Carnosine exhibits significant antiviral activity against Dengue and Zika virus

Hussin A. Rothan | Ammar Yasir Abdulrahman | Ahmad Suhail Khazali | Nurshamimi Nor Rashid | Teoh Teow Chong | Rohana Yusof

1 Department of Biology, College of Arts and Sciences, Georgia State University, Atlanta, GA, USA
2 Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
3 Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

Correspondence
Hussin A. Rothan, Department of Biology, College of Arts and Sciences, Georgia State University, Atlanta, 30303 GA, USA. Email: hrothan@gsu.edu
Rohana Yusof, Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. Email: rohana@um.edu.my

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INTRODUCTION

Dengue virus (DENV) and Zika virus (ZIKV) are mosquito-borne flaviviruses affecting millions of people in the tropical and subtropical regions. The World Health Organization (WHO) estimated 390 million DENV infections per year worldwide. Unlike DENV, ZIKV infection is much less prevalent but has been shown to cause serious neurological complications including microcephaly in newborns and Guillain-Barre syndrome in adults. DENV and ZIKV patients initially present with similar symptoms such as fever, arthralgias, myalgias, and conjunctivitis. Due to the lack of antiviral treatments, patients typically receive only antipyretics to alleviate the fever. Platelet level is monitored in dengue patients to assess disease progression, which may develop into dengue hemorrhagic fever and dengue shock syndrome.

Aedes aegypti and Aedes albopictus mosquitoes are the common vectors for DENV and ZIKV. These mosquitoes can carry and transmit...
both viruses simultaneously,\textsuperscript{2} creating significant practical difficulties in controlling and distinguishing the infections of these flaviviruses. Thus, broad-spectrum antiviral drugs that can target both DENV and ZIKV and attenuate the symptoms of both infections are of importance. Carnosine, an endogenous dipeptide composed of the amino acids β-alanine and L-histidine, possesses free radical scavenging properties and anti-inflammatory effects.\textsuperscript{6,7} Carnosine was shown to inhibit cytotoxic nitric-oxide induced proinflammatory condition and normalized host's immune response during influenza A pathogenesis.\textsuperscript{8} The effects of carnosine on DENV2 and ZIKV have never been reported. We hypothesized that carnosine could inhibit DENV2 and ZIKV. We found that carnosine significantly inhibited viral genome replication, ameliorated human liver cell viability post-infection, and demonstrated a significant prophylactic effect against DENV2.

2 | MATERIALS AND METHODS

2.1 | Virus and cells

DENV serotype 2 (isolate Malaysia M2) and ZIKV (MRS_OPY_Martineau_ParI_2015) were propagated by a single passage in C6/36 mosquito cells. Viral titer was determined via plaque assay using Vero cells. Vero cells (ATCC CCL-81) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and 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. L-Carnosine was purchased from Sigma (Sigma Aldrich) and dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO was lower than 1% in all cell culture-based assays.

2.2 | Docking studies

Carnosine peptide was modeled by Hyperchem 8.0.10. The PDB files of DENV2 and ZIKV proteins were downloaded from Protein Data Bank website. The binding capacity of carnosine with DENV or ZIKV viral proteins was assessed by protein-protein docking studies. Docking simulations were performed in an information-driven flexible docking approach for the modeling of biomolecular complexes using an available online HADDOCK server http://haddock.science.uu.nl/services/HADDOCK/haddockserver-easy.html.\textsuperscript{9} Final docking output files were analyzed for hydrogen bonds using PyMOL software version 1.3 (PyMOL 2010 Schrodinger, LLC).

2.3 | DENV2 NS2B-NS3 protease assay

This assay was carried out as we described previously.\textsuperscript{10} In brief, DENV2 serine protease was designed by joining the N-terminal of NS3 sequence to the C-terminal of NS2B via a linker (GGGGSGGGG). The DNA construct was synthesized and cloned into pQE30 expression vector. Recombinant NS2B-NS3pro (0.6 μM) was mixed with increasing concentrations of carnosine in 200-mM Tris-HCl pH 8.5. The mixtures were incubated at 37°C for 10 minutes; 50 μM fluorogenic BOC-Gly-Arg-Arg-AMC (Peptide Institute Inc., Japan) was added into the mixtures, and the reaction mixtures were further incubated at 37°C for 30 minutes. Substrate cleavage was detected using Ex/Em of 360/440 nm in triplicate samples using Varian Eclipse Cary fluorescence spectrophotometer. The readings were normalized against the no-inhibitor control, and the following equation was used to determine the protease percent activity. IC\textsubscript{50} value of carnosine was determined using a nonlinear regression model in GraphPad Prism 5.0 software.

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\text{protease activity} = \frac{\text{Intensity of enzyme activity} - \text{Intensity left after inhibition}}{\text{Intensity of enzyme activity}} \times 100.
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2.4 | Cytotoxicity assay

Huh7 cells (1 × 10\textsuperscript{4} cells per well of a 96-well plate) were treated with increasing concentrations (0, 25, 50, 100, and 200 μM) of carnosine for 72 hours. The cytotoxic effect of carnosine was determined using MTS assay as recommended by the manufacturer (Promega). The percentage of cell viability was calculated as follows: (absorbance of treated cells/absorbance of untreated cells) × 100%.

2.5 | Virus infection and titration

Huh7 cells were grown in a 24-well tissue culture plate (1.5 × 10\textsuperscript{5} cells/well) for 24 hours and then infected with DENV2 or ZIKV (MOI of 1) for 2 hours with gentle shaking after every 15 minutes. The virus inoculum was removed, and serially diluted carnosine treatments were added. The cells were incubated for 72 hours, and the supernatants were collected for titration using plaque formation assay as previously published.\textsuperscript{11} Briefly, 10-fold serial dilutions of the harvested supernatant were added to Vero cells monolayer in six-well plates (0.3 × 10\textsuperscript{6} cells) and incubated for 1 hour at 37°C. After washing, the cells were overlaid with the maintenance medium containing 1.1% carboxymethylcellulose. Viral plaques were stained with crystal violet dye after 5 days of incubation. Virus titer was calculated according to the following formula: Titer (pfu/mL) = number of plaques/volumes of the diluted virus added × well × dilution factor of the virus used to infect the cells in which the plaques were enumerated.

2.6 | Mode-of-inhibition assays

These assays were performed to determine the mechanism of antiviral activity of carnosine against DENV2 infection. Huh7 cells were grown for 24 hours under optimal conditions in a 24-well tissue culture plate (1.5 × 10\textsuperscript{5} cells/well). DENV2 (MOI of 1) was used for these assays. Pre-treatment experiment was carried out by applying 100 or 200 μM of carnosine to the cells for 4 hours prior to virus inoculation. Then, carnosine was removed, and viral inoculum was added for adsorption as mentioned previously. The cells were washed twice with serum-free DMEM. Fresh maintenance DMEM was added, and the cells were cultured for 24 hours in 5% CO\textsubscript{2}-supplemented incubator.
at 37°C. In simultaneous experiment, carnosine was mixed with DENV2 and added onto the cells for 2 hours with gentle shakings after every 15 minutes. Then, viral inoculum and carnosine were removed. After washing, fresh maintenance medium containing 100 or 200 μM of carnosine was added, and the cells were incubated for 24 hours. In postinfection treatment, the cells were inoculated with DENV2 for 2 hours as previously mentioned, then the cells were washed and incubated in fresh maintenance DMEM for 4 hours. Then, carnosine was added to the maintenance medium, and the cells were further incubated for 20 hours. For these experiments, virus infection positive control was the infected cells without carnosine treatment, and the negative control was noninfected cells treated with 1% DMSO. The supernatants were collected and stored at −80°C for viral load quantification using plaque formation assay.

2.7 | Immunostaining

Huh7 cells, grown on coverslips, were infected with DENV2 or ZIKV (MOI of 1) in the presence or absence of carnosine (100 or 200 μM). After 72 hours, cell monolayers were washed with cold PBS and fixed with ice-cold methanol for 15 minutes at −20°C. Indirect staining was carried out using mouse anti-dengue 2 (Millipore) primary antibody and rabbit antiserum against ZIKV NS3 and Alexa-Fluor 594-labeled goat anti-mouse or anti-rabbit IgG secondary antibodies. Hoechst 33342 (molecular probes) was used to stain the nuclei. The number of AF594 positive cells were manually counted and normalized to the total number of cells (based on nuclei staining).

2.8 | Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). Unequal variance t test was used to test for statistical significance between two groups, and one-way ANOVA was used to test for statistical significance among three groups. P values were denoted as follows: P < .05(*), P < .01(**), and P < .001 (***).

3 | RESULTS

3.1 | Prediction of carnosine interactions with viral proteins

Putative interactions between carnosine and DENV2 or ZIKV protease, and envelope proteins were predicted by protein-protein docking studies using publicly available viral proteins PDB files. Our docking studies revealed that carnosine may bind to several viral proteins as listed in Table S1 where carnosine showed the highest binding affinity towards ZIKV protease followed by DENV envelope, ZIKV envelope, and DENV2 protease.

3.2 | Carnosine inhibited DENV2 NS2B-NS3 protease activity

Next, we performed computational modeling and biochemical assay using recombinant DENV2 NS2B-NS3 protease that was previously generated in our lab.12 Computational modeling predicted that carnosine (stick structure) could interact with the catalytic triad residues (yellow) of DENV2 protease (Figure 1A,B). Carnosine was predicted to form hydrogen bonds with His107, Pro187, Ile92, Gly206, Ser190, and Gly208 of DENV2 NS2B-NS3 protease (Figure 1C). The residue numbers reported here are based on the full-length of our DENV NS2B-NS3 protease. We verified that His107 and Ser190 correspond to the reported DENV protease catalytic triad His51 and Ser135 residues.13 Carnosine interaction with the other catalytic residue, Asp130 (or the reported Asp75), was unclear. Nevertheless, the interactions with two of the catalytic triad residues may be sufficient and crucial in inhibiting DENV protease activity. Biochemical assay using fluorogenic substrates substantiated our computational modeling results where carnosine inhibited DENV2 NS2B-NS3 protease with an IC50 of 63.7 μM (Figure 1D).

3.3 | Carnosine protected liver cells against viral infection

Next, we assessed the potential antiviral effects of carnosine in vitro. We first established that exogenous carnosine treatment was not detrimental to human liver cells as the cell viability was more than 95% in all of the concentrations tested with a CC50 value of greater than 200 μM (Figure 2A,B).

The antiviral activity of carnosine was evaluated via plaque reduction assay. Carnosine inhibited the production of infectious viral particles by greater than 70% at the highest concentration tested with EC50 values of 52.3 and 59.5 μM for DENV2 and ZIKV, respectively (Figure 2A,B).

We observed some cytopathic effects such as irregular morphology, cell shrinkage, and cell death (not shown) of the infected Huh7 cells, which was consistent with reported studies.14,15 Thus, to verify the plaque reduction assay results, we measured the viability of the infected cells with or without exogenous carnosine treatment. DENV2 infection reduced cell viability to 33% but 100 and 200 μM carnosine treatment rescued cell viability to approximately 60% and 80% (Figure 2C). Similarly, Huh7 viability was only 40% after ZIKV infection but was ameliorated to 56% and 78% with 100 and 200 μM carnosine treatment (Figure 2D).

3.4 | Carnosine inhibited DENV2 genome replication and entry in liver cells

We performed mode-of-inhibition assays to determine the possible mechanism(s) of antiviral activities mediated by carnosine. The schematics of the assays are depicted in Figure 3A. For pre-treatment assay, the cells were pre-treated with carnosine for 4 hours followed
by DENV2 infection and subsequent viral quantification using plaque assay. The untreated samples showed 22.0 ± 1.5 × 10^5 pfu/mL, which was substantially reduced by 83.4% to 3.6 ± 0.8 × 10^5 pfu/mL with 200 μM carnosine treatment (Figure 3B). In the simultaneous carnosine treatment and viral infection assay, 200 μM carnosine significantly reduced virus titer to 2.8 ± 1.2 × 10^5 pfu/mL from 27.3 ± 5.2 × 10^5 pfu/mL in the untreated cells (89.7% reduction) (Figure 3C). Postinfection treatment showed similar antiviral effect (89% reduction) where at 200 μM of carnosine, the viral titer was 2.6 ± 1.1 × 10^5 pfu/mL compared with 23.8 ± 3.8 × 10^5 pfu/mL in the untreated samples (Figure 3D).

3.5 | Carnosine inhibited viral replication in Huh7 cells

The antiviral effects of carnosine were confirmed by immunostaining assay. In the no-carnosine samples, the fluorescent signals labeling DENV2 or Zika-NS3 were clearly visible in almost all of the infected cells (approximately 80%). Fluorescent signal was reduced in DENV-infected cells treated with 100-μM carnosine and undetected in cells treated with 200-μM carnosine. Similarly, in ZIKV-infected cells, reduction of fluorescence signal was observed in cells treated with carnosine but at a lower degree when compared with DENV2 samples (Figure 3E).

4 | DISCUSSION

Carnosine is an endogenous dipeptide that possesses significant antioxidant property. Antioxidant compounds have been shown to be tremendously beneficial in alleviating viral symptoms and improving patients’ conditions. A clinical trial assessing the effects of seven antioxidant compounds in chronic hepatitis C patients showed normalization of liver injury marker and histologic improvement in the patients. Similarly, supplementation with antioxidant vitamin E improved clinical, hematological, and biochemical parameters in children with dengue fever and dengue hemorrhagic fever. In addition to its antioxidant activity, carnosine also demonstrate anti-inflammatory property where it could directly interact with nitric oxide and protect cells against peroxynitrite-induced damage during influenza A (H1N1) pathogenesis. On the basis of these reports, we hypothesized that carnosine could mediate antiviral activity against DENV and ZIKV. We found that exogenous carnosine treatment significantly inhibited viral infection mainly by inhibiting viral genome replication and also interfering with DENV entry into host cells.
Hence, carnosine supplementation could be useful in preventing or alleviating DENV and ZIKV infection. It could also be utilized as a lead compound for the development of an effective antiviral agent. Peptide modification(s) may be required to improve carnosine stability especially in limiting its susceptibility to carnosinase and enhance its antiviral activity. These additional studies, however, are beyond the scope of the current study and will be addressed in the future.

In addition to its bioactivities, carnosine is safe as it is endogenously synthesized by carnosine synthase in a very slow process in skeletal muscle tissues, liver, and brain. Exogenous carnosine administration up to 2000 mg/kg was well-tolerated in vivo, which is consistent with our toxicity result that showed no changes in cell viability after carnosine treatment. In dengue and Zika patients, multi-organ dysfunctions affecting the liver, kidney, and brain have been documented, which might disrupt carnosine synthesis. Thus, exogenous carnosine administration or intake may be required to engender antiviral effects in the patients.

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Multi-organ dysfunction could also compromise carnosine homoeostasis as the brain and liver also secrete carnosinase to regulate carnosine level. Exogenous carnosine intake for ZIKV infection management in pregnant women requires further investigations as chronic exposure to exogenous carnosine was reported to delay zebrafish embryo development, accelerate hatching, and compromise larval survival. These effects are important as carnosinemia patients, a rare autosomal recessive disorder due to the lack of carnosinase, can also develop several neurological symptoms such as developmental delay and mental retardation. Thus, it is prudent to verify and establish the safe doses of carnosine in virus-infected young children, pregnant or nursing women, or patients with severe liver or kidney dysfunction.

We previously reported minimal cytotoxicity of several short synthetic peptides in human host cells. More importantly, the same short peptides exhibited antiviral effects against both DENV (our unpublished observation) and ZIKV replication. Low toxicity of the peptides and carnosine was presumably due to limited unwanted interactions, thus reducing potential side effects. The ability of these short peptides and carnosine to target both viruses was due to the homologous DENV and ZIKV NS2B-NS3pro sequence where the catalytic triad residues of the protease for both flaviviruses are conserved.

The computational data of this study predicted carnosine interaction with viral NS2B-NS3 serine protease and viral envelope that could lead to the inhibition of viral genome replication and viral entry. Carnosine interaction with viral serine protease was validated by the biochemical assay that showed protease inhibition with an IC50 of 63.7 μM. The inhibition on viral genome replication was further supported by the postinfection assay that showed 89% titer reduction. The highly similar virus titer in simultaneous and postinfection assays after carnosine treatment indicated that carnosine exhibited minimal virucidal effects. Carnosine also demonstrated some prophylactic effects where viral titer was reduced in dose-dependent manner in the pre-treatment assay indicating that carnosine may also exert its antiviral effect by interfering with virus entry into the cells.
computational studies indicated that carnosine could interact with the envelope proteins and potentially reduced virus binding to cellular receptors for entry. DENV and ZIKV share common receptors such as DC-SIGN, mannose receptor, heparan sulfate, and HSP90/HSP70 to gain entry into the cells. Polaprezinc, a zinc-chelated L-carnosine used to treat gastric ulcer, was reported to bind and inhibit HSP70 activity. Hence, it is plausible that carnosine could competitively bind to and inhibit HSP70 leading to reduced viral infection. Direct interactions between carnosine and other viral receptors have not been documented and require further investigation. The pretreatment assay may also indicate that carnosine accumulated in the cytoplasm and inhibited viral genome replication instead of preventing virus attachment. However, liver cancer cells express both carnosinase 1 and carnosinase 2 but at lower levels when compared with normal liver and human plasma. Furuta et al reported a complete conversion of L-carnosine to L-histidine within 15 minutes in vitro in the presence of human plasma, and Gardner et al reported carnosine half-life of the order of 1 minute in ex vivo human serum. Thus, significant accumulation of carnosine in the in vitro assay may be unlikely due to the short half-life of carnosine. Therefore, we believe carnosine inhibited DENV infection mainly by inhibiting viral genome replication and preventing virus entry into cells.

In conclusion, we demonstrated for the first time the potential antiviral activity of dipeptide carnosine against DENV2 and ZIKV. Further investigations are required to verify the safety of exogenous carnosine treatment, to evaluate the reproducibility of carnosine inhibitory effects against DENV and ZIKV in vivo, and to elucidate the exact or additional mechanisms of viral inhibition in vivo.
consideration should also be given in assessing carnosine safety during pregnancy, carnosine distribution, and its ability to cross placental barrier to inhibit ZIKV in utero and protect the fetus from Zika-mediated microcephaly.

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CONFLICT OF INTEREST
Authors declare that they have no conflict of interest.

ETHICAL APPROVAL
This article does not contain any study with human or animals.

REFERENCES


SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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