM710 META (Germany) laser scanning confocal microscope.

**Funding information**

The study was supported by the HMRF Commissioned Study Project Grant HKM-15-M11 to R.Y.K.

**Acknowledgements**

The Providence Foundation is acknowledged for its support for the establishment of LC-MS facilities in the Department of Microbiology, LKS Faculty of Medicine, HKU. We would like to thank Dr KH Sze and Y. Ke for confirming the structure of compounds by LC-MS and NMR. We also acknowledge the kind assistance of the Li Ka Shing Faculty of Medicine Core Facilities.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

All procedures and experiments using animals as described in this manuscript were conducted in accordance with the standard operating protocols of the biosafety level 2 animal facility approved by the Animal Ethics Committee of The University of Hong Kong.

**References**


Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.