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Inhibitors of cellular kinases with broad-spectrum antiviral activity for hemorrhagic fever viruses ☆

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ABSTRACT

Host cell kinases are important for the replication of a number of hemorrhagic fever viruses. We tested a panel of kinase inhibitors for their ability to block the replication of multiple hemorrhagic fever viruses. OSU-03012 inhibited the replication of Lassa, Ebola, Marburg and Nipah viruses, whereas BIBX 1382 dihydrochloride inhibited Lassa, Ebola and Marburg viruses. BIBX 1382 blocked both Lassa and Ebola virus glycoprotein-dependent cell entry. These compounds may be used as tools to understand conserved virus–host interactions, and implicate host cell kinases that may be targets for broad spectrum therapeutic intervention.

1. Introduction

Viral hemorrhagic fevers (VHFs) cause significant morbidity and mortality globally. The infectious etiologies of VHFs include members of several RNA virus families, including the *Arenaviridae*, *Bunyaviridae*, *Filoviridae*, and *Flaviviridae*. The leading cause of VHF worldwide is Lassa virus (LASV), an arenavirus endemic to West Africa that causes 300,000–500,000 infections annually (Ogbu et al., 2007). Ebola virus (EBOV), Marburg virus (MARV), Junin virus (JUNV), Alkhurma hemorrhagic fever virus (AHFV, called Alkhumra virus in some reports), and Crimean Congo hemorrhagic fever virus (CCHFV), as well as the encephalitic Nipah virus (NiV), cause sporadic outbreaks, often with high case-fatality rates (Aljofan, 2013; Kortekaas et al., 2010; MacNeil and Rollin, 2012; Madani, 2005). These highly pathogenic agents are all classified as biosafety level 4 (BSL-4) pathogens; there are no approved therapeutics or vaccines, and medical care for patients is generally only supportive. Despite the challenges inherent in studying BSL-4 agents, research into therapies for these viruses is critical because of the potential for large outbreaks with high case-fatality rates, as demonstrated by the 2013–2015 EBOV outbreak in West Africa.

Host cell kinases have been implicated in the replication of several BSL-4 viruses. One signaling pathway, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, was reported to be essential for the propagation of LASV and EBOV in cell culture. Inhibition of the PI3K/Akt pathway by the small molecule BEZ-235 impeded the budding of LASV virus-like particles (VLPs) (Urata et al., 2012). Another inhibitor, LY294002, blocked EBOV entry (Saeed et al., 2008) and an early event in JUNV infection (Linero and Scolaro, 2009). The replication of Andes virus, a bunyavirus, was blocked by temsirolimus, an inhibitor of mTOR, another kinase in the PI3K/Akt pathway (McNulty et al., 2013). We therefore hypothesized that a cellular kinase could be essential for the replication of multiple highly pathogenic viruses. Identifying such a kinase

Abbreviations: AHFV, Alkhurma hemorrhagic fever virus; BSL-4, biosafety level 4; CC_{50}, 50% cytotoxic concentration; CCHFV, Crimean Congo hemorrhagic fever virus; EBOV, Ebola virus; EC_{50}, 50% effective concentration; GFP, green fluorescent protein; LASV, Lassa virus; MARV, Marburg virus; NiV, Nipah virus; PBS, phosphate buffered saline; SI, selectivity index; VHF, viral hemorrhagic fever; VLP, virus-like particle; VSV, vesicular stomatitis virus.

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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might shed light on conserved virus–host interactions. In addition, therapies targeting such kinases could have broad-spectrum antiviral activity, a desirable property given the difficulty of developing therapies for individual hemorrhagic fever viruses. Here, we report the identification of 2 inhibitors of cellular kinases which impeded the replication of multiple highly pathogenic viruses.

2. Materials and methods

2.1. Biosafety

All work with infectious virus was conducted in a BSL-4 laboratory at the Centers for Disease Control and Prevention (CDC, Atlanta, GA). All laboratorians adhered to international practices appropriate for this biosafety level. Experiments involving cDNA encoding viral sequences were approved by the CDC Institutional Biosafety Committee.

2.2. Cell lines, viruses, and compounds

A549, Vero-E6, HeLa, and HT-1080 cells were from the CDC Biologics Branch and HEK-293 cells were from ATCC. These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) fetal calf serum (FCS; Hyclone, Thermo Scientific, Waltham, MA, USA) and penicillin–streptomycin (Life Technologies). Huh7 cells were from Apath, LLC (Brooklyn, NY, USA) and were propagated in DMEM, 10% (v/v) FCS, and 1 × non-essential amino acids (Life Technologies). Viruses were from the CDC Viral Special Pathogens Branch reference collection: LASV (strain Josiah); EBOV (strain Mayinga); AHFV (strain 200300001); CCHFV (strain IbAr10200). The Kinase Inhibitor Toolbox library and BIBX 1382 dihydrochloride were from Tocris Bioscience (Bristol, UK). The PI3K Signaling Inhibitor Library and OSU-03012 were from Selleck Chemicals (Houston, TX, USA). Compounds were diluted in dimethylsulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO, USA) as indicated.

2.3. Assays for antiviral activity and cell viability

To test for the inhibition of LASV replication, A549 cells were seeded at a density of 1×10^4 cells/well of a 96-well plate the day prior to infection. Compounds were added to the cells, and 1 h later, the cells were infected with LASV at an MOI of 0.2. After 48 h, the monolayers were fixed with 10% (v/v) formalin (Sigma–Aldrich) and γ-irradiated with 2×10^6 rads. The cells were permeabilized with 0.1% (v/v) Triton X-100 in phosphate buffered saline (PBS) for 10 min at room temperature, and LASV proteins were detected with monoclonal antibodies directed against the LASV glycoprotein and nucleoprotein (1:10,000 dilution in PBS supplemented with 2% w/v bovine serum albumin) and goat anti-mouse Alexa 488 (1:1000; Life Technologies). Cells were stained with CellMask Red and NucBlue (Life Technologies) and immunofluorescence microscopy was performed using the Operetta Imaging System (PerkinElmer, Waltham, MA).

The assay for the inhibition of AHFV-induced cytopathic effect in A549 cells was as described previously (Flint et al., 2014). The recombinant reporter viruses NiV-luc and EBOV and MARV expressing green fluorescent protein (GFP) reporter (EBOV-GFP and MARV-GFP) have also been described (Albariño et al., 2013; Lo et al., 2014; Towner et al., 2005). The assay for the inhibition of CCHFV-induced cytopathic effects was based on one described previously (Paragas et al., 2004). Briefly, compounds were added to SW13 cells in an 80% confluent monolayer in a 96-well plate, followed by infection with CCHFV at an MOI of 0.1, 1 h later. Cell viability, as measured by CellTiter-Glo (Promega, Madison, WI, USA), was measured 72 h post infection.

Cell viability was determined concurrently with the virus inhibition assays, but on compound-treated and mock-infected cells, using CellTiter-Glo (Promega) or PrestoBlue (Life Technologies) according to the manufacturer's instructions. Viability was also assessed by nuclei number, as determined by counting the NucBlue-stained organelles with Harmony image analysis software (PerkinElmer). For each assay, values were normalized to vehicle-only DMSO controls.

For compound titrations, GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used to fit a 4-parameter equation to semilog plots of the concentration–response data and to interpolate the concentration of compound that inhibited 50% of the virus replication (EC_{50}). The 50% cytotoxic concentration (CC_{50}) was similarly derived using viability data from mock-infected cells. The selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50}.

2.4. Viral titer reduction assay

Titer reduction assays for LASV and EBOV were performed in A549 and Huh7 cells, respectively. Cells were treated with compounds for 1 h prior to infection. Two days later, culture supernatants were harvested and virus titrations were performed in Vero-E6 cells. Three days post infection, the cells were fixed, permeabilized, and stained to visualize viral proteins. End-point viral titers were determined, and the 50% tissue culture infectious dose ($TCID_{50}$) was calculated using the method of Reed and Muench (Reed and Muench, 1938).

2.5. Quantitative reverse transcription polymerase chain reaction assay

Cells were seeded and treated with compounds for 1 h before infection with LASV at an MOI of 0.1. The medium was removed 24 h post-infection, lysis buffer (MagMax Total RNA isolation kit; Life Technologies) was added, and RNA was extracted using a MagMax-96 deep-well magnetic particle processor (Life Technologies). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with the Express One-Step Superscript qRT-PCR kit (Life Technologies) and analyzed on an Applied Biosciences 7500 real-time PCR machine (Life Technologies). LASV nucleoprotein RNA was quantitated using forward (5′-AATCAGTTCGGGACCATGC-3′) and reverse (5′-GTGTTGG GATACTTTGCTGTG-3′) primers and a probe oligonucleotide (5′-/5 6-FAM/AGTCAACCT/ZEN/GCCCCTGTTTTGTCA/Iowa Black FQ/-3′) from Integrated DNA Technologies (Coralville, IA). Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA, or 18S ribosomal RNA in Vero-E6 cells, were determined using control primer-probe sets (Life Technologies). Viral RNA levels were normalized to GAPDH or 18S RNA and expressed relative to infected, vehicle-treated controls.

2.6. VLP assembly assay

HEK-293 cells were transfected with plasmids encoding FLAG-tagged LASV Z protein, or the non-budding G2A mutant (Perez et al., 2004), using Lipofectamine 2000 (Life Technologies). Following overnight incubation, the transfection media was removed and compounds were added to a final DMSO concentration of 0.5%. After 48 h, cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Complete Protease Inhibitor, Roche, Indianapolis, IN, USA). The culture medium was clarified by centrifugation at 4000×g for 20 min, and VLPs were concentrated from the supernatants by centrifugation at 300,000×g through a 20%