Antiviral Research 100 (2013) 29–37

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

A small molecule multi-kinase inhibitor reduces influenza A virus replication by restricting viral RNA synthesis

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ARTICLE INFO

Article history:
Received 1 July 2012
Revised 8 July 2013
Accepted 15 July 2013
Available online 24 July 2013

Keywords:
Kinase inhibitor
Influenza virus
Viral RNA synthesis

ABSTRACT

Currently available drugs against influenza virus target the viral neuraminidase or the M2 ion channel. The emergence of viral strains resistant to these drugs has been widely described; therefore, there is an urgent need for novel antiviral drugs. Targeting of host factors required for viral replication is an attractive option for circumventing the problem of drug resistance. Several RNAi screens have demonstrated that host kinases are required for the replication of influenza virus. To determine whether compounds that inhibit these kinases can impair viral replication, we tested several kinase inhibitors for activity against influenza A virus. We demonstrate that the multi-kinase inhibitor ON108110 reduces replication of influenza A virus in a dose-dependent manner by suppressing viral RNA synthesis. In addition, ON108110 also inhibits other viruses including vesicular stomatitis virus and Newcastle disease virus, suggesting that this compound may represent a novel class of antiviral agents.

1. Introduction

Influenza A virus is a member of the *Orthomyxoviridae* family. It attaches to the cell surface via sialic acid-containing receptors and enters the cell via pH-dependent endocytosis. The acidic environment of the endosome triggers fusion of the viral and endosomal membranes, leading to the release of the viral ribonucleoprotein complex (vRNP) into the cytoplasm. The vRNPs, composed of the viral RNA (vRNA), nucleoprotein (NP) and the polymerase proteins (PB1, PB2 and PA), then dissociates from the matrix protein (M1) and enters the nucleus, where vRNA replication and transcription occur (Palese and Shaw, 2007). Newly synthesized vRNPs are subsequently exported from the nucleus through the chromosome region maintenance 1 protein (CRM1)-mediated pathway (Watanabe et al., 2001). Virus assembly is mediated by the M1 protein, via interactions with viral membrane proteins (hemagglutinin (HA), neuraminidase (NA) and the M2 ion channel) and vRNP complexes (Ali et al., 2000; Ye et al., 1999). Virion release from the cell surface is dependent upon neuraminidase activity of NA (Palese and Shaw, 2007).

Influenza virus continues to be a significant health and economic burden worldwide. Although drugs are currently available for the treatment of influenza A virus, several viral strains have developed resistance (Bright et al., 2006; Ison, 2011) which necessitates the development of novel antiviral compounds. Currently available anti-influenza drugs inhibit viral proteins such as NA or the M2 ion channel protein (Garman and Laver, 2004; Pinto and Lamb, 1995; Wharton et al., 1994); however, the error prone viral polymerase provides the virus with many opportunities to develop resistance. One way to circumvent drug resistance is to target host factors required for viral growth instead of viral proteins. As a first step toward this goal, we performed a genome-wide siRNA screen and identified 295 host factors required for the replication of influenza virus (Konig et al., 2010). The majority of these host factors are kinases, so we sought to further investigate whether kinase inhibitors can impair the replication of influenza virus.

We screened a panel of kinase inhibitors for inhibition of influenza viral growth, and herein, we demonstrate that the multi-kinase inhibitor ON108110 inhibits the replication of influenza A virus. In addition, ON108110 inhibits other viruses including vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV).

2. Materials and methods

2.1. Cells

A549 (human lung adenocarcinoma cell line) and LA4 (mouse lung adenoma cell line) cells were maintained in Dulbecco's

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http://dx.doi.org/10.1016/j.antiviral.2013.07.010

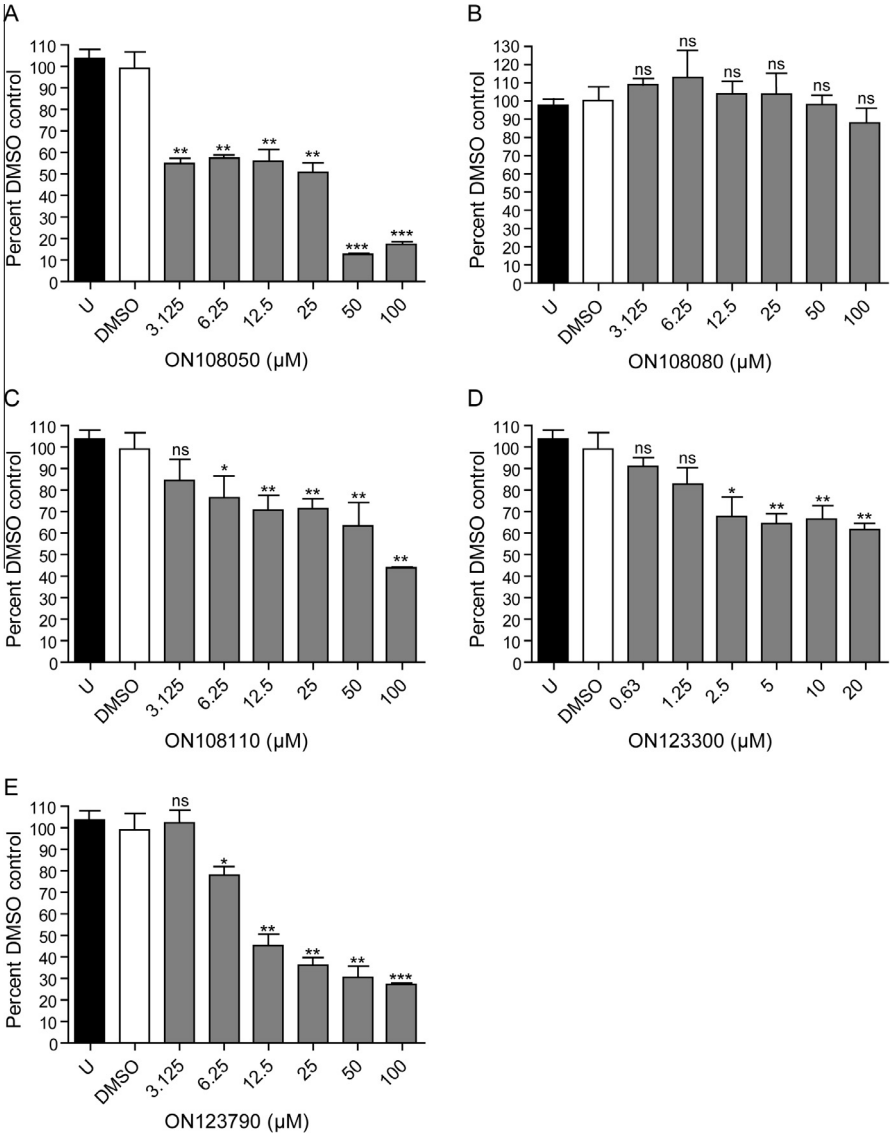

Fig. 1. Kinase inhibitors have varying effects on cell viability. A549 cells were treated with twofold serial dilutions of each kinase inhibitor (A–E, ON108050, ON108080, ON108110, ON123300 and ON123790), solvent DMSO alone or left untreated (U). The amount of DMSO was the same for all the dilutions. Cell viability was measured 24 h post-treatment using the Promega CellTiter-Glo assay. Error bars represent the standard deviation of three independent replicates, ns indicates not significant, $^{*}p$ value < 0.05, $^{**}p$ value < 0.001, $^{***}p$ value < 0.0001.

modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

2.2. Cell viability assay

A549 or LA4 cells were seeded in 96-well plates at 8000 cells per well one day prior to treatment with twofold serial dilutions of the compound of interest or the vehicle alone (DMSO). The amount of DMSO was equivalent for all dilutions of the compound. Cell viability was determined 24 h post-treatment using the CellTiter-Glo assay (Promega) according to the manufacturer's instructions.

2.3. Viral infection

A549 or LA4 cells were seeded in 24-well plates at 1×10^5 cells per well one day prior to treatment with twofold serial dilutions of the compound of interest or DMSO alone. Two to four hours post-treatment, cells were washed with phosphate buffered saline (PBS) and infected with influenza virus (A/WSN/33) at an MOI of 0.01, vesicular stomatitis virus (VSV-GFP) at an MOI of 0.0001, or Newcastle disease virus (NDV-B1 strain) at an MOI of 2 for one hour. After removing the viral inoculum, medium containing the compound of interest (or the DMSO control) was added back to the cells. The amount of virus in the supernatants was determined 24 h post-infection (hpi) using either a hemagglutination (HA) or a plaque assay. The hemagglutination assay was performed by making twofold serial dilutions of the supernatant with PBS in a 96-well V-bottom plate followed by addition of 0.5% chicken red blood cell suspension. The plate was incubated for 45 min at room temperature observed for hemagglutination and the number of HA wells was counted for each sample. Influenza A plaque assay was performed as previously described (Konig et al., 2010). Plaque assay for NDV-B1 was performed using DF1 cells (chicken embryo fibroblast cell line), and the plaques were visualized by immunostaining with an antiserum raised against the virus.

2.4. Entry assay

A549 cells were seeded in 96-well plates at 3×10^4 cells per well. The next day, cells were treated with ON108110, diphyllin

(as a positive control), or DMSO alone in medium supplemented with 4 μg/ml polybrene (Sigma–Aldrich) for 2–4 h. Subsequently, pseudoparticles bearing WSN–HA/NA proteins and encoding the Gaussia luciferase reporter gene were added to the media and incubated for 18 h to assay entry.

Cells were then washed four times, fresh medium was added and 24 h later, Gaussia luciferase activity was measured using the Renilla luciferase assay system (Promega). Diphyllin (ChemDiv 000A–1085) is a potent v-ATPase inhibitor that inhibits acidification of endosomes thereby blocking membrane fusion. Pseudoparticles were generated by transfecting 293T cells with plasmids encoding a minimal HIV pro-virus encoding the Gaussia luciferase reporter gene, HIV gag-pol and the viral envelope proteins (WSN–HA/NA) using Lipofectamine 2000 (Invitrogen). Twenty-four hours post transfection the pseudoparticles were collected from the media by centrifugation through a sucrose gradient.

2.5. Immunofluorescence and Immunoblots

A549 cells were seeded on cover-slips in a 24-well plate at 7.5×10^4 cells per well. The next day, cells were infected with influenza virus (A/WSN/33) at an MOI of 3. Cells were then washed and incubated with medium containing ON108110 at various concentrations. At 3, 5 and 7 hpi, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with cold methanol or 0.5% Triton X-100 in PBS, and stained with either a polyclonal rabbit anti-NP serum or a mouse monoclonal anti-NP mAb (AbD Serotec, MCA-400). Alexa Fluor 488 donkey anti-rabbit or anti-mouse IgGs were utilized as secondary antibodies (Invitrogen) as appropriate. 4′-6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. The coverslips were mounted on a slide using Prolong gold antifade reagent (Invitrogen). Cells were imaged using the Axioplan2IE or the AMG Evos fluorescence microscopes. To detect viral proteins Western blots were performed using a monoclonal anti-HA (4B2, CellSciences) or a rabbit polyclonal NS1-specific anti-serum (pAb 155). A mouse monoclonal against GAPDH (Thermo Scientific) was used to monitor host protein levels. Anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (GE Healthcare) were utilized where appropiate.

2.6. Kinase inhibition profiling

Kinase inhibition profiling was performed by Reaction Biology Corporation (Malvern, PA) using a standard panel of 300 kinases.

2.7. RT-PCR and quantitative RT-PCR (qRT-PCR)

Extraction of RNA from cells for subsequent qRT-PCR analysis was performed using the RNeasy Mini Kit (Qiagen) according to manufacturer's specifications.

A two-step qRT-PCR was performed to analyze cellular and viral genes as described before (Ortigoza et al., 2012). Briefly, first-strand synthesis of isolated RNA was achieved by reverse transcription using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's specifications. The resulting cDNA was used for quantitative PCR using LightCycler 480 SYBR Green I Master reagent (Roche) and the LightCycler 480 instrument (Roche) according to the manufacturer's specifications. Analysis of results was done using the delta delta CT method. Values were normalized using β-actin as the endogenous housekeeping gene. Primer sequences are available upon request.

Table 1
Activity of kinase inhibitors against influenza A virus.

Compound	Reduction in number of HA wells
ON108080 (3.13–100 μm)	0
ON108110 (3.13–25 μm)	5
ON123300 (0.63–1.25 μm)	0
ON123790 (3.13 μm)	2
ON123790 (6.25 μm)	5

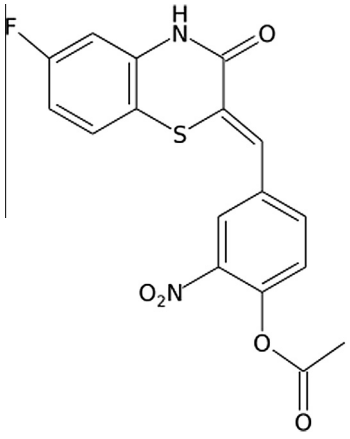

Fig. 2. Chemical structure of ON108110. Representation of the 2D structure of ON108110.

2.8. Influenza virus minigenome assay

For influenza A virus minigenome reporter assays, 293-T cells were transfected with Lipofectamine 2000 (Invitrogen). Transfections were performed in 24-well plates at cell concentrations of 2×10^5 cells/well and a lipid:DNA ratio of 2 μL:1 μg. 100 ng of DNA expression plasmids for WSN NP-Firefly reporter, SV40-Renilla reporter, WSN or PR8 PB1, WSN or PR8 PB2, WSN or PR8 PA, and WSN or PR8 NP (or empty vector for negative control) were co-transfected as described before (Ortigoza et al., 2012). Eighteen hours post transfection, cells were lysed and luciferase production was assayed with the Dual-Glo Luciferase Assay kit (Promega) according to the manufacturer's specifications.

Table 2
Kinase inhibtion profile of ON108110.

Kinase	IC50 (nM)	König et al.	Brass et al.	Saphira et al.
CAMKK1	26.66			
CAMKK2	92.9	Yes		
CDK1/cyclin A	48.52			
CDK1/cyclin B	5.26			
CDK2/cyclin A	20.5			
CDK4/cyclin D1	11.25	Yes		
CDK4/cyclin D3	14.78	Yes		
CDK5/p25	6.52			
CDK5/p35	12.37			
CDK6/cyclin D1	1.51			Yes
CDK6/cyclin D3	24.16			
CDK9/cyclin K	35.07			
CDK9/cyclin T1	7.67			
CK2a	2.6			
CK2a2	1.59			
DYRK1B	47.35	Yes		
DYRK2	57.96			
GSK3a	16.5		Yes	
GSK3b	18.41	Yes		Yes
HGK/MAP4K4	23.29	Yes		
MLK3/MAP3K11	87.4	Yes		
MUSK	56.14			
NLK	35.61			
PIM3	1.92			
SGK1	82.29	Yes		

3. Results

3.1. Screening of kinase inhibitors for activity against influenza A virus

Five kinase inhibitors were selected from a compound library based on a preliminary kinase inhibition profile and tested for their ability to inhibit influenza A virus. We wanted to test the compounds at concentrations that are not toxic to cells. To this end,

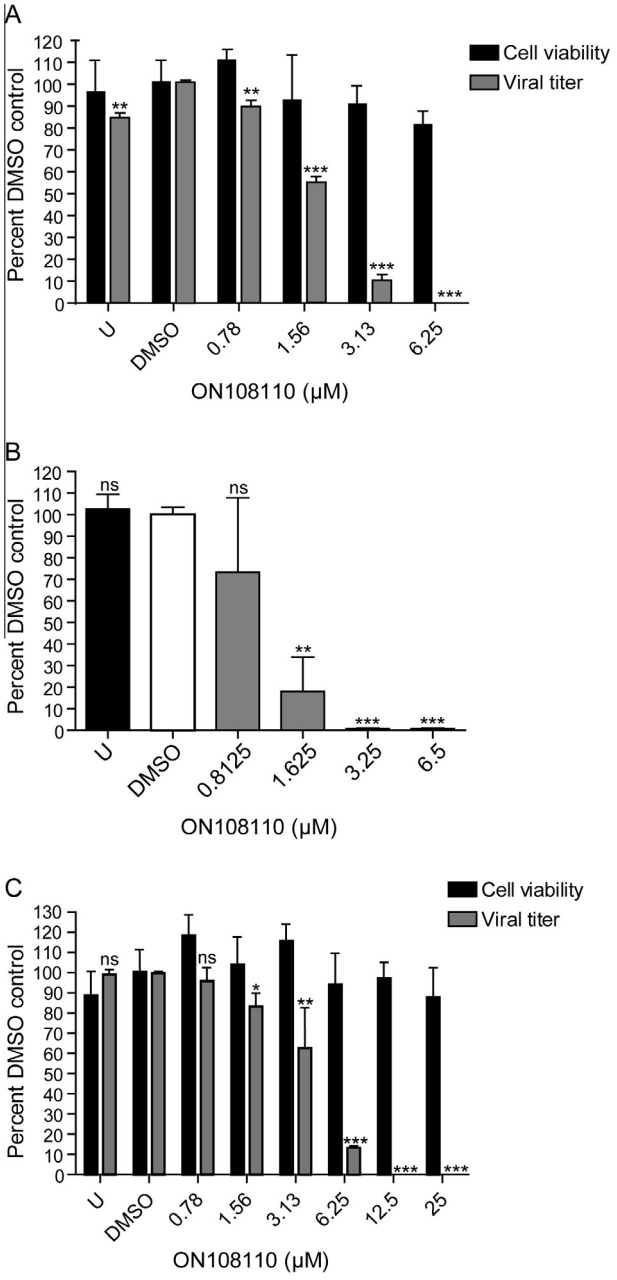

Fig. 3. ON108110 reduces influenza A virus replication in a dose-dependent manner. A549 (A and B) or LA4 (C) cells were treated with twofold serial dilutions of ON108110, or solvent DMSO, or left untreated (U). The amount of DMSO was the same for all the dilutions. Cell viability and viral titers were measured 24 h post-treatment. To determine cell viability, we measured the ATP within the cells. Plaque assays were performed to determine viral titers. Cell viability and viral titers were expressed as percent of the DMSO control. Cells were pretreated with compound or DMSO before the infection in A and C. In panel B, the cells were treated immediately after infection. Error bars represent the standard deviation of three independent replicates, ns indicates not significant, $^{*}p$ value < 0.05, $^{**}p$ value < 0.001, $^{***}p$ value < 0.0001.

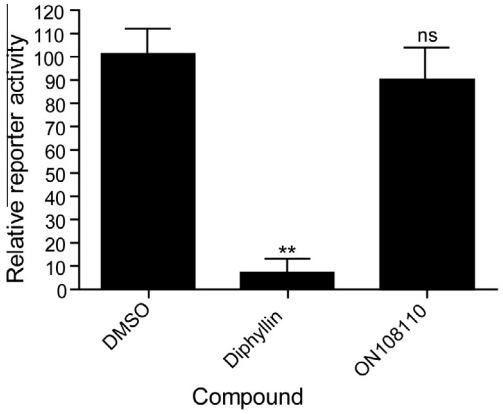

Fig. 4. ON108110 does not inhibit influenza A virus entry. A549 cells were treated with ON108110 (3.13 μM), or solvent DMSO, or diphyllin (2.1 μM) as a positive control. The cells were then incubated with pseudoparticles bearing influenza virus HA/NA proteins and encoding the Gaussia luciferase reporter. After 18 h, cells were washed and medium without compound was added. A luciferase assay was performed 24 h after addition of medium. Error bars represent the standard deviation of three independent replicates, ns indicates not significant, $^{*}p$ value < 0.05, $^{**}p$ value < 0.001, $^{***}p$ value < 0.0001.

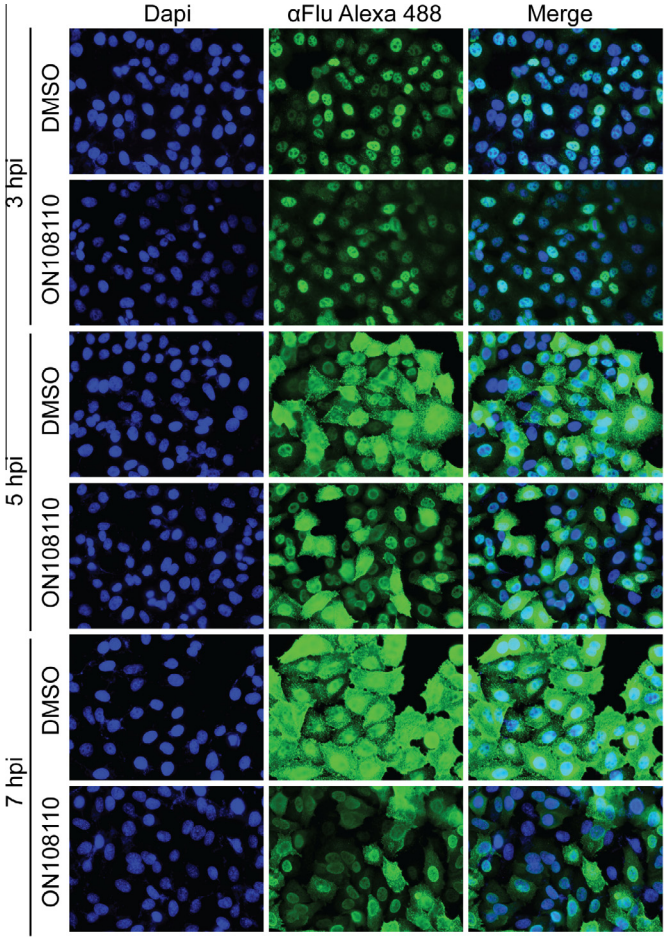

Fig. 5. ON108110 reduces influenza virus protein synthesis in A549 cells. A549 cells were pre-treated with ON108110 (3.13 μM) or solvent DMSO for 2 h. The cell medium was removed, and the cells were infected with influenza virus (A/WSN/33) at an MOI of 3. After 1 h the medium with compound or DMSO was added. At 3, 5 and 7 h post-infection, cells were fixed, permeabilized awnd stained with a polyclonal rabbit anti-influenza serum. 4′-6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. Results shown are representative images.

A549 cells were treated with twofold serial dilutions of each compound, and the volume of DMSO solvent was equivalent for all dilutions. Cell viability was determined 24 h post-treatment by measuring the ATP levels in the compound versus DMSO-treated cells. The results, expressed relative to the DMSO control, show that ON108050 was toxic to cells (55% viability at the lowest concentration tested) so this compound was excluded from further characterization (Fig. 1). However, ON108080, ON108110, ON123300 and ON123790 had varying effects on cell viability (Fig. 1B–E). To determine whether any of these compounds inhibit the replication of influenza virus (A/WSN/33), A549 cells were treated with each compound at concentrations that yielded at least 70% viability relative to the DMSO control. Two to four hours post-treatment, cells were infected with the virus at an MOI of 0.01 to allow multi-cycle replication. Supernatants were harvested 24 h post-infection (hpi) and a hemagglutination (HA) assay was performed to measure viral levels. The ability of the compounds to inhibit the virus was expressed as the reduction in the number of HA wells relative to the DMSO control (Table 1). While ON108080 and ON123300 did not inhibit the virus at any of the concentrations that were tested, ON108110 reduced the number of HA wells by five at a range of 3.13–25 μM. ON123790 reduced the number of HA wells by two at a concentration of 3.13 μM and by five at a concentration of 6.25 μM. Taken together with the results in Fig. 1, these studies show that ON108110 and ON123790 inhibit influenza virus at concentrations where cell viability is greater than 80%. Since ON108110 is the more potent of the two compounds, we performed additional studies aimed at further characterizing its antiviral properties. The chemical structure of ON108110 is represented in Fig. 2.

3.2. Kinase inhibition profile of ON108110

To obtain a more comprehensive understanding of the cellular factors affected by ON108110 we performed an extensive kinase inhibition profile. The analysis revealed that 25 kinases were significantly inhibited by ON108110 with an IC_{50} of less than 100 nM among 300 kinases that were tested (Table 2). We were

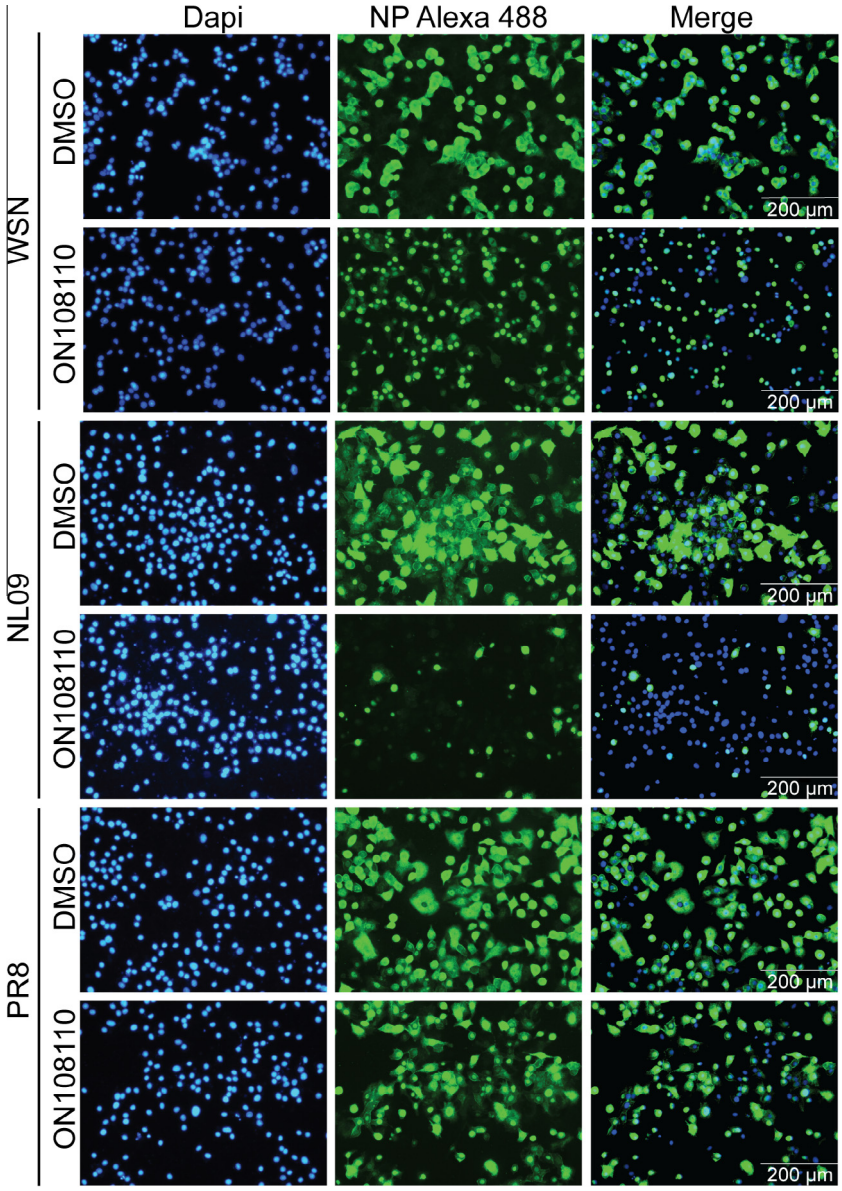

Fig. 6. ON108110 inhibits several influenza virus strains. A549 cells were infected with influenza virus A/WSN/33 (as a control), A/PR8/34 or A/NL/09 at an MOI of 3 in all cases. Following the infection, cells were treated with ON108110 (3.25 μM) or DMSO. At 5 h post-infection, cells were fixed, permeabilized and stained with a mouse monoclonal anti-NP antibody (AbD Serotec, MCA-400). 4′-6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. Results shown are representative images.

encouraged to find that among the 25 kinases identified, at least 8 (Table 2), were previously identified as required host factors for influenza replication in one or more studies (Konig et al., 2010; Shapira et al., 2009; Brass et al., 2009).

3.3. ON108110 reduces influenza A virus replication in a dose-dependent manner

To better quantify the degree to which ON108110 inhibits replication of influenza virus, A549 cells were treated with twofold serial dilutions of ON108110 (0.78–6.25 μM) for 2–4 h prior to infection with WSN virus at an MOI of 0.01. The amount of virus present in the supernatant at 24 hpi was quantified using a plaque assay. ON108110 inhibited viral replication in a dose-dependent manner (Fig. 3A). At the highest concentration tested (6.25 μM), the cell viability and amount of virus in the supernatant were 81% and 0.007%, respectively, relative to the DMSO control. We also demonstrated that ON108110 can inhibit influenza A virus replication when the cells were treated with the compound post-infection (Fig 3B).

To determine whether ON108110 inhibits the replication of influenza virus in a species-specific manner, we tested the compound in a mouse lung adenoma cell line (LA4). Cells were treated with twofold serial dilutions of ON108110 (0.78–25 μM) and infected with WSN virus. Fig. 3C shows that ON108110 inhibited, once more, viral replication in a dose-dependent manner. At the highest concentration tested (25 μM), the cell viability and amount of virus in the supernatant were 87% and 0.05%, respectively, relative to the DMSO control. Taken together, the results in (Fig. 3) indicate that ON108110 effectively inhibits viral replication in both human and mouse cells, although A549 (human) cells were more sensitive to the effects of the compound.

3.4. ON108110 does not inhibit influenza A virus entry

Next, we wanted to determine the step(s) of the influenza virus life cycle that are inhibited by ON108110. First, we tested whether ON108110 inhibits entry of influenza virus using a previously described protocol (Konig et al., 2010). Briefly, A549 cells were treated with ON108110 at 3.13 μM, a concentration that resulted in a high level of cell viability (90% relative to the DMSO control) and a viral inhibition of approximately 10-fold relative to the DMSO control. Two to four hours post-treatment, cells were incubated with pseudoparticles bearing WSN–HA/NA proteins and encoding the Gaussia luciferase reporter gene. Diphyllin, a compound previously shown to inhibit influenza virus entry (Konig et al., 2010), served as a positive control. Diphyllin is a v-ATPase inhibitor that prevents acidification of endosomes and therefore membrane fusion. Data in Fig. 4 indicate that ON108110 did not inhibit viral entry.

3.5. ON108110 reduces influenza A virus replication

To determine whether ON108110 affects the influenza virus protein levels, A549 cells were treated with the compound at a concentration of 3.13 μM for 2 h prior to infection with WSN virus at an MOI of 3. Cells were then washed several times and medium containing the compound was added back to the cells. At 3, 5 and 7 hpi, cells were fixed, permeabilized and stained with a polyclonal rabbit anti-influenza serum (Fig. 5). No differences could be seen at 3 hpi, confirming that ON108110 does not inhibit viral entry. However, at 5 and 7 hpi we detected a reduction in the signal intensity from the viral proteins. These results were corroborated by using a mouse monoclonal antibody for NP detection and by eliminating the pre-treatment (Fig. 6). The pattern observed for influenza

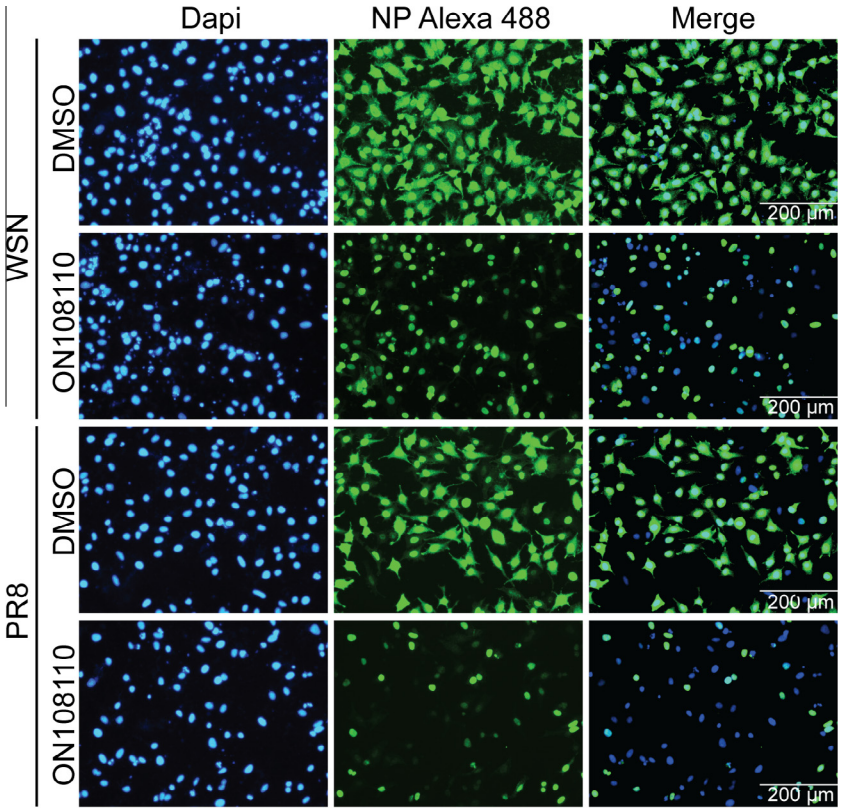

Fig. 7. ON108110 inhibits viral replication in HeLa cells. HeLa cells were infected with influenza virus A/WSN/33 or A/PR8/34 at an MOI of 3 in both cases. Following infection, cells were treated with ON108110 (3.25 μM) or DMSO. At 5 h post-infection, cells were fixed, permeabilized and stained with a mouse monoclonal anti-NP antibody (AbD Serotec, MCA-400). 4′-6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. Results shown are representative images.

WSN virus was also detected when cells were infected with influenza PR8 or NL/09 viruses (Fig. 6). This restriction in viral protein production is not limited to A549 cells as a similar result was obtained in HeLa cells (Fig. 7). The NP distribution observed in ON108110 treated cells (Figs. 5–7) could also reflect a block in the export of viral ribonucleoprotein complexes (vRNP).

To further explore this finding, viral protein levels were analyzed by western blot (Fig. 8A). At 5 and 7 hpi with influenza A/WSN virus, cells treated with ON108110 had reduced levels of influenza HA and NS1 proteins compared with the DMSO treated samples, indicating a reduction in viral protein synthesis. Next, we assayed the activity of the viral polymerase complex in the presence of ON108110. At 5 hpi, ON108110 treatment reduced RNA levels (either mRNA or genomic positive sense cRNA) of influenza A virus NP as assayed by qRT-PCR (Fig. 8B). This result was confirmed by assessing the function of the viral polymerase complex with a mini-genome assay (Fig. 8C and D). In this case, influenza virus NP, PB1, PB2 and PA (from influenza A/WSN or PR8 virus) were transfected into 293-T cells along with the WSN NP-Firefly luciferase reporter plasmid and an SV40-driven Renilla luciferase expressing plasmid. At 18–20 h after transfection, the ratio between the firefly and the Renilla luciferase signal was measured. The presence of ON108110 reduced expression of firefly luciferase by the viral polymerase complex in a dose dependent manner. The levels of Renilla luciferase were also affected. This effect may be due either to a specific inhibition of host RNA synthesis or to a nonspecific cytotoxic effect. However, the virus-specific transcript was reduced at higher levels as indicated by the firefly: Renilla luciferase ratios, suggesting that ON108110 preferentially inhibits vRNA replication.

3.6. ON108110 reduces the replication of vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) in a dose-dependent manner

Different viruses rely on the host machinery for their replication. Since ON108110 inhibits a variety of these host kinases, we hypothesized that this compound may inhibit other viruses. A549 cells were treated with twofold serial dilutions (0.78–12.5 μM) of ON108110 for 2–4 h prior to infection with VSV-GFP at an MOI of 0.0001. Supernatants were harvested 24 hpi and plaque assay were performed. Fig. 9A shows that ON108110 inhibits the replication of VSV in a dose-dependent manner at concentrations that are not cytotoxic. To determine whether ON108110 inhibits the replication of NDV, A549 cells were treated with twofold serial dilutions (0.78–25 μM) of ON108110 for 2–4 h prior to infection with NDV (B1 strain) at an MOI of 2. The amount of virus in the supernatant at 24 hpi was measured using a plaque assay. As shown in Fig. 9B, ON108110 inhibits the replication of NDV in a dose-dependent manner at concentrations that are not toxic to cells. ON108110, however, seems to inhibit VSV more effectively than NDV.

4. Discussion

Several RNAi screens performed by our group and other laboratories have demonstrated that several kinases are required for the

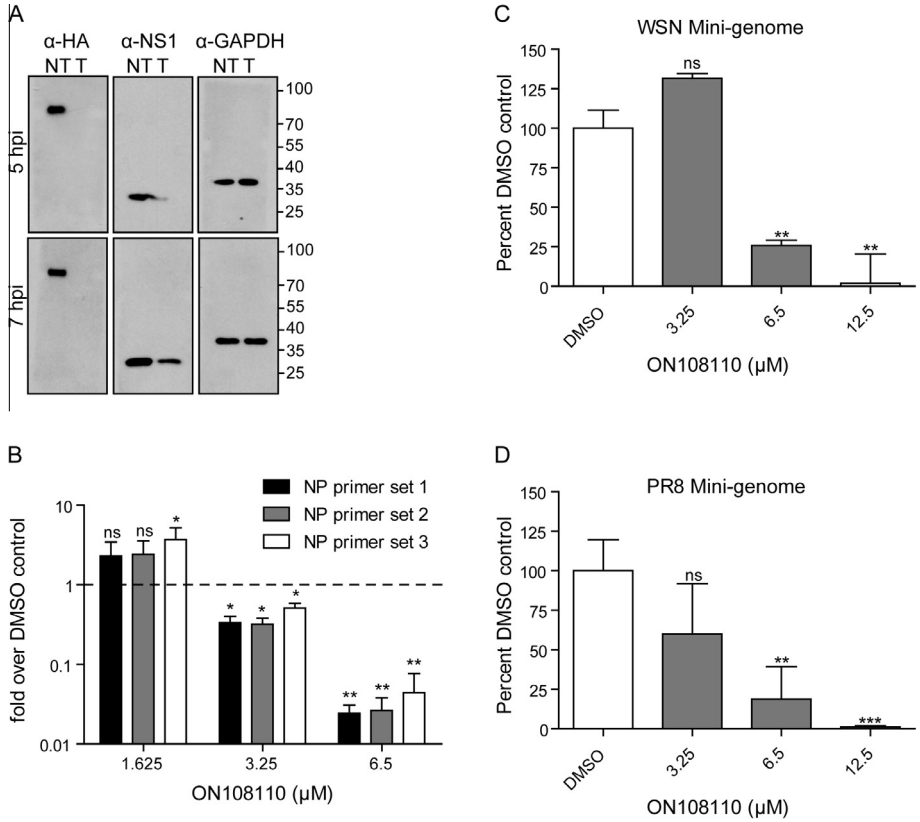

Fig. 8. ON108110 reduces viral RNA synthesis. (A) A549 cells were infected with influenza virus A/WSN/33 at an MOI of 3. Following the infection, cells were treated with ON108110 (3.25 μM) or DMSO. At 5 h post-infection, cells were lysed and cellular extracts probed by Western blot for influenza virus HA and NS1. GAPDH was used as a loading control. (B) Once again, A549 cells were infected with influenza virus A/WSN/33 at an MOI of 3. Following the infection, cells were treated with ON108110 (at the indicated concentration) or DMSO. At 5 h post-infection, RNA was extracted and Influenza virus RNA levels were measured by qRT-PCR using three sets of NP-specific primers. (C and D) Influenza A virus mini-genome activity in 293-T cells transfected with the influenza virus firefly luciferase mini-genome reporter, Renilla luciferase control reporter, PB1, PB2, PA, and NP protein expressing plasmids. Treatment with ON108110 or DMSO alone was performed during transfection. Luciferase activity was assayed 18–20 h post transfection. Firefly luciferase levels were normalized with the Renilla luciferase signal. Values are represented as% activity relative to the DMSO control. Error bars in panels B, C and D represent the standard deviation of three independent replicates, ns indicates not significant, $^{*}p$ value < 0.05, $^{**}p$ value < 0.001, $^{***}p$ value < 0.0001.

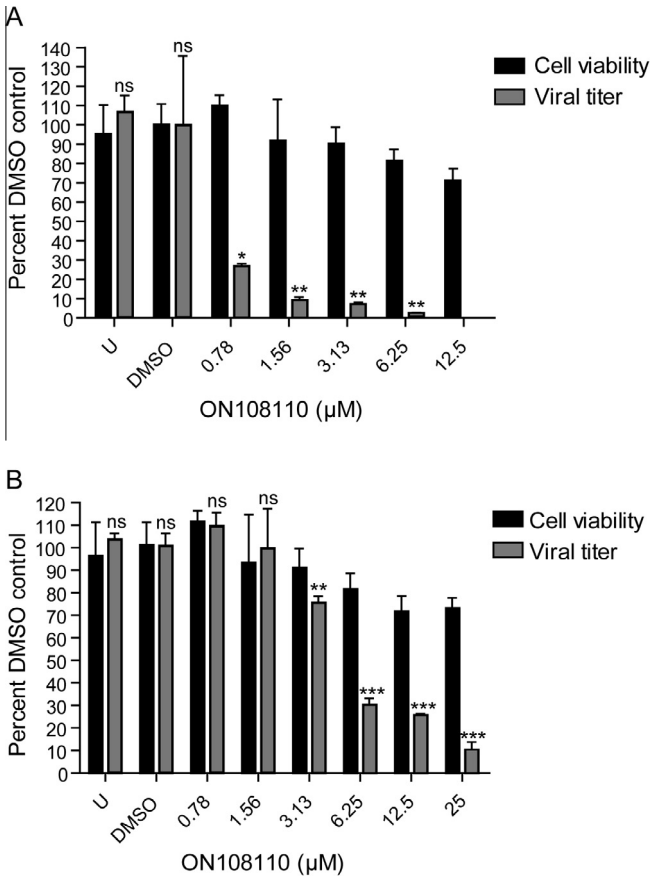

Fig. 9. ON108110 reduces the replication of vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) in a dose-dependent manner. A549 cells were treated with twofold serial dilutions of ON108110, or solvent DMSO, or left untreated (U). The amount of DMSO was the same for all the dilutions. Cell viability was measured 24 h post-treatment using the Promega CellTiter-Glo assay. Another set of ON108110-treated cells were infected with VSV-GFP at an MOI of 0.0001 (A) or NDV (B1 strain) at an MOI of 2 (B). Supernatants were harvested 24 h post-infection and plaque assay was performed. Cell viability and viral titer were expressed as percent of the DMSO control. Error bars represent the standard deviation of three independent replicates, ns indicates not significant, $^{*}p$ value < 0.05, $^{**}p$ value < 0.001, $^{***}p$ value < 0.0001.

replication of influenza virus (Konig et al., 2010; Karlas et al., 2010; Brass et al., 2009; Shapira et al., 2009; Hao et al., 2008). In this study, we demonstrate that the multi-kinase inhibitor ON108110 impairs the replication of influenza virus by reducing the polymerase complex activity and, therefore, viral RNA and protein synthesis. Not surprisingly we found that ON108110 inhibits at least 8 kinases that were previously identified as host factors required for influenza replication (Konig et al., 2010). These results are similar to those of other groups who have identified kinase inhibitors that reduce the replication of influenza virus. Kumar et al. (2011) showed that receptor tyrosine kinase inhibitors AG879 and tyrphostin A9 blocked replication of influenza virus in cells. Karlas et al. (2010) demonstrated that the CDC-like kinase 1 (CLK1) inhibitor TG003 reduced influenza virus growth. A recent study also showed that the c-Jun N-terminal kinase (JNK) inhibitors SP600125 and AS601245 reduced influenza virus replication by suppressing viral protein and RNA synthesis (Nacken et al., 2012). Previously, our group also demonstrated that the calcium/calmodulin-dependent kinase (CAMK2B) inhibitor KN93 inhibited influenza virus replication (Konig et al., 2010). ON108110 targets host factors and therefore it is not surprising that host RNA transcription could also be affected. However, the viral polymerase complex is far more sensitive to the effects of ON108110 than the host transcriptional machinery thereby limiting cytotoxicity.

As previously mentioned, ON101180 seems to act primarily on viral replication. However, our results can not exclude an effect of ON101180 on the nuclear export of vRNPs. Kinase inhibitors have previously been described to block the propagation of influenza A viruses by interfering with nuclear export of vRNPs (Pleschka et al., 2001; Watanabe et al., 2001; Mazur et al., 2007). Pleschka et al. reported that inhibition of Raf signaling resulted in nuclear retention of vRNP and impaired function of the nuclear-export protein (NEP/NS2). The CRM1 inhibitor leptomycin B (Watanabe et al., 2001) and acetylsalicylic acid (Mazur et al., 2007) can also inhibit influenza A vRNP nuclear export. This phenomenon is not limited to influenza A virus, as a MEK inhibitor can restrict influenza B virus propagation by interfering with the viral nuclear export process (Ludwig et al., 2004). Interestingly, no resistant virus variants emerged in the presence of the MEK inhibitor demonstrating that influenza viruses cannot easily adapt to the missing cellular function (Ludwig et al., 2004).

In addition, to influenza A virus, we found that ON108110 inhibits two other negative sense RNA viruses (VSV and NDV), suggesting that ON108110 inhibits one or more kinases required for the replication of these three viruses. A recent RNAi screen identified 72 host factors that are required for VSV infection (Panda et al., 2011). None of the kinases that ON108110 inhibited in our panel were identified as targets in this study, suggesting that other host factors could be involved in the life cycle of VSV. The role of host factors in the life cycle of NDV is poorly understood, and knockdown of the kinases that are inhibited by ON108110 via siRNA-mediated targeting may lead to the identification of some important host factors.

In conclusion, we have demonstrated that ON108110 inhibits influenza A virus, VSV and NDV. Analogs of this compound need to be designed and synthesized to improve its pharmacodynamic properties for *in vivo* experiments. This is particularly important for the development of novel anti-influenza agents for potential use against drug-resistant strains of the virus. In addition, ON108110 might represent an appealing starting point for the development of novel, broad-spectrum antiviral agents targeting host factors essential for viral replication.

Acknowledgments

We thank Silke Stertz for helpful discussions and Matthew Evans for generously providing reagents. We also thank Chen Wang for technical support. This work was partially supported by Center for Research on Influenza Pathogenesis (CRIP) grant HHSN266200700010C.

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