Modulation of inflammation and pathology during dengue virus infection by p38 MAPK inhibitor SB203580

Yilong Fu, Andy Yip, Peck Gee Seah, Francesca Blasco, Pei-Yong Shi * Maxime Hervé *

Novartis Institute for Tropical Diseases, Singapore 138670, Singapore

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ABSTRACT

Dengue virus (DENV) infection could lead to dengue fever (DF), dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). The disease outcome is controlled by both viral and host factors. Inflammation mediators from DENV-infected cells could contribute to increased vascular permeability, leading to severe DHF/DSS. Therefore, suppression of inflammation could be a potential therapeutic approach for treatment of dengue patients. In this context, p38 MAPK (mitogen-activated protein kinase) is a key enzyme that modulates the initiation of stress and inflammatory responses. Here we show that SB203580, a p38 MAPK inhibitor, suppressed the over production of DENV-induced pro-inflammatory mediators such as TNF-α, IL-8, and RANTES from human PBMCs, monocytic THP-1, and granulocyte KU812 cell lines. Oral administration of SB203580 in DENV-infected AG129 mice prevented hematocrit rise and lymphopenia, limited the development of inflammation and pathology (including intestine leakage), and significantly improved survival. These results, for the first time, have provided experimental evidence to imply that a short term inhibition of p38 MAPK may be beneficial to reduce disease symptoms in dengue patients.

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1. Introduction

The mosquito-borne dengue virus (DENV) annually causes about 390 million human infections, leading to 96 million cases with manifest symptoms (Bhatt et al., 2013). DENV infection is either asymptomatic, self-limited known as dengue fever (DF), or lead to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) that is characterized by plasma leakage (Mackenzie et al., 2004). In vitro studies have shown that DENV replicates in monocytes, macrophages, mast cells, and dendritic cells (Kou et al., 2008; King et al., 2002; Marovich et al., 2001; Wu et al., 2000). Over production of pro-inflammatory vasoactive cytokines or chemokines from DENV-infected cells such as TNF-α, IL-8, and RANTES, could trigger the increase of vascular permeability in DHF/DSS patients (Chareonsrisuthigul et al., 2007; Pang et al., 2007). These observations suggest that inhibitors of inflammatory cytokines could prevent plasma leakage in dengue patients. In support of this hypothesis, treatment of DENV-infected mouse anti-TNF-α antibodies was shown to prevent severe pathological symptoms, including death (Shresta et al., 2006).

Mitogen-activated protein kinases (MAPKs) are members of intracellular kinases that play critical roles in cell signaling and gene expression. The p38 MAPK cascade is stimulated by various pro-inflammatory cytokines, such as TNF-α, and activation of the p38 MAPK signaling culminates in increased expression of pro-inflammatory molecules like TNF-α, IL-6, IL-8, and metalloproteinases (MMPs) (Ono and Han, 2000; Cuadrado and Nebreda, 2010). Although inhibition of these inflammatory mediators has been studied in chronic inflammatory syndromes for decades, it is only recently that such approach has been developed for treatment of patients with acute inflammation (Schieven, 2009). Along this line, SB203580, a p38 MAPK inhibitor, suppresses in vitro production of TNF-α and reduces pro-inflammatory cytokines in mice and rats (Birrell et al., 2000; Yong et al., 2009; Escott et al., 2000).

Two MAPKs, p38 and ERK1/2, were recently found to be activated during DENV infection in endothelial cells (Huerta-Zepeda et al., 2008), suggesting that MAPKs could be involved in DENV
pathogenesis. P38 and JNK (Jun NH(2)-terminal kinase) pathways were also shown to be activated in DENV-infected macrophages. However, it is not known whether these inhibitors would be beneficial during DENV infection in vivo. The goal of this study was to investigate the effect of p38 MAPK inhibitor (SB203580) on DENV infection in vitro and in vivo.

2. Materials and methods

2.1. Compound, cell line, virus, and antibody

SB203580 (Selleck Chemicals LLC) was used at various concentrations up to 5 μM for in vitro assays. For in vivo study, SB203580 was suspended in 1% carboxymethyl cellulose sodium salt (CMC, Sigma). Cell lines, BHK-21, C6/36, THP-1, KU812, were obtained from ATCC and maintained as instructed. Isolated cryopreserved human PBMCs were obtained from AllCells, LLC. DENV-2 strain D2Y98P-PP1 (gift from Pr. Alonso, National University of Singapore, NUS) and clinical isolates MY10340 (from Shamala Devi, University of Malaya, Malaysia) were propagated in mosquito C6/36 cells. Chimeric 4G2 monoclonal antibody was a kind gift of Paul A. MacAray (NUS).

2.2. Propagation of DENV and plaque assay

For propagation of D2Y98P-PP1 and MY10340 strains, C6/36 cells were infected at an MOI of 0.01. After 1 h of infection, the cells were washed and cultured at 28 °C in RPMI 1640 supplemented with 5% FBS. At days 5, 6, and 7, cell supernatants were harvested, centrifuged at 4000 rpm for 10 min at 4 °C (to remove cell debris), and stored at −80 °C. Viral titers were determined by plaque assay (Fu et al., 2014). Viral titers were expressed as plaque forming units per milliliter (PFU/ml).

2.3. Infection of cells with DENV

PBMCs, THP-1, and KU812 cells were cultured as previously described (Fu et al., 2014). After pre-incubation of 4G2 antibody (IgG1, 1/5000e) with DENV for 30 min, the antibody–virus mixture was used for infection (MOI of 1). For SB203580 treatment, cells were incubated with various concentrations of the drug in 2% FBS-RPMI. The cells were then incubated with 5% CO2. At 48 h.p.i., the supernatant was collected, centrifuged at 4000 rpm for 10 min, and subjected to plaque assay. The amount of TNF-α, IL-8, and RANTES in the supernatant was determined by ELISA (R&D Systems, Minneapolis, MN).

2.4. Cell counting kit-8 (CCK-8) assay

Cell viability assays were conducted according to the manufacturer’s instructions. Human PBMCs, THP-1, and KU812 cell suspensions (5 × 103 cells in 200 μl of RPMI-1640 supplemented with 10% FBS) were transferred into 96-well plates, and treated with different concentrations of SB203580. At 48 h post-treatment, the medium was replaced by CCK-8 reagent (10 μl CCK-8 and 100 μl RPMI-1640), and the cells were incubated 2–3 h. Absorbance at 450 nm was measured for each well.

2.5. Mouse infection and compound treatment

AG129 mice were obtained from B&K Universal. All mice experiments were performed in compliance with national laws and institutional policies and with the approval of the Institutional Animal Care and Use Committee. Nine to ten week-old mice were administered with 5 × 105 PFU of D2Y98P-PP1 virus via the subcutaneous route (0.1 ml in PBS). At day 3 post-infection, mice were orally dosed with vehicle (1% CMC, 10 ml/kg) or with SB203580 (60 mg/kg) once daily for 5 days. Blood samples were obtained by retro-orbital puncture under anesthesia. Blood aliquot were collected in K2EDTA tubes (Becton–Dickinson) and were immediately centrifuged at 600g for 4 min to obtain plasma. Then, 20 μl of plasma was diluted in 980 μl of RPMI 1640 medium and snap frozen in liquid nitrogen for plaque assay analysis.

2.6. Vascular permeability assay and hematology

Vascular leakage was assessed using Evans Blue dye as a marker for albumin extravasation as previously described (Tan et al., 2010, 2011). Briefly, 0.2 ml of Evans blue dye (0.5% w/v in PBS, Sigma Aldrich) was injected intravenously into anesthetized mice. After 1.5 h, the animals were euthanized and perfused with PBS. The tissues were harvested and weighed prior to dye extraction using N,N-dimethylformamide (Sigma; 4 ml/g of tissue wet weight) at 37 °C for 24 h after which absorbance was read at 620 nm. Data were expressed as decrease in optical density (OD) at 620 nm per gram of wet tissue compared to the untreated control. Mouse whole blood was immediately analyzed for cell counts using automated hematology analyzer (Sysmex pocH-100i). Various blood parameters were assessed including red blood cells (RBC) and hematocrit (HCT).

2.7. Detection of inflammatory mediators

Mouse blood samples were obtained and allowed to clot at room temperature before centrifuging at 2000g for 20 min. Serum samples were then frozen at −80 °C freezer. The levels of TNF-α, Interleukin-6 (IL-6), and MMP-9 in the serum samples were measured using R&D Systems ELISA kits.

2.8. Statistical analysis

All statistical analysis was done with Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Data were analyzed by Student’s t test. A two-tailed P < 0.05 was considered significant. For the Kaplan–Meier survival curve, Wilcoxon test was used to compare treated and untreated groups.

3. Results

3.1. SB203580 does not interfere with DENV replication in cell culture

To assess whether SB203580 affected DENV replication, we treated DENV-infected human PBMCs, monocytes (THP-1), and pre-basophilic cells (KU812) with SB203580 and compared virus production in culture supernatant. A sub-neutralizing concentration of monoclonal antibody 4G2 was used to enhance viral infection (Fu et al., 2014) as immune cells are usually poorly susceptible to DENV infection in vitro (Brown et al., 2009; Halstead and O'Rourke, 1977). As summarized in Table 1, treatment with SB203580 did not significantly affect viral titers of DENV-2 strains MY10340 and D2Y98P-PP1 in PBMCs, THP-1, and KU812 cells. Among the three cell types, KU812 produced the highest virus yield at 48 h post-infection.

3.2. SB203580 inhibits DENV-induced pro-inflammatory cytokine secretion in vitro

Previous studies demonstrated that pro-inflammatory cytokines, such as TNF-α, were over-expressed in DENV-infected PBMCs (Carr et al., 2003; Kelley et al., 2011; Sun et al., 2011). We
then investigated the impact of drug treatment on the secretion of cytokines from DENV-infected PBMCs, THP-1, and KU812 cells. In infected PBMCs, significant increases of TNF-α, IL-8, and RANTES were detected at 48 h p.i.; and SB203580 inhibited significantly the infection-induced TNF and IL8 production in a dose dependent manner (Fig. 1a). Similarly, SB203580 suppressed the production of TNF-α (P < 0.05) and RANTES, two major inflammatory mediators produced upon DENV infection in THP-1 (Fig. 1b) and KU812 cells (Fig. 1c). To exclude the possibility that the suppression of cytokine production was due to compound-mediated cytotoxicity, we incubated PBMCs, THP-1, and KU812 cells with SB203580 (up to 5 μM) and we observed that it did not affect cell viability (Fig. 1d). These results indicate that the decrease of cytokine and chemokine production after SB203580 treatment is most likely due to an anti-inflammatory effect rather than cytotoxicity.

3.3. SB203580 therapy affected blood parameters and inflammation in DENV-infected mice

We then evaluated the effect of SB203580 on DENV-infected AG129 mice. On day 3 p.i., the mice were orally dosed once a day for 5 consecutive days with vehicle or 60 mg/kg of SB203580 (Fig. 2a). Blood samples were harvested before treatment and after the last dosing, i.e., on day 3 and day 7, respectively; the samples were quantified for viral titers, hematological parameters, and cytokine production. As expected, high viremia (medium of 1 × 10^6 PFU/ml) was detected on day 3 p.i. (Fig. 2b). On day 7 p.i., three out of ten treated animals had detectable virus, whereas only one animal showed detectable level of virus in control group (Fig. 2b). SB203580 prevented significantly (P < 0.05) the increase of RBC concentration (Fig. 3a) and the rise of HCT (Fig. 3b) on day 7 post-infection. In addition, the infection-induced lymphopenia was not observed in the treated animals (Fig 3c). For cytokine production, SB203580 significantly decreased viral infection-induced serum levels of TNF-α (Fig. 4a) and IL-6 (Fig. 4b) on day 7 post-infection (P < 0.05). The circulating serum level of MMP-9 was not modified on day 7 p.i., but a significant reduction was observed in the treated animals during moribund stage at day 11 (Fig. 4c). These data suggest that SB203580 treatment could limit the hematological disorders and systemic inflammation associated with DENV infection.

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Virus strain</th>
<th>SB203580 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>THP-1</td>
<td>MY</td>
<td>11.8 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>PP1</td>
<td>19.5 ± 0.9</td>
</tr>
<tr>
<td>KU812</td>
<td>MY</td>
<td>2050 ± 50</td>
</tr>
<tr>
<td></td>
<td>PP1</td>
<td>625 ± 35</td>
</tr>
<tr>
<td>PBMCs</td>
<td>MY</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>PP1</td>
<td>11.8 ± 1.3</td>
</tr>
</tbody>
</table>

* The cells were infected with antibody–virus complex (MOI = 1) for 1 h and then treated with various concentrations of SB203580 for 48 h. The amount of virus produced in the culture fluid was evaluated by plaque assay and expressed as mean PFU/ml ± SD (×1000), where n = 3.

Fig. 1. In vitro effect of SB203580 on DENV-infected immune cells. PBMCs (a), THP-1 (b), and KU812 (c) cells were infected with antibody-DENV-2 complex (MOI = 1) for 1 h and then treated with SB203580 for 48 h. TNF-α, IL-8, and RANTES concentrations in culture media were measured by ELISA. The cytokine levels are shown with the presence or the absence of indicated concentrations of SB203580. Each cytokine value represents the mean ± SD in each group (n = 3). Two DENV-2 strains, MY10340 and D2Y98P-PP1 (PP1), were used for infections as indicated. The cell viability of human PBMCs, THP-1, and KU812 cells exposed to different concentrations of SB203580 for 48 h was determined by CCK-8 assay (d). The results are expressed as percentages of viable cells compared to the viable cells without drug treatment. Statistical significance compared treated cells with cells treated with vehicle control (P < 0.05, Student’s t test).
3.4. Impact of SB203580 on leakage and survival of DENV-infected mice

Vascular leakage is a hallmark of disease in dengue patients. Different organs from the DENV-infected AG129 mice were evaluated using Evan’s blue dye extrusion assay (Fig. 5) (Tan et al., 2010). No difference in Evan’s blue dye extrusion was observed in spleens (Fig. 5a) and in livers (Fig. 5b) between treated and control animals. In contrast, SB203580 significantly reduced dye extrusion in intestine of mice at moribund stage (Fig. 5c) and significantly improved the survival of the infected animals ($P = 0.0229$, Fig. 5d). Specifically, 100% of the treated animals survived, whereas only 67% of the mock-treated animals survived on day 10 post-infection ($P < 0.05$). These results indicate that treatment with p38 MAPK inhibitor decreases vascular leakage in intestine and improves survival of DENV-infected AG129 mice.

4. Discussion

Using human PBMCs, stable cell lines, as well as mouse model, we showed here that p38 MAPK inhibitor SB203580 suppressed inflammation and limited disease development during DENV infection. In vitro infection of human myeloid cells with DENV induces secretion of various chemokines, including IL-8, MCP-1, and RANTES (Bosch et al., 2002; Rothman, 2011). We observed here that viral infection induced cytokine and chemokine production in PBMCs, THP-1 cells as well as KU812 cells and that SB203580 inhibited p38 MAPK-mediated TNF-α secretion from human...
PBMCs and monocytes. In addition, SB203580 also blocked IL-8 and RANTES production from PBMCs and from KU812 cell line at submicromolar concentrations. These chemokines in blood and in PBMCs are thought to contribute to disease pathogenesis as high concentrations of IL-8 and RANTES were found in fluids from patients with DSS and DHF (Raghupathy et al., 1998). Our results here are in agreement with previous findings which demonstrated that DENV-2 infection of KU812 cells can induce the production of IL-8 and RANTES in vitro (King et al., 2002; Medin et al., 2005; Chen and Wang, 2002; Bosch et al., 2002). This observation suggested a potential role very early on of granulocytes in both recruiting and activating T lymphocytes during infection but also during a secondary DENV infection (King et al., 2000). We showed here that the KU812 cell line was the most permissive immune cells to DENV infection. As this type of cell line predominantly expresses FcγRII, it is possible that the virus entry through this receptor during antibody-dependent enhancement might be one of the most efficient physiological processes to favor cell infection in vivo.

It was reported that p38 MAPK affected the viral yield in vitro (Ceballos-Olvera et al., 2010). However, in the present study, we did not observe any impact on the DENV-2 replication in PBMCs, THP-1, and KU812 cells. The discrepancy could result from a different timing of treatment: In the previous study, macrophages were incubated with the inhibitor prior to infection, while in our study the cells were treated 1 h after infection. This suggests that the activation of MAPKs may occur within the first hour of DENV infection. As such, the use of SB203580 therapy provided a way to block the chemokine production in vivo as shown in Fig. 5.

![Fig. 5. Effect of SB203580 therapy on leakage and survival. AG129 mice were infected with 5 x 10^5 PFU and orally dosed for 5 days with SB203580 (60 mg/kg, QD) or with control vehicle (1% CMC, 10 ml/kg) starting from day 3. At day 10 p.i., mice were intravenously injected with Evan’s blue dye and sacrificed 1.5 h later. Organs were harvested and processed for Evan’s blue dye quantification. Dots representing OD620 nm absorbance per gram of wet tissue of spleen (a), liver (b), and intestine (c) for each animal are shown and compared with untreated control group. The lines indicate the average of each parameter. Survival rates in AG129 mice infected with 5 x 10^5 PFU and orally treated with 60 mg/kg of SB203580 (n = 15) (d). Statistical significance is compared with untreated control (\(P < 0.05\)) through a Student’s t test for Fig. 5a, b and c and for the Kaplan–Meier survival curves, Wilcoxon test was used to compare treated and untreated groups.](image-url)
infection, and administration of SB203580 later on may not interfere with DENV replication in cell culture. We also recently observed that the addition of TNF, activator of the P38 MAPK signaling pathway, to PBMCs in vitro does not affect the virus replication (Chen et al., 2014). However, we detected slightly more viruses in the SB203580 treated animals compared to control AG129 mice. Recently, p38 MAPK inhibition has been described to limit the apoptosis of infected cells (Nagila et al., 2013). In that context, delaying apoptosis or inflammation in vivo may favor more viruses to be produced after 5 days of therapy. The treatment of PBMCs with IL10 favored the replication of the virus, suggesting that other signaling pathways are also involved in the regulation of virus replication (Chen et al., 2014).

Several adverse effects have been reported for p38 MAPK inhibitors in pre-clinical and clinical studies (Wong et al., 2012; Fischer et al., 2011; Yong et al., 2009). However, in our study, the doses used in vitro and in vivo did not appear to be toxic: No apparent effect was observed on cell viability in vitro; and the oral administration of 60 mg/kg of SB203580 once daily for 5 days was well tolerable and had no effect on mice body weight. One of the major differences with those studies is that we have performed a very short term regimen with a considered safe dose, so in this context, the development of side effects and toxicity inherent to P38 inhibition in vivo will be rather very limited. Extensive evidences from both preclinical animal models and clinical studies have highlighted the overproduction of pro-inflammatory cytokines in DHS/DSS patients (Levy et al., 2010; Chen et al., 2007; Gagnon et al., 2002). Subcutaneous injection of DENV-2 strain D2Y98P-PPI led to viral inoculums-dependent clinically relevant manifestations, including a rise in HCT, elevated pro-inflammatory cytokine levels, such as TNF-α, IL-6, and MMP-9 and increased vascular permeability (Tang et al., 2010). P38 MAPK-dependent mechanisms promote TNF-α and MMP-9 production, increases endothelial permeability and triggers vascular leakage by extracellular matrix remodeling and disruption of endothelial cell integrity (Luplertlop et al., 2006). Here, we showed that oral administration of SB203580 limited the changes of RBC/HCT blood parameters induced by DENV infection, decreased the circulation of pro-inflammatory cytokines and reduced the leakage in intestine, resulting in a better survival. The marked decrease of dye extrusion in the intestine level during SB203580 treatment also confirmed the major role of P38 MAPK in vascular inflammation and epithelial dysfunction of such organ (Mihaescu et al., 2010). While a slight increase of virus has been observed in treated animals, the onset of symptoms and the recovery phase in this model were not altered up to 4 days after therapy. This suggested that SB203580 limited the symptoms and inflammation during a critical period, between day 3 and day 7, where the host was developing signs of diseases, rather than delaying the onset of the symptoms.

In summary, this study showed, for the first time, that short term p38 MAPK inhibition could lead to an efficient control of the development of the disease in DENV-infected mice. Although p38 MAPK has been extensively investigated as a target to develop inhibitors for chronic inflammatory syndromes, it is only recently that this therapeutic alternative has been described to limit vascular and acute systemic inflammation in various syndromes. Thus, such strategy could represent an alternative strategy for an acute viral infection.

Conflict of interest

All authors agree there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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