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Effect of Vandetanib on Andes virus survival in the hamster model of Hantavirus pulmonary syndrome

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ABSTRACT

Hantavirus pulmonary syndrome (HPS) is a severe disease caused by hantavirus infection of pulmonary microvascular endothelial cells leading to microvascular leakage, pulmonary edema, pleural effusion and high case fatality. Previously, we demonstrated that Andes virus (ANDV) infection caused up-regulation of vascular endothelial growth factor (VEGF) and concomitant downregulation of the cellular adhesion molecule VE-cadherin leading to increased permeability. Analyses of human HPS-patient sera have further demonstrated increased circulating levels of VEGF. Here we investigate the impact of a small molecule antagonist of the VEGF receptor 2 (VEGFR-2) activation in vitro, and overall impact on survival in the Syrian hamster model of HPS.

The hantaviruses are rodent-borne RNA viruses in the Family *Bunyaviridae*, genus *Hantavirus* and cause two distinct and severe diseases in humans. Hemorrhagic Fever with Renal Syndrome (HFRS) caused by Old-World group hantaviruses such as Hantaan (HTNV) virus, and Hantavirus Pulmonary Syndrome (HPS) caused by New-World hantaviruses including Sin Nombre virus (SNV) or Andes virus (ANDV) (Schmaljohn and Hjelle, 1997; Schmaljohn, 2007). HPS is characterized by rapid onset interstitial pneumonitis, variable mononuclear cell infiltration, and interstitial and alveolar edema (Bustamante et al., 1997; Nolte et al., 1995). The primary target for virus replication is pulmonary microvascular endothelial cells which can support virus growth without any obvious cytopathic effects (Hepojoki et al., 2014; Srikiatkhachorn and Spiropoulou, 2014). A potential mechanism used by ANDV to increase microvascular permeability has been linked closely with elevated secretion of potent vasoactive mediators such as vascular endothelial growth factor (VEGF) (Gavrilovskaya et al., 2008; Gorbunova et al., 2011; Shrivastava-Ranjan et al., 2010). These increases lead to internalization and degradation of vascular endothelial cadherin molecule (VE-Cadherin) and eventual disruption of endothelial cell to cell adherens junctions and loss of vascular barrier function (Gorbunova et al., 2010; Shrivastava-Ranjan et al., 2010). Work in our group and by others has further revealed that in severe cases of HPS, patients have elevated circulating levels of multiple vasoactive cytokines/chemokines including VEGF, implicating these as potential key factors in HPS pathogenesis (Bondu et al., 2015; Morzunov et al., 2015; Shrivastava-Ranjan et al., 2010). We report here the use of Vandetanib, a tyrosine-kinase inhibitor targeting VEGF-receptor 2 (VEGFR-2) activation, and assess its impact on pathogenesis and survival in a hamster model of severe HPS.

To determine if Vandetanib, a small molecule inhibitor developed initially as a cancer therapeutic, reviewed in (De Luca et al., 2014), (Catalogue # V-9402, Lot BTB104 and BTB105; LC Laboratories, Woburn, MA USA; www.LClabs.com) had potency against ANDV a series of in vitro experiments in human microvascular endothelial cells (HMVEC-lung) cells were conducted to examine

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the ability of the compound to block VEGFR-2 phosphorylation and VE-cadherin degradation. All in vitro work reported here utilized the ANDV (isolate Chile-9717869; passaged 4 times in VeroE6 cells) and was conducted in a BSL-3 laboratory following guidelines in the Biosafety in Microbiological and Biomedical Labortories (BMBL) and Centers for Disease Control and Prevention standard operating procedures and protocols. Previously, pathogenic hantaviruses have been shown to enhance VEGFR-2 phosphorylation (Gorbunova et al., 2010). To confirm whether Vandetanib inhibits VEGFR-2 signaling pathways, confluent monolayers of HMVEC-L were starved for growth factors and then pretreated with either vehicle control or Vandetanib (5 μM) for 1 h (Fig 1A). Cells were then infected with ANDV at MOI of 0.1. Twenty four hours post infection, cells were stimulated with VEGF 50 ng/ml for 15 min or left unstimulated. Cells lysates were analyzed by western blotting using phospho-specific VEGFR-2 antibody, VEGFR-2-PY1214 (Invitrogen). As expected, ANDV infection induced VEGFR-2 phosphorylation in HMVEC-L cells (Fig. 1A lanes 3, 5) (Gavrilovskaya et al., 2008; Gorbunova et al., 2011), and the addition of Vandetanib strongly suppressed phosphorylation of VEGFR-2, without affecting its overall expression (Fig. 1A lanes 2, 4 and 6) (Fig. 1A).

Previously we had shown that blockage of VEGFR-2 activation by monoclonal antibodies prevented VEGFR-2 mediated degradation of VE-cadherin, a key endothelial cell adherens junction protein (Shrivastava-Ranjan et al., 2010). To determine if Vandetanib could also prevent this degradation, confluent primary HMVEC-L cells were infected with ANDV (MOI = 1.0) or were untreated (mock) or treated with ANDV inactivated by gamma irradiation as controls (Fig. 1B). Vandetanib (5 μM) was added immediately following 1 h adsorption of the virus and remained present during the entire course of the infection. At 24-hr post infection, cell lysates were analyzed by Western blotting for the expression of VE-cadherin. As previously reported, ANDV-infected cells showed a decrease in VE-cadherin levels (Fig. 1B lane 3) in comparison to mock- or inactivated virus-treated cells (Fig. 1B lanes 1 and 2 respectively). Vandetanib treatment of ANDV infected cells caused a 3-fold increase of VE-Cadherin (Fig. 1B lane 6) compared to DMSO-treated ANDV infected control cells (Fig. 1B lane 3) as determined by densitometric quantification analysis normalized to actin (internal control). These results taken together, further confirm the previously reported observation that ANDV-induced reduction of VE-cadherin is linked to the phosphorylation and function of VEGFR-2 (Gavrilovskaya et al., 2008; Gorbunova et al., 2010, 2011; Shrivastava-Ranjan et al., 2010).

Given the ability of Vandetanib to block VEGFR-2 phosphorylation and reduce ANDV-infection mediated VE-cadherin degradation we sought to determine what efficacy this compound may have in the Syrian golden hamster model of severe HPS. This model is characterized by acute onset respiratory disease approximately 8–14 days post-infection featuring pulmonary edema and pleural effusion that is associated with microvascular leakage and cardiopulmonary shock (Campen et al., 2006; Hooper et al., 2001; Safronetz et al., 2012). All animals (females aged 10–12 weeks) were group housed in an ABSL-4 laboratory in isolator-caging systems (Thoren Caging, Inc., Hazleton, PA, USA) with a HEPA-filtered inlet and exhaust air supply. The CDC is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) fully accredited research facility and all experiments described here were approved by the CDC Institutional Animal Care and Use Committee (IACUC) and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). For all infections 2000 PFU of ANDV (isolate Chile-9717869; passaged 4 times in VeroE6 cells and diluted in DMEM) was given intramuscularly. Vandetanib was delivered once per day oral gavage and was diluted

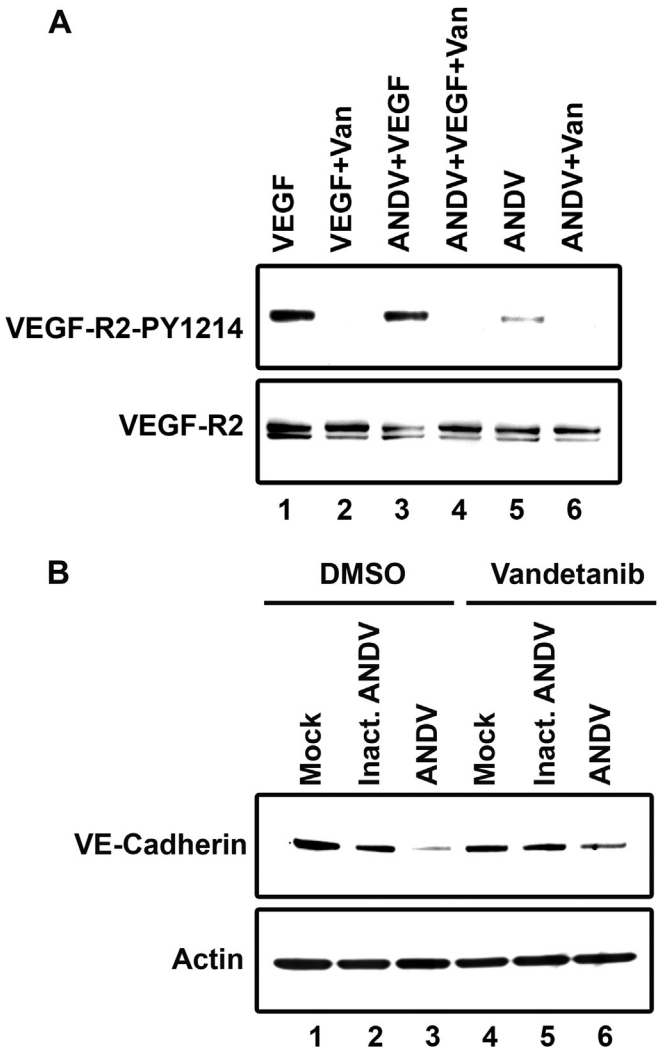

Fig. 1. A. Effect of Vandetanib on ANDV induced VEGF-R2 phosphorylation in vitro. Confluent monolayers of HMVEC-L were starved for growth factors for 1 h and treated with either DMSO or Vandetanib (5 μM). After 1 h, cells were infected with ANDV at MOI of 0.1. Twenty four hours post infection, cells were either left unstimulated or stimulated with VEGF 50 ng/ml for 15 min. Cells lysates were analyzed by western blotting using phospho-specific VEGFR-2 antibody, VEGF-R2-PY1214 (Invitrogen). After detection of phospho-VEGFR2, blot were stripped and re-probed with total VEGF-R2 antibody. **B**. Effect of Vandetanib on ANDV induced VE-Cadherin degradation. Confluent primary HMVEC-L cells were infected with ANDV (MOI = 1.0) or were untreated (mock) or treated with ANDV inactivated by gamma irradiation as controls. Vandetanib (5 μM) was added immediately following 1 h adsorption of the virus and remained present during the entire course of the infection. At 24-hr post infection, cell lysates were analyzed by Western blotting for the expression of VE-cadherin. After detection of VE-Cadherin, blot were stripped and re-probed with actin antibody. Comparable results were obtained from at least three independent experiments.

in 1% Tween80 and sterile de-ionized water. Vandetanib treatment was initiated 5 days prior to infection and continued throughout the course of the experiment.

Animals were randomly assigned to 2 main arms of the study, an initial dose-escalation trial to determine overall drug efficacy and potential adverse effects among groups of 10 animals each given either 10, 25, 50, or 100 mg/kg doses, and a later serial euthanasia trial (25 mg/kg) to more closely monitor virus growth kinetics and indicators of disease progression. At necropsy, the pleural effusion present in the thoracic cavity of each animal was carefully collected and quantitated from all surviving animals and terminal cases. In addition, samples of lung, liver, spleen, blood, and other organs