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## 2016-2017 12-month Progress Report

### Treatment of Dengue Virus Infection With Repurposed Pharmaceuticals That Inhibit Cellular Autophagy

Karla Kirkegaard, Ph.D., Principal Investigator

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#### 1. Introduction

Dengue virus causes untreatable viral infections that are hazardous to our armed forces. As described in the original proposal for this funding, although spautin-1 inhibits the formation of infectious dengue viral particles in cultured cells, it is toxic to mice. With the goal of finding an existing compound for 'repurposing' against dengue virus, we collaborated with the laboratory of Vijay Pande (Stanford University), to identify compounds similar to spautin-1 that had already been tested for safety in humans. A list of compounds with structural similarity to spautin-1 was identified. After testing several of these compounds for inhibition, we had obtained preliminary results that one, vandetanib, inhibited dengue growth in tissue culture.

An our Statement of Work, written on 01/01/2016, we proposed three Specific Aims. Progress on these after 12 months is described below.

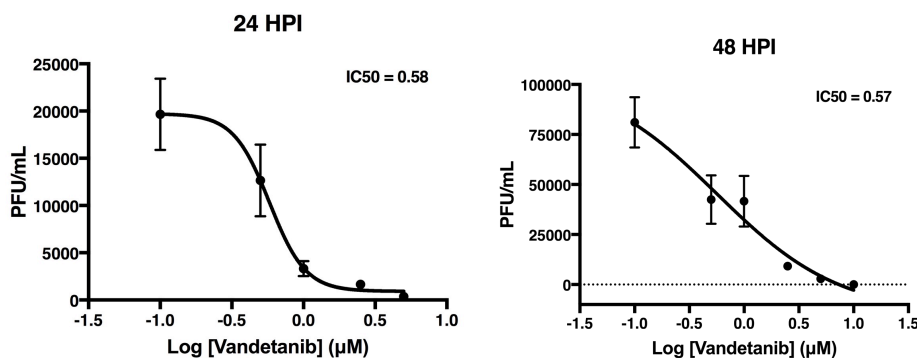
## 2. Keywords

Autophagy  
Dengue virus  
Repurposing  
Antiviral  
Starvation and disease susceptibility

## 3. Key Research Accomplishments (based on outline from Statement of Work)

Specific Aim 1: determine whether vandetanib is an inhibitor of dengue virus and obtain animal approval.

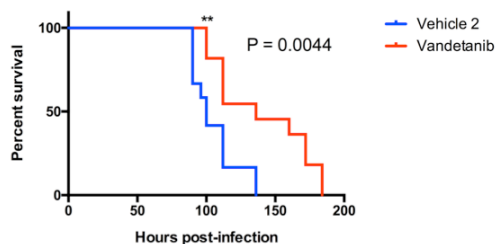
By 12 months, we completed Specific Aim 1, confirming that vandetanib was an inhibitor of dengue virus.



**Figure 1. Inhibition of dengue virus by vandetanib.**

A range of concentrations of vandetanib was tested for its inhibition of dengue virus at an MOI of .05 PFU/cell at 24 hours (left) and 48 hours (right) post-infection of Huh-7 cells.

Upon approval of our ACURO protocol, we were able to perform experiments in mice. In AG129 strains, although the route of treatment and dosing needs to be optimized, it is clear that oral vandetanib decreased the rate of mortality from dengue virus infection. Thus, vandetanib is a novel inhibitor of dengue virus whose identification was supported by this funding. This has been disclosed to the Intellectual Property Office at Stanford University.



**Figure 2. Inhibition of murine pathogenesis by vandetanib.**

C57Bl/6 mice were treated twice daily with vandetanib at a dose of 15 mg/kg.

Specific Aim 2: Approaches to determine the mechanism of vandetanib inhibition of dengue virus.

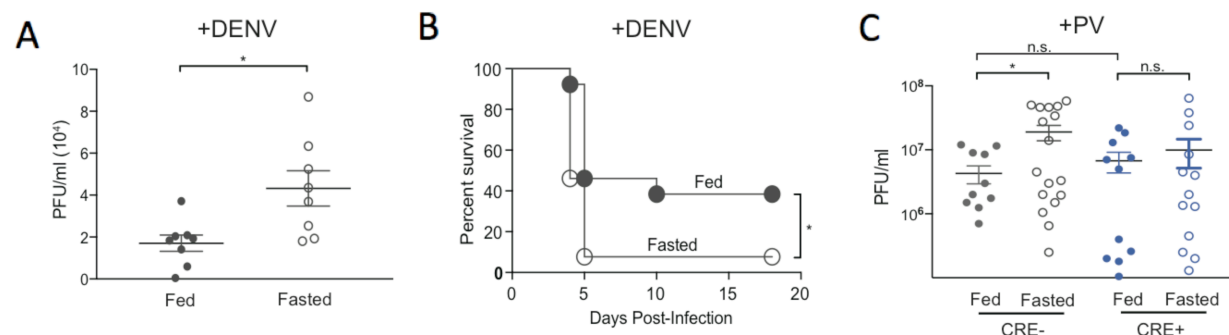
To approach the mechanism of vandetanib in a different way, we asked which components of the canonical autophagy pathway are used by dengue virus and, in addition, the closely related Zika virus. A panel of CRISPR knock-outs was generated in HeLa cells. Seven autophagy-related genes involved with distinct steps in the autophagy pathway, including the initiation complexes that either contain ULK1 and FIP200 or VPS34 and Beclin-1; lipid-scavenger ATG9; LC3, which is associated with autophagic membranes by conjugation to a lipid molecule; and ATG5, part of the complex LC3 conjugation system.

By 12 months, we constructed multiple ULK1, FIP200, VPS34, Beclin-1, ATG9, LC3 and ATG5 knockout lines in HeLa and Huh-7 cells, and were prepared to test them for their ability to support dengue virus infection.

We were unable to recover viral variants resistant to vandetanib or spautin-1. This is, of course, very good for the prospects of vandetanib as an anti-viral compound. However, the lack of drug-resistant variants takes away one avenue for target identification.

Specific Aim 3

For this Aim, we constructed mice for which it was possible to conditionally delete ATG5, required for conventional autophagy. We amplified this difficult-to-raise colony and prepared to test both the effect of ATG5 knockout on dengue growth and the effect of conventional autophagy on starvation-induced exacerbation of disease. As shown in Figure 3, fasting increases dengue virus growth, and now we were in a position to test whether this was a direct effect of the autophagy pathway, as we have found for poliovirus infection.



**Figure 3. Effect of 48-hour fasting on the growth and pathogenesis of dengue virus and poliovirus. (A)** C57BL/6 *Ifnar*<sup>-/-</sup> *Ifngr*<sup>-/-</sup> mice were either fed normally or fasted for 48 hours before infection with dengue virus. All were then fed normally and splenic titer was determined after four days. **(B)** AG129 mice, which show increased dengue pathogenesis, were or were not fasted for 48 hours before infection. Time of death or extreme morbidity was monitored for each group. **(C)** A similar increase in titer with pre-fasting was also observed during poliovirus infection in wild type mice that expressed the poliovirus receptor, but not in mice conditionally ablated for ATG 5.

## 5. Changes/Problems

- a. Our inability to select for vandetanib-resistant variants made it impossible to use such variants to determine target identification. Instead, we derived CRISPR/Cas9-mediated knock out lines for autophagy genes in both HeLa and Huh-7 cells. In this way, we could show that the inhibition of vandetanib on dengue virus growth in cultured cells is VPS34-dependent.
- b. Vandetanib is currently used in end-stage cancer treatment, where side effects are more tolerable than other cases. We will need to work to balance the toxicity of this molecule with its efficacy. We would like to work with its developer to test molecules that are similar in structure for their efficacy on this new target.
- c. During the course of this work, the impact of Zika virus became apparent. Thus, we will use our knockout lines to investigate the dependence of Zika virus on the autophagy pathway.

## 6. Reportable Outcomes

The Stanford Office of Technology and Licensing has been approached with respect to the patentability of the inhibition of dengue virus pathogenesis by vandetanib, and an official disclosure filed.

The preparation of the following manuscripts has been initiated.

Emma Abernathy, Roberto Mateo, Karim Mazjoub, Nick van Buuren, Claude Nagamine, Jan Carette and Karla Kirkegaard (2018). Differential utilization of autophagy components by positive-strand RNA viruses. In preparation

David Constant, Bowen Liu, Roberto Mateo, Steven Kearnes, Paul Novick, Karim Majzoub, Ritica Prasad, Claude Nagamine, Jan Carette, Vijay Pande and Karla Kirkegaard (2018). A targeted computational screen of the SWEETLEAD database for inhibitors of dominant drug targets reveals FDA-approved compounds with anti-flaviviral activity. In preparation.

## 7. Conclusions and impact

We have identified a new lead compound for the inhibition of dengue virus infection. Vandetanib inhibits dengue virus in a VPS34-dependent mechanism that appears to be gain-of-function, as it is more inhibitory than the loss of this protein. In the process, we have shown that vandetanib, or similar molecules, will also be promising compounds for Zika infection. For Zika, inhibitors of ULK1 kinase, involved in a different branch of autophagy signaling, will also be promising.

If infectious disease is exacerbated by induction of the autophagy pathway, then starvation and many over-the-counter drugs that induce autophagy, can lead to greater disease severity. *Perhaps even more relevant to our military is the ability of exercise to promote autophagy in affected tissues: during the poliovirus epidemics, for example, one of the few variables that correlated with paralytic disease was athleticism.* Experiments to test whether supplementation of simple nutrients, those that signal the induction of autophagy, can block viral infection in scenarios such as those shown in Figure 2 will be an additional component of these studies.

## 8. Participants & Other Collaborating Organizations

All participants were employees of Stanford University:

Karla Kirkegaard, Ph.D.

Claude Nagamine, M.D., Ph.D.

Roberto Mateo, Ph.D.

David Constant, Ph.D.

**8. References**  
Not applicable.

**9. Appendices**  
Not applicable.