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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

Joshua C. Johnson[a,†], **Osvaldo Martinez**[b,e,†], **Anna N. Honko**[a], **Lisa E. Hensley**[a,c], **Gene G. Olinger**[a,c], and **Christopher F. Basler**[e,*]

[a]Virology Division, United States Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD 21702

[e]Dept. of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029

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 IC50 of 4.73μM (SB202190), 8.26μM (p38kinhIII) and 8.21μM (SB203580) and primary human monocyte-derived dendritic cells (MDDCs) with an IC50 of 2.67μM (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.

Keywords

Ebola virus; p38 MAPK; inhibitor; dendritic cell; THP-1; entry; antigen-presenting cell

[*]Corresponding Author: Christopher F. Basler, Dept. Microbiology, Box 1124 Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place New York, NY 10029, Tel (212) 241-4847, Fax (212) 534-1684, chris.basler@mssm.edu.
[b]Current Address: Dept. of Biology, Winona State University, Winona, MN 55987
[c]Current Address: National Institute of Allergy and Infectious Diseases, Integrated Research Facility, Frederick MD 21702
[†]Equal contribution

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1. Introduction

Zaire Ebola virus (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; Lee et al., 1999; Lee et al., 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 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 150mM stock concentrations in DMSO and diluted to final concentrations of 15uM to 1uM 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 0.05mM 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 100uL 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 H.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 hour 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 ($0.7–1\times10^6$ cells/ml) in DC media (RPMI (Life Technologies, Carlsbad, CA) containing 100units/ml of penicillin, 100g/ml streptomycin, 55μM 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 ciproflaxocin (Sigma Aldrich, St. Louis, MO) for 5 to 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 250μL total volume per well for approximately 1 hour.

Human DCs were then infected using EBOV-eGFP at an MOI of 1 or 5 for 1 hour 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 50000 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 1X Antibiotic-Antimycotic (Life Technologies, Carlsbad, CA). 200 uL 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 hour with gentle rocking every 15 minutes. Following incubation, 2mL of primary overlay was added containing equal parts of 2X Modified Eagle medium (MEM, 2X Temin's) (Gibco) supplemented with 10% Heat inactivated FBS, 4mM (2X) Glutamax (Life Technologies, Carlsbad, CA) and 2X 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 second integration time. Percent viability was quantified by normalizing data to untreated cells and comparing cells cultured in the presence of inhibitors for 72 hours to cells prepared using the ToxiLight 100% cell lysis kit.

3.0 VLP production and isolation

293T cells were co-transfected by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) (ratio of 1:1 DNA:plasmid) and plasmids encoding EBOV VP40 fused to β-lactamase

(pcDNA BLA-VP40) or EBOV VP40 fused to green fluorescent protein (pCAGGS GFP-VP40) and either EBOV GP or vesicular stomatitis virus glycoprotein (VSV G) at a ratio of 3:2 (VP40 to envelope glycoprotein plasmid). VLPs were harvested 3 days post-transfection and the 293T cell supernatant was purified by layering over 20% sucrose in NTE buffer (100 mM NaCl, 20 mM Tris-hydrochloride pH 7.5, and 1 mM ethylenediaminetetraacetic acid [EDTA]) and pelleting at 25,000 rpm in an SW-28 rotor (approximately 80,000 g) for 2 hours at 4°C. VLPs were then gently washed without resuspension with cold NTE or PBS and re-centrifuged. VLP were finally resuspended in a total of 150 μL NTE and stored on ice at 4°C prior to use.

3.1 Entry assays

Entry assays were performed as outlined in a previous study (Martinez et al., 2010). Briefly, different VLP preparations were normalized to equivalent levels of total β-lactamase activity. VLPs were added to target cells (10^5 cells/well/96 well plate) and centrifuged ("spinoculated") at 1850 rpm in a tabletop centrifuge for 45 minutes at 4°C before incubation at 37°C in 2% RPMI medium for indicated times. Target cells were then loaded for 1 hour at room temperature with a fluorescent CCF2-AM substrate (Life Technologies, Carlsbad, CA) that accumulates in the cytosol. Cells were harvested and analyzed using the violet laser of an LSR II flow cytometer. Live cells were sorted (5000 events) using side scatter and forward scatter properties, to identify live cells. Cells were then assayed for green (uncleaved CCF2-AM substrate) or blue fluorescence, which occurs upon excitation of the cytosolic CCF2-AM substrate which is cleaved by VLP delivered β-lactamase. Those cells fluorescing blue as compared with control (for example, mock control) were scored as entry positive.

3. Results

3.1 p38 MAPK inhibitors block EBOV replication in differentiated THP-1 cells

We tested whether p38 MAPK inhibitors would affect EBOV replication and EBOV-induced cytokine production in human APCs. We first tested whether inhibitor treatment would block infection of the human THP-1 cell line, a model cell line for monocytes/macrophages. Although undifferentiated monocytic-like THP-1 human cells are nonpermissive for EBOV infection, phorbol 12-myristate-13 acetate (PMA)-treatment induces their differentiation into permissive macrophage-like cells (Dube et al., 2008; Martinez et al., 2013a).

Differentiated THP-1 cells were infected at an MOI of 0.1 with recombinant EBOV expressing EBOV-eGFP (Towner et al., 2005). Pyridinyl imidazole p38MAPK inhibitors SB202190, p38inhK III, SB203580 as well as the control pyridinyl imidazole compound SB202474 that does not inhibit p38 MAPK, were added to cells approximately 1 hour prior to infection. DZNep, previously demonstrated to suppress EBOV replication in cell culture (Bray et al., 2000) served as a positive control for inhibition. 3 days post-infection cellular GFP fluorescence was assayed as a marker of viral replication. The data demonstrated inhibition by the p38 MAPK inhibitors (Figure 1A). The IC_{50} concentrations for each treatment, summarized in Table 1, further demonstrate significant inhibition by the p38

MAPK inhibitors but by not control compound SB202274. Cells were greater than 90% viable at all chemical concentrations tested, with the exception of SB202190, which yielded 82% viability at 15μM (data not shown). These experiments were repeated three times with similar results. Taken together, these data indicate that p38 MAPK inhibitors block EBOV replication. Cell supernatants were also measured for infectious virus by plaque assay following infection at a multiplicity of 0.1. Modest inhibition was seen in the presence of the p38 MAPK inhibitors, but not the control inhibitor, correlating with the GFP expression data (Figure 1B). The inhibition was dose-dependent with greater antiviral activity at the higher concentrations. A significant reduction in viral output, as compared to the control, was only seen with concentrations of greater than 5μM. Pretreatment with 10μM of p38 MAPK inhibitors SB202190 resulted in a slightly greater than half-log reduction in viral titer. Pretreatment using 15μM of each of the p38 MAPK inhibitors demonstrated similar suppression of replication. Since p38 MAPK inhibitor SB202190 consistently demonstrated the greatest inhibitory activity (Figure 1 and Table 1) this inhibitor was selected for use in subsequent experiments.

3.2 p38 MAPK inhibitor SB 202190 inhibits EBOV replication in human MDDCs

Next we sought to determine if p38 MAPK inhibition would similarly block EBOV infection of primary MDDCs. Human MDDCs were infected in triplicate with EBOV-eGFP at an MOI of 1 or 5 for either 48 or 72 hours in the presence of decreasing concentrations p38 MAPK inhibitors. Two relatively high MOIs were utilized to determine if the magnitude of viral input affected the result and because infectivity of DCs is lower than on Vero cells where the virus was originally titered. DZNep and control pyridinyl imidazole SB202474 were used as positive and negative controls, respectively. At each indicated time point, cells were harvested and GFP fluorescence was quantified by flow cytometry as a correlate of viral infection and replication. Figure 1(C–F) shows the percentage of infected (% GFP positive) MDDCs at MOIs of 1 and 5 at 48 and 72 PI. After 48 hours at MOI of 1, SB202190 treatment reduced the number of GFP fluorescent cells from about 50% to roughly 10% at concentrations ranging from 7.5μM to 15μM. While less profound, the percentage of fluorescent cells was also decreased at the lower concentrations assayed. This trend was reproduced at the 72-hour time point for the MOI of 1, although the overall percentage of GFP-positive cells was higher (maximum GFP-positive cells was 60% rather than 50%) for both SB202190 and control treated cultures (Figure 1C & 1E). At the higher MOI of 5, the inhibition was less prominent, particularly at lower concentrations of compound, but a dose dependent inhibition was maintained. At the 48-hour time point at MOI of 5, the 7.5μM and 15μM concentrations of SB202190 reduced the percentage of GFP-positive cells from 60% to 35% and 20%, respectively (Figure 1D). Unlike the infections performed using an MOI of 1, extending the infection to 72 hours at the higher MOI did not result in an increase in the percentage of GFP-positive cells. At an MOI of 5, DZNep also had a much weaker inhibitory effect than at an MOI of 1 (Figure 1C–F). All inhibitor concentrations tested resulted in greater than 96% viability in MDDCs (data not shown). These data demonstrate that the p38 MAPK inhibitor SB202190, but not the control compound, inhibits EBOV replication in primary human MDDCs (IC_{50} of 2.67μM, Table 1) as was observed in the THP-1 model.

3.3 p38 MAPK inhibitor treatment of MDDCs inhibits EBOV induced cytokine production

To evaluate the effect of p38 MAPK inhibitor treatment on cytokine production, MDDCs pre-treated with DZNep, control SB202474 or p38 MAPK inhibitor SB202190 were infected with EBOV-eGFP (MOI=1), and the supernatants were analyzed for cytokine production 48 hours later. The levels of IFN-α and the IFN-induced cytokine IP-10, as well as the proinflammatory cytokines TNF-α, IL-6, IL-12, and cytokine antagonists IL-2R and IL-1RA were determined (Figure 2A–C).

IFN-α and IP-10 production were potently suppressed by SB202190 treatment relative to control compound SB202474 (Figure 2A). The suppression by SB202190 treatment exceeded that mediated by DZNep (Figure 2A), despite the effective inhibition of virus replication by this compound (Table 1 and Figure 1). Although relatively low levels of TNFα, IL-6 and IL-12 were detected, SB202190 also reduced these cytokines in the supernatants. For these, significant suppression relative to control SB202474, was again seen at drug concentrations that did not result in effective inhibition of EBOV-eGFP replication.

Additionally, because hypersecretion of chemokines and growth factors has been associated with fatal outcome in human cases (Wauquier et al., 2010), the levels of G-CSF, MIP-1α, MIP-1β, IL-8 and RANTES were also measured (Figure 2D, E). SB202190 effectively inhibited MIP-1a, MIP-1b and RANTES production even at drug concentrations as low as 3.75μM, while significant inhibition of G-CSF was seen at 15 μM.

3.4 p38 MAPK inhibitor SB202190 blocks EBOV GP, but not VSV G mediated entry into human MDDCs

We next tested whether p38 MAPK inhibition could block EBOV at the level of entry by using an established virus-like particle (VLP)-based assay (Martinez et al., 2010; Martinez et al., 2013b; Simmons et al., 2003). MDDCs were pretreated for 30 minutes with 10μM of control SB202474 or p38 MAPK inhibitor SB202190. After drug treatment, MDDCs were incubated with VLPs pseudotyped with EBOV GP or VSV G and assayed for entry (Figure 3A). Shown is the relative entry normalized to the entry seen in control compound treated cells. The relative amount of EBOV GP, but not VSV G mediated entry was substantially reduced in p38 MAPK inhibitor SB202190-pretreated MDDCs. This experiment was repeated and similar results were obtained. These results suggest that the block in MDDC EBOV infection and replication may be due in part to a block of viral entry.

3.5 p38 MAPK inhibitor SB202190, but not control, inhibits human MDDC uptake of FITC-dextran

EBOV is endocytosed into cells via macropinocytosis (Aleksandrowicz et al., 2011; Hunt et al., 2011; Mulherkar et al., 2011; Nanbo et al., 2010; Saeed et al., 2010). One possible mechanism by which the p38 MAPK inhibitors might affect EBOV entry would be through inhibition of macropinocytosis. We therefore tested whether uptake of FITC-dextran (10KDa), an indicator of macropinocytosis, is affected by 30 minute pre-treatment with 10μM of control SB202474 or p38 MAPK inhibitor SB202190. MDDCs were incubated with FITC-dextran for 30 minutes at 37°C, washed and then assayed for VLP binding and

uptake (Figure 3B). Uptake by SB202190-pretreated MDDCs was significantly less efficient (approximately 60% decrease) as compared to mock and control pretreated MDDCs. Taken together, these data suggest that the p38 MAPK inhibitor-induced block in viral entry occurs in part due to a block in endocytosis.

4. Discussion

EBOV disease is associated with excessive inflammatory cytokine production, therefore, therapies that control this host response might prove beneficial. However, suppression of inflammation could also promote virus replication. Our study identifies small molecules that block the viral-induced production of potentially damaging inflammatory cytokine response while also suppressing virus replication. The development of drugs that could potently suppress replication while also decreasing inflammation could mitigate concerns that the immune suppressing activity would lead to uncontrolled virus spread. Therefore, this study suggests a unique strategy for anti-filovirus therapeutic development.

We examined the impact of the p38 MAPKs on EBOV infection in macrophages and DCs because these cells play a critical role in the pathogenesis of EBOV the disease. These cells are infected in vivo. For example, Geisbert et al. demonstrated that in infected cynomolgus macaques, DCs stain positive for EBOV antigen early after infection and throughout the course of disease, suggesting that DCs are both early and sustained targets of viral infection (Geisbert et al., 2003). In vitro, EBOV productively infects primary monocytes, macrophages and DCs and leads to their deregulation (Bosio et al., 2003; Feldmann et al., 1996; Mahanty et al., 2003; Stroher et al., 2001; Wahl-Jensen et al., 2011). That p38 MAPK signaling is important for EBOV replication in macrophages and DCs has precedence. EBOV infection induces MAPK signaling cascades in a GP-dependent manner (Martinez et al., 2007; Wahl-Jensen et al., 2011), and p38 MAPK is thought to play dual roles in DC maturation and induction of inflammatory cytokines (Ayala et al., 2000; Lee et al., 2009; Terrazas et al., 2011).

p38 MAPK signaling is activated in response to, among other things, environmental stress (Risco and Cuenda, 2012; Roux and Blenis, 2004) and plays an important role in a number of viral infections. For example, p38 MAPK activation and signaling is important for viral entry of reovirus and respiratory virus (Huang et al., 2011; Marchant et al., 2010). p38 MAPK signaling has also been implicated as playing a role in the viral transcription and translation of a number of viruses including hepatitis C virus, avian reovirus, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, rotavirus, Kaposi sarcoma-associated herpesvirus, encephalomyocarditis virus and HIV-1 (Cohen et al., 1997; George et al., 2012; Hirasawa et al., 2003; Jafri et al., 2007; Ji et al., 2009; Lee and Lee, 2012; Pan et al., 2006; Wei et al., 2009); and inhibition of p38 MAPK signaling has been shown to directly inhibit virus infection (Chang et al., 2008; Ludwig et al., 2003; Wei et al., 2009). EBOV infection induces MAPK signaling cascades in a GP-dependent manner, and p38 MAPK is thought to play dual roles in DC maturation and induction of inflammatory cytokines (Ayala et al., 2000; Lee et al., 2009; Terrazas et al., 2011). These facts motivated our analysis of p38 MAPK inhibitors on viral replication and entry. Our data identify inhibition of viral entry as one mechanism contributing to suppression of EBOV replication.

This is likely due, at least in part, to suppression of macropinocytosis of virus particles, based on the FITC-dextran uptake assay. However, based on the available data, we also cannot exclude a contribution from other post-entry inhibitory mechanisms.

The p38 MAPK inhibitors tested here suppressed EBOV-induced cytokine production. That this does not simply reflect suppression of virus infection by the compounds is based upon the observation that DZNep, which more potently suppressed replication, was less effective than SB202190 in suppressing cytokine responses (Figure 2). Furthermore, p38 MAPK inhibitors are known to inhibit inflammatory responses (Han et al., 1994; Kumar et al., 2003; Lee et al., 1994). Fatal outcome in EBOV infection is associated with the presence of high levels of inflammatory cytokines (Wauquier et al., 2010). In vitro, infected macrophages produce excess levels of inflammatory cytokines that increase vascular endothelial cell junction permeability (Feldmann et al., 1996). Furthermore, induction of chemokines from infected macrophages has been been postulated to play a role in recruitment of additional targets of viral infection (Baize et al., 2002; Bray and Geisbert, 2005). Because p38 MAPK signaling is linked to inflammatory disease (reviewed in (Schieven, 2005, 2009)) and pyridinyl imidazole p38 MAPK inhibitors have been shown to exhibit anti-inflammatory activities (Gallagher et al., 1997; Lantos et al., 1984; Lee et al., 1988; Lee et al., 1999; Lee et al., 1994), we tested how p38 MAPK inhibitors might affect EBOV-induced cytokine and chemokine secretion. We show that p38 MAPK inhibitors ablated proinflammatory cytokine, chemokine and growth factor production from EBOV treated MDDCs at similar concentrations as were previously used to inhibit APC cytokine production (Mikkelsen et al., 2009; Mitchell et al., 2010; Puig-Kroger et al., 2001; Wilflingseder et al., 2004). Therefore, these findings suggest a potential therapeutic role for p38 MAPK pyridinyl imidazole inhibitors or derivatives of these compounds for the treatment of EBOV infection via multiple mechanisms: blocking or impairing EBOV infection of target APCs, as well as countering the detrimental inflammatory cytokine response generated by any escaping virus.

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Highlights

- Pyridinyl imidazole inhibitors of p38 MAP kinases inhibit ebolavirus replication in macrophages and dendritic cells
- Pyridinyl imidazole inhibitors of p38 MAP kinases inhibit ebolavirus induced cytokine production
- Pyridinyl imidazole inhibitors of p38 MAP kinases inhibit Zaire ebolavirus entry into dendritic cells

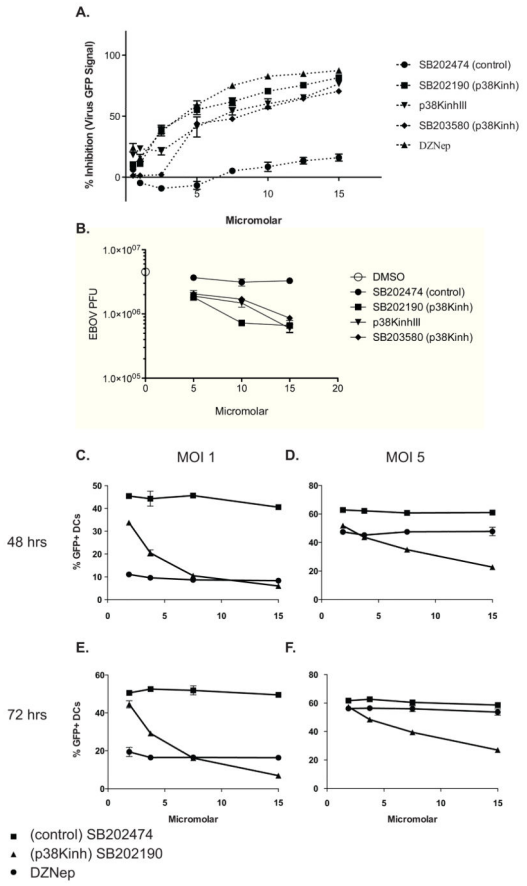

Figure 1. Methylsulfanylimidazole inhibitors of p38 MAPK inhibit Ebola virus replication in THP-1 cells and human MDDCs

(A) THP-1 cells were seeded in triplicate in 96-well cell culture plates and pretreated with DZNEP, control compound SB202474, or the p38 MAPK inhibitors SB202190, p38inhK III or SB203580 at decreasing concentrations. Cells were then infected with recombinant Ebola virus expressing eGFP (EBOV-eGFP) at a MOI of 0.1 and incubated at 37°C, 5% CO_2 for 3 days prior to fluorescent measurement at 515 nm. Inhibitor effect is expressed as % inhibition of infected, untreated THP-1 cells. (B) Supernatants from DMSO, control SB202474 and p38 MAPK SB202190, p38inhK III, SB203580 inhibitor-treated THP-1s three days post-infection with $EBOV_{GFP}$ were tested for EBOV titers by plaque assay. Plaque-forming units (PFU) are shown for supernatants. Human monocyte-derived dendritic cells (MDDCs) were pretreated with DZNEP, control compound SB202474 or p38 MAPK inhibitor SB202190 at decreasing concentrations. Cells were then infected in triplicate with EBOV-eGFP at an MOI of 1 (C and E) and 5 (D and F), the cells were washed to remove free virus and fresh media containing inhibitors was replaced. Cells were harvested at 48 (C and D) and 72 (E and F) hours and percentage of GFP+ MDDCs is shown.

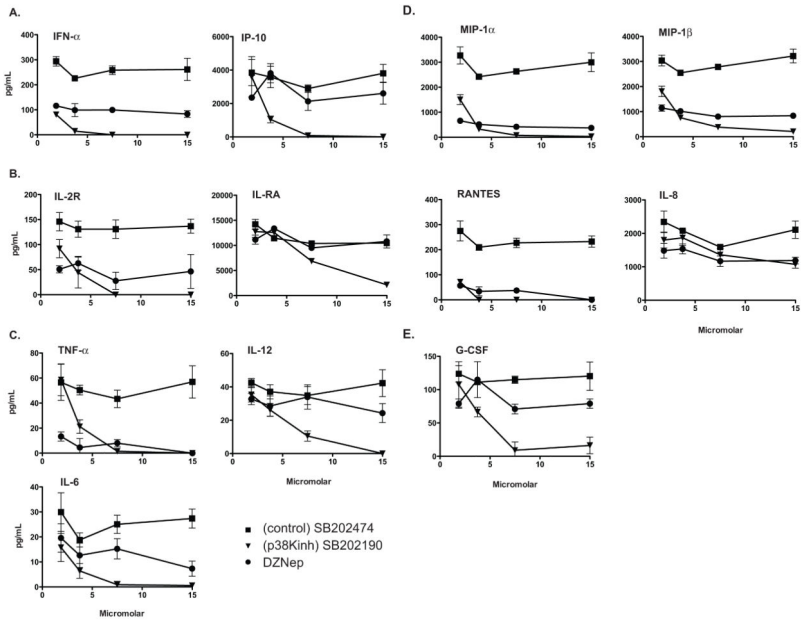

Figure 2. p38 MAPK inhibitor SB 202190 inhibits cytokine production from EBOV treated MDDCs

Supernatants from DZNEP, control SB202474 and p38 MAPK SB202190 inhibitor-treated MDDCs forty-eight hours PI with (MOI 1) EBOV-eGFP were tested for the levels of (A) interferon-alpha and IP-10, (B) IL-2R and IL-1RA as well as (C) TNF-α, IL-6 and IL-12, (D) chemokines MIP-1, MIP-1beta, IL-8 and RANTES and (E) G-CSF.

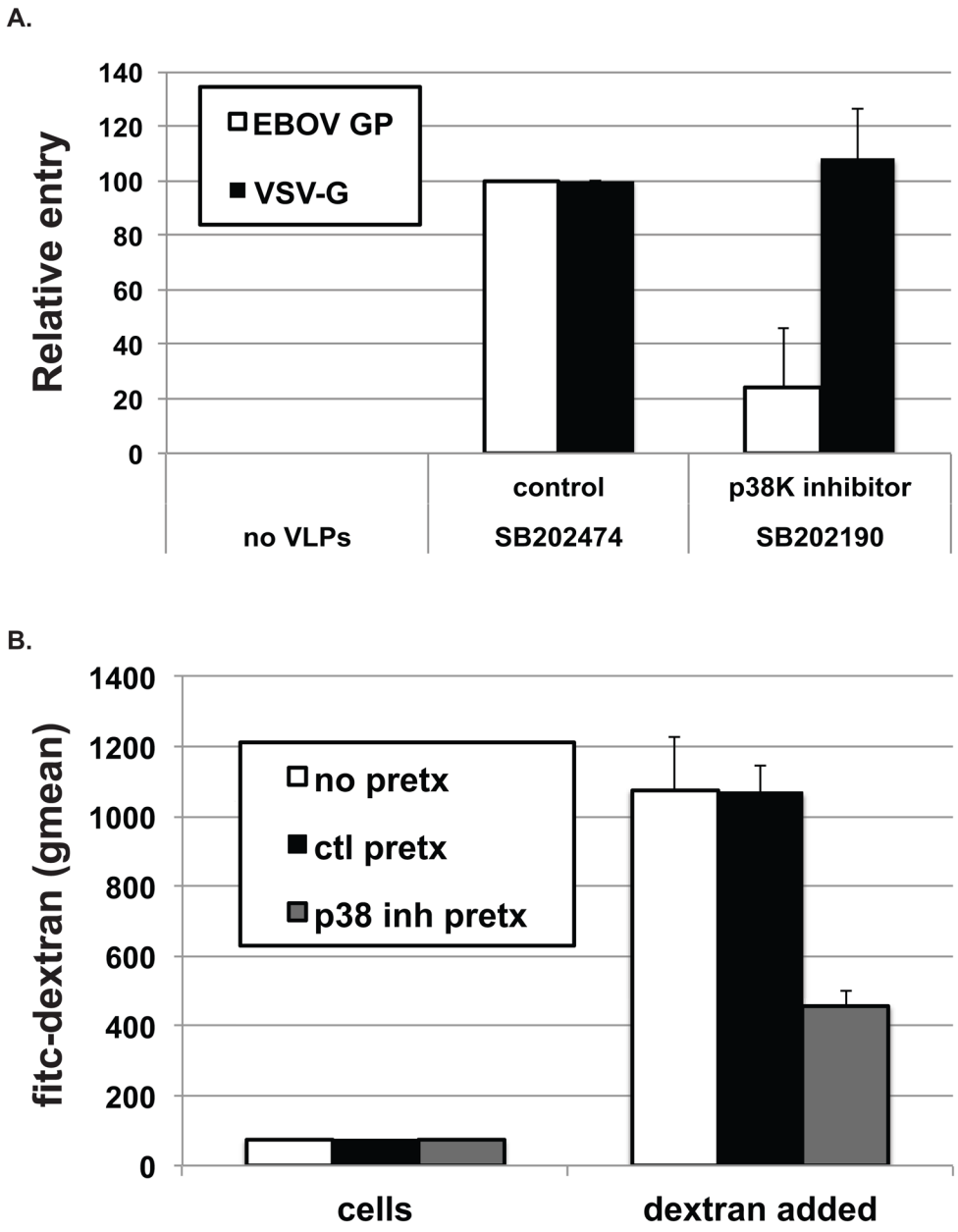

Figure 3. p38 MAPK Inhibitor SB 202190 inhibits EBOV GP-mediated, but not VSV G mediated entry into human DCs

Human DCs were pretreated with 10-μM mock, control SB202474 or p38 MAPK SB202190. An entry assay was performed using virus-like particles (VLPs) that contained a chimeric VP40-β-lactamase enzyme. VLPs pseudotyped with either EBOV GP or VSV G were incubated with human DCs for 3.5 hours. Cells were then loaded with a cytoplasmic substrate that fluoresces blue upon cleavage by β-lactamase. Cells whose cytoplasm is penetrated by VLPs (entry positive) will fluoresce blue when assayed by flow cytometry. The number of cells positive for EBOV-GP (blue bar) and VSV G (black bar) mediated

entry in the presence of control inhibitor was set at 100. This experiment was repeated with similar results. Shown is the average of four replicates.

Table 1

IC_{50} of tested compounds in MDDCs and differentiated THP-1 cells.

Compound	Target cell inhibition [a]	
	Differentiated THP-1	MDDCs
SB202190	4.7μM	2.7μM
SB203580	8.2μM	ND
p38KinhIII	8.3μM	ND
DZNep	3.4μM	<1μM
SB202474	>15μM	>15μM

[a] IC_{50} values, as determined by inhibition of GFP expression by EBOV-GFP, were calculated for p38 MAPK inhibitors and controls using Graphpad Prism 5.0 software. ND- not determined