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Small molecule grp94 inhibitors with antiviral activity against Dengue and Zika virus

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Abstract

Two major flaviviruses, dengue virus (DENV) and Zika virus (ZIKV), cause severe health and economic burdens worldwide. Recently, genome-wide screenings have uncovered the importance of the Hrd1 ubiquitin ligase-mediated endoplasmic reticulum (ER)-associated degradation (ERAD) pathway for flavivirus replication in host cells. Here we report the identification of the compound Bardoxolone methyl (CDDO-me) as a potent inhibitor of the Hrd1 ubiquitin ligase-mediated ERAD, which possesses a broad-spectrum antiviral activity against both DENV and ZIKV. Cellular thermal shift assay (CETSA) suggested that CDDO-me binds to grp94, a key component of the Hrd1 pathway, at a low nanomolar concentration, whereas interaction was not detected with its paralog Hsp90. CDDO-me and the grp94 inhibitor PU-WS13 substantially suppressed DENV2 replication and the cytopathic effects caused by DENV and ZIKV infection. The antiviral activities of both compounds were demonstrated for all four DENV serotypes and four ZIKV strains in multiple human cell lines. This study defines grp94 as a crucial host factor

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for flavivirus replication and identified CDDO-me as a potent small molecule inhibitor of flavivirus infection. Inhibition of grp94 may contribute to the antiviral activity of CDDO-me. Further investigation of grp94 inhibitors may lead to a new class of broad-spectrum anti-flaviviral medications.

Keywords

ERAD; CDDO-me; Dengue virus; Zika virus; grp94; Antiviral

Introduction

Dengue virus (DENV) and Zika virus (ZIKV) (family *Flaviviridae*, genus *Flavivirus*) are pathogens borne by *Aedes* mosquitoes and disseminated worldwide. There are approximately 390 million cases of human DENV infections each year and the infections cause a range of symptoms from mild fever to dengue hemorrhagic fever with a mortality rate of 2–5% (Bhatt et al., 2013; Morra et al., 2018; Schaffner and Mathis, 2014; Horstick et al., 2014). ZIKV infection, which typically is asymptomatic or only causes mild symptoms, became a global health emergency in recent years due to its unprecedented high prevalence in several regions and its association with severe neurological complications including catastrophic microcephaly in newborns and Guillain-Barre syndrome in adults (Solomon and Mallewa, 2001; Pyke et al., 2014; Tappe et al., 2014; Rothan et al., 2019).

Currently there is no approved vaccine for ZIKV. The recently approved vaccine for DENV has regional and age based restrictions due to the limitation of vaccine efficacy and the potential deadly side effects (Hueston et al., 2017; Castanha et al., 2017; Dejnirattisai et al., 2016; Rothan et al., 2018). Moreover, epidemics of ZIKV infections have mostly occurred in the DENV endemic areas and there is evidence to show co-infection of these two flaviviruses, which makes vaccine development even more complicated (Shan et al., 2018; Dejnirattisai et al., 2016; Rothan et al., 2018). There are currently also no approved antiviral drugs specific for treatment or prophylaxis of either DENV or ZIKV infection. Traditionally antivirals are developed to target viral pathogens directly and specifically. However, antivirals that target host cell components that are essential for viral infection or replication represent an alternative approach (Plummer et al., 2015; Barrows et al., 2016; Boldescu et al., 2017; Scaturro et al., 2018). Host factor-targeted antivirals would address not only two limitations associated with vaccines: 1) evasion of immunity caused by viral mutations (Schein et al., 2005) (Chiappelli et al., 2014; Maillard et al., 2014; Silveira et al., 2016; Chang et al., 2016; Sulczewski et al., 2018), and 2) DENV and ZIKV co-infection (Shan et al., 2018; Dejnirattisai et al., 2016; Rothan et al., 2018), as these flaviviruses exhibit the same lifecycle progression and require similar host factors (Gerold et al., 2017; Wang and Zhang, 2017; Puschnik et al., 2017). Thus, targeting the shared host factors would have a broad-spectrum of anti-flavivirus activity in co-infected patients (Boldescu et al., 2017).

Flaviviruses use the endoplasmic reticulum (ER) for viral proteins production and new virion assembly (Romero-Brey and Bartenschlager, 2016). Recently, genome-scale RNAi and CRISPR/Cas9 screenings have identified many host factors that are required for DENV,

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West Nile Virus (WNV), and ZIKV replication (Krishnan et al., 2008; Mairiang et al., 2013; Ma et al., 2015; Zhang et al., 2016; Marceau et al., 2016; Marceau et al., 2016; Boldescu et al., 2017; Scaturro et al., 2018), including proteins in the Hrd1 complex (Krishnan et al., 2008; Mairiang et al., 2013; Ma et al., 2015; Scaturro et al., 2018). The Hrd1 complex mediates a protein quality control mechanism in the ER by which misfolded proteins are dislocated from the ER lumen to the cytosol for degradation by the proteasome, a process known as ER-associated degradation or ERAD (Vembar and Brodsky, 2008). Although how the Hrd1 complex is involved in flavivirus replication is currently not understood, its essential role in flaviviral replication suggests that it is an attractive target for developing broad spectrum anti-flaviviral agents. In this study, we identified a small molecule CDDO-me that inhibits ER-to-cytosol protein dislocation and has broad-spectrum anti-flaviviral activities in vitro. Furthermore, we identified grp94 (Christianson et al., 2008), an ER luminal chaperone and a critical component of the Hrd1 ubiquitin ligase complex, as a potential new target for CDDO-me. CDDO-me and the grp94 inhibitor PU-WS13 (Patel et al., 2013) exhibited potent antiviral activities against both DENV and ZIKV replications at low nanomolar concentrations and protected human cells from viral cytopathic effects.

Materials and Methods

Viruses, cells, antibodies, and other materials

All viruses were obtained from Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA, USA). Monkey plasmas neutralizing DENV or ZIKV were provided by Dr. Gregory Gromowski, Viral Diseases Branch, Walter Reed Army Institute of Research. *Aedes albopictus* mosquito C6/36 cells (ATCC CRL-1660), African green monkey kidney epithelial Vero cells (CCL-81), human embryonic kidney HEK-293 cells (CRL-1573), SH-SY5Y cells (CRL-2266), U-251 MG cells (HBT-17), and HeLa cells (CCL-2) were obtained from ATCC (Manassas, VA, USA). Huh-7 cells were provided by Dr. Hongbing Wang, University of Maryland School of Pharmacy. All cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as growth medium or 2% FBS as maintenance medium. Bortezomib (BTZ), CDDO-me, and PU-WS13 were purchased from Sigma-Aldrich (St. Louis, MO, USA), Adooq Biosciences (Irvin, CA, USA), EMD Chemicals (San Diego, CA, USA), respectively. The sources for antibodies are: Anti-grp94 antibody (Affinity Bioreagents, Golden, CO, USA), anti-OS9 antibody (Thermo Fisher Scientific, Waltham, MA, USA), Anti-Hsp70 and anti-Tubulin antibodies (Enzo Life Sciences, Farmingdale, NY, USA), anti-Hsp90 and anti-BiP antibodies (BD Biosciences, San Jose, CA, USA), Anti-DENV2 E protein and anti-DENV2 NS3 protein antibodies (GeneTex, San Antonio, TX, USA).

Live-cell imaging and quantification of drGFP

Live-cell imaging of protein dislocation was performed using the previously developed drGFP assay (Zhong and Fang, 2012). Briefly, drGFP HeLa cells reporting NHK dislocation (NHK-drGFP cells) were seeded in chamber slides (177402 Lab-Tak). After overnight culture, the cells were washed once with PBS and then treated with BTZ (1 μM) alone or BTZ (1 μM) together with CDDO-me (1 μM). Live cell images were acquired immediately after addition of the inhibitors and then every 30 min under a 40x objective lens mounted on

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a Nikon TRE fluorescence microscope equipped with a high-sensitivity CCD camera (QuantEM 512SC, Photometrics, Tucson, AZ), environment control units and an Autofocus module.

Cellular Thermal Shift Assay (CETSA)

This assay was performed as previously described (Martinez Molina et al., 2013; Martinez Molina and Nordlund, 2016). In brief, cells were trypsinized and washed with PBS. The cell lysates were diluted with appropriate buffer and divided into aliquots that were treated with CDDO-me (1 μM). After 30 min incubation at room temperature, the respective lysates were divided into smaller (50 μL) aliquots and heated individually at different temperatures for 3 min (Veriti Thermal Cycler, Applied Biosystems, Foster City, CA, USA) followed by cooling for 3 min at room temperature. The heated lysates were centrifuged at 20,000 × *g* for 20 min at 4°C to separate the soluble fractions from precipitates. The supernatants were transferred to new tubes and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting.

Immunoblotting analysis

A previously reported protocol was used (Zhong and Fang, 2012). Briefly, equal amounts of protein samples were separated by SDS-PAGE and transferred onto PVDF membrane. Non-specific binding sites were blocked with 5% milk for 1 hr at room temperature followed by incubation with the specific primary antibody. Blots were incubated with the HRP-conjugated secondary antibody (1:5000) for 1 hr. ECL Immunoblotting Substrate (Thermo Scientific Pierce) was used to detect membrane-bound antibodies. Immunoblots were imaged by Fluorchem M System (ProteinSimple, San Jose, CA, USA).

Virus infection and titration

Huh-7 cells were seeded at 3×10^5 cell/well in 12-well plates for 24 hrs using DMEM media containing 2% FBS. Then, the cells were infected with either DENV2 or ZIKV at various multiplicity of infection and treated with the test compounds. The culture supernatant was collected at 48 hrs post-infection, and plaque assay was used to measure virus titer as we previously described (Rothan et al., 2014; Rothan et al., 2016). In brief, a 10-fold serial dilution of culture supernatant collected from virus-infected cells was added to fresh Vero cells grown in 6-well plates (5×10^5 cells) and incubated for 1 hr at 37°C. The cells were overlaid with DMEM (maintenance medium) containing 0.5% agarose. Viral plaques were stained with crystal violet dye after a 5-day incubation. Virus titers were calculated according to the following formula: Titer (pfu/ml) = number of plaques/volume of the diluted virus added to the well × dilution factor of the virus used to infect the well in which the plaques were counted.

Immunostaining

Huh-7 cells were seeded on a glass coverslip in 12-well plate and infected with DENV2 at a multiplicity of infection (MOI) of 0.1. Cells were then treated with 100 nM each compound and after 48 hrs fixed using 4% paraformaldehyde for 1 hr at 4°C. Viral particles were stained using anti-flavivirus group antigen-antibody (clone D1–4G2–4-15, EMD Millipore)

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at 1:1,000 in blocking buffer for 1 hr, washed three times and incubated with goat anti-mouse immunoglobulin G (IgG) Alexa 488 (Life Technologies) and DAPI (Insitus) for 30 min. After three washes with PBS, cells were visualized using confocal microscopy. Virus infectivity was analyzed by selecting five random fields of view for each condition. ImageJ software was used for fluorescence dye quantification, and the percentage of infected cells was calculated by dividing the number of Alexa-488-positive cells by the number of DAPI-positive cells.

Cell viability assay

Huh-7 cells (1×10^4 cells per well of a 96-well plate) were treated with increasing concentrations (25, 50, 100, 200 and 400 nM) of the compound for 72 hrs. The cytotoxic effects of the test compounds were determined based on MTT (3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide). The percent cell viability was calculated as follows: 100% - (Absorbance of treated cells / Absorbance of untreated cells) × 100%. For calculation of the adherent to apoptotic cell percentage, the apoptotic cells were collected from the supernatant, and the adherent cells were trypsinized, and both cells were counted using TC™ Automated Cell Counter (Bio-Rad, Portland, ME, USA).

Real-time cell proliferation assay

Effect of CDDO-me treatment or antibody neutralization on the proliferation of ZIKV-infected cells was assessed using IncuCyte Live-Cell Analysis System (Essen BioScience, Ann Arbor, MI). Vero cells were seeded in 96-well plates and allowed to grow to 10–30% confluence. To infect cells, growth media was removed and 250 pfu of virus was added to each assay well for a MOI of approximately 0.02. Virus and cells were incubated together for 1 hr before the removal of viral supernatant followed by the addition of maintenance media containing the respective treatments in duplicate. Proliferation was measured through quantitative kinetic processing metrics derived from images acquired from each well every 3 hours and presented as a percentage of culture confluence over time. The wells were imaged over 90 hours (DENV infected cells) or 110 hours (ZIKV infected cells).

Real-Time RT-PCR

Huh-7 cells were plated in 24-well plates (in triplicate for each condition), infected at a MOI of 0.1, and treated with 100 nM CDDO-me or PU-WS13 and 50 μg/ml Heparin as an inhibitor for virus entry and 10 μM of 2’CMA as a viral polymerase inhibitor. Viral RNA was harvested using the QIAamp viral RNA extraction mini kit (Qiagen, USA) at 48 post-infection (hpi). Real-time quantitative reverse transcription and PCR (qRT-PCR) was performed and analyzed on a CFX96™ Real Time System (Bio-Rad (Portland, ME, USA) using DENV2 forward primer 5’ACATCTCAAGTGCAGGCTGA3’, and reverse primer 5’GTCTCCGAATGGAGGTTCTG3. Viral RNA levels were normalized to GAPDH RNA levels.

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Results

Bardoxolone methyl (CDDO-me) is an Hrd1-mediated protein dislocation inhibitor

CDDO-me is a noncytotoxic and multifunctional synthetic triterpenoid that has potential applications for various diseases (Wang et al., 2014). Its multifunctionalities could be attributed to its ability to inhibit multiple targets in cells, including Keap1 (Wang et al., 2014; Wong et al., 2016), IKKβ (Ahmad et al., 2006), Arp2/3 (To et al., 2010), USP7 (Qin et al., 2016), Hsp90 (Qin et al., 2015), and Lon protease (Bernstein et al., 2012). In addition, CDDO-me also induces ER stress in cells (Zou et al., 2008; Jeong et al., 2015), indicating a buildup of misfolded proteins in the ER, but the underlying mechanism is not known. To address this question, we used the previously established drGFP assay (Zhong and Fang, 2012) to determine the effects of CDDO-me on ER-to-cytosol protein dislocation in live cells. A time-lapse imaging of mutant α−1-antitrypsin protein (NHK) dislocation in HeLa cells revealed that treatment with the proteasome inhibitor Bortezomib (BTZ) to block the degradation of dislocated NHK caused a time-dependent increase in GFP fluorescence (drGFP), an indicator of NHK dislocation (Fig. 1A, upper panel). The increase in NHK dislocation was significantly diminished when CDDO-me was applied along with BTZ (Fig. 1A, lower panel). A dose response experiment showed that CDDO-me inhibited NHK dislocation with an IC_{50} of 0.39 μM (Fig. 1B). CDDO-me did not affect the expression of S11-NHK and S1–10 (Fig. S1), suggesting that the CDDO-me-induced decreases in drGFP is due to its inhibition of NHK dislocation.

grp94 is a potential molecular target for CDDO-me

As a dislocation inhibitor, we expect that CDDO-me targets one of the key regulators in the dislocation pathway. We, therefore, used the cellular thermal shift assay (CETSA) to identify its potential target(s). CETSA is a well-established assay for detecting interaction between a small molecule and protein, based on the biophysical principle of ligand-induced thermal stabilization of a target protein (Martinez Molina et al., 2013; Martinez Molina and Nordlund, 2016). Ligand-stabilized protein target can be detected in soluble cellular fraction by immunoblotting or mass spectrometry (Martinez Molina et al., 2013; Martinez Molina and Nordlund, 2016). Using CETSA, we found that CDDO-me increased the thermal stability of grp94 compared to the vehicle control (Fig. 1C and 1D) while other related chaperones, including Hsp90, Hsp70, and BiP/grp78, showed little to no changes under the same condition as revealed by immunoblotting (Fig. 1C). Previous studies have shown that knockdown of grp94 expression inhibited degradation of NHK, indicating an important role of grp94 in the ERAD pathway (Christianson et al., 2008). Thus, CDDO-me may target grp94 to inhibit dislocation.

grp94 is essential for virus replication

Several proteins in the Hrd1 pathway are required for Dengue and/or Zika replication (Krishnan et al., 2008), but it is not known whether grp94 is required. Therefore, we determined the effects of grp94 knockdown on DENV2 replication in Huh-7 cells. The results showed that grp94 knockdown markedly inhibited DENV2 replication as indicated by decreased viral particle production and viral protein (Envelope and NS3) synthesis (Fig. 2A–C). Interestingly, CDDO-me treatment almost recapitulated the antiviral results by grp94

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knockdown (Fig. 2A–C), which support the possibility that CDDO-me is an inhibitor of grp94. However, CDDO-me is known to have multiple molecular targets in cells, including Keap1 (Wang et al., 2014; Wong et al., 2016), IKKβ (Ahmad et al., 2006), Arp2/3 (To et al., 2010), USP7 (Qin et al., 2016), Hsp90 (Qin et al., 2015), and Lon protease (Bernstein et al., 2012). Therefore, CDDO-me can simultaneously affect different cellular functions and may inhibit DENV replication by multiple mechanisms. Indeed, an additive effect of CDDO-me treatment and siRNA knockdown of grp94 or its binding partner OS9 on the decrease in DENV E protein production (Fig. S2), suggesting that mechanisms in addition to grp94 inhibition were involved in the antiviral activity of CDDO-me.

To further determine the involvement of grp94 in viral replication, we assessed the effects of PU-WS13, a well characterized specific inhibitor of grp94 (Patel et al., 2013), on DENV2 replication. Indeed, treatment of DENV2-infected Huh-7 cells with PU-WS13 also reduced DENV2 particles and viral protein expression as seen in grp94 knockdown and CDDO-me treated cells (Fig. 2A–C). In addition, a real-time cell viability assay showed that CDDO-me protected Vero cells against ZIKV and DENV infection. The cell viability assay is a dye-free method that allows for drug-induced cytotoxicity and drug-induced changes in proliferation to be observed concurrently for a nuanced approach to drug toxicity screening. After the initial growth curve, virus-induced cell death due to viral infection is observed as a decrease in confluence while a maintenance of confluence is indicative of viral protection. The results demonstrated the antiviral effect with the CDDO-me treatment, in a dose-dependent manner comparable to that by virus neutralizing antibody treatment. CDDO-me protected Vero cells against ZIKV infection with efficacy that was comparable to 5% ZIKV-neutralizing plasma (Fig. 2D, E). CDDO-me showed a significant inhibitory effect on proliferation kinetics at higher drug concentrations (0.25 μM – 2 μM), but did not show a significant effect on proliferation at low concentrations. Protection against ZIKV infection was seen at low CDDO-me concentrations including 0.03 μM, 0.06 μM and 0.13 μM. Weak or partial protection was seen at 0.02 μM. Similar antiviral effect was also seen against DENV infection (Fig. S3).

PU-WS13 and CDDO-me exhibited a broad spectrum of anti-DENV and anti-ZIKV activity

We showed that the knockdown of grp94 or treatment with either CDDO-me or PU-WS13 led to significant reduction in viral titers of DENV2 and ZIKV in Huh-7 cells (Fig. 3A). These anti-viral activities of CDDO-me and PU-SW13 were translated across multiple DENV serotypes and ZIKV strains (Fig. 3B, C), and also across several human cell lines (Fig. 3D, E). These findings indicate that grp94 inhibitors have a broad spectrum of anti-DENV and ZIKV activity and may be considered as drug candidates to combat DENV and ZIKV infections and co-infections. Next, we determined the potencies of the antiviral activities of CDDO-me and PU-WS13 (Fig. 4). We infected Huh-7 cells with DENV or ZIKV and treated the cells with increasing concentrations of the compounds. Viral replication was measured 48 hours post-infection (hpi) (Fig. 4A, B). A significant reduction in viral replication was observed at 100 nM, and the half-maximal effective concentrations (EC_{50}) were 17 nM for CDDO-me and 27 nM for PU-WS13 against DENV infection (Fig. 4A) and 15 nM for CDDO-me and 25 nM for PU-WS13 against ZIKV inhibition,

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respectively (Fig. 4B). Furthermore, we observed a significant reduction in viral plaque formation of DENV- or ZIKV-infected Huh-7 cells (Fig. 4C, D).

Because CDDO-me leads to cell-cycle arrest, reduces proliferation, and apoptosis in a wide variety of human cancer cells (Chintharlapalli et al., 2005; Konopleva et al., 2002; Gao et al., 2007; Samudio et al., 2008), we determined the effects of increasing concentrations of CDDO-me and PU-WS13 on Huh-7 proliferation. The half-maximum cytotoxic concentration (CC_{50}) in Huh-7 cells was 0.5 μM for CDDO-me and more than 2 μM for PU-WS13 (Fig. 2D, E), indicating that the antiviral effects could not be explained by reduced proliferation. The therapeutic indices (CC_{50}/EC_{50}) for CDDO-me and PU-WS13 in Huh-7 cells were ~29.4 and 80, respectively. This finding suggests that the antiviral activities of the grp94 inhibitors is due to inhibition of DENV and ZIKV replication and not due to a general cytotoxic effects.

PU-WS13 and CDDO-me inhibited virus replication at a late stage of the virus life cycle

To dissect which steps in the viral life-cycle require grp94 in human cells, we performed a time-of-addition experiment using CDDO-me, PU-WS13, the NS5 polymerase inhibitor 2'C-methyladenosine (2'CMA,) and the entry inhibitor heparin (Fig. 5A). The activity of grp94 inhibitors on DENV replication was comparable to 2'CMA. The grp94 inhibitors and 2'CMA suppressed viral production (Fig. 5B) and RNA synthesis (Fig. 5C) in human cells. As expected, heparin only blocked viral production and viral RNA replication when added before or concurrently with DENV infection. In contrast, 2'CMA was effective only when added concurrently with or after infection. Both CDDO-me and PU-WS13 considerably inhibited DENV production and viral RNA replication at the last three treatment regimes, suggesting grp94 is required for post-entry steps of the viral life cycle (Fig. 5B, C), which is inhibited by CDDO-me and PU-WS13.

Discussion

In this study, we identified CDDO-me as novel dislocation inhibitor with a broad-spectrum anti-DENV and anti-ZIKV activity. CDDO-me may exert these effects by targeting the ER luminal chaperone grp94, which is strongly supported by the fact that PU-WS13, a characterized grp94 inhibitor that is structurally unrelated to CDDO-me, phenocopied the antiviral activities of CDDO-me. The present study also identified grp94 as a novel host factor for DENV and ZIKV, suggesting that small molecule inhibitors of grp94 may be developed as broad-spectrum anti-DENV and anti-ZIKV drugs. While ER dislocation is a critical cellular function for both infected and non-infected cells, previous studies have shown that dislocation inhibitors can be tolerated in vivo. For CDDO-me, it entered clinical developments for the treatment of chronic kidney disease associated with type 2 diabetes but failed in phase III clinical trial due to heart-related adverse effects (Masullo et al., 2016). In contrast to the chronic kidney condition, DENV and ZIKV cause acute infections, and therefore do not require long-term treatment. Thus, CDDO-me has a great potential to be developed as an anti-DENV and anti-ZIKV drug since it has passed phase I and phase II trials (Masullo et al., 2016). In addition, inhibitors of three other key dislocation regulators, BiP, p97/VCP and Npl4, are under preclinical development as anti-cancer drugs all of which

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are well tolerated in mice (Anderson et al., 2015; Cerezo et al., 2016; Skrott et al., 2017). Therefore, inhibition of dislocation is a novel and promising strategy to develop broad-spectrum anti-flaviviral drugs.

Although several molecular targets have been reported for CDDO-me, including Hsp90 (Qin et al., 2015), a cytosolic counterpart of grp94, inhibition of grp94 is likely to contribute to the anti-viral effects of CDDO-me, since grp94 knockdown and treatment with PU-WS13, a grp94 inhibitor structurally unrelated to CDDO-me, both potently inhibited DENV and ZIKV replication. A mechanistic understanding of how grp94 regulates DENV and ZIKV replication is not available, however, our results indicate that grp94 is required for post-entry steps of the viral life cycle (Fig. 5). We speculate that grp94 may play a quality control role for viral proteins. Normally, the grp94 chaperone binds to OS9 lectin to deliver misfolded protein to the Hrd1 complex that in turn forwards misfolded protein to the proteasome for degradation (Christianson et al., 2008; Seidler et al., 2014). Therefore, CDDO-me may interrupt the function of grp94 that is necessary for misfolded viral protein degradation by ERAD. Accumulation of misfolded viral proteins would interfere with viral replication. In support for this possibility, previous studies have reported that flaviviruses exploit host cell protein machinery to translate its genomic RNA to viral proteins, fold viral proteins, and alter proteostasis (Valk et al., 1999; Perera and Kuhn, 2008; Mayer, 2005; Nagy and Pogany, 2011). The impact of grp94 on flavivirus replication could also be on the protein folding function of grp94. grp94 is not only an essential chaperone for ERAD, it also facilitates protein folding in the ER (Vembar and Brodsky, 2008; Marzec et al., 2012; Hebert and Molinari, 2007). Flavivirus replication requires host cell chaperones, including the newly identified grp94, for appropriate folding of viral multi-domain proteins (Valk et al., 1999; Perera and Kuhn, 2008; Mayer, 2005; Nagy and Pogany, 2011).

Previous studies have shown an essential role of grp94 in promoting replications of other viruses, such as retrovirus (Valk et al., 1999), herpes simplex type virus 1 (HSV-1) (Ramakrishnan et al., 1995), HCV (Lee et al., 2008) and HBV (Lim et al., 2005). The retention of secreted HCV E2 envelope proteins in the ER frequently induces grp94 expression to attenuate ER stress and inhibit cell apoptosis (Liberman et al., 1999). Knockdown of grp94 during HCV infection inhibits cell apoptosis which in turn promotes virus replication (Mayer, 2005). Interestingly, the data presented in this study revealed that in the context of DENV2 and ZIKV infection, silencing of grp94 has the opposite effect, and inhibits replication. CDDO-me and a previously identified grp94 inhibitor, PU-WS13, both showed significant anti-DENV2 and anti-ZIKV activity.

In conclusion, our study for the first time identified the importance of grp94 in flavivirus replication. We have demonstrated that PU-WS13 and CDDO-me have a broad spectrum of anti-flavivirus activity, and inhibition of grp94 may contribute to the antiviral activity of CDDO-me. However, CDDO-me has multiple molecular targets, it is entirely possible that CDDO-me inhibits DENV and ZIKV replication via targeting multiple proteins in addition to grp94. Further studies are required to define the mechanism of grp94 action in DENV and ZIKV replication and test the anti-DENV and ZIKV activity of grp94 inhibitors in animal models.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- CDDO-me inhibits ER-to-cytosol protein dislocation potentially by targeting the ER luminal chaperone grp94.
- grp94 is a novel host factor required for DENV and ZIKV replication.
- The specific grp94 inhibitor PU-WS13 and CDDO-me exhibit a broad-spectrum anti-DENV and anti-ZIKV activity in vitro.
- PU-WS13 and CDDO-me significantly suppressed the cytopathic effects caused by DENV and ZIKV infection.

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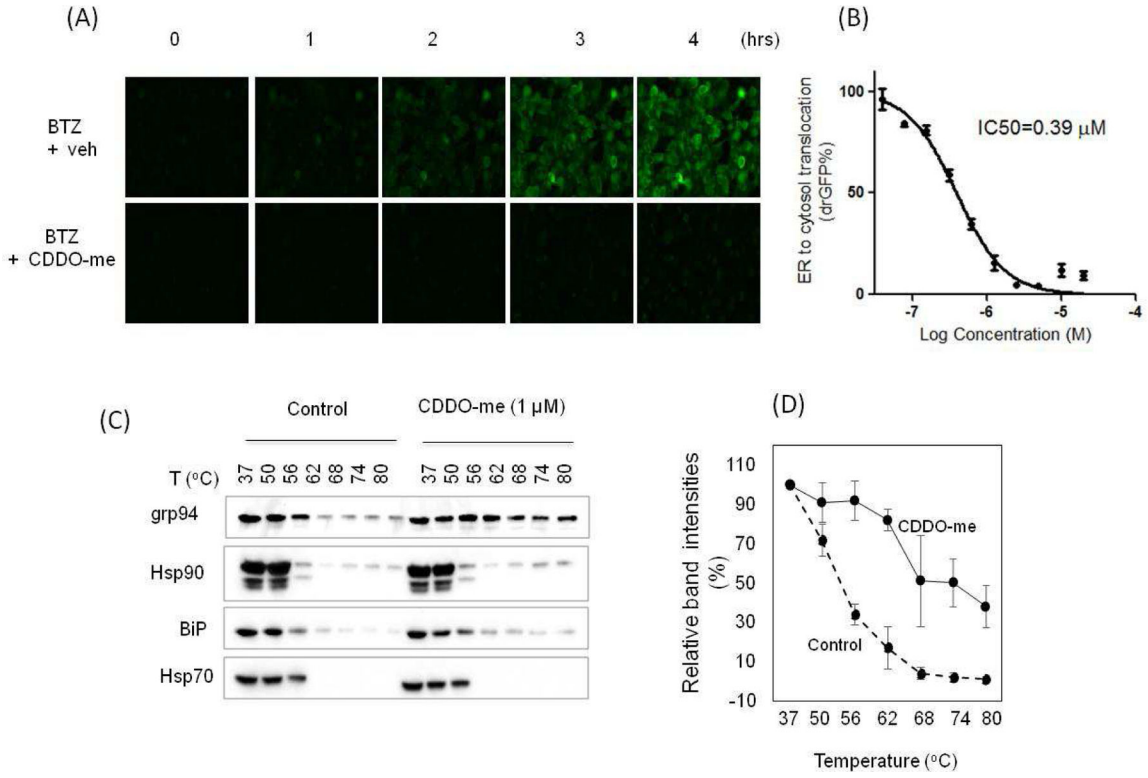

Figure 1. CDDO-me is a dislocation inhibitor that may target grp94.

(A) Time-lapse imaging of mutant α−1-antitrypsin protein (NHK) dislocation by drGFP in HeLa cells revealed that CDDO-me is an inhibitor of protein dislocation. Cells were treated with 1 μM bortezomib (BTZ) + 1 μM CDDO-me or DMSO (veh) and dislocation was monitored over 4 hrs **(B)** CDDO-me inhibited ER to cytosol dislocation of NHK at an IC_{50} of 0.39 μM. **(C)** Examining CDDO-me target engagement by CETSA. (**D)** Densitometric analysis of grp94 band intensities in CETSA were quantified and expressed as mean +/− SEM, n=3.

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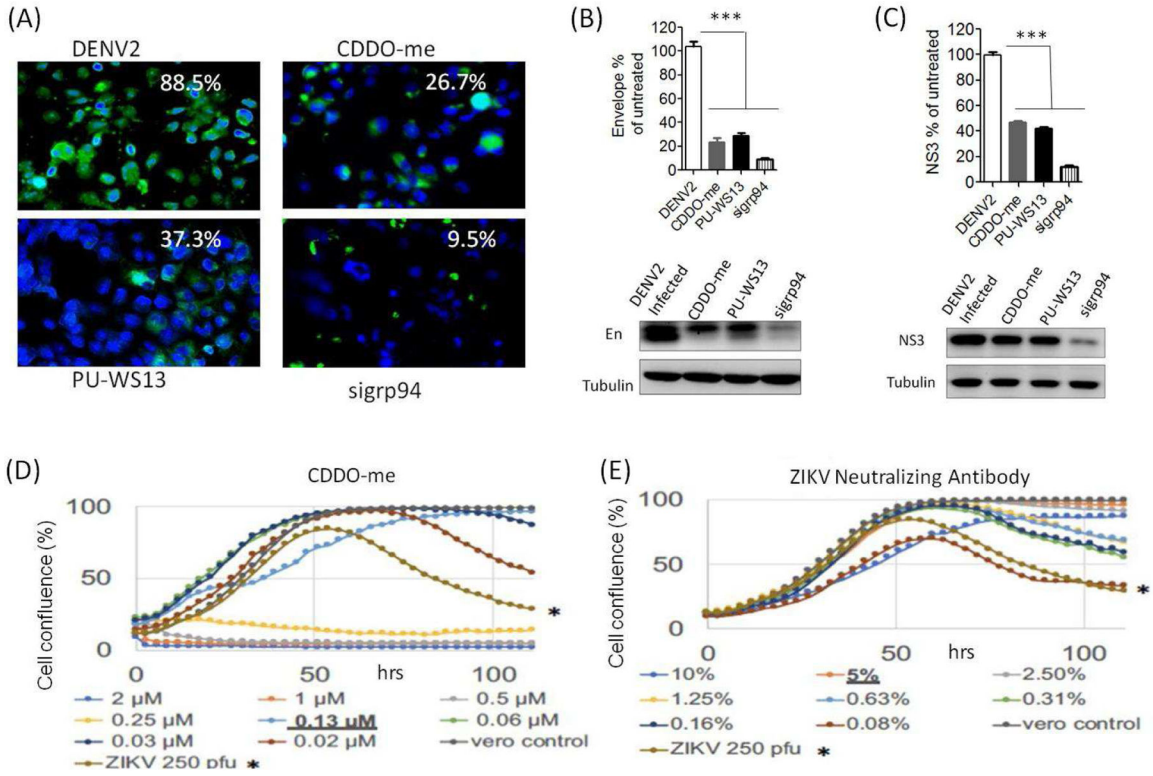

Figure 2. Pharmacologic and genetic inhibition of grp94 reduced virus replication and protected cells from death.

Infection with DENV2 were performed at MOI of 0.1 and the cells were treated with 100 nM of the test compounds for 48 hrs. **(A)** grp94 knockdown, CDDO-me, and PU-SW13 reduce virus particle production in Huh-7 cells. **(B and C)** grp94 knockdown and treatment with CDDO-me or PU-WS13 reduced the expression levels of DENV2 envelope and NS3 proteins in Huh-7 cells. (**D** and **E**) ZIKV infected Vero cells were treated with different dilutions of CDDO-me or neutralizing antibody. Cell confluence was monitored over 110 hours. * denotes the cytopathic effects caused by infection with 250 pfu ZIKV (MOI of approximately 0.02). CDDO-me at 0.13 μM protected ZIKV-infected Vero cells with the effects comparable to that with 5% ZIKV-neutralizing antibody. Densitometric analysis of the band intensities were quantified and expressed as mean +/− SEM, n=3. One-Way ANOVA with Bonferroni's post test. *** p<0.001.

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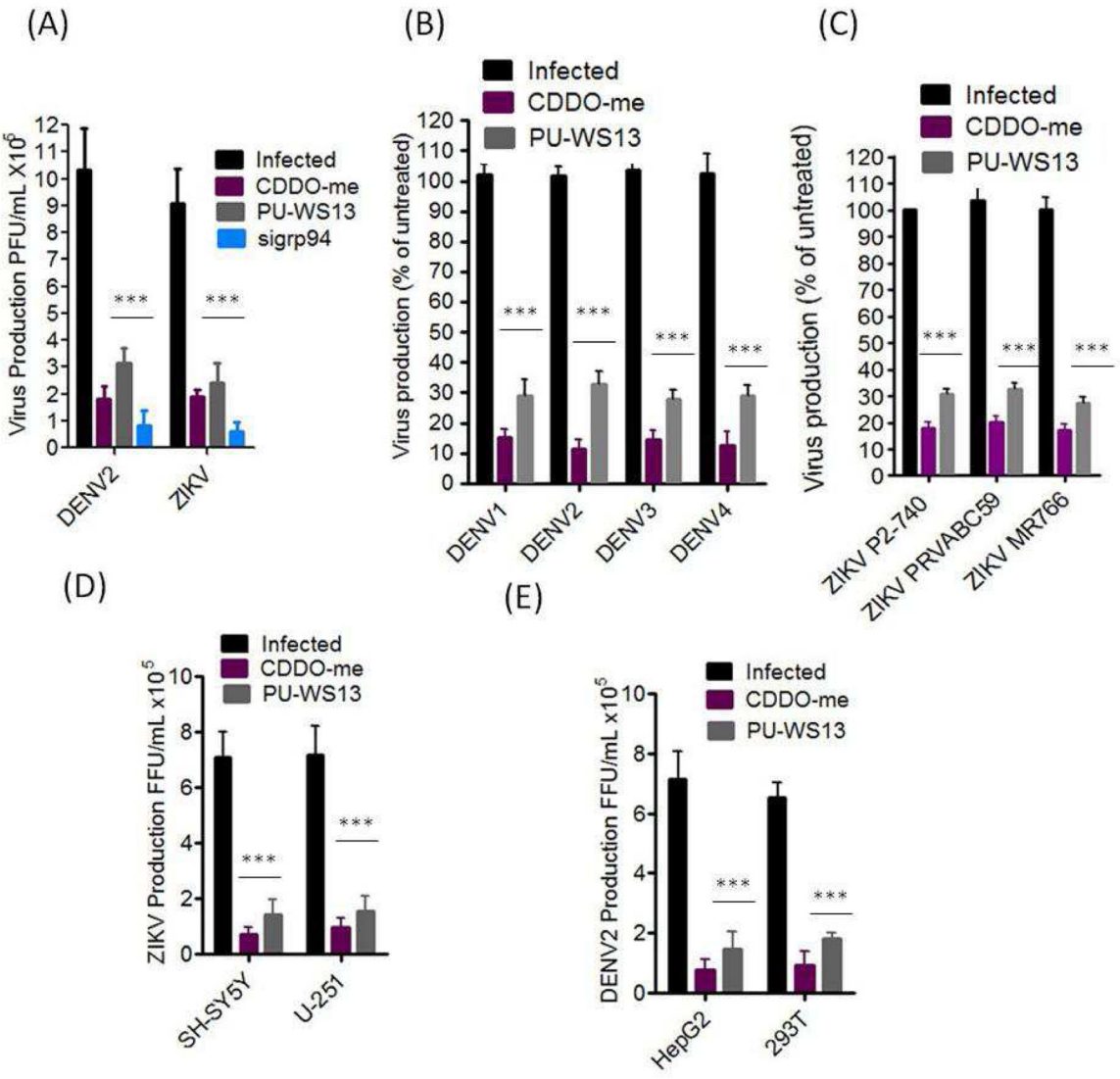

Figure 3. Broad-spectrum antiviral activity of PU-WS13 and CDDO-me against different DENV serotypes and ZIKV strains.

(**A**) Knockdown of grp94 or treatment with either CDDO-me or PU-SW13 led to a significant reduction in viral titers of DENV2 and ZIKV in Huh-7 cells. (**B** and **C**) grp94 inhibitors significantly reduced the virus titers of different DENV serotypes and ZIKV strains. (**D** and **E**) CDDO-me and PU-WS13 inhibited ZIKV and DENV2 replication in different permissive cell lines. All virus infections were performed at MOI of 0.1 and the cells were treated with 100 nM of the indicated compound for 48 hrs. One-Way ANOVA with Bonferroni's post test. *** $p<0.001$.

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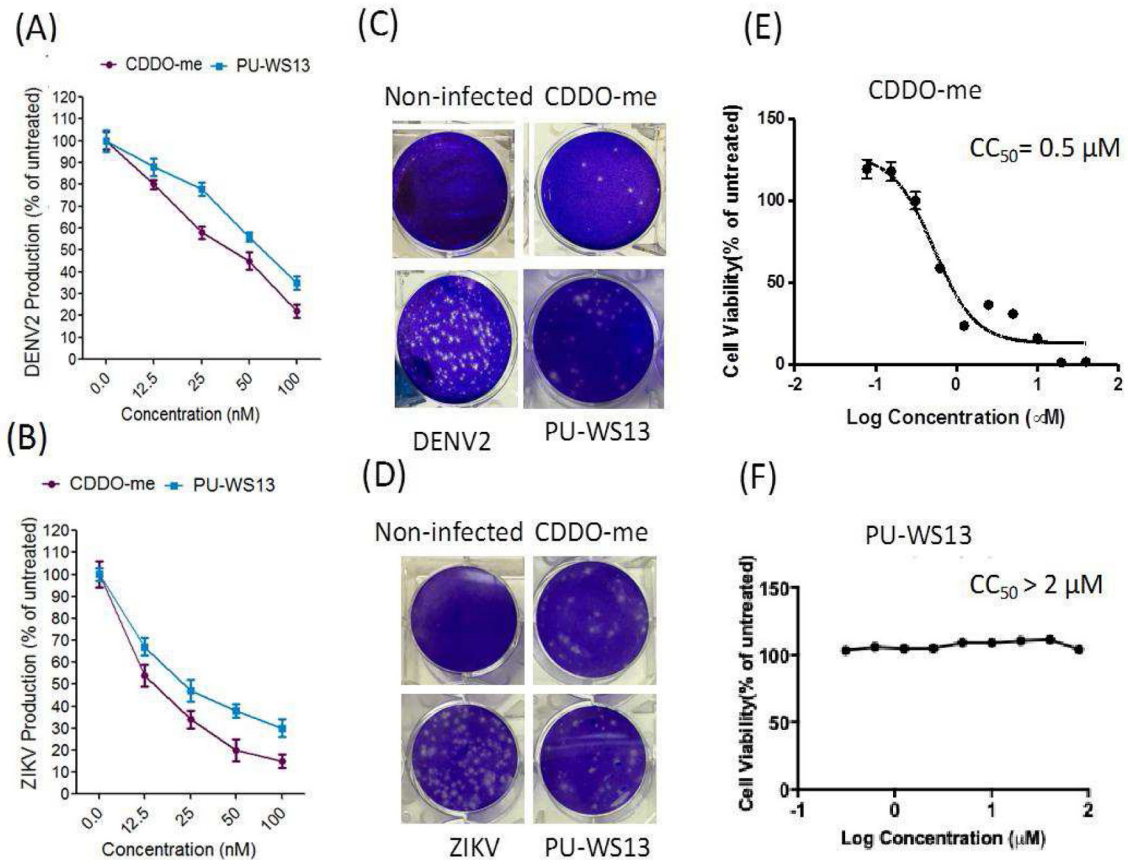

Figure 4. PU-WS13 and CDDO-me showed dose-dependent antiviral activity and inhibited DENV2 and ZIKV in Huh-7 cells.

(A and **B)** Huh-7 cells were infected with DENV or ZIKV (MOI, 0.1) and treated cells with increasing concentrations of the indicated compounds and viral replication was measured 48 hrs post-infection. The percentages of DENV2 and ZIKV production were remarkably reduced with increasing concentrations of the test compounds (**C** and **D)** The test compounds at 100 nM exhibited remarkable reduction in virus plaque formation at 48 hrs post-infection. (**E** and **F**) Huh-7 cell viabilities were measured 72 hrs after CDDO-me or PU-WS13 treatment.

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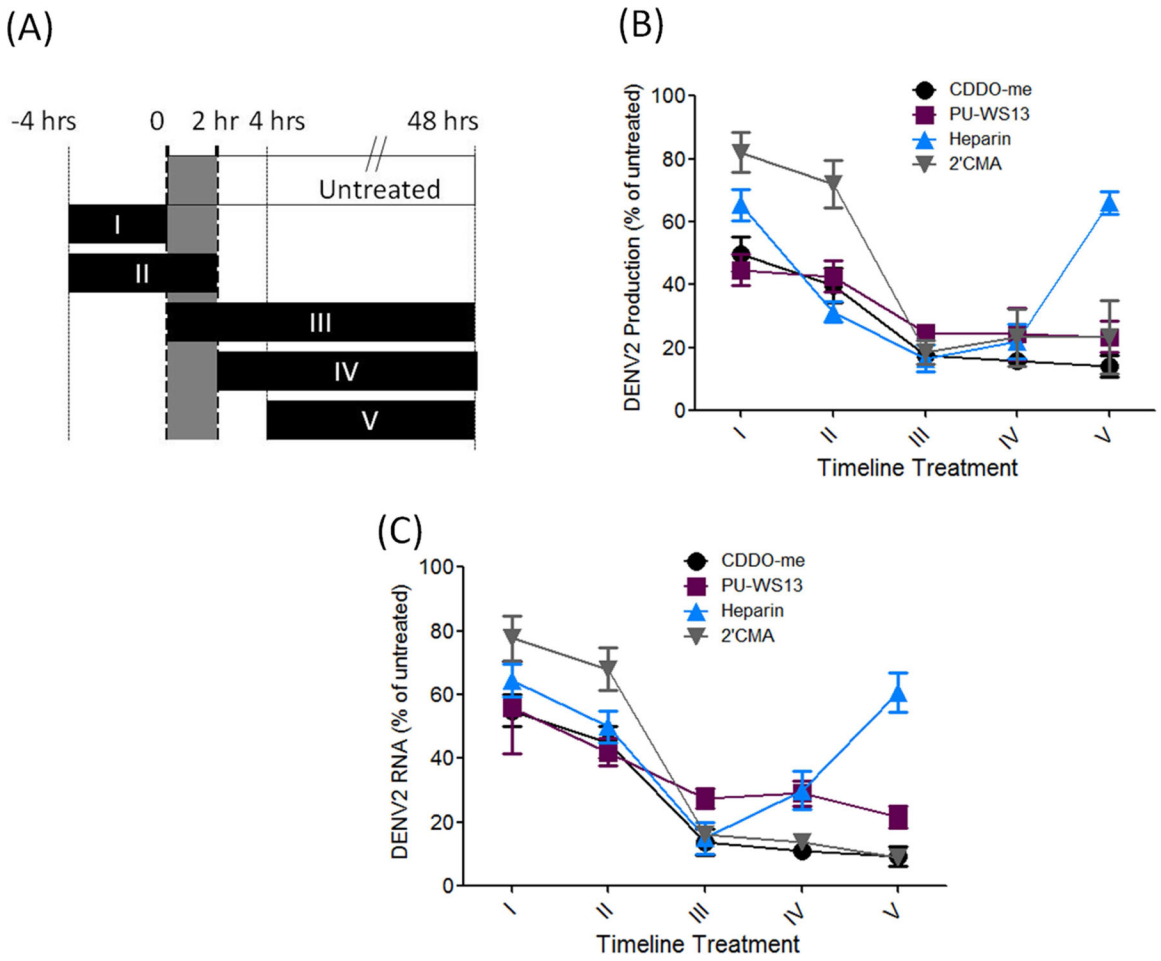

Figure 5. PU-WS13 and CDDO-me interfered with the late stage of virus life cycle.

Huh-7 cells were plated in 24-well plates (in triplicate for each condition), infected at a MOI of 0.1, and treated with 100 nM PU-WS13 and 50 μg/ml Heparin as an inhibitor for virus entry and 10 μM 2'CMA. **(A)** Experimental timeline to assess viral stage at which compounds have effects. 4 hrs before infection, 0–2 hrs during infection, 4 hrs post infection and 48 hrs post infection. **(B)** PU-WS13 or CDDO-me was applied to determine their effects on the virus life cycle. The compounds inhibited DENV production measured by plaque formation assay at post-entry stages, similar to NS5 polymerase inhibitor, 2'C-methyladenosine (2'CMA). (**C**) Viral RNA levels measured by qRT-PCR were remarkably decreased after applying the test compounds at the post-entry stages of virus life cycle.