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Pyridinyl imidazole inhibitors of p38 MAP kinase impair viral entry and reduce cytokine induction by *Zaire ebolavirus* in human dendritic cells

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ABSTRACT

Antigen presenting cells (APCs), including macrophages and dendritic cells, are early and sustained targets of Ebola virus (EBOV) infection *in vivo*. Because EBOV activates mitogen-activated protein kinase (MAPK) signaling upon infection of APCs, we evaluated the effect of pyridinyl imidazole inhibitors of p38 MAPK on EBOV infection of human APCs and EBOV mediated cytokine production from human DCs. The p38 MAPK inhibitors reduced viral replication in PMA-differentiated macrophage-like human THP-1 cells with an IC_{50} of 4.73 μM (SB202190), 8.26 μM (p38kinhIII) and 8.21 μM (SB203580) and primary human monocyte-derived dendritic cells (MDDCs) with an IC_{50} of 2.67 μM (SB202190). Furthermore, cytokine production from EBOV-treated MDDCs was inhibited in a dose-dependent manner. A control pyridinyl imidazole compound failed to inhibit either EBOV infection or cytokine induction. Using an established EBOV virus-like particle (VLP) entry assay, we demonstrate that inhibitor pretreatment blocked VLP entry suggesting that the inhibitors blocked infection and replication at least in part by blocking EBOV entry. Taken together, our results indicate that pyridinyl imidazole p38 MAPK inhibitors may serve as leads for the development of therapeutics to treat EBOV infection.

1. Introduction

Zaire ebolavirus (EBOV) is an enveloped, negative-sense RNA virus belonging to the family *Filoviridae* that causes an often fatal hemorrhagic disease in humans (Khan et al., 1998). Dendritic cells (DC) and macrophages, both antigen-presenting cells (APCs), are early and sustained targets of EBOV infection (Geisbert et al., 2003). It has been hypothesized that infection of APCs and their subsequent deregulation, which is manifested in part by uncontrolled secretion of inflammatory cytokines, leads to an ineffective antiviral host response (Bray and Geisbert, 2005; Martinez et al., 2012).

APCs exposed to EBOV or virus-like particles (VLPs) expressing the EBOV glycoprotein (GP) activate MAPK signaling (Martinez et al., 2007; Wahl-Jensen et al., 2011). Microarray analysis of human macrophages exposed to EBOV demonstrated the upregulation of genes known to be activated by p38 and ERK 1/2 MAPK signaling pathways (Wahl-Jensen et al., 2011). Furthermore, similar genes were upregulated from macrophages treated with non-replicating VLPs that express the VP40 protein and GP, consistent with a previous study demonstrating EBOV VLPs induce NF-κB and MAPK signaling in human DCs (Martinez et al., 2007). Further, overexpression of GP in 293 cells modulates MAPK activity (Zampieri et al., 2007). Importantly, an siRNA screen identified canonical phosphatidylinositol-3-kinase and MAPK signaling networks, among others, as important for EBOV infection (Kolokoltsov et al., 2009). Altogether these studies suggest that MAPK signaling plays an important role in EBOV infection of APCs.

Pyridinyl imidazole inhibitors of p38 MAPK exhibit anti-inflammatory properties and have been shown, for example, to block inflammatory cytokine production in the monocytic/macrophage cell line THP-1 (Gallagher et al., 1997; Lantos et al., 1984; Lee et al., 1988, 1999, 1994). Since exposure of APCs to EBOV has been shown to activate MAPKs, we sought to evaluate how inhibition of p38 MAPK signaling would influence EBOV infection. Furthermore, because p38 MAPK signaling mediates inflammatory cytokine production, and because EBOV infection is characterized as having a

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deregulated immune response, including deregulated cytokine production, we also tested if p38 MAPK inhibition would inhibit EBOV-induced cytokine production (Bray and Geisbert, 2005; Hoenen et al., 2006; Kumar et al., 2003). We show that p38 MAPK chemical inhibitors SB202190, SB203580 and p38inhK III impair EBOV replication and cytokine induction. Furthermore, target cell pretreatment with SB202190 blocked EBOV GP-mediated entry by inhibiting viral particle uptake suggesting that p38 MAPK inhibitors block EBOV infection, at least in part, by blocking the entry step of the virus.

2. Methods and materials

2.1. Preparation of p38 MAPK inhibitors

p38 MAPK pyridinyl imidazole inhibitors SB202190, p38inhK III, SB203580; control compounds SB202474 (all from EMD Millipore, Billerica, MA) and 3-Deazaneplanocin A (DZNep) (kindly provided by Dr. Victor E. Marquez, National Cancer Institute) were prepared as 150 mM stock concentrations in DMSO and diluted to final concentrations of 15–1 μM in 0.66% DMSO (Sigma Aldrich, St. Louis, MO).

2.2. Culture and differentiation of human THP-1 cells

THP-1 cells (ATCC, Catalogue # TIB-202) were grown in RPMI-1640 media (ATCC, Manassas, VA) supplemented with 10% FBS (Life Technologies, Carlsbad, CA) and 0.05 mM 2-mercaptoethanol (Life Technologies, Carlsbad, CA) at 37 °C, 5% CO_2. Prior to infection, cells were plated in 96-well, black, clear bottom cell culture plates (Corning Catalog #3904) at a density of 5×10^4 cells per well in 100 μL volume and differentiated overnight with 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, St. Louis, MO).

2.3. EBOV Mayinga strain expressing enhanced green-fluorescent protein

All work with Ebola virus was performed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) at Fort Detrick, Frederick, MD, USA within biosafety level 4 containment. A recombinant EBOV engineered to express enhanced green-fluorescent protein (Ebola virus *Homo sapiens*-rec/COD/1976/Mayinga-eGFP) was used for all experiments utilizing infectious virus. The generation and rescue of the full-length eGFP clone (derived from an Ebola virus, family *Filoviridae*, species *Zaire ebolavirus*, GenBank accession No. NC002549) was described previously (Towner et al., 2005).

2.4. Antiviral activity assays in THP-1 cells

PMA-differentiated THP-1 cells were pretreated for 1 h with p38 MAPK inhibitors prior to infection with EBOV-eGFP at a multiplicity of infection (MOI) of 0.1. At 2–3 days post-infection (PI), total GFP fluorescence was quantified per well using a SpectraMAX M5 Spectrofluorometer (Molecular Devices) at 515 nm as a measure of EBOV infection and replication.

2.5. Isolation and differentiation of monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Histopaque; Sigma Aldrich, St. Louis, MO) from anonymous, blood bank blood (New York Blood Center). CD14+ cells were isolated immunomagnetically (Miltenyi Biotec, Auburn, CA) and cultured ($0.7–1 \times 10^6$ cells/mL) in DC media (RPMI (Life Technologies, Carlsbad, CA) containing 100 units/mL of penicillin, 100 g/mL streptomycin, 55 μM β-mercaptoethanol) and 4% human serum AB (GemCell, Gemini Bio-Products, West Sacramento, CA) supplemented with 500 U/mL human granulocyte–macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ), 500 U/mL human interleukin-4 (IL-4; Peprotech), 1 ng/mL ciprofloxacin (Sigma Aldrich, St. Louis, MO) for 5–7 days at 37 °C to produce immature DCs. By day 5, immature DCs expressed surface CD11c and HLA-DR, but low to no CD14 (data not shown).

2.6. MDDCs treatment and infection

Prior to infection, MDDCs were pretreated with MAPK inhibitors at final concentrations ranging from 15 μM to 1 μM in 250 μL total volume per well for approximately 1 h. Human DCs were then infected using EBOV-eGFP at an MOI of 1 or 5 for 1 h at 37 °C and 5% CO_2. After infection, cells were washed 3 times with PBS and replenished with media containing inhibitors. Cells were then incubated for 2–3 days, harvested by gently scraping the wells and 50,000 events (cells/sample) were analyzed for GFP fluorescence by flow cytometry as a marker of EBOV infection.

2.7. Measurement of infectious virus by plaque assay

Supernatants harvested from THP-1 cells were assayed for infectious virus titer by preparing 10-fold dilutions in minimal essential medium containing 5% certified, United States origin, heat-inactivated fetal bovine serum (HI-FBS) (Life Technologies, Carlsbad, CA) and 1× Antibiotic–Antimycotic (Life Technologies, Carlsbad, CA). 200 μL of inoculum was added in triplicate to 6-well plates of Vero E6 cells at 95–100% confluence and incubated at 37 °C in 5% CO_2 for 1 h with gentle rocking every 15 min. Following incubation, 2 mL of primary overlay was added containing equal parts of 2× Modified Eagle medium (MEM, 2× Temin's) (Gibco) supplemented with 10% Heat inactivated FBS, 4 mM (2×) Glutamax (Life Technologies, Carlsbad, CA) and 2× Antibiotic–Antimycotic preheated to 37 °C and 1% SeaKem ME Agarose (Lonza) that had been preheated to liquid and allowed to cool to 50 °C. The overlay was allowed to solidify at room temperature and cells were placed back at 37 °C and 5% CO_2 for 7 days. Following incubation, 2 mL of secondary overlay containing a final concentration of 0.5% agarose and 4% Neutral Red solution (Life Technologies, Carlsbad, CA) was added to stain viable cells and visualize virus plaques. Cells were placed back in the incubator and plaques were counted the following day.

2.8. Cytokine quantification

MDDC supernatants were assayed for the presence of cytokines using a magnetic BioPlex Human Cytokine Group I 13-Plex Cytokine Assay (Bio-Rad) according to the manufacturer's instructions. A standard curve was prepared by rehydrating pre-mixed, lyophilized cytokine standard provided with the assay kit followed by serial dilution. Assay plates were washed using a Bio-Plex Pro Wash II Station with a magnetic plate carrier attached. Data were acquired using a Bio-Rad Bio-Plex 3D system and analyzed using Bio-Plex Manager 6.0 software and a 5-parameter logarithmic fit.

2.9. Evaluating cell viability

Cell viability was determined by assaying cell supernatants for the presence of adenylate kinase using the ToxiLight cell viability kit (Lonza, Walkersville, MD). Positive control wells containing no viable cells were prepared using the ToxiLight 100% cell lysis reagent. Luminescence was read using a SpectraMAX M5 at a 1 s integration time. Percent viability was quantified by normalizing