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Feasibility and biological rationale of repurposing sunitinib and erlotinib for dengue treatment

Szu-Yuan Pu[a,1], Fei Xiao[a,1], Stanford Schor[a], Elena Bekerman[a], Fabio Zanini[c], Rina Barouch-Bentov[a], Claude M. Nagamine[d], Shirit Einav[a,b,*]

[a] Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University School of Medicine, Stanford, CA, USA
[b] Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA
[c] Department of Bioengineering, Stanford University, Stanford, CA 94305, USA
[d] Department of Comparative Medicine, Stanford University School of Medicine, Stanford, CA, USA

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ABSTRACT

There is an urgent need for strategies to combat dengue virus (DENV) infection; a major global threat. We reported that the cellular kinases AAK1 and GAK regulate intracellular trafficking of multiple viruses and that sunitinib and erlotinib, approved anticancer drugs with potent activity against these kinases, protect DENV-infected mice from mortality. Nevertheless, further characterization of the therapeutic potential and underlying mechanism of this approach is required prior to clinical evaluation. Here, we demonstrate that sunitinib/erlotinib combination achieves sustained suppression of systemic infection at approved dose in DENV-infected IFN-α/β and IFN-γ receptor–deficient mice. Nevertheless, treatment with these blood-brain barrier impermeable drugs delays, yet does not prevent, late-onset paralysis; a common manifestation in this immunodeficient mouse model but not in humans. Sunitinib and erlotinib treatment also demonstrates efficacy in human primary monocyte-derived dendritic cells. Additionally, DENV infection induces expression of AAK1 transcripts, but not GAK, via single-cell transcriptomics, and these kinases are important molecular targets underlying the anti-DENV effect of sunitinib and erlotinib. Lastly, sunitinib/erlotinib combination alters inflammatory cytokine responses in DENV-infected mice. These findings support feasibility of repurposing sunitinib/erlotinib combination as a host-targeted antiviral approach and contribute to understanding its mechanism of antiviral action.

1. Introduction

Dengue virus (DENV) is a global threat estimated to infect 390 million people annually (Bhatt et al., 2013). The majority of individuals infected with any of the four DENV serotypes present with acute dengue fever. A fraction (~5–20%) of dengue patients will progress to severe dengue manifested by bleeding, shock, organ failure, and death. The greatest risk factor for severe dengue is secondary infection with a heterologous DENV serotype causing antibody-dependent enhancement (ADE) (Katzelnick et al., 2017; Wang et al., 2017). ADE has also been hindering the development of effective dengue vaccines, with recent data indicating increased dengue severity in vaccinated children (Halstead and Russell, 2016). Moreover, there are currently no approved therapeutics for the treatment of dengue.

The current "one drug-one bug" antiviral approach is not easily scalable to address the unmet clinical need posed by emerging viruses and is often associated with emergence of drug resistance (Bekerman and Einav, 2015). Host-targeted antivirals have a potential for broad-spectrum coverage and a higher barrier to resistance (Bekerman and Einav, 2015). The host-targeted approach is particularly attractive for the treatment of emerging viral infections lacking any treatment, given the opportunity to repurpose already approved, safe drugs that modulate specific host functions (Schor and Einav, 2018).

One cellular process that is usurped by multiple viruses is intracellular membrane traffic, which relies, in part, on the interactions between adaptor protein (AP) complexes and transmembrane cargo. The host cell kinases AP2-associated protein kinase 1 (AAK1) and cyclin G–associated kinase (GAK) regulate clathrin-associated adaptor-cargo trafficking in the endocytic and secretory pathways (Ricotta et al., 2002; Umeda et al., 2000; Zhang et al., 2005), in part by recruiting AP2 and AP1, respectively, to relevant membranes and enhancing their binding affinity for cargo (Ghosh and Kornfeld, 2003; Ricotta et al.,

* Corresponding author. Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University School of Medicine, 300 Pasteur drive, Lane building, Rm L127, Stanford, CA, 94305, USA.
E-mail address: seinav@stanford.edu (S. Einav).

[1] Denotes equal contribution.

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2002). We and others have reported roles for APs in the lifecycles of multiple unrelated viruses (Bekerman et al., 2017; Bhattacharyya et al., 2011; Neveu et al., 2012, 2015; Ohno et al., 1997; Xiao et al., 2018). Our work demonstrated that through AP1 and AP2 phosphorylation, AAK1 and GAK regulate early and late stages of the viral lifecycle, thereby representing “master regulators” of viral infection (Bekerman et al., 2017; Neveu et al., 2012, 2015; Xiao et al., 2018). Moreover, we reported that sunitinib and erlotinib, approved anticancer drugs with potent binding to AAK1 and GAK (dissociation constant [KD] of 11 and 3.1 nM, respectively (Karaman et al., 2008)), block entry and late stages in the lifecycle of the 4 DENV serotypes and multiple viruses *in vitro* (Bekerman et al., 2017; Kovackova et al., 2015; Neveu et al., 2012, 2015; Xiao et al., 2018). Treatment with combinations of sunitinib and erlotinib revealed synergistic inhibition of DENV2 with a high genetic barrier to resistance (Bekerman et al., 2017). In murine models of dengue and Ebola, 5-day treatment with the individual drugs provided partial or no protection, yet, consistent with our *in vitro* synergy results, sunitinib/erlotinib combinations significantly protected mice from morbidity and mortality (Bekerman et al., 2017). Nevertheless, DENV-infected mice were monitored for 10–12 days only and relatively high drug doses were used. To support planned efforts to study the safety and efficacy of sunitinib/erlotinib combinations in dengue patients and potentially in Ebola patients in future outbreaks (ClinicalTrials.gov NCT02380625), it is therefore important to examine efficacy in preventing disease relapse, efficacy of lower dosing regimens, and antiviral effect in primary human cells. Moreover, whereas our mechanistic studies indicated that inhibition of intracellular viral trafficking mediates the anti-DENV effect of sunitinib and erlotinib, other mechanisms of antiviral action were incompletely characterized. Here, we integrate *in vitro*, *ex vivo* and *in vivo* data to address these gaps in knowledge and provide further evidence for the therapeutic potential of this repurposed approach and insight into its mechanism of action (MOA).

2. Materials and methods

2.1. Virus constructs

DENV2 (New Guinea C strain) *Renilla* reporter plasmid used for *ex vivo* assays was a gift from Pei-Yong Shi (Xie et al., 2013; Zou et al., 2011). DENV 16681 plasmid (pD2IC-30P-NBX) used for transcriptomics was a gift from Claire Huang (CDC) (Huang et al., 2010). Mouse-adapted N124D/K128E DENV2 PL046 used for *in vivo* studies was a gift from Sujan Shresta (LIAI).

2.2. Cells

Huh7 (Apath LLC), BHK-21 (ATCC), and T-REx 293 (ThermoFisher) cells were grown in DMEM (Mediatech) supplemented with 10% FBS (Omega Scientific), nonessential amino acids, 1% L-glutamine, and 1% penicillin-streptomycin (ThermoFisher Scientific) (5% CO_2, 37 °C). C6/36 cells were grown in Leibovitz's L-15 media (CellGro) supplemented with 10% FBS and 1% HEPES (0% CO_2, 28 °C).

2.3. Generation of MDDCs

(Rodriguez-Madoz et al., 2010). Buffy coats were obtained from the Stanford Blood Center. CD14$^+$ cells were purified from by EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion (Stemcell Technologies). Cells were seeded in 6-well plates (2×10^6 cells/well), stimulated with 500 U/ml G-CSF and 1000 U/ml IL-4 (Pepro tech), and incubated at 37 °C for 6 days prior to DENV infection (MOI 1).

2.4. Virus production

DENV RNA was transcribed *in vitro* by mMessage/mMachine (Ambion) kits and electroporated into BHK-21 cells. Supernatant was harvested at day 10 and titered by plaque assays on BHK-21 cells or used to inoculate C6/36 cells to amplify the virus. For mice studies, supernatant was concentrated 100-fold (50,000 × *g*).

2.5. In vivo dengue studies

AG B6 mice were generated by crossing C57BL/6.129S2-*Ifnar1*^{tm1Agt}/Mmjax (N10) with C57BL/6.129S7-*Ifngr1*^{tm1Agt}/J (N11). Age-matched, male and female mice at 8–10 weeks of age were used. Mouse adapted DENV2 (3×10^3 or 3×10^4 pfu produced in BHK-21 cells) was inoculated retro-orbitally under general and local anesthesia. Sunitinib (Selleckchem) and erlotinib (LC Laboratories) (both > 95% pure by mass spectrometry) were dissolved in 10% Captisol. 100–200 µl of drugs or vehicle were administered intraperitoneally once daily starting at inoculation for 5 days. Mice were monitored twice daily. Moribund animals were euthanized by CO_2 inhalation. Whole blood was obtained retro-orbitally under general and local anesthesia. Tissues were harvested following euthanasia.

2.6. Virus-inclusive single-cell RNA-Seq

As described (Zanini et al., 2018), Huh7 cells were infected with DENV at time 0 at MOI 0, 1, or 10, then harvested at different time points, sorted and lysed into single wells. Whole genome mRNA and viral RNA (vRNA) were reverse transcribed and amplified from each cell. Cells were then screened for virus infection by qPCR. Libraries were made and sequenced on an illumina NextSeq 500 with a coverage of ~400,000 reads per cell.

2.7. Gain-of-function assays

Doxycycline-inducible cell lines were established to overexpress AAK1 or GAK via the Flp-In™ recombination system (ThermoFisher) (Torres et al., 2009). T-REx293 cells with a pFRT/lacZeo site and pcDNA™6/TR were co-transfected with puromycin-resistant vector encoding Flp-In™ recombination target site and pOG44 plasmid containing Flp recombinase followed by puromycin selection. Eight hours post-induction with doxycycline, cells were treated with the drugs, infected with DENV (MOI = 0.01) and incubated for 72 h prior to luciferase and viability assays.

2.8. RNA extraction and quantification

RNA extraction and quantification of DENV, AAK1 and GAK RNA were performed as described (Bekerman et al., 2017).

2.9. Viability assays

AlamarBlue® reagent (Invitrogen) was used according to manufacturer's protocol. Fluorescence was detected at 560 nm on InfiniteM1000 plate reader (Tecan).

2.10. Luminex assay

Luminex assay (Stanford Human Immune Monitoring Center). Mouse 38-plex magnetic bead kits (eBioscience/Affymetrix) were added to a 96-well plate and washed in a Biotek ELx405 washer. Serum samples were added in duplicate and incubated at RT for 1 h followed by overnight incubation at 4 °C with shaking. Following washing, biotinylated detection antibody was added for 75 min at RT with shaking, followed by washing and addition of streptavidin-PE. After 30-minute incubation at RT and washing, reading buffer was added. Plates were read by Luminex 200. Custom assay control beads (Radix Biosolutions) were added to all wells.

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