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Coordination of different complexes to copper(II) and cobalt(III) metal centers enhances Zika virus and dengue virus loads in both arthropod cells and human keratinocytes

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Running title: copper(II) and cobalt(III) containing compounds promote Zika virus and dengue virus infections

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ABSTRACT

Trace elements such as copper and cobalt have been associated with virus-host interactions. However, studies to show the effect of conjugation of copper(II) or cobalt(III) metal centers to thiosemicarbazone ligand(s) derived from either food additives or mosquito repellent such as 2-acetylethiazole or citral, respectively, on Zika virus (ZIKV) or dengue virus (serotype 2; DENV2) infections have not been explored. In this study, we show that four compounds comprising of thiosemicarbazone ligand derived from 2-acetylethiazole viz., (E)-N-ethyl-2-[1-(thiazol-2-yl)ethylidene]hydrazinecarbothioamide (acetylethTSC) (compound **1**), a copper(II) complex with acetylethTSC as a ligand (compound **2**), a thiosemicarbazone ligand-derived from citral (compound **3**) and a cobalt(III) complex with a citral-thiosemicarbazone ligand- (compound **4**) increased DENV2 and ZIKV replication in both mosquito C6/36 cells and human keratinocytes (HaCaT cells). Treatment of both cell lines with compounds **2** or **4** showed increased dengue viral titers at all three tested doses. Enhanced dengue viral plaque formation was also noted at the tested dose of 100 μM, suggesting higher production of infectious viral particles. Treatment with the compounds **2** or **4** enhanced ZIKV and DENV2 RNA levels in HeLa cell line and primary cultures of mouse bone marrow derived dendritic cells. Also, pre- or post treatments with conjugated compound **2** or **4** showed higher loads of ZIKV or DENV2 envelope (E) protein in HaCaT cells. No changes in loads of E-protein were found in ZIKV-infected C6/36 cells, when compounds were treated after infection. In addition, we tested bis(1,10-phenanthroline)copper(II) chloride ([Cu(phen)$_2$]Cl$_2$, (compound **5**) and tris(1,10-phenanthroline)cobalt(III) chloride ([Co(phen)$_3$]Cl$_3$, (compound **6**) that also showed enhanced DENV2 loads. Also, we found that copper(II) chloride dehydrate (CuCl$_2$•2H$_2$O) or cobalt(II)

chloride hexahydrate (CoCl$_2$•6H$_2$O) alone had no effects as "free" cations. Taken together, these findings suggest that use of Cu(II) or Co(III) conjugation to organic compounds, in insect repellents and/or food additives could enhance DENV2/ZIKV loads in human cells and perhaps induce pathogenesis in infected individuals or individuals pre-exposed to such conjugated complexes.

KEY WORDS: Zika virus, dengue virus, viral replication, copper(II) and cobalt(III),

thiosemicarbazones, 1, 10-phenanthroline

IMPORTANCE

Mosquito-borne diseases are of great concern to the mankind. Use of chemicals/repellents against mosquito bites and transmission of microbes has been the topic of interest from many years. Here, we show that thiosemicarbazone ligand(s) derived from 2-acetylethiazole or citral or 1,10-phenanthroline upon conjugation with copper(II) or cobalt(III) metal centers enhances dengue virus (serotype 2; DENV2) and/or Zika virus (ZIKV) infections in mosquito, mouse and human cells. Enhanced ZIKV/DENV2 capsid mRNA or envelope protein loads were evident in mosquito cells and human keratinocytes, when pre-treated with compounds before/after infections. Also, treatment with copper(II) or cobalt(III) conjugated compounds increased viral titers and number of plaque formations. These studies suggest that conjugation of compounds in repellents/essential oils/natural products/food additives with copper(II) or cobalt(III) metal centers may not be safe, especially in tropical and subtropical places, where several dengue infection cases and deaths are reported annually or in places with increased ZIKV caused microcephaly.

INTRODUCTION

Insect bites, food or crop damage and transmission of pathogens to humans, animals and plants has led to the extensive use of insecticides, pesticides and insect attractants or repellents [1,2]. Use of these substances has substantially been increased in military personnel and in agriculture or horticulture practices. Over a decade, several of these chemicals have been used extensively in all parts of the world. Some synthetic compounds or chemicals are of major concern to the environment and human health and they have been withdrawn due to the adverse effects and serious human health hazards [3-8]. One of the most recent study tested that several of the repellents including citronella candles had no effects and did not reduced mosquito attraction to humans [9]. The disadvantages of some insecticides have promoted the use of plant-based essential oils and their derived natural products, belonging to several herb species that poses repellent activities [7,10]. Some of the essential oils, herbal skin products and non-natural inorganic insecticides (the contact insecticides that are toxic to insects upon direct contact) have been shown to contain metals such as arsenates, copper, cobalt and fluorine compounds in small amounts and sulfur as a major component [11]. Also, both copper sulfate and cobalt chloride has been shown to improve the varieties in rice and vegetables that are mostly consumed in Australia and some parts of Asia [12]. In addition, complex mixtures of volatile organic compounds produced from plant-based essential oils or natural products such as citral have been extensively used as repellents to prevent transmission of human pathogens through biting arthropods such as mosquitoes [7,10,13].

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Mosquitoes are the primary vectors responsible for the transmission of arthropod-borne flaviviruses that cause microcephaly (Zika Virus), dengue hemorrhagic fever, yellow fever, West Nile neuroinvasive disease (WNND) and Japanese encephalitis. With regard to the global impact from arthropod-borne diseases, dengue is the most important human pathogen (that exist as four serotypes; DENV-1, 2, 3 and 4) that should be addressed promptly. According to the 2009 WHO criteria, dengue cases have been classified according to the levels of severity. Dengue infections without warning signs or with warning signs (such as abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, liver enlargement, increasing hematocrit with decreased platelet numbers and lethargy) were considered as less severe. However, dengue infections with severe plasma leakage, bleeding or organ failures were considered as severe cases [14,15]. Approximately 20,000-25,000 deaths and 50-100 million dengue infections with 500,000 hospitalizations are reported, annually [16]. Also, there are no fully effective vaccines available for human use and prevention of dengue [17,18]. Nevertheless, recently, WHO immunization group (Strategic Advisory Group of Experts; SAGE) has recommended the use of partially effective dengue vaccine (a live attenuated tetravalent dengue vaccine developed by Sanofi Pasteur; CYD-TDV, named Dengvaxia) that has been licensed and used in 11 countries including Brazil, Mexico, Singapore, Thailand and Indonesia [19]. Related to dengue, Zika virus (ZIKV) is an emerging infectious agent transmitted and spread by the daytime active *Aedes* mosquitoes, such as *A. aegypti* and *A. albopictus*. ZIKV can be transmitted by blood transfusions, sexual and placental leakage from the mother to the fetus [20,21]. An important public health concern has been declared due to global epidemic of ZIKV infections. The association between ZIKV infection during pregnancy and intrauterine fetal infection, microcephaly, brain damage, congenital malformation syndrome and evidences from laboratory

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models of fetal infections are reported [20-22]. The best prevention practice is the use of mosquito nets, protective clothing such as long-sleeve shirts and full pants and insect repellents to avoid the mosquito bites. In high endemic areas, it becomes important to use repellents on a routine basis to prevent bites from infected mosquitoes.

Several reports suggest that trace elements such as copper and cobalt play an important role in the host immunomodulatory activities during viral infections [23,24]. Any changes in the levels of trace elements could impact disease severity [25]. Essential oils from plants and herbs can inactivate enveloped and non-enveloped viral replication and exhibit antiviral activities [26-29]. Due to the strong antiviral and antimicrobial activities, novel citral-based compounds (the well-known natural insect repellent) are developed continuously [26-29]. Studies that show the effect of conjugation of citral-based compounds with different trace elements on ZIKV and dengue virus replication has not been explored. The purpose of the current study is to analyze the effects of copper(II) and cobalt(III) metal conjugations with organic compounds (that are used in repellents or food additives) on DENV2/ZIKV infections. The four compounds analyzed in this study are selected based on the structural activity relationship (SAR) in order to determine the best efficacy in inhibition of the virus. It has been reported that when "free" thiosemicarbazone ligands are coordinated to a metal center, the efficacy in the activity is enhanced in comparison to the "free" ligands. In this study, we show that treatment of mosquito (C6/36 cells) and human keratinocytes (HaCaT cells), HeLa cells and primary cultures of mouse bone marrow derived dendritic cells with thiosemicarbazones coordinated to Cu(II) or Co(III) metal centers enhances ZIKV and DENV2 infections. Our data suggests that use of coordinated

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version with metal centers in the repellent formulations may aid better replication of ZIKV and

DENV2 in the human cells upon transmission by the biting mosquitoes.

ACCEPTED MANUSCRIPT

MATERIALS AND METHODS

Synthesis of the ligands and the compounds. Analytical or reagent grade chemicals were used throughout this study. All the chemicals including solvents were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) or other commercial vendors, and used as received. Microanalyses (C, H, N) were performed by Intertek Chemical and Pharmaceuticals, 291 Route 22 East, Salem Industrial Park, Building #5, Whitehouse, NJ 08888, U.S.A. The FT IR (Fourier Transform infrared) spectrum was acquired in the range of 4000-400 cm^{-1} using the Attenuated Total Reflectance (ATR) accessory (with a diamond crystal) on a Nicolet 6700 FT IR spectrometer. ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were acquired on a Bruker AVANCE III 400 MHz spectrometer with deuterated dimethyl sulfoxide-d$_6$ (DMSO-d$_6$) as solvent. All ^{1}H NMR spectra were processed with the Spectrus Processor 2012 software, which is available from Advanced Chemistry Development (ACD, Inc., 8 King Street East, Suite 107, Toronto, Ontario M5C 1B5, Canada).

The "free" salts and complexes.

The "free" CuCl$_2$.2H$_2$O and CoCl$_2$.6H$_2$O salts were obtained from Sigma-Aldrich Corporation.

Synthesis of (E)-N-ethyl-2-[1-(thiazol-2-yl)ethylidene]hydrazinecarbothioamide (acetylethTSC) **1** *and [Cu(acetylethTSC)Cl]Cl•0.25C$_2$H$_5$OH* **2** *(Compounds* **1** *and* **2***, respectively)*

(*E*)-*N*-ethyl-2-[1-(thiazol-2-yl)ethylidene]hydrazinecarbothioamide (acetylethTSC) **1** was synthesized and characterized as by Lewis *et al.*; [30] while the complex, [Cu(acetylethTSC)Cl]Cl•0.25C$_2$H$_5$OH **2** (where acetylethTSC = (*E*)-*N*-ethyl-2-[1-(thiazol-2-

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yl)ethylidene]hydrazinecarbothioamide) was synthesized and characterized as by Sandhaus *et al.*
[31].

*Synthesis of citralEtTSC•0.25C$_2$H$_5$OH **3** (Compound **3**)*

Citral (3.0 g, 3.377 ml, 20 mmol) and 4-ethyl-3-thiocarbazide (2.4 g, 20 mmol) were mixed in a
250 ml round bottom flask, followed by anhydrous ethanol (100 ml) and five drops of glacial
acetic acid. The reaction mixture was refluxed with stirring for three hours and evaporated to
dryness. The resulting oil was allowed to stay at room temperature until it solidified to leave the
resulting product. Yield = 4.8 g (91%). Calc. C$_{13.5}$H$_{24.5}$N$_3$O$_{0.25}$S, C, 61.21; H, 9.32; N, 15.86%.
Found: C, 60.82; H, 9.34; N, 15.76%. FT IR (v/cm^{-1}): 3164 (m) (-N^{1}H), 3054 (m) (-N^{2}H), 1059
(m) (N-N), 1543 (m) (TSC (C=N)), (1296) (m) and (813) (m) (C=S). ^{1}H NMR (400 MHz;
DMSO-d$_6$); δ_H = 1.16 (t, J^t = 7.10 Hz, 3H, -CH$_3$), 2.42 (s, 3H, CH$_3$CH=N), 3.61 (d, 2H, CH$_2$),
7.79 (d, J^d = 3.21 Hz, CH$_{ar}$), 7.88 (d, J^d = 3.19 Hz, CH$_{ar}$), 8.08 (d, J^d = 3.24 Hz, CH$_{ar}$), 8.30 (s, H,
aminic NH), 10.58 (s, 1H, hydrazinic-NH), and 13.2 (s, H-bonded hydrazinic-NH) ppm.

*Synthesis of [Co(phen)$_2$(citralEtTSC)](NO$_3$)$_3$•0.25H$_2$O•1.5C$_2$H$_5$OH **4** (Compound **4**)*

At first, [Co(phen)$_2$CO$_3$]Cl•5H$_2$O was synthesized using the procedure as reported by
Kashiwabara *et al.* [32]. First, we prepared [Co(phen)$_2$(H$_2$O)$_2$](NO$_3$)$_3$, then
[Co(phen)$_2$(H$_2$O)$_2$](NO$_3$)$_3$•2H$_2$O was synthesized by the acid hydrolysis of
[Co(phen)$_2$CO$_3$]Cl•5H$_2$O using the synthesis as reported by Ye *et al.* [33]. Finally,
[Co(phen)$_2$(H$_2$O)$_2$](NO$_3$)$_3$•2H$_2$O was placed in an oven set at 110 °C for 24 hours in order to
produce anhydrous [Co(phen)$_2$(H$_2$O)$_2$](NO$_3$)$_3$. [Co(phen)$_2$(H$_2$O)$_2$](NO$_3$)$_3$ (0.80 g, 1.2 mmol) and
citralEtTSC•0.25C$_2$H$_5$OH (0.32 g, 1.2 mmol) in anhydrous ethanol (410 ml) were refluxed in a
500 ml round bottom flask with stirring at 110 °C under argon for 24 hours. The mixture was
rotary evaporated to dryness. The solid was washed with ether and collected. Yield = 1.0 (91%).

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Calc. $C_{40}H_{48.5}CoN_{10}O_{10.75}S$, C, 51.53; H, 5.24; N, 15.02%. Found: C, 51.53; H, 4.57; N, 14.57%. Complexes **5** and **6**, $[Cu(phen)_2]Cl_2$ and $[Co(phen)_3]Cl_3$ were synthesized respectively as by the literature procedure [34,35].

All tested compounds were completely solubilized in DMSO and were prepared as 1 ml of 100 mM solutions (compounds **1**, **3** and **4**) or 20 mM stocks of compound **2** (due to low solubility issues) or 0.1 mM stock of Compound **5** and **6** were prepared. The indicated doses 1, 10 and 100 µM were prepared from the stock solutions and used as test doses throughout the study. The compound stocks and dilutions were kept as recommended by the previous studies.

Cell Culture and Infection. Mosquito (C6/36) cells and Vero (African Green Monkey kidney) cell line were purchased from American Type Culture Collection (ATCC). Human keratinocytes (HaCaT cells) were obtained from Dr. Loree Heller, Center for Bioelectrics. C6/36 or HaCaT cells were plated in a 24/12 well plate at a density of 1×10^5 cells per well in complete MEM or DMEM medium containing 10% FBS, L-glutamine and Pen/Strep and kept at either 30º C or 37 º C with 5% CO2, respectively. Cells were either treated with respective compounds **1**, **2**, **3** or **4** (with indicated doses; 1, 10 or 100 µM) for 24 hours before or after infection with ZIKV/DENV2 (multiplicity of infection, MOI = 1) for 24-hour. Compounds **5** and **6** or the "free" Cu(II) and Co(III) salts (with indicated doses; 1, 10 or 100 µM) were also treated for 24 hours followed by infection with DENV2 (multiplicity of infection, MOI = 1) for another 24-hour. ZIKV strain, VR-1838, was obtained from ATCC or DENV2 (Serotype 2); El Salvador TVP2176 strain was obtained from Dr. John F. Anderson, Connecticut Agricultural Experiment Station. DMSO-treated or untreated (uninfected or infected) cells served as controls.

RNA extraction, cDNA synthesis and Quantitative Real-time PCR (Q-PCR) Analysis. Total

RNA from (1 x 10^5) of either C6/36 cells or HaCaT cells, first treated with the compounds (for

24 hours) using different doses (1, 10 or 100 µM) followed by infection with DENV2/ ZIKV

(MOI 1; 24 hour post infection) or post-treated (24 hours) with respective compounds (indicated

doses) after DENV2/ZIKV infection (MOI 1; 24 hours) were generated using Aurum Total RNA

Mini kit (BioRad, Hercules, CA) following manufacturer instructions. RNA was converted to

cDNA using iScript cDNA synthesis kit (BioRad). The generated cDNA was used as template

for the amplification of DENV2/ZIKV; capsid/NS5 transcripts, respectively, from cDNA with

primers as described [36,37]. As an internal control and to normalize the amount of template,

either mosquito or mouse or human *beta actin* amplicons were quantified using published

primers [36]. Q-PCR data showing DENV2/ZIKV loads were presented by normalizing

expression of capsid/ NS5 gene transcripts to either mosquito or mouse or human beta actin. Q-

PCR was performed using iQ-SYBR Green Supermix and CFX96 touch System (BioRad).

Standard curves were prepared using 10-fold serial dilutions starting from 1 to 0.00001 ng/µl of

known quantities of DENV2/ ZIKV, *capsid/ NS5,* and respective beta-actin fragments. Following

are the oligonucleotides used in this study: DENV2 capsid, 5'

AATATGCTGAAACGCGAGAGAAACCGCG 3' and 5' CTCTTCAGTATCCCTGCTGTTGG

3'; ZIKV NS5, 5' AARTACACATACCARAACAAAGTGGT 3' and 5'

TCCRCTCCCYCTYTGGTCTTG 3'; human actin, 5' AGCCTCGCCTTTGCCGA 3' and 5'

CTGGTGCCTGGGGCG 3'; mouse actin, 5' CCCTAGGCACCAGGGTGTGA 3' and 5'

GGGGTGTTGAAGGTCTCAAACA 3'; mosquito actin, 5' CCATGTACGTCGCCATCCA 3'

and 5' GCGGTGGCCATTTCCTG 3'; human IFN-beta, 5'

CAGCAATTTTCAGTGTCAGAAGCT 3' and 5' TCATCCTGTCCTTGAGGCAGT 3'; and

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human TNF-alpha, 5' CCCAGGCAGTCAGATCATCTT 3' and 5'

TCTCAGCTCCACGCCATT 3'.

Western Blotting. Total proteins from 1 x 10^5 of either C6/36 cells or HaCaT cells infected with

DENV2 (MOI 1; 24 hour post infection) or with ZIKV (MOI 1 or 5; for 24 hour) and then

followed by treatments with respective compounds at different doses (1, 10, 100 μM) for another

24-hour period or vice versa (treated with compounds and then infected; in a similar manner as

described above) were collected in ice-cold modified RIPA buffer (G-Biosciences, BioExpress,

Kaysville, UT). Total proteins were estimated using BCA kit (Pierce, ThermoScientific). Total

lysates from C6/36 or HaCaT cells (25 μg) were separated on 4-20% gradient or 12% SDS-

PAGE gels (NuSep; BioExpress). Following gel electrophoresis, blotting (on nitrocellulose

membranes) and blocking (5% milk buffer or BSA NAP-blocker solution; G-Biosciences,

BioExpress) was processed and blots were incubated with 4G2 primary antibody (non-reducing

conditions), obtained from Dr. Michel Ledizet, L2 Diagnostics, Inc. Monoclonal Anti-Flavivirus

Group Antigen, Clone D1-4G2-4-15 (Catalog Number; NR-50327), antibody obtained from BEI

resources was used to detect ZIKV E-protein. Mouse monoclonal IgG conjugated with HRP

(Santa Cruz Biotechnologies, Dallas, TX) were used as secondary antibodies for detection. Total

protein profiles serve as loading controls and not for comparison between the compounds. The

differences in protein profile gels are due to use of either stain-free gels images after run, or

staining of gels with either colloidal Coomassie blue or R-250 Coomassiee blue solutions. ECL

detection of antibody binding was performed with WesternBright ECL kit (Advansta,

BioExpress). Blots were scanned using Chemidoc MP imaging system and recommended Image

Lab software from the manufacturer (BioRad).

Virus Titration Assays. For the titration assay, briefly, C6/36 or HaCaT cells were seeded into 96 well plates and at a density of 1 x 10^5 cells/well in 225 μl of either MEM or DMEM complete medium with 10% FBS, pen/Strep and L-Glutamine. Cells were incubated overnight at either 30 ° C or 37 ° C with 5% CO2, respectively. Cells were treated with respective compounds **1-4** at doses of 1, 10 or 100 μM (for 24 hours), followed by infection with DENV2 (MOI 1) for another 24 hours. Each of the compound and respective doses were kept as six independent replicates in addition to the uninfected, infected and infected-DMSO treated controls. Following 24 hour post-infection, cells were fixed with acetone-PBS mixture (3:1) for 20 min at -20 ° C. Immediately after fixation, plates were air dried and washed with 1 x PBS and blocked with 5% FBS-PBS-0.05% Sodium Azide for 15 min at RT. After blocking, cells were incubated with 4G2-monoclonal antibody for overnight at 4º C. Cells were washed three times with PBS and incubated with Alexa-594 labeled mouse secondary antibody for 1 hour at RT, followed by washes (3X) with PBS. Plates were analyzed using EVOS fluorescence system (Invitrogen/Thermoscientific) and cells were scored for fluorescence or the presence or absence of infection in comparison to the positive (Infected and infected-DMSO-treated) or negative (uninfected) controls. Percentages were calculated from ratio of fluorescent cells and no fluorescent cells and as manual countings from eight microscopic fields. We collected eight images for each treatment and respective doses and representative images are shown in Supplementary information. Infection indicated by red fluorescence was determined as average from collected images and percentages were plotted in comparison to the infected-DMSO-treated controls.

Viral Plaque Formation Assay. Plaque assays were performed as described [36]. For the plaque formation assay, briefly, Vero cells were seeded into 6 well plates and at a density of 1.5 x 10^6 cells/well in DMEM complete medium with 10% FBS. Cells were incubated overnight at 37 ° C with 5% CO2 and then treated with compounds **1-4** at doses of 100 μM (for 24 hours), followed by infection with DENV2 (MOI 1) for 2 hours. Following post-infection, medium was removed and warm 2% Seaplaque agarose (Lonza) overlay with complete DMEM media (1:1 ratio) containing antibiotic and antimycotics solution (1% each; Sigma) was added. Plates were incubated for additional 5 days, at 37 °C and 5% CO2. After an incubation period, plaques were stained with 0.03% of Neutral Red (Sigma) for 4 hours; stain was removed to count plaques. Each of the compounds (at the dose of 100 μM) was kept as two independent replicates in addition to the controls (infected and infected-DMSO treated). Representative images are shown for each compound and controls. Plaques counting are averages from three independent readouts.

Isolation and culturing of mouse bone marrow-derived dendritic Cells. Bone marrow cells were isolated from femurs and tibias of C57BL/6 mice, and red blood cells were lysed with ammonium chloride. Remaining cells were plated at a density of 7 x 10^6 per 100-mm cell culture dish in 10ml of α-MEM media (HyClone) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals), 2 mM L-glutamine, dextrose, essential and non-essential amino acids, sodium pyruvate, sodium bicarbonate, antibiotics, and 2-β-mercaptoethanol. Granulocyte-macrophage colony- stimulating factor (GM-CSF; Peprotech; 5ng/ml) was added at the time of plating, and again after 72 hours of incubation. Experiments using animals were in accordance with protocols from the Institutional Animal Care and Usage Committee (as per the approved protocol 14-009). For infection and treatment with compounds, we re-plated dendritic cells into

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24 well plates as 1 x 10^5 cells/ml in each well and infected with either DENV2/ZIKV (MOI = 1), respectively. After 24h post infection, dendritic cells were treated with all four compounds at different doses (1, 10, 100 μM) for another 24 hour, and collected for RNA extractions.

Statistics. Statistical significance of differences observed in data sets was analyzed using Prism6 software (GraphPad, La Jolla, CA) and Microsoft Excel. For data to compare two means, the non-paired, two-tail Student *t* test was performed for all analysis. P values of <0.05 were considered significant in all tests, error bars represent mean (+SD) values. Statistical test and P values are shown.

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RESULTS

Synthesis and Schemes of compounds. (*E*)-*N*-ethyl-2-[1-(thiazol-2-yl)

ethylidene]hydrazinecarbothioamide (acetylethTSC) compound **1** was synthesized as described

by Lewis *et al.*; [30] while the compound **2**, [Cu(acetylethTSC)Cl]Cl•0.25C$_2$H$_5$OH 2 (where

acetylethTSC = (*E*)-*N*-ethyl-2-[1-(thiazol-2-yl)ethylidene]hydrazinecarbothioamide) was

synthesized as described by Sandhaus *et al.* [31] (Scheme 1, Supplementary Information).

Compounds **3** and **4** were synthesized as described in (Scheme 2, Supplementary Information).

The ^{1}H NMR spectrum of compound **3** (citralEtTSC•0.25C$_2$H$_5$OH) is shown in Supplementary

Fig. 1A. The thiosemicarbazone ligand derived from 2-acetylethiazole (compound **1**), 2-

acetylthiazole thiosemicarbazone ligand-copper(II) complex (compound **2**), had been

synthesized in [31]. Compounds **3** and **4** that are thiosemicarbazone ligands derived from citral

without (compound **2**) or with Co(III) complex (compound **4**), respectively, were synthesized in

this study as shown in schemes 1 and 2. Complexes **5** and **6**, [Cu(phen)$_2$]Cl$_2$ and [Co(phen)$_3$]Cl$_3$,

respectively, were synthesized in this study as by the literature procedure [34,35]. Schemes 3 and

4 in Supplementary information show the synthesis of complexes **5** and **6**. The ^{1}H NMR

spectrum of complexes **5** and **6** and [Co(phen)$_3$]Cl$_3$ are shown in Supplementary Fig. 1B and 1C.

The methods used to synthesize these compounds are validated and are routinely been used in

the biochemistry laboratory [31].

Thiosemicarbazone and 1, 10-Phenanthroline ligands conjugated with Cu(II) or Co(III)

complexes enhance dengue virus loads. We determined the dengue virus loads in order to

investigate any interference of these compounds in virus inhibition. C6/36 cells or HaCaT cells

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were infected with DENV2 (MOI = 1) for 24 hours, followed by 24-hour of incubations with respective compounds at different doses of 1, 10 and 100 µM concentrations. Q-PCR data showed that treatment of C6/36 cells with compounds **1** and **3** (100 µM) and compounds **2** and **4** (10 and 100 µM) concentrations significantly ($P<0.05$) enhanced the DENV2 capsid mRNA transcripts in comparison to the infected-DMSO control (Fig. 1A). Immunoblotting analysis showed increased dengue envelope (E) protein loads in C6/36 cells treated with compound **2** or **4** at all concentrations (1, 10 and100 µM) in comparison to DMSO-treated control (Fig. 1B). Human keratinocytes treated with compounds **2** and **4** (10 and 100 µM) also showed significant ($P<0.05$) increase in dengue capsid mRNA transcripts in comparison to the infected-DMSO-treated control (Fig. 1C). However, compound **1** showed significantly increased dengue capsid mRNA transcripts at 100 µM treatment (Fig. 1C). Immunoblotting analysis with HaCaT cells revealed that compounds **2** and **4** (at 10 and 100 µM) showed dramatic increase in dengue E-protein levels in comparison to the infected-DMSO treated control (Fig. 1D). Increased dengue virus E-protein loads were evident in compound **1**-treated cells even at 100 µM concentrations in comparison to DMSO-treated controls. Total protein profiles serve as loading control and are not shown for comparison to the infected (untreated) or infected-DMSO treated samples (Fig. 1B & 1D). In addition to compounds **2** and **4**, we tested complexes **5** and **6** to find if coordination of copper(II) or cobalt(III) to these 1, 10-phenanthroline compounds also enhances DENV2 loads upon treatment. We found that in both mosquito and HaCaT cells, complexes **5** and **6** also enhanced DENV2 loads upon 24-hour treatment followed by 24 hour post-infection (Supplementary Fig. 2A and 2B). Viral loads were enhanced in a dose dependent manner when complexes **5** and **6** were treated at increasing concentrations of 1, 10, 100 µM (Supplementary Fig. 2A and 2B). In order to include the possibility that the inorganic salts containing Cu(II) or

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Co(III) may itself enhance the DENV2 loads, we tested the $CuCl_2.2H_2O$ and $CoCl_2.6H_2O$ salts independently in both mosquito and HaCaT cells. We found that in both cell lines the salts alone did not enhanced the DENV2 loads at the similar treatments (with tested doses of 1, 10, 100 µM) and infection times (Supplementary Fig. 2C and 2D). This data suggested that Cu(II) or Co(III) salts alone has no effects as free cations.

Cu(II) or Co(III) metal centers with thiosemicarbazone ligands enhance production of infectious viral particles. To further confirm the enhanced viral replication and increased DENV2 loads upon treatment with compounds (**2** and **4**), we performed the virus titration and plaque formation assays as described in methods. In both mosquito and HaCaT cells, virus titration assay showed percentage of infected cells (determined by fluorescent signals). Both compounds **2** and **4**, showed significantly ($P<0.05$) increased percentage of infected cells at all tested doses of 1, 10 and 100 µM, for 24 hours of treatment followed by another 24 hour post-infection period (Fig. 2A and 2B). All four compounds (**1-4**) at 100 µM treatment showed increased production of viral infectious particles in both mosquito and human cells (Fig. 2A and 2B). However, compounds **2, 3** and **4** showed increased virus loads even at lower concentration of 10 µM in both cells (Fig. 2A and 2B). Representative images are shown for increased number of infected cells with pre-treatment of compounds **1-4** in both mosquito and HaCaT cells in comparison to their respective infected-DMSO treated controls (Supplementary Figures 3 and 4). The plaque formation assay further confirmed the increased production of viral plaques in Vero cells treated with compounds **1-4** at 100 µM (Fig. 2C and 2D). The plaques formed in the presence of compounds **3** and **4** were large with bigger diameter in comparison to the plaques formed in infected-DMSO-treated controls (Fig. 2C). Also at 100 µM treatments, compound **2**

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showed several but smaller plaques in comparison to compound **1**. The numbers of plaques

formed were significantly increased upon treatment with compounds **2** and **4** in comparison to

the compounds **1** and **3** (Fig. 2D). This data further confirmed that treatment with compounds

containing Cu(II) or Co(III) metal centers increased production of infectious viral particles. Also,

we analyzed the antiviral/cytokine response in DENV2-infected HaCaT cells treated with

different compounds. Treatment of HaCaT cells with compound **1** (10 μM), showed significantly

decreased levels of IFN-beta transcripts in comparison to infected-DMSO-treated control

(Supplementary Fig. 5A). No differences were evident with the treatment with other compounds.

In addition, we noted that treatment of HaCaT cells with compounds (**1**, **2** and **4**) at 1 μM

concentration showed significantly (P<0.05) decreased levels of TNF-alpha transcripts

(Supplementary Fig. 5B). Due to the observation of increased viral replication upon treatment

with compound **4**, we determined whether treatment with this compound elicits similar effect in

additional human cell lines such as HeLa cells. QPCR analysis revealed that HeLa cells

treatment with compound **4** at 100 μM showed significantly (P<0.05) increased DENV2 loads, in

comparison to the infected DMSO-controls (Supplementary Fig. 5C).

Thiosemicarbazone ligands conjugated with Cu(II) or Co(III) enhance DENV2 and ZIKV

loads in primary cultures of murine bone marrow derived dendritic cells. We next

determined DENV2/ZIKV loads in the primary cultures of mouse dendritic cells isolated from

bone marrow derived precursor cells. Dendritic cells were infected with either DENV2 or ZIKV

(MOI = 1) for 24 hours, and then treated with compounds **1-4** at different doses (1, 10 and 100

μM) for another 24 hours. QPCR analysis showed that both DENV2 (Fig. 3A) capsid transcripts

and ZIKV NS5 transcripts (Fig. 3B) were significantly (P<0.05) increased in Cu(II) conjugated

compound **2** (both 10 and 100 μM treatments), however, unconjugated compound **1** showed

significant ($P<0.05$) increase in only ZIKV loads. Compound **3** treatments (at 100 μM) showed

enhanced viral loads in both DENV2 and ZIKV infected dendritic cells (Fig. 3A & 3B). In

dendritic cells infected with DENV2, treatment with compound **4** showed a significant ($P<0.05$)

increase in virus loads. However, treatments with compound **4** (100 μM) showed enhanced

DENV2/ZIKV loads in comparison to their respective controls (Fig. 3A & 3B).

Treatment with copper(II) conjugated compound post infection with ZIKV enhances viral

burden in both mosquito and human cells. In C6/36 cells infected with ZIKV (MOI = 1; for

24 h), followed by treatment with compounds **1-4** (at 1, 10 and 100 μM) for another 24 hours, we

found that ZIKV loads (NS5 mRNA transcripts) were significantly ($P<0.05$) enhanced upon

treatment with compound **2** (at 100 μM) (Fig. 4A). Although, no detectable levels of E-protein

were evident in the same samples at MOI = 1 infectious dose (Fig. 4B). It was noted that in

HaCaT cells, under similar conditions (ZIKV infection, MOI = 1, for 24hours; followed by

compounds (**1-4**) treatments at 1, 10 and 100 μM for another 24 hours), ZIKV NS5 mRNA

transcripts were significantly ($P<0.05$) increased upon treatment with compound **2**, at both 10

and 100 μM concentrations (Fig. 4C). Also, ZIKV E-protein loads were elevated upon treatment

with compound **2** (at 100 μM) in comparison to the infected DMSO-treated controls (Fig 4D).

Other compounds and their tested doses did not show any significant changes at either mRNA or

protein levels (Fig. 4). We did not detected any ZIKV E-protein in infected and infected DMSO-

treated controls due to very low infection with MOI = 1. Total protein profiles serve as loading

controls (Fig. 4B & 4D). These results suggest that treatment with Cu(II) conjugated compound

2 post infection with ZIKV enhance viral burden, whereas without conjugation the same compound **1** did not showed any enhancement in ZIKV loads.

Since we did not find any detectable levels of E-protein in infected (untreated) or infected DMSO-treated controls at MOI = 1 of ZIKV infection, we performed additional experiments with higher infectious doses of MOI = 5. We first infected C6/36 cells or HaCaT cells with ZIKV at higher multiplication of infection of 5 for 24 hours, followed by treatments with compounds (**1-4**) at different doses of 1, 10, and 100 µM concentrations. We detected E-protein in all compound treatments including compound **2**, and there were no differences in comparison to the infected DMSO-treated controls (Fig. 5A). When HaCaT cells were infected with MOI = 5 of ZIKV in a similar manner, we detected E-protein in all samples including the infected (untreated) and infected DMSO-treated controls (Fig. 5B). We also included sodium salicylate (NaSal) compound that inhibits flavivirus replication [38] as control. NaSal-treated (5mM) cells showed inhibition of ZIKV replication and lower E-protein loads in both C6/36 cells and HaCaT cells (Fig. 5A & 5B). Total protein profiles serve as loading controls in both C6/36 cells and HaCaT cells (Fig. 5A & 5B).

Pre-treatment with compounds 2 or 4 before infection with ZIKV enhance viral burden in both mosquito and human cells. To analyze if treatment with compounds before infection also enhance ZIKV replication in C6/36 and HaCaT cells, we first treated cells with compounds **1**-**4** (at concentrations of 1, 10 and 100 µM) for 24 hours and then infected with ZIKV (MOI = 1) for another 24 hours. In C6/36 cells, treatment with Cu(II) or Co(III) conjugated- compounds **2** (10 and 100 µM) or **4** (100 µM) significantly (P<0.05) increased ZIKV NS5 transcripts in

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comparison to the infected (untreated) or infected DMSO-treated controls (Fig. 6A). It was also

noted that ZIKV E-protein loads were enhanced in C6/36 cells treated with compound **2** (10 and

100 µM) or **4** (100 µM) (Fig. 6B). In HaCaT cells, inductions in ZIKV loads were also noted in

treatments with both Cu(II) or Co(III) conjugated compounds **2** and **4** (100 µM), respectively at

100 µM concentration. In addition compound **1** (100 µM) also showed significant ($P<0.05$)

induction in ZIKV NS5 loads in HaCaT cells (Fig. 6C). Immunoblotting showed that ZIKV E-

protein loads were enhanced upon treatment with compound **2** (100 µM) in comparison to the

infected DMSO-treated control (Fig. 6D). No significant changes were observed with other

compounds and the tested doses (Fig. 6D). Total protein profiles serve as loading controls (Fig.

6C & 6D). E-protein levels were since not detected in infected DMSO-treated or infected

untreated controls, we next analyzed effect of these compounds on ZIKV infection with higher

infectious doses of MOI = 5. We treated C6/36 cells and HaCaT cells with compounds (**1-4**) at

different doses of 1, 10, and 100 µM concentrations for 24 hours and then infected with ZIKV at

higher infectious dose (MOI = 5) for another 24 hours. With increased infectious dosage both

C6/36 cells and HaCaT cells, showed E-protein in all samples including the infected (untreated)

and the infected DMSO-treated controls (Fig. 7). In C6/36 cells, no differences were noted

between compounds **1-4**, and in comparison to their respective infected DMSO-treated controls

(Fig. 7A). However, a dose dependent enhancement of ZIKV loads was evident in HaCaT cells

upon treatment with compounds **1-4** (Fig. 7B). Sodium salicylate treated (5mM) C6/36 cells and

HaCaT cells serve as controls and show inhibition of ZIKV replication and lower E-protein

levels (Fig. 7A & 7B). Total protein profiles serve as loading controls in both C6/36 cells and

HaCaT cells (Fig. 7A & 7B). Collectively, these results suggest that Cu(II) or Co(III) conjugated

compounds, enhance ZIKV and DENV2 infection in mosquito cells and human keratinocytes.

DISCUSSION

Mosquito-borne diseases such as malaria, filariasis, arbovirus encephalitis and dengue are some of the major human health problems in tropical and subtropical countries. Recently, there has been an outbreak of ZIKV and Yellow Fever virus infections in the Americas. One of the potential interventions to combat mosquito-borne diseases is the continuous use of repellents in children and adults [7]. Our study suggests that use of Cu(II) or Co(III) containing metal centers in future preparations of food additives/repellents against mosquitoes may enhance replication and production of infectious viral particles of both DENV2 and ZIKV in the human cells upon transmission by the biting arthropods. Use of some insect repellents has been discouraged due to their adverse effects [3-8]. Safe use of alternatives like repellents of natural origin such as essential oils and their derived complex products has been preferred. Several studies have shown that plant based essential oils such as lemongrass; allspice oils, citral and other compounds are effective in controlling insect nuisance, mosquito bites and exhibit repelling properties [1,29,39-41]. Repellent bioassays have revealed that the activity of lemon essential oils is due to the presence of citral component [42,43]. Our study has shown that thiosemicarbazone ligands derived from citral and 2-acetylethiazole and their conjugation with Co(III) or Cu(II), enhance ZIKV and DENV2 loads at both mRNA and protein levels, respectively. Testing of additional complexes **5** and **6** with 1, 10-phenanthroline ligands coordinated to Co(II) and Co(III) also showed similar increase in viral replication and DENV2 loads. Testing of "free" Cu(II) and Co(III) salts alone did not show any significant increase in DENV2 loads, suggesting no effects of free cations on viral replication. Our data also suggested that conjugation with these metal

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complexes might affect the antiviral or antimicrobial properties of such compounds. The reduced loads of IFN-beta suggested a diminished antiviral response in human cells (Supplementary Fig. 5A).

Several studies have shown that plant compounds have antiviral activities against dengue virus and other enveloped viruses [44-47]. However, conjugation of Co(III) or Cu(II) to such compounds may increase DENV2 loads in mosquito and HaCaT cells suggesting that both arthropod and human cells are susceptible to metal centers that may interfere and enhance DENV2 replication. In addition to human keratinocytes, human epithelial cells (HeLa cells) showed similar increase in DENV2 replication at 100 μM doses, suggesting similar activity of the compound **4** in enhancing viral loads (Supplementary Fig. 5C). Primary cultures of mouse bone marrow derived dendritic cells infected with DENV2/ZIKV also suggested that compounds **2** and **4** increased viral replication at 100 μM concentration in other mammalian cells (Fig. 3B & 3C). Infection with ZIKV, followed by treatment with compounds also showed similar results with compound **2**, where 10 and 100 μM induced the ZIKV loads in both C6/36 cells and HaCaT cells (Fig. 4A & 4C). Although, upon treatment with compound **2**, the ZIKV E-protein loads were higher in HaCaT cells (Fig. 4C & 4D), suggesting high susceptibility and less tolerance in presence of ZIKV with treatment of compounds conjugated to Cu(II) containing complexes. Increased infection with ZIKV (MOI = 5) showed that the E-protein loads in both mosquito and human keratinocytes had no changes in comparison to the loads of E-protein from the infected (untreated) or infected DMSO-treated controls. This data revealed that with higher infectious dose of MOI =5, and post-treatment of compounds after infection may moderately allow compounds to interfere in the enhancement of viral replication.

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Pre-treatments with compounds **2** and **4** followed by ZIKV infection showed increased viral loads (NS5 mRNA transcripts) in both mosquito and human keratinocytes (Fig. 6A & 6C). However, the observation of higher loads of ZIKV E-protein in mosquito cells indicated greater replication of ZIKV in mosquito cells at all tested doses of compounds **2** and **4** (Fig. 6B). It was also noted that ZIKV loads in HaCaT cells (that were first treated with compounds **1**, **2** and **4**, followed by infection) were much higher when compared to the viral loads in HaCaT cells that were exposed to compounds after virus infection (Figs. 4C & 6C). It is assumed that when these Cu(II) or Co(III) conjugated compounds **2** & **4** are applied to normal human skin, there are greater chances of encountering enhancement in the ZIKV replication upon bites from infected mosquitoes. It was noted that HaCaT cells treated with compounds **1-4** for 24 hours, then followed by ZIKV infection at higher infectious doses (MOI = 5), showed a dose dependent increase of ZIKV E-protein levels with all tested compounds and their respective increasing doses (Fig. 7B). This data suggested that pre-exposure of human keratinocytes to these conjugated compounds might better enhance ZIKV loads when confronted through the infected mosquito bite. It is also intriguing to note that treatment with compound **2**, followed by infection (with higher infectious dose of MOI = 5) with ZIKV, dramatically increased the viral loads in HaCaT cells (Figs. 7B), suggesting conjugation of Cu(II) or Co(III) complexes interference with ZIKV replication. Irrespective of the treatment procedures (infection followed by treatment with these compounds or pretreatment of compounds followed by infection) incubations lead to enhancement in DENV2/ZIKV loads in both arthropod and human cells. This enhancement of DENV2/ZIKV infection suggest that Cu(II) or Co(III) complexes in food additives or repellent sprays, lotions or any other forms might be disadvantageous on human cells and perhaps on other

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animals and aquatic environments. The mechanism of action of these compounds in enhancing

DENV2/ZIKV loads in human keratinocytes is a future perspective. In summary, the present

study shows that pre/post treatment with organic compounds containing Cu(II) or Co(III) metal

complexes enhances ZIKV/DENV2 infection in mosquito, mouse and human cells.

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AUTHOR CONTRIBUTIONS

SD, SK and HS performed experiments. MJC, AH, JFA (Jessa), AM and AAH prepared compounds tested in this study. CS and PJK provided bone marrow derived dendritic cells used in this study. SD, GN, AAH and HS discussed, analyzed and interpreted the data. HS planned, designed, coordinated and compiled the entire study. LH, ML and JFA provided important reagents. All authors edited and approved the manuscript. HS supervised overall investigations, wrote the paper.

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FIGURE LEGENDS

Figure 1. Treatment with organic compounds post infection enhances dengue virus loads.
Q-PCR and immunoblotting analysis showing the DENV2 capsid transcripts or envelope E-protein loads in C6/36 mosquito cells (A & B) or human keratinocytes (HaCaT cells) (C & D) infected with DENV2 (MOI = 1) for 24 hours followed by treatment with compounds **1**-**4** at different concentrations (1, 10 and 100 µM) for another 24 hours, respectively. DENV2 capsid mRNA transcript levels (A & C) were determined by Q-PCR upon treatment with compounds in comparison to the infected-DMSO controls. UT indicates untreated cells; M shows marker lane and µM means micro molar. Uninfected (UI) or infected (I) cells treated with DMSO are included as controls. DENV2 E- protein was detected in panels B & D and upper band indicates glycosylated form. Total protein profiles are shown as loading control and not for comparison. The differences in protein profiles are due to use of Colloidal Coomassie blue staining in panels with compounds **1** and **4** and Coomassie blue R-250 staining solution in panels with compounds **2** and **3**. Statistics was performed using the Student's two-tailed test. The $P<0.05$ is indicated with an asterisk to show significance in comparison to DMSO-treated control and error bars indicate standard deviation.

Figure 2. Treatment with compounds 2 and 4 increase DENV2 infectious titers and viral plaques. Virus titration assays in mosquito (A) and HaCaT (B) cells showing percentages of infected cells upon 24-hours treatment with compounds (**1-4**) at doses of 1, 10 and 100 µM, followed by infection with DENV2 (MOI 1) for another 24 hours. Percentage of infected cells were analyzed from eight different microscope fields in comparison to the infected-DMSO-

treated controls and plotted as percentages. (C) Plaque formation assay performed in Vero cells treated with compounds **1-4** at 100 µM for 24 hours followed by infection with DENV2 (MOI 1) for 2 hours and incubation for another 5 days to form plaques are shown. A representative image of a 6-well plate with plaques from different groups is shown. Plaques from compounds treated groups were compared to the infected-DMSO-treated control. Ruler at the bottom determines the scale for the plaques from the representative image. (D) Quantitative assessment of number of plaques from compound (**1-4**) treated (100 µM) and infected-DMSO-treated is shown. Representative data from two independent experiments is shown. P value determined by Student's two-tail *t* test is shown.

Figure 3. Treatment of compounds enhances DENV2 and ZIKV loads in primary cultures of mouse bone marrow derived dendritic cells.

Q-PCR analysis showing DENV2 loads in primary cultures of mouse bone marrow derived dendritic cells showing DENV2 (A) and ZIKV loads (B). 1 x 10^5 Cells were infected with MOI = 1 of either DENV2 or ZIKV for 24 hours and followed by treatments with compounds **1-4** at different concentrations (1, 10 and 100 µM) for another 24 hours, respectively. DENV2 capsid mRNA transcript (A) and ZIKV NS5 mRNA transcript levels (B) were determined by Q-PCR upon treatment with compounds in comparison to the infected-DMSO controls. UT indicates untreated cells and µM means micro molar. Uninfected (UI) or infected (I) cells treated with DMSO are included as controls. ns indicates not significant. Statistics was performed using the Student's two-tailed test. The P<0.05 is indicated with an asterisk to show significance in comparison to DMSO-treated control and error bars indicate standard deviation.

Figure 4. ZIKV loads are induced following post-treatment with organic compounds. Q-PCR and immunoblotting analysis showing the ZIKV NS5 mRNA or E-protein loads in mosquito C6/36 cells (A & B) or human keratinocytes (HaCaT cells) (C & D) infected with ZIKV (MOI = 1) for 24 hours followed by treatment with compounds **1**-**4** at different concentrations (1, 10 and 100 µM) for another 24 hours, respectively. ZIKV NS5 mRNA transcript levels (A & C) were determined by Q-PCR upon treatment with compounds in comparison to the infected-DMSO controls. ZIKV E- protein was detected in panels B & D. Total protein profiles serves, as loading controls are not shown for comparison. UT indicates untreated cells and (UI) or (I) indicates uninfected or infected cells, respectively. M shows marker lane. Cells infected and treated with DMSO are included as controls. ns indicates not significant. Statistics was performed using the Student's two-tailed test. The P<0.05 is indicated with an asterisk to show significance in comparison to DMSO-treated control and error bars indicate standard deviation.

Figure 5. ZIKV loads are induced with higher infection following post-treatment with organic compounds. Immunoblotting analysis showing the ZIKV E- protein in C6/36 cells (A) or human keratinocytes (HaCaT cells) (B) infected with ZIKV (MOI = 5) for 24 hours followed by treatment with compounds **1**-**4** at different concentrations (1, 10 and 100 µM) for another 24 hours, respectively. ZIKV E- protein was detected in panels B & D and upper band indicates glycosylated form. Total protein profiles serve as loading controls. UT indicates untreated cells and (UI) or (I) indicates uninfected or infected cells. M shows marker lane. Cells infected and DMSO-treated are included as controls.

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Figure 6. Pre-treatment with organic compounds further enhanced ZIKV loads. Q-PCR and immunoblotting analysis showing the ZIKV NS5 mRNA or envelope E-protein loads in C6/36 cells (A & B) or HaCaT cells (C & D) treated with compounds **1-4** at different concentrations (1, 10 and 100 µM) for 24 hours followed by infection with ZIKV (MOI 1) for another 24 hours, respectively. ZIKV NS5 mRNA transcript levels (A & C) were determined by Q-PCR upon treatment with compounds in comparison to the infected-DMSO controls. UT indicates untreated cells and ns indicate not significant. Uninfected (UI) or infected (I) cells treated with DMSO are included as controls. M shows marker lane. Total protein profiles are not shown for comparison and serve as loading controls. Statistics was performed using the Student's two-tailed test. The P<0.05 is indicated with an asterisk to show significance in comparison to DMSO-treated control and error bars indicate standard deviation.

Figure 7. ZIKV loads are induced with higher infection dose following pre-treatment with organic compounds. Immunoblotting analysis showing the ZIKV E- protein in C6/36 mosquito cells (A) or human keratinocytes (HaCaT cells) (B) that were treated with compounds **1-4** at different concentrations (1, 10 and 100 µM) for 24 hours and followed by infection with ZIKV (MOI = 5) for another 24 hours, respectively. ZIKV E- protein was detected in panels B & D and upper band indicates glycosylated form. Total protein profiles serve as loading controls and M indicates marker lane. UT indicates untreated cells and (UI) or (I) indicates uninfected or infected cells. Cells infected and treated with DMSO are included as controls.
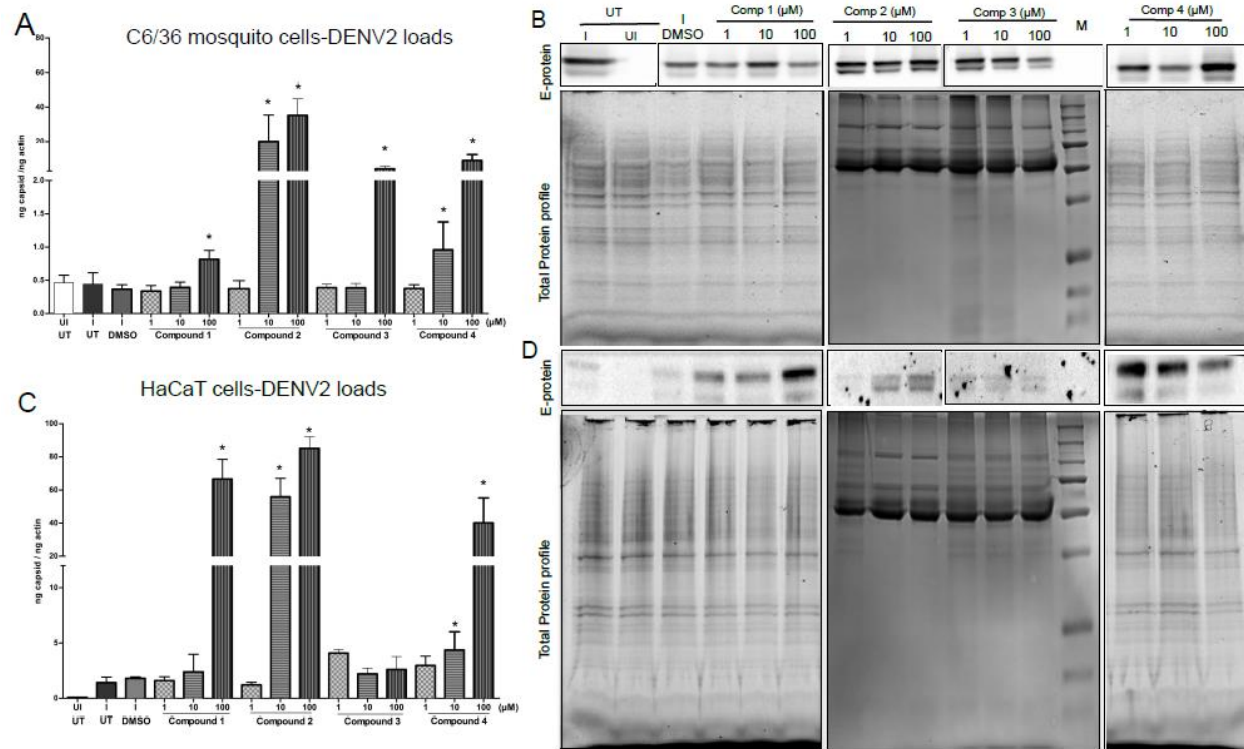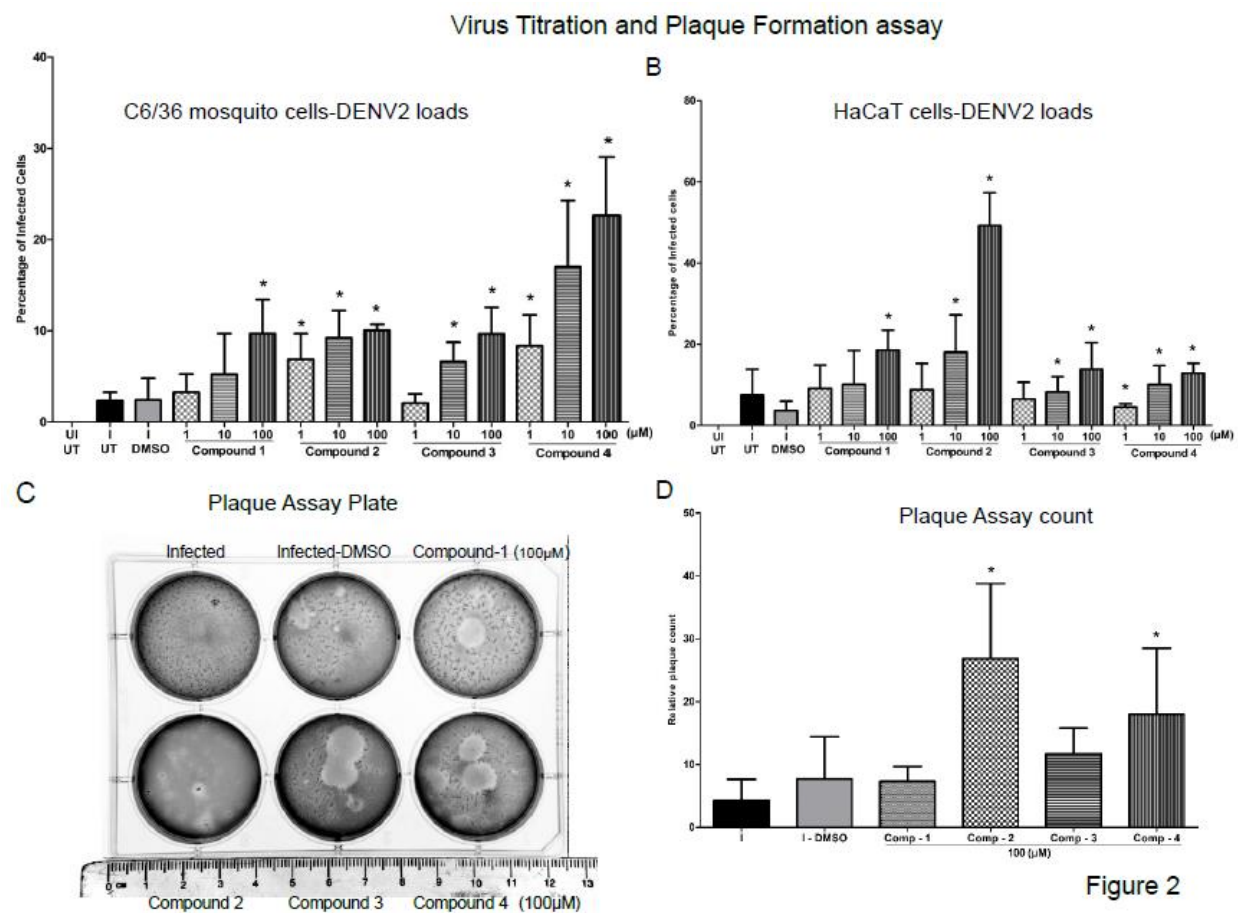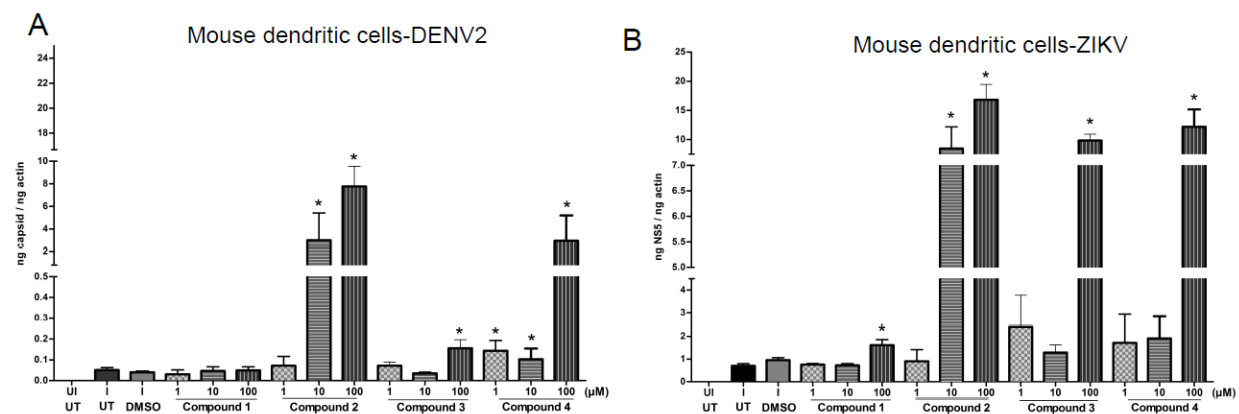
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FIGURES

Figure 1

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Virus Titration and Plaque Formation assay

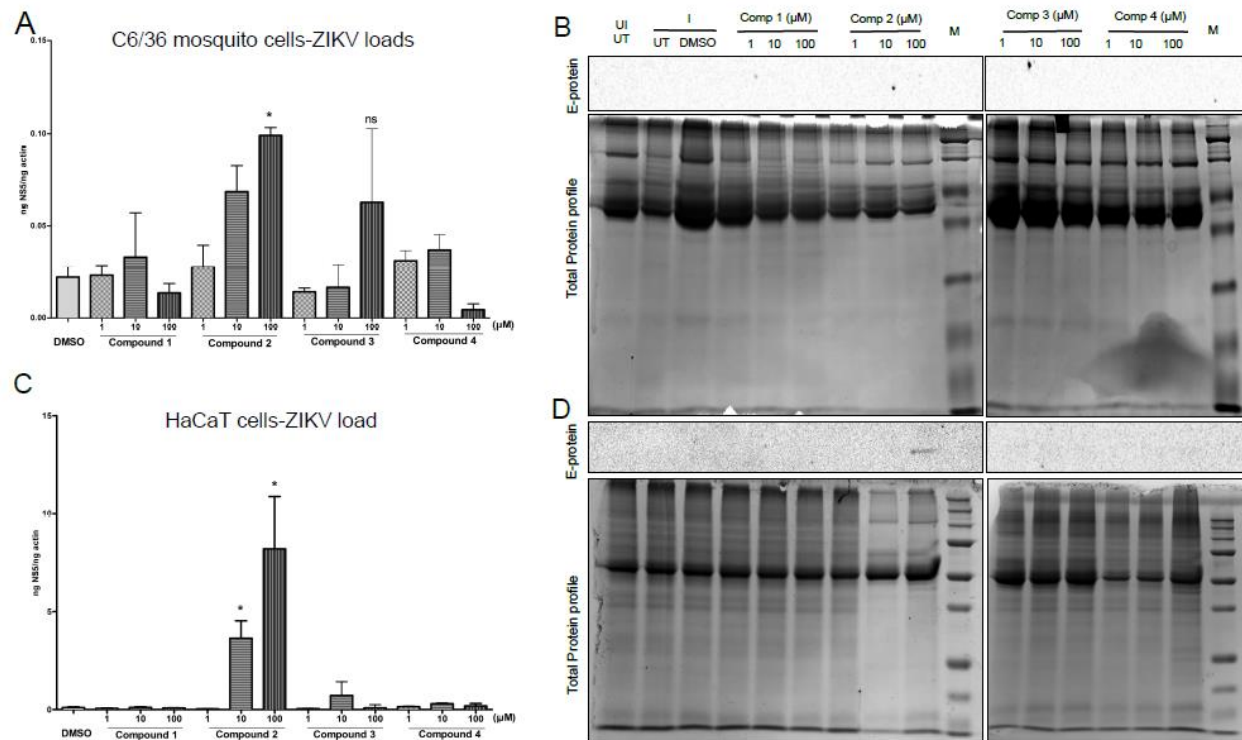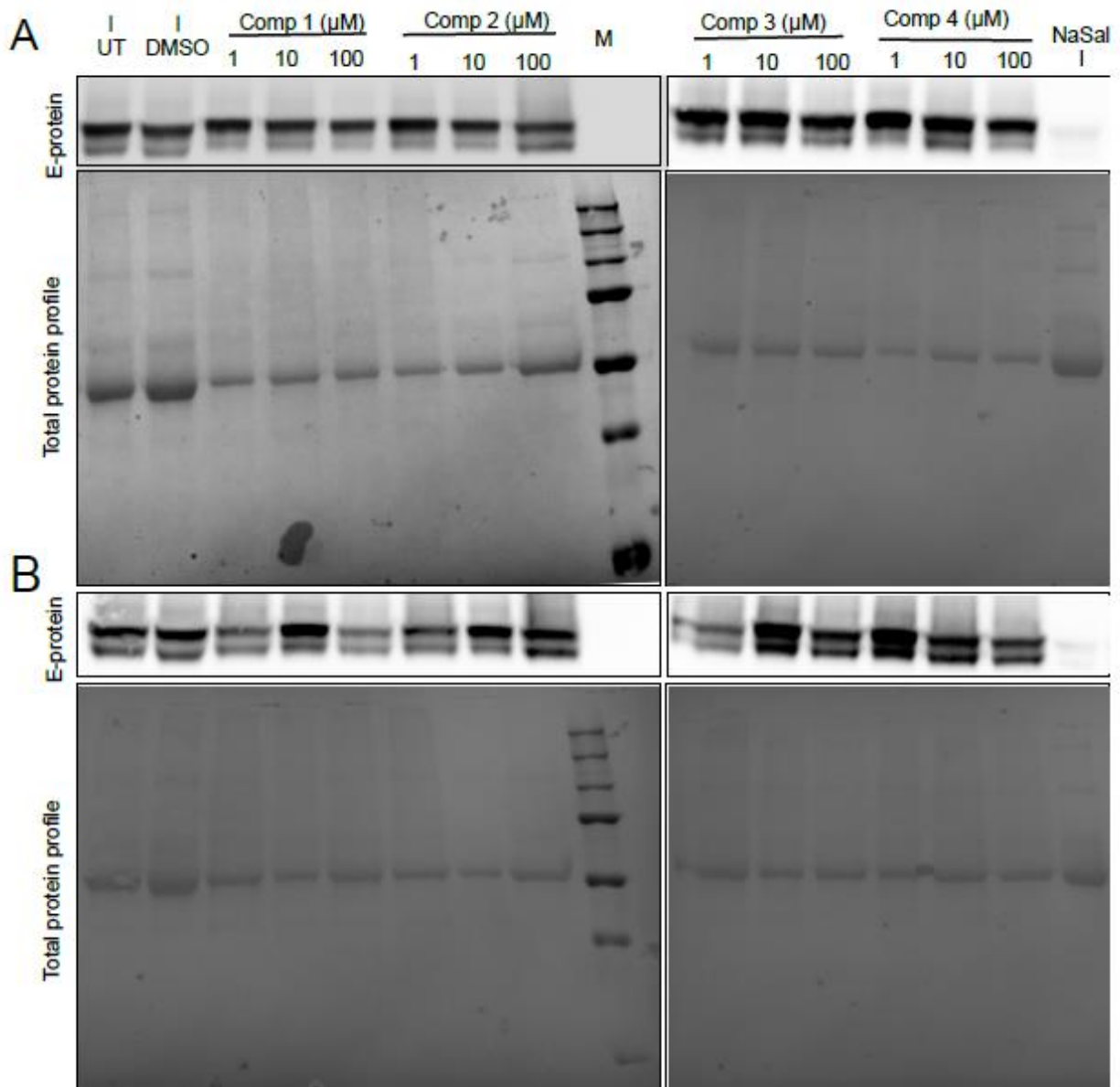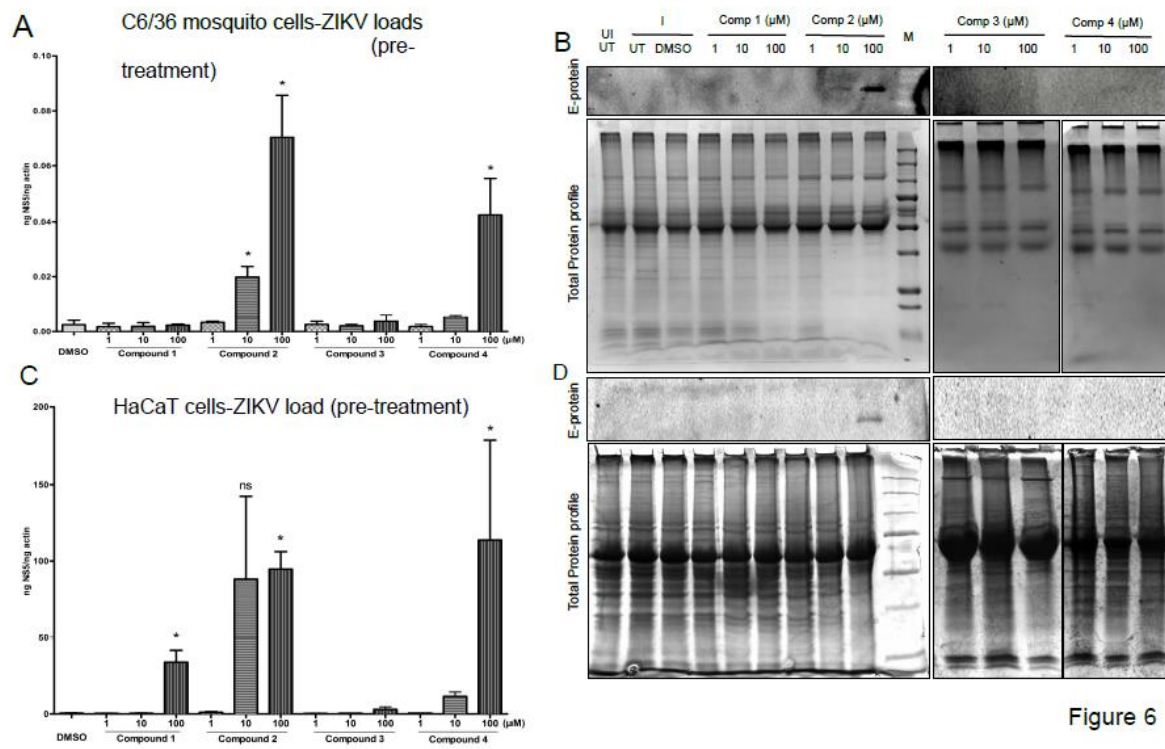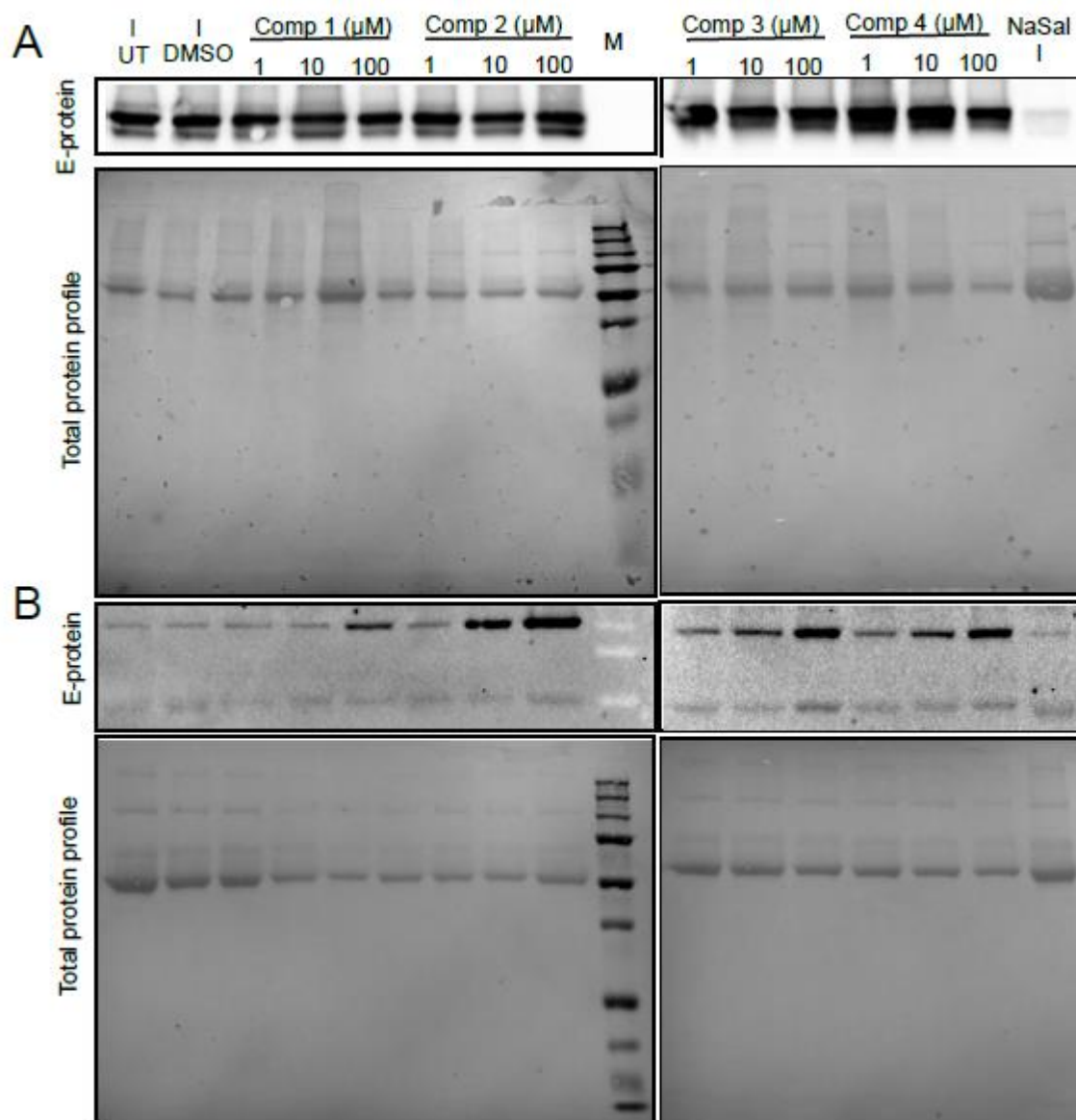Figure 2

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Figure 3

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Figure 4

Figure 5

Figure 6

Figure 7

HIGHLIGHTS

Mosquito-borne diseases are of great concern to the mankind. Use of chemicals/repellents against mosquito bites and transmission of microbes has been the topic of interest from many years. Here, we show that thiosemicarbazone ligand(s) derived from 2-acetylethiazole or citral or 1,10-phenanthroline upon conjugation with copper(II) or cobalt(III) metal centers enhances dengue virus (serotype 2; DENV2) and/or Zika virus (ZIKV) infections in mosquito, mouse and human cells. Enhanced ZIKV/DENV2 capsid mRNA or envelope protein loads were evident in mosquito cells and human keratinocytes, when pre-treated with compounds before/after infections. Also, treatment with copper(II) or cobalt(III) conjugated compounds increased viral titers and number of plaque formations. These studies suggest that conjugation of compounds in repellents/essential oils/natural products/food additives with copper(II) or cobalt(III) metal centers may not be safe, especially in tropical and subtropical places, where several dengue infection cases and deaths are reported annually or in places with increased ZIKV caused microcephaly.