

Original Research Article

Antiviral evaluation of an Hsp90 inhibitor, gedunin, against dengue virus

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Abstract

Purpose: To evaluate the antiviral potential of a tetranortriterpenoid, gedunin, against dengue virus (DENV) replication by targeting the host chaperone, Hsp90.

Methods: The compound, gedunin, was tested against the replication of DENV *in vitro* using BHK-15 cells transfected with DENV-2 subgenomic replicon. Molecular docking of gedunin with Hsp90 protein was performed for evaluation of mode of action, using the program, Autodock vina.

Results: *In vitro* antiviral data showed that gedunin significantly ($p < 0.05$) reduced DENV replication with EC_{50} of 10 μ M. Further, *in silico* molecular docking data revealed strong interaction of gedunin with the ATP/ADP binding site of the host protein, Hsp90, with an estimated average free binding energy of -8.9 kcal/mol.

Conclusion: The results validate gedunin as a potential antiviral candidate. Further *in vitro* assays and *in vivo* viral challenge studies are required to confirm the exact mode of action and pharmacological profile of gedunin in DENV infections.

Keywords: Dengue virus replication, Hsp90, Gedunin, Antiviral, Molecular docking

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INTRODUCTION

Dengue virus belongs to the family *Flaviviridae* and has recently emerged as a significant human pathogen causing 50-100 million infections per year globally [1]. Due to complexity of the immune response towards vaccines and the need to control the four virus serotypes simultaneously, the development of vaccines has been challenging [2]. Several studies have previously suggested that both viral and host proteins essential for viral replication can be exploited for antiviral development [1]. Like many cellular proteins, the viral proteins also require host chaperones as support for proper functions [3]. Hsp90 is an ATP dependent heat shock protein, which is highly conserved among many

species from yeast to humans and involved in the regulation of several signaling, oncogenic and cell cycle protein [4,5]. The dependence of several oncogenes and other signaling processes involved in cancer progression on Hsp90 makes it a potential target against cancers [6,7]. It was shown that gedunin as an anticancer agent inhibits the function of Hsp90 protein, resulting in the degradation and improper functioning of the client proteins, similar to other Hsp90 inhibitors [8]. Numerous Hsp90 inhibitors, identified as anticancer agents such as radicicol, geldanamycin (GA), and GA derivatives, block Hsp90 activity by binding to the N-terminal ATP pocket of the protein and inhibiting ATPase activity [9]. Geldanamycin was shown to be active against replication of a range of viruses,

including herpes simplex virus type 1 (HSV-1), severe acute respiratory syndrome coronavirus and Zaire EBOV *in vitro* [10,11].

It has been observed for several viruses, including Ebola, Influenza, DENV, and Japanese encephalitis virus (JEV), that many proteins of these viruses require Hsp90 for their folding, assembly, and maturation, making it an attractive target for antiviral development [3]. Other studies have suggested that Hsp90 also binds to the viral components mainly to the RNA polymerase affecting the assembly and nuclear transport of polymerase [12]. Researchers have found a tetranortriterpenoid compound called gedunin, obtained from the Indian neem tree (*Azadirachta indica*), to be active against the 90 kDa Hsp90 by binding directly to its helper protein p23 leading to its inactivation [13,14]. Neem has been found to possess antiviral activity against DENV, Ebola virus and coxsackie B virus, a group of enteroviruses [15]. Furthermore, *in vitro* studies and high throughput assays with gedunin and gedunin derivatives confirmed that these compounds inhibit Ebola virus replication and protein expression as well as their host cell binding [16]. However, the mechanism by which gedunin disrupts the function of Hsp90 or its client proteins consequently leading to reduction of viral replication in cell cultures, is still not completely understood. This study was conducted to evaluate the inhibitory potential of gedunin against DENV-2 replicon in cell culture. Further, molecular docking was performed in order to investigate the possible interaction of gedunin with Hsp90. The results of this study will aid in the development of more effective therapeutics to treat dengue virus infections.

EXPERIMENTAL

Cell culture, replicon construct and test compound

BHK-15 cells were grown in minimal essential medium (MEM) containing 10 % fetal bovine serum (FBS) at 37°C incubator in the presence of 5 % CO₂. DENV-2 replicon containing the non-structural genes and *R. luciferase* as a reporter was used for the replicon assays to test the compound against dengue replication. The replicon construct and the compound were obtained from the Department of Biological Sciences, Purdue University, Indiana, USA. The test compound, gedunin, was suspended in DMSO and four dilutions (1, 10, 50 and 100 µM) of the compound based on the efficacy of these concentrations were tested against DENV replicon *in vitro* in BHK cells.

RNA transcription and electroporation

E. coli DH5-α cells were transformed with DENV2-R.luc-Rep, transformed colonies were inoculated in LB media containing 100 µg ampicillin overnight in a 37 °C shaking incubator. Plasmid DNA was purified using midiprep (Qiagen) kit and linearized by Xba-I digestion. Linearized DNA was purified with GFX columns and used as template for *in vitro* transcription using T7 RNA polymerase. Replicon RNA was purified with the RNeasy kit (Qiagen).

For RNA electroporation, 2×10⁶ BHK cells were washed twice before being resuspended in 0.4 ml of room temperature PBS containing 10 - 20 µg of RNA. The cells were pulsed twice consecutively, using the electroporation apparatus (BioRad Gene-Pulser II), at 25 µF, 1.5 kV, and 200 Ohms in a 0.2 cm cuvette. Electroporated cells were incubated at room temperature for 3 min and 1 mL of 2.5 % FBS-MEM was added to the cells. Cells were resuspended and the contents of the cuvette were then transferred to 9 mL of 2.5 % FBS-MEM. The cells were mixed well, plated into a 96-well plate (100 µl per well) and incubated in a 5 % CO₂ incubator for 2 h at 37 °C.

Compound treatment

After 2 h of incubation, media was aspirated off from the electroporated cells in a 96 wells plate and drug dilutions were added at final concentrations of 1, 10, 50 and 100 µM in 2.5 % FBS-MEM, while 1 % DMSO and untreated cells were used as negative controls. Three replicates were used for each dilution. Cells were grown at 37 °C for 48 h in 5 % CO₂ incubator.

Luciferase assay

The cell extracts were taken at 48 h post-infection, washed with PBS and then lysed with *Renilla luciferase* lysis buffer. These cells were incubated at room temperature for 30 min with gentle rocking and stored at -80 °C till further use. Luciferase activity was measured using standard Promega's *Renilla luciferase* assay system. The frozen extracts (lysates) were thawed and then homogenized by gentle pipetting. For the luciferase activity, 10 µL of lysate from each well was transferred to an opaque plate. Luminescence was then measured after adding 50 µL of luciferase substrate to the cells using SpectraMaxL microplate reader (SoftMax Pro 5.4.4 software, Molecular Devices) and activity was detected in relative light units (RLU).

Cytotoxicity assay

BHK-15 cells at 90 % confluence were treated with gedunin, diluted in 2 % FBS-MEM, for the same range of concentrations as used for replicon assays in a 96-well plate, and incubated at 37 °C in the presence of 5 % CO₂. At 48 h post-treatment, the compound dilutions were aspirated from the cells, and washed with MEM. Cell toxicity of the compound was then measured using Quick Cell Proliferation Assay kit. Substrate from the kit was diluted appropriately and 100 µL of the substrate was added to each well. Plate was then incubated at 37 °C for 2 h and the optical density (OD₄₅₀) was measured using a microplate reader, SpectraMax M5 (SoftMax Pro 4.8 software, Molecular Devices). The OD₄₅₀ obtained for cells treated with the drug dilutions were compared to the cells treated with 1 % DMSO alone as negative control.

Molecular docking studies

Preparation of ligand

Chemical structure of gedunin was obtained from PubChem chemistry database, which has been reported to inhibit Hsp90 protein [8]. The structure of the compound was then drawn in ChemSketch software 11.02 (Advanced Chemistry Development, Inc. ACD/Labs) and 3-D structure optimization was done by adding hydrogen atoms to the ligand. The refined structure was saved in .mol format and then converted to pdb file format using ArgusLab 4.0.1 (Mark Thompson and Planaria Software LLC). Then torsions of the ligand were stabilized, non-polar hydrogens were added and PDBQT file was prepared using AutoDock Tool for further analysis through molecular docking.

Preparation of Hsp90 as a target protein

The structure of Hsp90 in complex with ADP in the N-terminal ADP/ATP binding site (PDB ID: 1AM1) was retrieved from RSCB Protein Data Bank [17]. Ligand molecule was initially removed for the purpose of docking. The protein structure was adjusted and optimized by adding hydrogen atoms and removing water molecules using AutoDockTools 1.5.6 (The Scripps Research Institute, La Jolla, USA). The grid box for the receptor was then set to cover the entire molecular surface and the output file was generated in PDBQT format.

Molecular docking

Molecular docking was then initiated using AutoDock Vina 1.1.2 [18] and the predicted

binding affinity of the ligand with the receptor was calculated based on the scoring function in kcal/mol. After docking, the docked complex having a conformation with the lowest binding affinity value was selected and saved in pdb format. The docked complexes were analyzed using UCSF Chimera version 1.8.1 (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco). Two-dimensional and three-dimensional interaction patterns of the docked complex were determined in order to study the amino acids involved in the ligand-protein binding site using Discovery Studio (Version 4.1.0).

Statistical analysis

The RLU values from the luciferase assay were plotted against the log transformation of the compound concentrations and the effective concentration (EC₅₀) was determined using GraphPad Prism version 5 software (GraphPad Software, San Diego California, USA). Data are presented as mean ± standard deviation (SD, n = 3). The data were evaluated statistically using one-way analysis of variance (ANOVA) and $p < 0.05$ was considered significant.

RESULTS

Inhibition of dengue virus subgenomic replicon in cell culture

The inhibitory activity of gedunin was evaluated against DENV replication in a replicon based assay. Here, four different dilutions of the compound (1, 10, 50 and 100 µM) were tested against dengue virus replication using the DENV replicon containing the reporter gene (*R. luciferase*), while DMSO (1 %) treated cells were used as negative control in the inhibition assays. Luciferase assays were performed with the cell extracts using standard *R. luciferase* substrate. Cells treated with 1 % DMSO were used as negative control for inhibition. The relative light units (RLU) were plotted against the log transformation of the concentrations of the compound and a concentration dependent virus inhibition was observed (Figure 1). Upon an increase in the concentration of gedunin, a significant reduction of reporter activity was observed and almost complete inhibition was observed at higher concentrations such as 50 and 100 µM. EC₅₀ value was determined using GraphPad Prism software. Gedunin exhibited significant reduction in the luciferase activity compared with the control indicating the inhibition of genome replication with EC₅₀ of 10 µM.

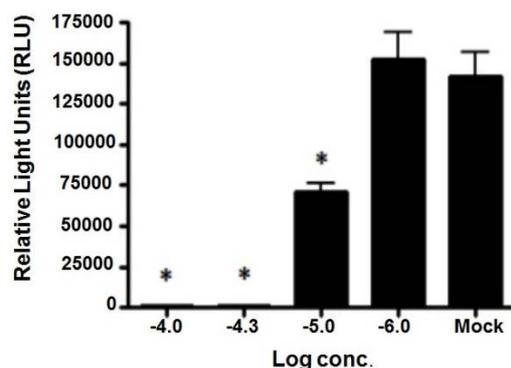


Figure 1: Inhibitory activity of gedunin against DENV-R.luc2A-RP replicon. Test concentrations indicating significant inhibition are represented by asterisk; * $p < 0.05$ using one way ANOVA). Mock indicates treatment with 1 % dimethyl sulfoxide; $n = 3$; error bars indicate standard deviation

Each of these compound concentrations were then tested for cytotoxicity in BHK-15 cells at 90 % confluence using the quick cell proliferation assay. Cytotoxicity assays were also performed for each concentration in a 96 well format and optical densities were determined at 450 nm using micro-titer plate reader. The optical densities obtained with the drugs were compared with that of the DMSO treated and untreated cells and there was no toxicity observed within the range of the concentrations used up to 100 μM .

Gedunin-Hsp90 docking results

Gedunin was selected as the ligand for this study on the basis of its previous roles in modulation of the Hsp90 activity in cancers and antiviral studies. Structures of gedunin (Figure 2) and Hsp90 (PDB: 1AM1, Figure 3) were processed for computational analysis using AutoDock Tool, then gedunin was docked with Hsp90 at its N-terminal binding pocket using AutoDock vina.

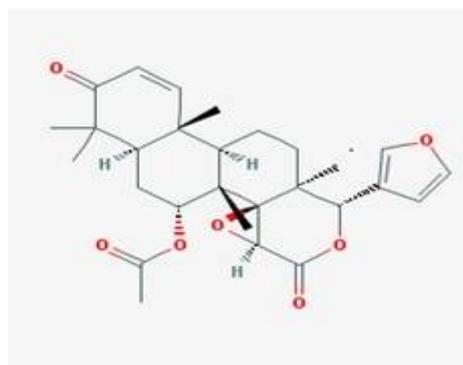


Figure 2: Structure of gedunin

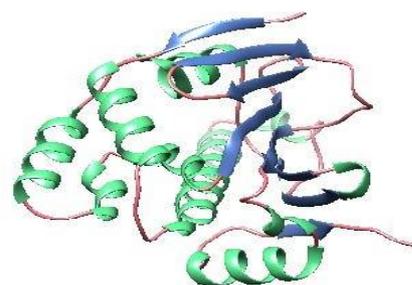


Figure 3: Crystal structure of Hsp90 (PDB 1AM1)

Initially, the protein with attached ATP in the N-terminal domain was selected for analyzing whether the ligand binds in the ATP binding site or not. The ligand was later removed using Chimera software. These docking results showed that gedunin successfully binds in the ATP binding pocket of the Hsp90 molecule at N-terminal (Figure 4). Thus, the active conformation of the ligand and the best binding mode for the active site cavity were obtained. The conformation of the docked complex with the lowest binding energy values was selected and saved in pdb format. According to the docking results, gedunin exhibited a good average binding affinity (-8.9 kcal/mol) and interacted potentially with the amino acids within the binding pocket of the Hsp90 at its N-terminal.

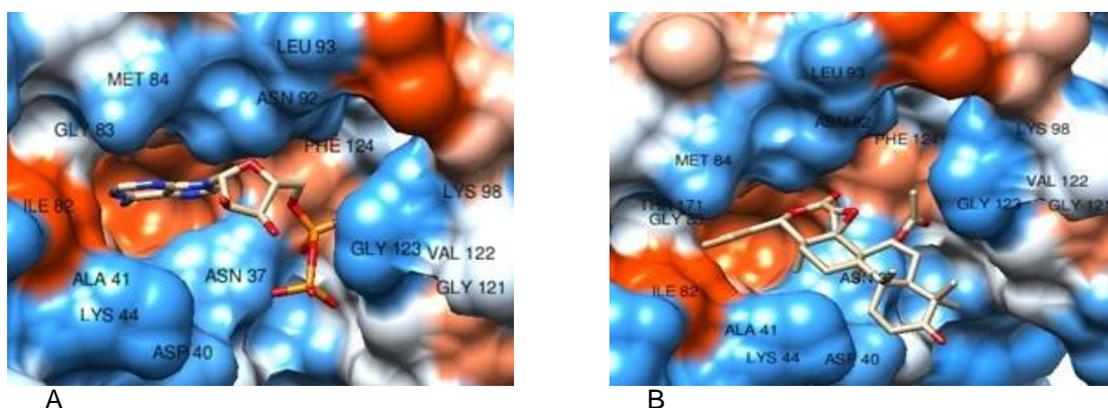


Figure 4: Three dimensional representation of the binding pocket. **(A)** Surface representation of the N-terminal ATP-binding site of Hsp90 with the bound ATP molecule **(B)** AutoDock predicted model of bound conformation of the compound gedunin at the ATP binding site of Hsp90 that demonstrated dengue antiviral activity in cell culture

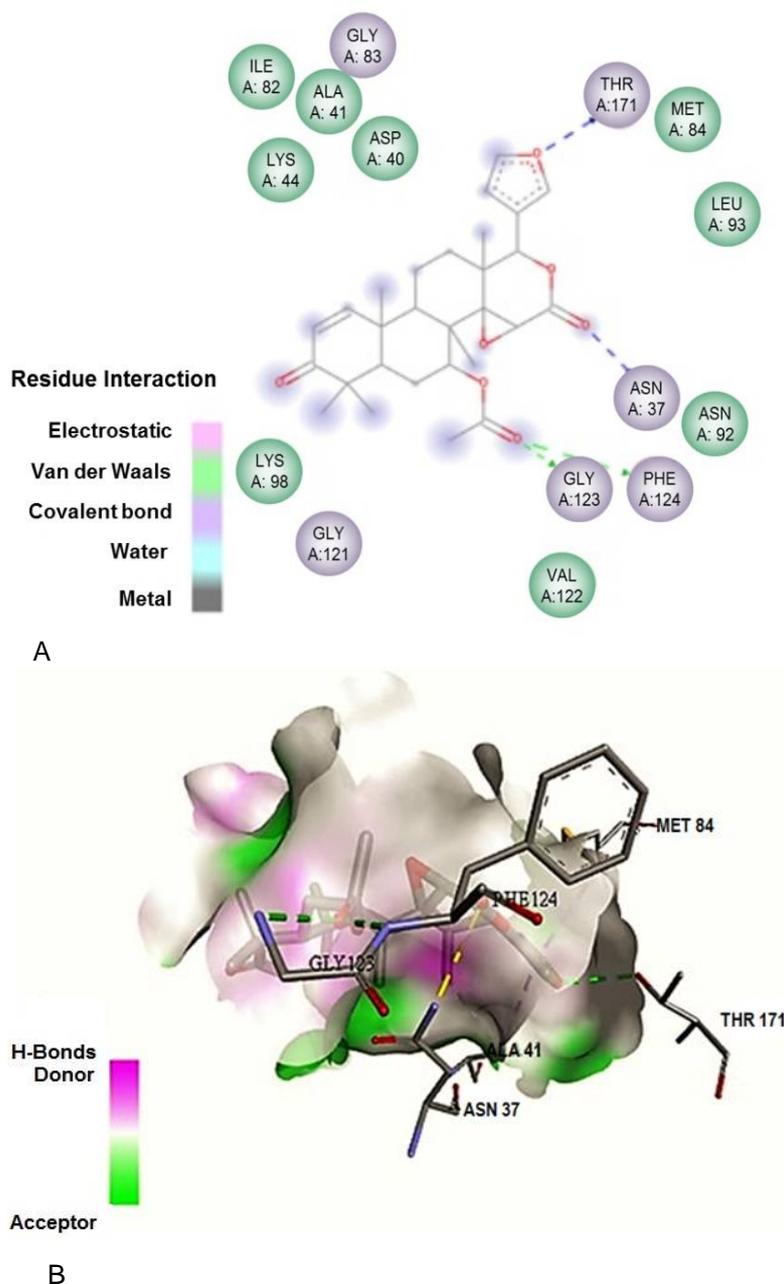


Figure 5: The interaction pattern of gedunin with the active site of host chaperone Hsp90 (PDB 1AM1) generated by the program, Discovery Studio 4.1.0. **(A)** Two dimensional interaction of the protein-ligand complex. The legend inset represents the type of interaction between the ligand atoms and the amino acid residues of the protein. **(B)** Three dimensional docking of the compound in the binding pocket showing the hydrogen bond interactions

The possible protein-inhibitor interactions of gedunin with the active site of Hsp90 molecule are shown in Figure 5A. Amino acid residues at the binding site interacting with the gedunin are Asn 37, Asp 40, Ala 41, Lys 44, Ile 82, Gly 83, Met 84, Asn 92, Leu 93, Lys 98, Gly 121, Val 122, Gly 123, Phe 124, and Thr 171. The compound also formed potential hydrogen bond interactions with the amino acids at the active site (Figure 5B). Amino acid residues involved in hydrogen bonding are Asn 37, Ala 41, Met 84, Gly 123, Phe 124, and Thr 171.

DISCUSSION

The aim of our work was to evaluate the antiviral property of the compound gedunin from the Indian neem plant against dengue virus and propose its potential mechanism of action through molecular docking studies. Gedunin has been shown to bind to the host chaperone machinery of Hsp90 and blocks its activity [14]. Hsp90s are host chaperone proteins which act as ATPases and consists of homodimeric

complexes. Each monomer consists of a C terminal domain involved in dimerization, a middle domain and an N-terminal ATP binding domain [19]. Hsp90 plays important role in helping mutated proteins in several cancers. It is involved in stabilization of a number of oncogenic kinases, including HER2, EGFR, BCR-ABL, B-Raf and EML4-ALK [20]. The host protein Hsp90 plays an essential role in the replication of various viruses, making it an important target for antiviral drugs. An antibiotic geldanamycin (GA), which was initially characterized as an anticancer agent, has been reported to exhibit broad-spectrum antiviral activity *in vitro* against several viruses by targeting the ADP/ATP binding site of Hsp90 [21]. The target viruses include herpes simplex virus type-1 (HSV-1) [10], severe acute respiratory syndrome corona virus, vaccinia virus [23], influenza virus, vesicular stomatitis virus [22], hepatitis C virus [24] and ebola virus [11].

Gedunin, identified as HSP-90 inhibitor was therefore tested against the dengue replicon *in vitro* in BHK-15 cells. Development of subgenomic replicons of the virus offer great potential to study the replication process, as well as to study the effects of antiviral drugs/inhibitors on the replication due to their non-infectious nature [25]. Different concentrations of the compound gedunin were tested against dengue virus replication using the DENV replicon, containing the reporter gene (*R. luciferase*) expressed in a replication dependent manner. It was observed that gedunin significantly reduced replication of dengue virus in cell culture. The exact mechanism of antiviral action of gedunin is not very clear yet. Several studies have shown that it inhibits the activity of the host chaperone Hsp90. Hsp90 has been believed to facilitate viral protein folding and activity of non-structural proteins such as polymerase, protease and helicase as well as the structural proteins [3]. The polymerase of several viruses require Hsp90 for genome replication which include influenza virus A [12], herpes simplex virus [26], flock house virus [27] and vesicular stomatitis virus and treatment with Hsp90 inhibitors such as geldanamycin and 17-AAG lead to degradation of polymerase complexes [28,22].

Molecular docking analysis was then carried out using AutoDock vina software to evaluate the binding capability of gedunin with Hsp90 molecule and propose its possible mechanism of action. The obtained results suggested that the Hsp90 inhibitor gedunin directly binds in the N-terminal ATP binding site of the protein and forms significant non-covalent and hydrogen bond interactions. In a study with geldanamycin,

it was shown that the compound binds specifically in the ATP/ADP binding site of Hsp90 and inhibits the protein function. In a study conducted by Petrikaite and Matulis [7], they also suggested that the majority of Hsp90 inhibitors blocks ATPase activity of Hsp90 by binding in the N-terminal ATP. These results correlate with the findings of the computational analysis of the inhibitor and the host chaperone in the current study. Recently, Hsp90 was shown to be an essential host protein for the replication of several negative strand RNA viruses [11]. Other studies have shown that host factors are involved in JEV life cycle and RNA virus replication and identified other host factors such as Hsp70 and GRP78 including Hsp90 in the secretion medium from JEV infected BHK-12 cells [29].

CONCLUSION

Using *in vitro* technique and molecular docking studies, a novel compound that inhibits DENV replication has been successfully identified. The findings of the study indicate that gedunin has a significant inhibitory activity against dengue virus *in vitro* and may prove to be a good lead compound for the development of an anti-dengue compound targeting the Hsp90 protein. Further investigations on the mechanism by which gedunin induce client protein degradation and inhibits dengue virus *in vitro* are required. Furthermore, the approach used in the present study may afford additional drug candidates for the development of therapies against dengue infections.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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