Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Identification of a novel multiple kinase inhibitor with potent antiviral activity against influenza virus by reducing viral polymerase activity

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ARTICLE INFO

Article history:
Received 30 April 2014
Available online xxxx

Keywords:
Anti-influenza viral drug
Compound library screening
vRNP activity
Kinase inhibitor
Target database search
KINOMEscan

ABSTRACT

Neuraminidase inhibitors are the only currently available influenza treatment, although resistant viruses to these drugs have already been reported. Thus, new antiviral drugs with novel mechanisms of action are urgently required. In this study, we identified a novel antiviral compound, WV970, through cell-based screening of a 50,000 compound library and subsequent lead optimization. This compound exhibited potent antiviral activity with nanomolar IC_{50} values against both influenza A and B viruses but not non-influenza RNA viruses. Time-of-addition and indirect immunofluorescence assays indicated that WV970 acted at an early stage of the influenza life cycle, but likely after nuclear entry of viral ribonucleoprotein (vRNP). Further analyses of viral RNA expression and viral polymerase activity indicated that WV970 inhibited vRNP-mediated viral genome replication and transcription. Finally, structure-based virtual screening and comprehensive human kinome screening were used to demonstrate that WV970 acts as a multiple kinase inhibitor, many of which are associated with influenza virus replication. Collectively, these results strongly suggest that WV970 is a promising anti-influenza drug candidate and that several kinases associated with viral replication are promising drug targets.

1. Introduction

Seasonal influenza infection causes large scale economic burdens, and pandemics such as those caused by the highly pathogenic avian influenza A subtypes, including H5N1 and H7N7 could result in millions of deaths [1,2]. Thus, anti-influenza drugs have been developed for prevention and treatment of influenza infections. Two classes of anti-influenza drugs are currently approved for clinical use, M2 channel blockers and neuraminidase (NA) inhibitors [3]. However, the clinical uses of M2 channel blockers are limited since all currently circulating influenza A virus strains are resistant to these drugs [4]. Moreover, the emergence of NA inhibitor-resistant viruses has also been reported [4,5]. If NA inhibitor-resistant viruses become more widespread then there will be few effective means of treating influenza infections. Therefore, new antivirals based on novel mechanisms of action are urgently needed.

One potential approach is to target host factors involved in influenza replication, which may provide a novel antiviral strategy to counteract viral drug resistance. Recent reports revealed that a large number of host factors are involved in influenza replication and that the majority of these are kinases [6]. Indeed, several kinase inhibitors including Ca^{2+}/calmodulin-dependent protein kinase (CAMK), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), receptor tyrosine kinases (RTKs) and MAPK/ERK kinase (MEK) exhibit potent anti-influenza activity [7].

Structure-based virtual screening is one of the most convenient and promising approaches for identifying potential binding partners for test compounds, and can provide targets that have a high likelihood of binding to the query compound [8]. In addition, a comprehensive human kinome screening system (KINOMEscan) was used to provide quantitative data on binding selectivity between test compounds and more than 450 human kinases [9]. In this study, we identified a novel antiviral compound, WV970, using a cell-based influenza A infection assay and optimized the compound by assessing structure–activity relationships (SAR). The mechanism of action was further characterized, wherein WV970 behaved as a multiple kinase inhibitor.

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http://dx.doi.org/10.1016/j.bbrc.2014.05.058
0006-291X/

2. Materials and methods

2.1. Compounds

The chemical library for cell-based screening was purchased from ChemBridge, and hit compound RK188 was purchased from Sundia MediTech. WV635 and WV970 were synthesized by Wakunaga Pharmaceuticals and a stock solution of 10 mM was prepared in dimethyl sulfoxide (DMSO). Oseltamivir carboxylate and nucleozin were purchased from Kemprotec and Shanghai Haoyuan Chemexpress, respectively.

2.2. Cells and viruses

Madin-Darby canine kidney (MDCK) cells, Vero cells and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing penicillin, streptomycin, and glutamine (PSG, GIBCO Industries Inc.) and 10% fetal bovine serum (Sigma–Aldrich). Influenza A viruses, including A/WSN/1933 (H1N1), A/Udorn/307/1972 (H3N2), A/ck/Yamaguchi/7/2004 (H5N1), and A/Anhui/1/2013 (H7N9), and influenza B viruses, including B/Yamagata/16/88 and B/Nagasaki/1/87, were propagated in MDCK cells. Japanese encephalitis virus (JEV) (Mie/41/2002 strain) and dengue virus type I (NIID 02-20) were propagated in Vero cells.

2.3. Random screening of compounds with MDCK cell-based influenza A infection assays

MDCK cells (3×10^4 cells/well) were seeded in 96-well plates and incubated at 37 °C in 5% CO_2 overnight with DMEM containing 10 μM library compounds, and then inoculated with influenza A virus (A/WSN/1933) at a multiplicity of infection (MOI) of 0.005 with 1 μg/ml of tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Trypsin-TPCK, Worthington Biochemical Corporation). After a 48 h incubation, the cells were washed twice with phosphate-buffered saline (PBS), and 10 μl of WST-1 reagent (Takara Bio) was added into each well. The plate was incubated at 37 °C for 1 h, and absorbance was measured at 450 nm. Inhibition (%) of virus-induced cytopathic effects (CPEs) was evaluated by comparing test compound-treated cells to DMSO-treated cells.

2.4. Plaque assays of antiviral activity

Plaque assays for influenza viruses were performed as previously described [10]. For plaque assays with JEV and dengue virus, Vero cells (6×10^5 cells/well) were seeded in 6-well plates and incubated at 37 °C in 5% CO_2 for 24 h. Cells were washed once with PBS and inoculated with approximately 200 plaque forming units (PFU) of JEV or dengue virus. After incubating for 1 h at 37 °C in 5% CO_2, 2 ml of DMEM containing 1% methylcellulose was overlaid onto each well. Cells were incubated at 37 °C in 5% CO_2 for 5 days after JEV inoculation or 7 days after dengue virus inoculation. After the removal of DMEM containing methylcellulose, cells were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet. The 50% inhibitory concentration (IC_{50}) was then evaluated by counting the number of plaques.

2.5. Cell toxicity

Cell toxicity was measured as previously described [10] using a WST-1 assay. The 50% cytotoxic concentration (CC_{50}) was evaluated by comparing test compound-treated cells to DMSO-treated cells.

2.6. Indirect immunofluorescence

Indirect immunofluorescence was performed as described previously [11] with a primary anti-NP monoclonal antibody (MAb) (Santa Cruz Biotechnology) and subsequent secondary Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Invitrogen). Samples were then incubated with PBS containing Hoechst 33342 (Immuno-Chemistry Technologies LLC) for nuclear staining. Prepared samples were observed under a confocal laser-scanning microscope (FV 1000, Olympus).

2.7. RNA fluorescence in situ hybridization (FISH) analysis

RNA FISH was performed according to published protocols [12]. Probes targeting the PB2 segment of the influenza virus were designed by Stellaris RNA FISH Probe Designer and purchased from Biosearch Technologies. Prepared samples were observed under a confocal laser-scanning microscope (FV 1000, Olympus).

2.8. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was extracted from MDCK cells (1.2×10^6 cells/well) infected with A/WSN/1933 at an MOI of 10 using TRIzol® LS Reagent (Invitrogen).

RT-PCR was performed using SuperScript™ III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions and specific primers for the following three targets using a previously described protocol [13]: viral genome RNA (vRNA) NP (F), 5′-GGCCGTCATGGTGGCGAATGAATGGACGGAGAACAAGGATTGC-3′; viral mRNA NP (R), 5′-CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTCTTTAATTGTC-3′; and mRNA MDCK actin (R), 5′-GGAACCGCTCGTTGCCAATG-3′.

For qPCR, each cDNA was used with 2 × SYBR GreenER qPCR SuperMix (Invitrogen) and ABI PRISM, according to the manufacturer's instructions. The specific primers used are as follows: vRNA NP (F), 5′-GGCCGTCATGGTGGCGAAT-3′; vRNA NP (R), 5′-CTCAATATGAGTGCAGACCGTGCT-3′; viral mRNA NP (F), 5′-CGATCGTGCCCTCCTTTG-3′; viral mRNA NP (R), 5′-CCAGATCGTTCGAGTCGT-3′; mRNA MDCK actin (F), 5′-CGTGCGTGACATCAAGGAAGAAG-3′; and mRNA MDCK actin (R), 5′-GGAACCGCTCGTTGCCAATG-3′. Data were analyzed using the ΔΔCT method. MDCK actin was used as a control.

2.9. Mini-genome assays

Mini-genome assays were performed using the expression plasmids PB1/pCAGGS, PB2/pCAGGS, PA/pCAGGS, NP/pCAGGS and vNP-luc/pHH21, as described previously [10].

2.10. Western blot analysis

Western blot analysis was performed with an anti-WSN polyclonal antibody (Ab) and HRP-conjugated goat anti-rabbit IgG (Amersham Bioscience) or anti-β-actin MAb (Sigma–Aldrich) and HRP-conjugated goat anti-mouse IgG (Amersham Bioscience), as described previously [11].

2.11. LASSO (ligand activity by surface similarity order) screening with the ChemSpider database

Target molecule searches using the structure-based virtual screening database, ChemSpider (Royal Society of Chemistry), were conducted to identify potential binding partners for test compounds. LASSO scores ranges from 0 to 1.0, where a score of

Please cite this article in press as: Y. Sasaki et al., Identification of a novel multiple kinase inhibitor with potent antiviral activity against influenza virus by reducing viral polymerase activity, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.05.058