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Short Communication

A potent prolyl tRNA synthetase inhibitor antagonizes Chikungunya and Dengue viruses

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ABSTRACT

Arboviruses represent a group of pathogens that can spread efficiently throughout human populations by hematophagous arthropod vectors. The mosquito-borne (re)emerging Chikungunya and Dengue viruses belong to the alphavirus and flavivirus genus, respectively, with no approved therapeutics or safe vaccines for humans. Transmitted by the same vector *Aedes* spp., these viruses cause significant morbidity and mortality in endemic areas. Due to the increasing likelihood of co-circulation and co-infection with viruses, we aimed to identify a pharmacologically targetable host factor that can inhibit multiple viruses and show that a potent antagonist of prolyl tRNA synthetase (halofuginone) suppresses both Chikungunya and Dengue viruses. Host tRNA synthetase inhibition may signify an additional approach to combat present and future epidemic pathogens.

Arthropod-borne pathogens are spread from blood-feeding invertebrates to host vertebrate species, and mosquitos constitute a major vector group responsible for transmission of arboviruses. Chikungunya virus (CHIKV) and Dengue virus (DENV) are positive-sense RNA viruses in the alphavirus and flavivirus genera, respectively, that are spread by *Aedes* spp. mosquitos (Higgs and Vanlandingham, 2015; Shaw and Catteruccia, 2018). Both cause fever, rash, headaches, and general malaise, but CHIKV can cause chronic joint inflammation and arthritis-like symptoms beyond the acute phase (Schwartz and Albert, 2010), while DENV can lead to hemorrhage and death (Wiwanitkit, 2010).

CHIKV had been confined to Africa and Asia, but in the past decade, the virus has spread to the western hemisphere to cause widespread epidemics (Burt et al., 2017). The typical vector responsible for transmission of CHIKV was *Aedes aegypti*, but a mutation in the viral envelope led to enhanced replication in the *Aedes albopictus* mosquitos present in temperate regions, potentially expanding the range of transmission (Tsetsarkin et al., 2007). CHIKV-induced arthropathy is due to viral replication and the ensuing host inflammatory response (Suhrbier et al., 2012), including inflammasome activation (Chen et al., 2017) and T-cell mediated pathogenesis (Amdekar et al., 2017).

DENV has four serotypes, and antibody-dependent enhancement caused by cross-reactive but sub-neutralizing antibodies has hampered the development of safe vaccines that are equipotent against all four serotypes (Acosta and Bartenschlager, 2016a; Rey et al., 2018). In 2010, there were an estimated 96 million symptomatic cases of dengue infection worldwide, and a significant portion of the human population, especially in the tropics, remains at risk (Bhatt et al., 2013). Unlike CHIKV, normal clinical sequelae of DENV includes mortality.

For both CHIKV and DENV, there are no approved therapeutics aside from palliative care and pain reduction (Fischer et al., 2014; Lee et al., 2017), although many direct virus targeting compounds have been investigated (Boldescu et al., 2017; Powers, 2018). Due to a potent innate immune response and sterilizing immunity, the acute phase of CHIKV and DENV infection is relatively short compared to the duration of chronic infection by other viruses, such as HIV or hepatitis C virus, so the risk of developing escape mutants to a given antiviral compound is lessened. However, direct virus-targeting pharmacophores may be less effective against multiple strains of viruses, or even ineffective across genera. In an epidemic setting where multiple arboviruses might co-circulate, it would be advantageous to find a means to inhibit viruses from different families. Furthermore, homotypic reinfections with genetically similar DENV have been documented (Forshey et al., 2016), suggesting natural infection or vaccination may not offer complete protection in all cases.

Host-targeting antivirals are another class of therapeutics. For instance, metabolism, subcellular trafficking, and cytoskeletal organization are avenues that can be discretely manipulated to hinder various steps of the viral life cycle (Acosta and Bartenschlager, 2016b; Ching

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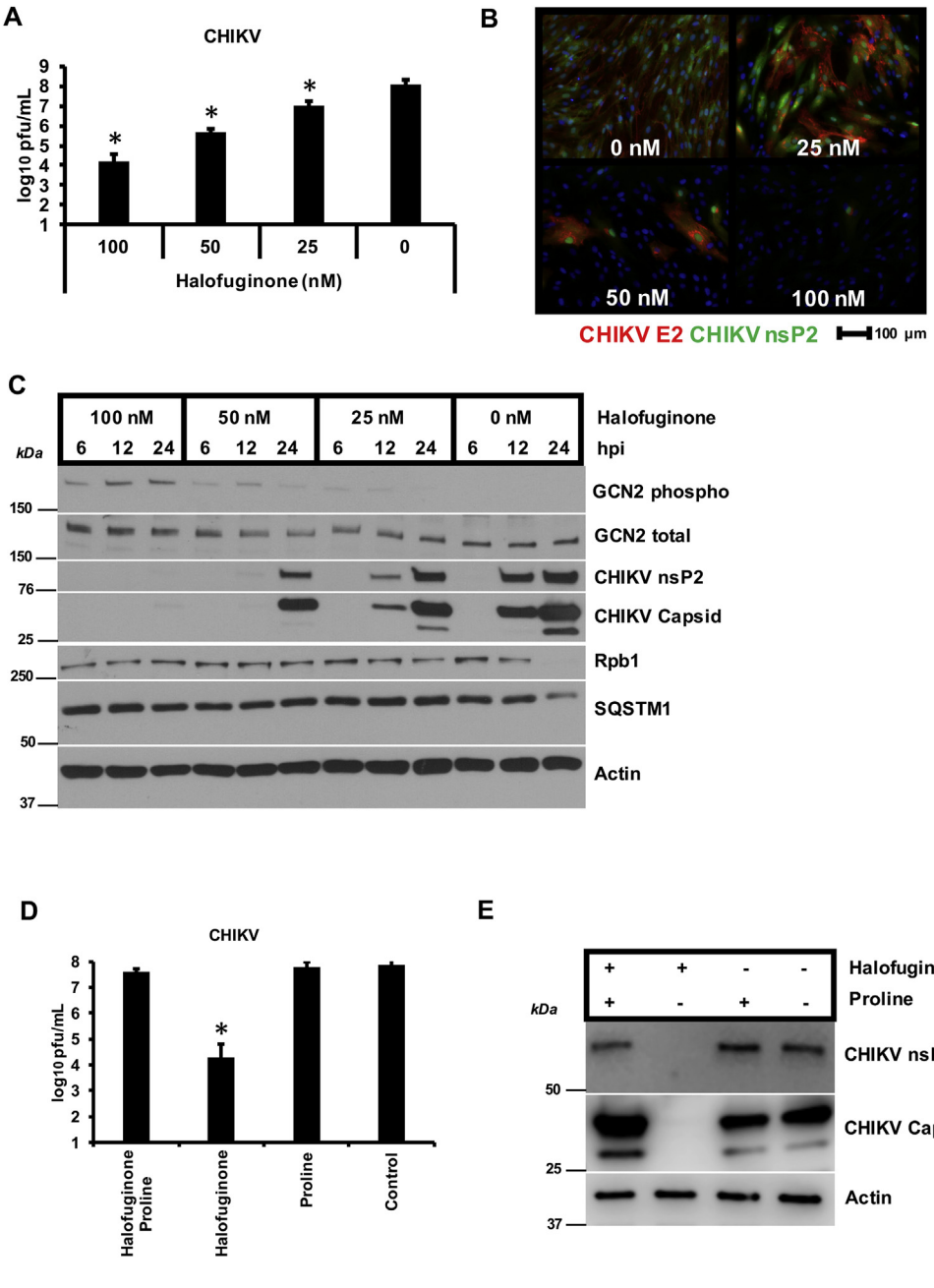

Fig. 1. Halofuginone inhibits CHIKV replication. (A) HFFs were infected with CHIKV (MOI 1) and treated with indicated concentrations of halofuginone at 1 hpi. Supernatants were titered at 24 hpi. (B) Similar to (A) except cells were processed for indirect immunofluorescence to detect CHIKV E2 (red) and CHIKV nsP2 (green). Nuclei are stained with Hoechst. (C) HFFs were infected with CHIKV (MOI 1) and treated with halofuginone at 1 hpi. Whole-cell lysates were harvested at 6, 12, and 24 hpi for western blot analysis. (D) HFFs were infected with CHIKV (MOI 1) and treated with halofuginone (100 nM) or proline (4 mM). Supernatants were titered at 24 hpi. (E) Similar to (D) except whole-cell lysates were subjected to western blot analysis. Values are the means ± standard deviation (SD). Significant values are defined by $^{*}P < 0.05$.

et al., 2017; Dohner and Sodeik, 2005; Pombo and Sanyal, 2018). Aside from the decreased chances of emergence of resistance compared to a pathogen-specific approach, host-targeting compounds have the potential to simultaneously inhibit multiple viruses, provided that the host pathway is an essential component of the replication strategies (Lou et al., 2014). In pragmatic terms, host-targeting compounds designated to be safe may be advantageous in the event of an outbreak caused by a previously uncharacterized virus where vaccines would not be readily available.

Viruses must hijack the host translational machinery to synthesize viral proteins (Hoang et al., 2018). For this reason, a common host response to viral infection is shutting down translation, such as through protein kinase R activation and inhibition of translation initiation (Garcia et al., 2007). Another endogenous pathway that controls translation initiation is the amino acid response. When the intracellular concentration of one or more amino acids is low, the cognate tRNA remains uncharged and binds to GCN2 kinase (also known as EIF2AK4) (Dong et al., 2000). The phosphorylated, active GCN2 then blocks translation initiation in a manner similar to protein kinase R (Pakos-Zebrucka et al., 2016).

Halofuginone is a potent, orally-available synthetic derivative of the plant compound febrifugine (Pines et al., 2000). By binding to and inhibiting the prolyl tRNA synthetase domain of the bifunctional enzyme EPRS (glutamyl-prolyl-tRNA synthetase), halofuginone causes buildup of uncharged prolyl tRNAs, forcing the cell to trigger the amino acid response and to suppress bulk translation even when proline levels are sufficient (Keller et al., 2012; Yao and Fox, 2013).

To determine the effect of EPRS inhibition on CHIKV replication, we chose to test human foreskin fibroblasts (HFFs) because fibroblasts are physiologically relevant *in vivo* targets (Couderc et al., 2008). HFFs were infected with CHIKV and treated with different concentrations of halofuginone starting at 1 hpi. Supernatants were harvested and cells were fixed with 2% paraformaldehyde at 24 hpi. Halofuginone exhibited dose-dependent inhibition of viral progeny production (Fig. 1A), leading to an approximately 3 $\log_{10}$ fold reduction at 100 nM. Likewise, indirect immunofluorescence assay to visualize viral proteins

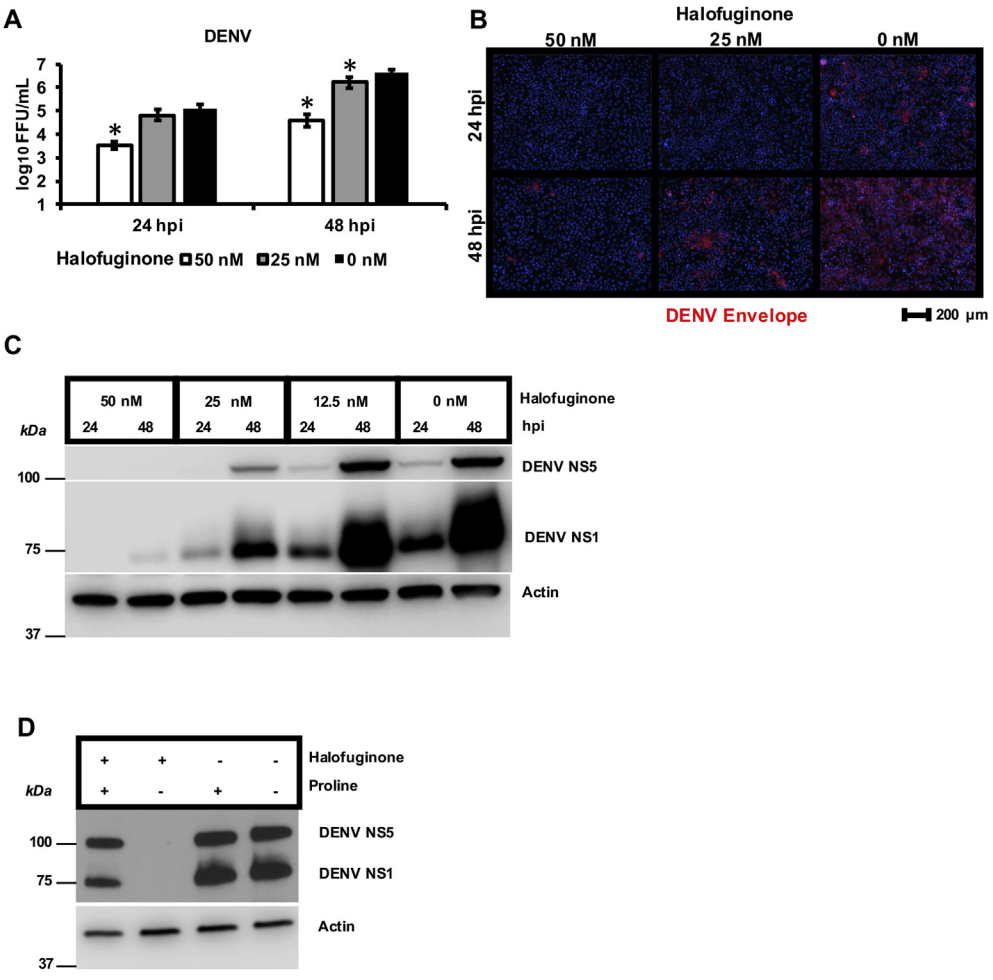

Fig. 2. Halofuginone inhibits DENV replication. (A) Huh7 cells were infected with DENV (MOI 0.5) and treated with halofuginone at 1 hpi. Supernatants were titered at 24 and 48 hpi. (B) Similar to (A) except infected cells were detected by indirect immunofluorescence assay using pan-flavivirus monoclonal antibody (clone 4G2). (C) Huh7 cells were infected with DENV (MOI 0.5) and treated with halofuginone. Protein samples were analyzed at 24 and 48 hpi to detect viral protein accumulation. (D) Similar to (C) except cells were treated with halofuginone (50 nM) or proline (4 mM). Protein samples were harvested at 48 hpi for detection of DENV NS5 and NS1. Values are the means ± standard deviation (SD). Significant values are defined by $^{*}P < 0.05$.

E2 and nsP2 showed dose-dependent reduction in replication-permissive cells (Fig. 1B). Pretreatment with halofuginone for 16 h led to even greater inhibition of viral titer (results not shown), but we performed all subsequent studies with posttreatment alone. There was no cytotoxicity as assessed by LDH release assay (Supplemental Fig. 1).

Western blot analysis also showed that halofuginone dose-dependently inhibited viral protein synthesis. HFFs were infected as described above, and whole-cell lysates were harvested at 6, 12, and 24 hpi. As expected, halofuginone treatment led to an increase in phosphorylated GCN2 with maximal induction at 100 nM (Fig. 1C), consistent with accumulation of uncharged prolyl tRNA. Both the early non-structural nsP2 protein and late structural capsid protein were repressed by halofuginone, and as a consequence, Rpb1, which is normally degraded by nsP2 in order to cause host transcriptional shutoff (Akhrymuk et al., 2012), remained steady. SQSTM1/p62, the receptor for proteins targeted for autophagosomal degradation, such as CHIKV capsid (Judith et al., 2013), also remained stable in the presence of halofuginone, whereas DMSO treated cells showed gradual downregulation of SQSTM1/p62. Thus, halofuginone efficiently inhibited CHIKV replication at a relatively early step.

Halofuginone binds to EPRS, and proline, the endogenous substrate of EPRS, should compete with halofuginone for interaction. To confirm that the antiviral effect of halofuginone is due to competitive disruption of aminoacylation, infected HFFs were treated with halofuginone or DMSO in the presence or absence of excess proline. Supernatant titration at 24 hpi showed that excess proline completely rescued the defect in virus replication (Fig. 1D), and the rescue was further verified by western blot analysis of CHIKV nsP1 and capsid levels (Fig. 1E).

DENV is another globally prevalent arbovirus. To determine whether halofuginone is effective against this flavivirus, we infected the permissive Huh7 cell line (Lin et al., 2000) and observed that the compound also led to dose-dependent inhibition of viral progeny release into the culture media (Fig. 2A). Indirect immunofluorescence assay showed that treated cells could not form fluorescent foci, indicative of failure to spread cell-to-cell proficiently (Fig. 2B). Viral protein levels were drastically reduced after exposure to halofuginone (Fig. 2C), including the NS1 protein that is responsible for vascular leakage during severe disease (Beatty et al., 2015). Similar to what was observed with CHIKV, proline co-treatment permitted DENV protein synthesis (Fig. 2D). These results show that halofuginone inhibits both arboviruses through EPRS inhibition. We tested additional arboviruses and confirmed that halofuginone also inhibited replication of O'nyong'nyong virus (ONNV), an alphavirus closely related to CHIKV, and Zika virus (ZIKV) in co-infected HFFs (Fig. 3), which were infected at a higher MOI due to lower susceptibility.

Although halofuginone is active against both virus families, targeting multiple host pathways may lead to even greater antiviral effects. Interferon signaling is an endogenous mechanism to hinder viral replication, and although injected PEGylated interferon has been utilized in different pathogenic settings (Palumbo, 2009), such an approach may not be practical in areas with less developed healthcare infrastructure. In contrast, RO8191 is a synthetic, orally-available IFNAR2 agonist that engenders an antiviral state like *bona fide* interferon, and importantly, this compound induces interferon stimulated genes (ISGs) without significant elevation of pro-inflammatory genes (Konishi et al., 2012).

To evaluate whether halofuginone can be used in combination with RO8191, we first confirmed the efficacy of the compound in our system

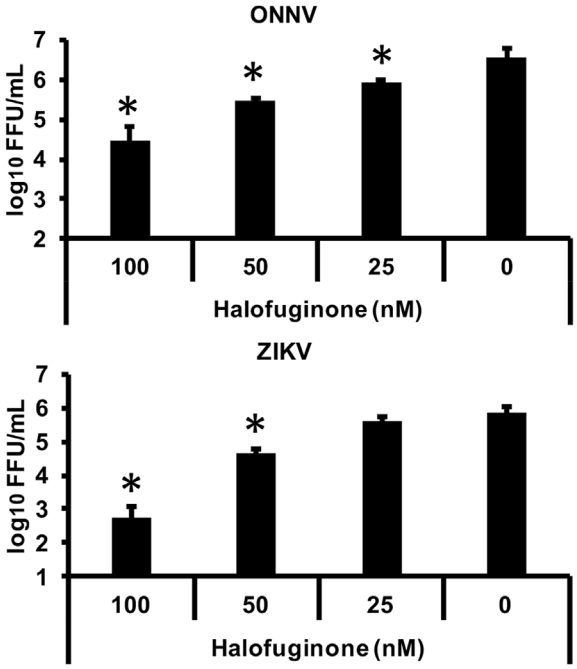

Fig. 3. Effect of halofuginone on co-infection with related viruses. HFFs were co-infected with ONNV (MOI 10) and ZIKV (MOI 10) and treated with halofuginone at 2 hpi. Supernatants were titered at 24 hpi. Fluorescent focus units (FFU) of ONNV and ZIKV were measured by using cross-reactive monoclonal antibody against CHIKV E2 and pan-flavivirus monoclonal antibody, respectively. Values are the means ± standard deviation (SD). Significant values are defined by $*P < 0.05$.

and showed that RO8191 dose-dependently increased STAT1 and ISG15 levels (Fig. 4A, C). Next, we pretreated HFFs or Huh7 cells with the lowest dose of RO8191 (0.33 μM) or DMSO for 24 h and then infected HFFs with CHIKV or Huh7 cells with DENV. After 1 h post infection, cells were washed and incubated with or without halofuginone in the absence of any RO8191. Viral titration at 24 hpi showed that while each compound inhibited replication individually, successive exposure to both led to an even greater effect (Fig. 4B, D). Co-treatment of compounds post-infection also displayed augmented effects, although total efficacy was less (Supplemental Fig. 2). These results show that even if a single compound fails to reach maximal effective dose, targeting multiple pathways consecutively may still yield protective antiviral effect.

CHIKV and DENV continue to pose a significant challenge for multiple reasons. There is a lack of safe vaccine against the four serotypes of DENV due to complications of antibody-dependent enhancement (Acosta and Bartenschlager, 2016a), and even for CHIKV which has a single serotype (Sahadeo et al., 2015), there is reduced impetus for human vaccine clinical trials, with one factor being that CHIKV is usually not lethal and infection leads to protective immunity.

Another complicating factor that delays response to an arbovirus epidemic is unpredictability of emergence. CHIKV acquired mutations that allowed replication in a mosquito species that previously exhibited low vector competence (Tsetsarkin et al., 2016), broadening the potential range of transmission. Although this particular virus lineage was not responsible for the large outbreaks in the Americas (2013–2015), the threat continues to persist in the Pacific, Southeast Asia, and Latin America (Pyke et al., 2018). Most recently, the formerly obscure Zika virus caused a large global epidemic. Two key mutations in the viral genome have been identified, which increased virus acquisition by *Aedes* mosquitos (NS1 mutation) (Liu et al., 2017) and enhanced infection of neural progenitor cells to contribute to fetal microcephaly (prM mutation) (Yuan et al., 2017).

Thus, every arbovirus has unique biological and practical elements that need to be taken into account to limit spread, and this is compounded by increased likelihood of co-circulation and co-infection of pathogens. Because viruses require the host cell resources for replication, therapeutically targeting a common essential factor could be beneficial to inhibit multiple viruses even if their replication strategies are otherwise divergent.

Host translational machinery is an absolute requirement for virus production, and one way to inhibit translation is to activate an endogenous mechanism that suppresses global protein synthesis. GCN2 is a sensor of uncharged tRNAs which accumulate during times of amino acid deprivation, and this kinase shuts down bulk translation to prevent

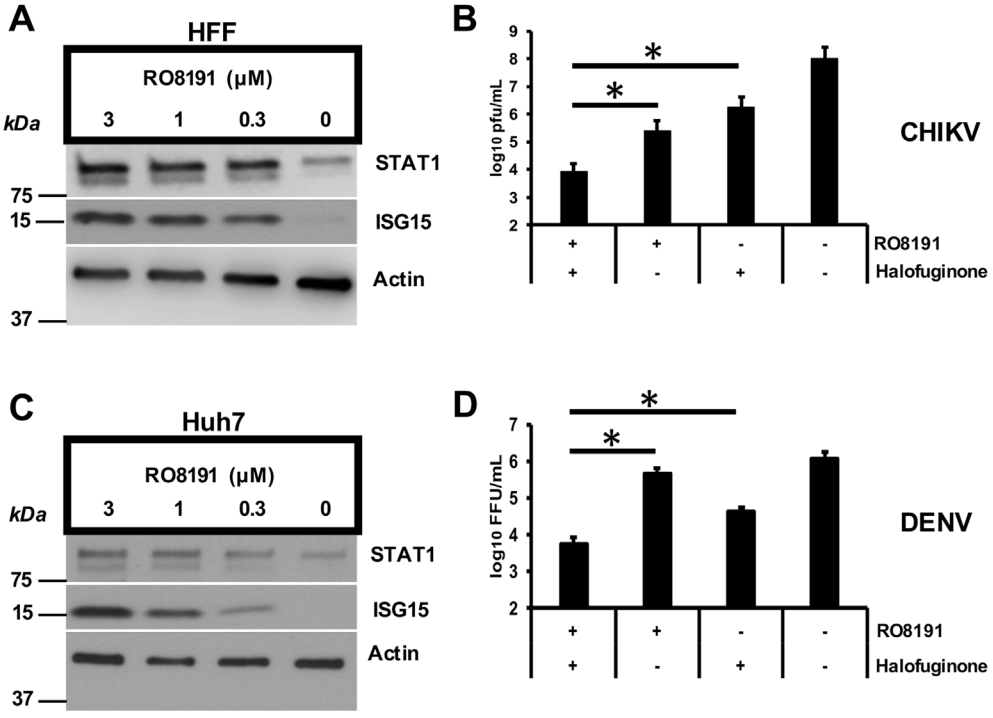

Fig. 4. Synergistic activity of halofuginone with IFNAR2 agonist. (A) HFFs were treated with RO8191 for 24 h, and protein samples were immunoblotted against interferon stimulated genes. (B) HFFs were pretreated 24 h with RO8191 (0.33 μM) or DMSO and inoculated with CHIKV (MOI 1). After 1 h viral adsorption period, cells were washed and replaced with media containing halofuginone (50 nM) or DMSO. Protein samples and supernatants were harvested at 24 hpi. (C) Similar to (A) except Huh7 cells were treated with RO8191 for 24 h. (D) Similar to (B) except Huh7 cells were infected with DENV (MOI 0.5). Values are the means ± standard deviation (SD). Significant values are defined by $*P < 0.05$.

wasteful energy expenditure (Dong et al., 2000). In fact, limitation of a single amino acid can activate GCN2 (Peng et al., 2012).

We tested halofuginone, an inhibitor of EPRS (Keller et al., 2012), against CHIKV and DENV because it is potent and orally-available, which may be practical criteria in clinical settings. Halofuginone indeed inhibited both alphaviruses and flaviviruses in a proline-dependent manner (Figs. 1–3). It was previously shown that GCN2 knockout cells and mice were more susceptible to Sindbis virus replication (Berlanga et al., 2006), but thus far, pharmacological activation has not been tested against mosquito-borne viruses. We demonstrate that a single compound that prevents aminoacylation inhibits RNA viruses of epidemic potential, and it is likely that halofuginone would inhibit replication of other emerging arboviruses, such as those spread by ticks (e.g., tick-borne encephalitis virus or Powassan virus).

Halofuginone can also exert anti-inflammatory effects. Recently, it was shown that treatment of murine macrophages with halofuginone leads to sequestration of IL-1β mRNA into translationally inactive riboclusters, thereby suppressing the inflammasome (Battu et al., 2018). Since inflammasome activation plays a significant role in CHIKV pathogenesis (Chen et al., 2017), halofuginone may be efficacious against both viral replication and the subsequent arthralgia-inducing host inflammation. Furthermore, halofuginone downregulates NF-κB signaling pathway (Luo et al., 2018), which contributes to DENV-induced hemorrhage (Lin et al., 2014). Interestingly, both protein-deficient diet and halofuginone protected against ischemia reperfusion injury, leading to improved organ function concomitant with lower inflammatory markers (Peng et al., 2012). It remains possible that modifying dietary protein intake or composition can ameliorate virus-induced inflammation and shock.

Although inhibiting host translation may lead to toxic effects, halofuginone and similar derivatives have been shown in murine models to inhibit malaria (Herman et al., 2015), reduce cardiac stress (Qin et al., 2017), suppress viral myocarditis (Sun et al., 2016), and improve hepatitis B virus-induced liver inflammation (Zhan et al., 2017). Furthermore, commercial preparations of halofuginone (Stenorol and Halocur) are used in livestock to inhibit parasite growth (Pines and Spector, 2015), and a clinical trial for a slow-release form of halofuginone has been approved by the FDA to treat Duchenne muscular dystrophy (Pines and Spector, 2015). Thus, with proper dosing or synergy with other antiviral compounds, such as an orally-available interferon agonist (Fig. 4), targeting host aminoacylation may be an effective strategy against arboviruses that gain a foothold in naïve human populations.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Contributions

J.H., E.F.: conceived and designed the project. J.H., A.J.: performed the experiments. J.H. analyzed the data. J.H., E.F.: drafted and revised the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.11.017.

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