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Computational design of a sulfoglucuronide derivative fitting into a hydrophobic pocket of dengue virus E protein

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ABSTRACT

We performed first-principles calculations based on the *ab initio* fragment molecular orbital method on dengue virus envelope protein with a hydrophobic ligand, octyl-β-D-glucose to develop an entry inhibitor. As several polar amino acid residues are present at the edge of the pocket, the glucose moiety was chemically modified with hydrophilic groups. Introduction of both sulfated and carboxylated groups on glucose enhanced not only binding affinity to the protein but also inhibition of dengue virus entry. Octyl-2-*O*-sulfo β-D-glucuronic acid may serve as a molecular probe to study the dengue virus entry process.

1. Introduction

Dengue fever and dengue hemorrhagic fever caused by dengue virus (DENV) infection are major public health concerns around the world [1–3]. The DENV RNA genome encodes a single polyprotein that is co- and post-translationally processed into three structural (C, PrM and E) and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) proteins by host- and virus-derived proteases [4]. Among the structural proteins, envelope glycoprotein (E protein) contributes to virus entry events, such as adsorption to host cells, followed by virus-host membrane fusion [5–7]. Conformational rearrangement of the protein induced by the low pH in the endosome of host cells is essential for membrane fusion. A hydrophobic pocket of the protein proximal to the hinge region between domains I and II affects the pH threshold for membrane fusion and changes into a β-hairpin structure. In post-fusion events, virus particles are disassembled and the viral genome is released into the cytoplasm, followed by its translation and replication [5,7,8].

No vaccines or anti-DENV drugs are clinically available at present. Several classes of antiviral agents have been developed, including compounds targeting two viral enzymes, NS3 protease and NS5 RNA-dependent RNA polymerase [9–11], compounds against *N*-linked glycan biosynthesis of viral prM, E and NS1 proteins [12–14], compounds targeting viral E protein functions, such as virus adsorption and fusion [15–21], and compounds targeting host factors, including protein kinases, molecules responsible for cholesterol biosynthesis and immune responses [22].

Inhibition of virus entry into host cells is an effective means of preventing virus infection [22]. Dengue virus-host cell membrane fusion is induced by pH-dependent conformational changes and subsequent rearrangement from a dimer to a trimer of the E protein on the virus particles. This conformational change is generated in a hinge region between domain I and domain II of the protein. A hydrophobic pocket occupied by octyl-β-D-Glc is located in the hinge, suggesting that substances fitting the pocket may become fusion inhibitors that prevent the conformational change of the protein, and subsequent rearrangement essential for virus-host cell membrane fusion [23]. Recently, virtual screening approaches of chemical compound libraries demonstrated that small inhibitors targeting DENV E protein can effectively prevent membrane fusion between the virus and host cells [24–26]. However, the precise

Abbreviations: DENV, dengue virus; NS, non-structural; GlcA, glucuronic acid.

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modes of action of these compounds remain to be determined. Based on the physiochemical properties affecting bioavailability and biological activity related with efficacy and unfavorable side effects of these compounds, we postulated that further exploration of fusion inhibitors by different approaches would be beneficial for the development of anti-DENV agents. In the present study, we designed, synthesized and evaluated sulfo-GlcA derivatives on the basis of the E protein structure with a hydrophobic ligand, octyl-β-D-Glc. We performed first-principles calculations based on the *ab initio* (total electronic) fragment molecular orbital (FMO) method at the correlated RI-MP2/cc-pVDZ level on the whole complex structure of the protein with a hydrophobic ligand, octyl-β-glucoside, which fits into the pocket for development of fusion inhibitors. The correlated FMO calculations can adequately estimate not only electrostatic but also van der Waals (hydrophobic) interactions between the ligand and amino acid residues in the protein [27]. As several polar amino acid residues, such as glutamic acid, serine, threonine and lysine, are located at the edge of the pocket, chemical modifications of the glucose moiety of octyl-β-glucoside with hydrophilic groups were performed for generation of hydrophobic ligands with more potent inhibitory activity. Infection assay demonstrated that the introduction of both sulfated and carboxylated groups at the C2 and C6 positions on glucose, respectively, significantly enhanced the inhibition of dengue virus entry into the host cells. The compound, octyl-β-2′-*O*-sulfated glucuronide described in this report may also serve as a molecular probe to study the dengue virus entry process.

2. Materials and methods

2.1. Computational analysis

The amino acid sequence of the E protein of DENV-1 strain, Laos/03, which was used for the focus-forming assay in this study, was deduced by nucleotide sequencing of the E gene. The DENV1 strain, Tahi01, which had a complete match to the sequence of the Laos/03 E protein, was used for the computational analyses discussed below. The three-dimensional structure of the target protein was built by homology alignment modeling using the MOE 2010.10 program on the basis of the crystal structure (PDB ID: 1OKE) [23]. The structure was refined by Refmac5 of the CCP4 suite (R-factor = 0.261, R-free = 0.308) [28]. Hydrogen atoms were automatically added at pH 7.0 by the protonate 3D program in the MOE. All hydrogen atomic coordinates were optimized by the conjugate gradient method with the AMBER99 force field under the Born solvent model. N- and C-termini were ionized $-NH_3^+$ and $-COO^-$, respectively. The initial structure of octyl-2-*O*-sulfo β-glucuronic acid was modeled from octyl-β-glucose in the X-ray crystal structure. The structure of octyl-2-*O*-sulfo β-glucuronic acid in the hydrophobic pocket was optimized at the traditional HF/6-31G* level for the partial model system with the Gaussian09 program [29]. The FMO calculations were performed on the complex of DENV1 E protein with octyl-2-*O*-sulfo β-glucuronic acid as described previously [30]. Interfragment interaction energy (IFIE) analysis was performed for identification of the amino acid residues in the hydrophobic pocket interacting with synthetic compounds [27]. Binding affinities of these compounds to DENV1 E protein were theoretically evaluated as the total scores of all the IFIE with each compound [31]. Molecular Dynamics (MD) simulations of the complex were performed using the AMBER12 program package with the standard AMBER99SB and GAFF force fields for the target DENV E protein and inhibitors, respectively [32]. The time step and temperature were 1 fs and 300 K, respectively. The isothermal–isobaric (NPT) ensemble and Langevin thermostat were applied to the system. Total simulation time was 10 ns. Pressure was set at 1.0 bar. The protonated complex was moved to the center of a cubic box ($1.2 \times 1.2 \times 1.2\ nm^3$), and transferable intermolecular potential three-point model (TIP3P) water molecules were solvated into the box at a solvent density of $1.0\ g/cm^3$ [33,34]. Electrostatic interactions over long distances were evaluated by the standard particle mesh Ewald method [35].

2.2. Focus-forming assay

Inhibition of virus infection by synthetic compounds was determined by focus-forming assay as described previously [36]. Briefly, BHK-21cells were seeded onto 96-well plastic plates and cultured for 24 h at 37 °C in DMEM supplemented with 5% FBS. Virus–compound premixtures were inoculated onto the cells for 2 h at 37 °C. After removal of the virus solution, the overlying medium was added, and plates were incubated at 37 °C for 41–43 h. Infectious foci were immunochemically detected. Virus infectivity was determined as focus-forming units (FFU).

3. Results and discussion

3.1. Computational designation of compounds fitting into the hydrophobic pocket in DENV1 E protein

A structural biology study showed that the hydrophobic ligand, octyl-β-D-Glc, binds to the pocket located in domain I of DENV E protein [23]. We proposed a theoretical modeled structure of DENV1 E protein with octyl-β-D-Glc as a lead compound for the design of anti-viral agents (Fig. S1). Based on the template protein structure, 1OKE, we fit the ligand into the hydrophobic pocket of the protein (Fig. 1). We analyzed significant interactions among octyl-β-D-Glc and amino acid residues of the pocket. The octyl-group of the ligand apparently fitted to several hydrophobic amino acids of the pocket, such as V50, F193, L198, L207, I270, F279, A280 and G281. On the other hand, the glucose portion of the ligand interacted with Q271 alone. This sugar residue did not apparently interact with any other hydrophilic amino acid residue around the edge of the pocket. It seems that this portion could move in an unrestricted manner. Therefore, we hypothesized that introduction of hydrophilic functional groups on glucose residues, such as sulfation and carboxylation, could contribute to tighter binding of ligands to the hydrophobic pocket. The obtained compounds were expected to inhibit dengue virus infection with more potent activity than octyl-β-D-Glc. We chemically synthesized and tested four derivatives (compounds **1**–**4**) and octyl-β-D-Glc as controls for anti-DENV activity (Fig. S2).

3.2. Octyl-2-O-sulfo β-D-GlcA inhibited DENV1 infection to BHK-21 cells

Table 1 shows a list of octyl-β-D-Glc derivatives used in this study. Compounds **1** and **2** are sulfated at different positions of GlcA. Compounds **3** and **4** have sulfur introduced at different positions on the Glc residue. The purity and assignment of the compounds were confirmed by ^{1}H-nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HR-MS) analyses. Table 1 also summarizes the inhibitory activities of five compounds on DENV1 infection at a concentration of 500 μM. Dengue virus 1 infection is significantly inhibited by treatment of the virus with sulfated and carboxylated octyl-β-D-Glc derivatives. Focus-forming assay demonstrated that compound **1** inhibited infection of the cells by DENV1 most potently. The assay also showed that compound **1** dose-dependently inhibited DENV infection with an IC_{50} value of 116 μM (Fig. 2). The lead compound **5**, octyl-β-D-Glc, slightly inhibited

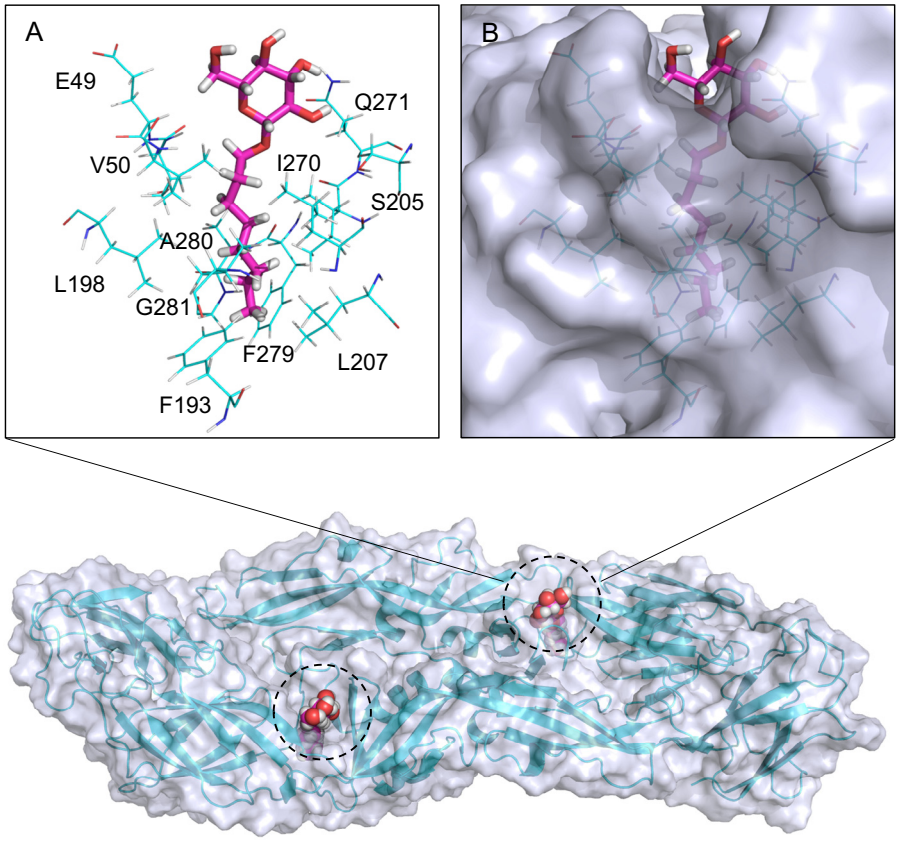


Fig. 1. Homology-modeled structure of DENV1 E protein with a hydrophobic ligand, octyl-β-D-Glc. The protein structure was built by homology alignment modeling on the basis of the crystal structure of the DENV E protein (PDB ID: 1OKE) as described in the Section 2. The structure was also refined by the Refmac5 algorithm. Amino acid residues located within 4 Å from octyl-β-D-Glc are shown in (A). A hydrophobic pocket of E protein is shown by the water-accessible surface in (B). Dotted circles indicate a hydrophobic pocket including octyl-β-D-Glc.

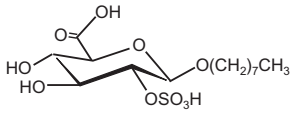
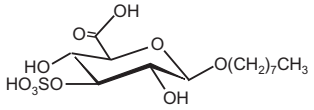
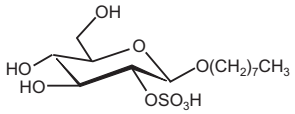
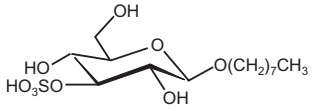
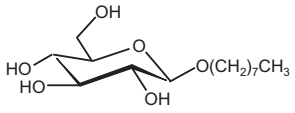
DENV1 infection. Analysis of the structure-inhibitory activity relationship yielded findings regarding the chemical structures related to enhancement of the inhibition of DENV infection. First, in comparison of the inhibitory effect with compounds **1** and **2**, 2-*O*-sulfation was favored over 3-*O*-sulfation of GlcA. Second, as compounds **3** and **4** show slight enhancement of the inhibitory effect on DENV infection to BHK-21 cells, the carboxyl group on GlcA contributed significantly to the inhibition. This finding also suggests the importance of carboxylate as an anionic group at the 6-position of the glycoside. Morphological observations and MTT assay indicated that 2-*O*-sulfated GlcA did not show cytotoxicity on BHK-21 cells up to 2 mM. The introduction of both sulfated and carboxylated groups on the Glc residue decreased cytotoxicity as compared with the lead compound **1** (Fig. S3). This strongly suggests that compound **1** does not show either cellular toxicity or viral membrane lysis by a detergent-like effect.

Several lines of evidence demonstrated that small inhibitors targeting DENV E protein effectively prevent membrane fusion between the virus and host cells. *In silico* screening of chemical small-molecule library identified a compound that inhibited DENV2 infection of the cells with an EC_{50} concentration of 119 nM [24]. The authors proposed that the compound binds the hydrophobic pocket of the E protein based on docking simulation analyses. Other studies using similar methodologies showed that compounds inhibited infection by flaviviruses, including DENV, West Nile and yellow fever viruses, in the IC_{50} concentration range of 1–500 μM [17,25,26]. Very recently, high-throughput screening of chemical small-molecule library by blocking assay in fusion peptide binding to the E protein identified a potent inhibitor that prevented rearrangement of the protein from a dimeric to a trimeric form [37]. The inhibitory mechanism of action of compound **1** was similar to those of other fusion inhibitors described previously. However, this compound showed several unique properties: generation by theoretical design on the basis of the crystal structure of DENV E protein including octyl-β-D-Glc, a single sulfated group introduced at the 2-*O*-position of GlcA, and a carboxyl group as an active portion. Although the inhibitory activity of the compound was lower than those of similar types of inhibitors under our focus-forming assay conditions, the inhibitory activity may be augmented by further modification of sugar residues as well as octyl-portion according to the structure–activity relationship determined in this study. As sulfo GlcA derivatives are highly soluble, stable and have low toxicity, they are potential candidates as therapeutic agents in combination with other types of inhibitor in dengue patients.

*3.3. FMO calculation demonstrated a possible mechanism on the interaction of E protein with the compound **1***

We investigated the molecular mechanism underlying the enhancement of anti-DENV activity of sulfo GlcA derivatives by first-principles calculations based on FMO-IFIE analysis. Octyl-portions of the compounds **1** and **2** made effective contact and formed stable van der Waals interactions with hydrophobic amino acid residues in the hydrophobic pocket of the DENV1 E protein (Figs. 3 and S4). Two of three oxygen atoms of the sulfo group in the compound **1** formed stable hydrogen bonds with the hydroxyl group in S205 and hydrogen atom in the side chain of L198, respectively. The other formed an intermolecular hydrogen bond with the 3-hydroxyl group of GlcA. The hydroxyl group at the C3 position

Table 1
Anti-dengue virus activities of test compounds.

Compound (**No.**)	Structure	Relative infectivity (%)
Octyl 2-*O*-sulfo-β-D-GlcA (**1**)		18.4 ± 8.3
Octyl 3-*O*-sulfo-β-D-GlcA (**2**)		34.8 ± 8.6
Octyl 2-*O*-sulfo-β-D-Glc (**3**)		64.9 ± 8.3
Octyl 3-*O*-sulfo-β-D-Glc (**4**)		62.4 ± 12.5
Octyl β-D-GlcA (**5**)		76.7 ± 17.9

Relative infectivity (%) was calculated after normalizing data to control cell viability based on vehicle treatment. Values are shown as averages ± standard deviation obtained from at least three independent experiments carried out in triplicate. Compound numbers are indicated in bold.

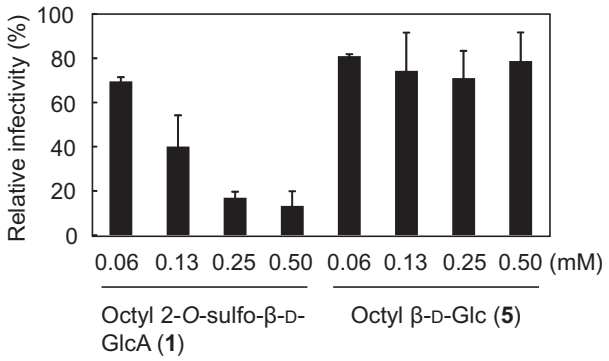


Fig. 2. Inhibitory effects of octyl-2-*O*-sulfo β-D-GlcA (compound **1**) and octyl-β-D-Glc (compound **5**) on DENV1 infection of BHK-21 cells, evaluated by focus-forming assay as described in the Section 2. Values indicate means ± SD of relative infectivity in the presence of compound at the indicated concentrations to virus alone as control. Bars show standard deviation of triplicate measurements in each experiment. The data represent three independent experiments.

in compound **1** interacted with the NH_2 group in Q271. These interaction mediated through hydrogen bonds of the sulfo group seems to become a stable hydrogen bond network. Formation of an additional hydrogen bond between the carboxyl group in compound **1** and side chain of E49 contributed to stabilization of the complex of the E protein with the compound. On the other hand, the hydroxyl group at the C3 position in octyl-β-D-Glc formed only a single hydrogen bond with Q271. In the case of compound **2**, similar hydrogen bonds formed with E49, S205 and Q271 residues. However, all interactions were apparently weaker than those of compound **1** due to the longer distances of the hydrogen bonds. FMO calculation demonstrated several significant interaction energies of K47, L198, S205, Q271 and H282 residues interacted ionically with compound **1** (Fig. S5A). The same calculations also showed similar interaction energies of E49 and S205 with octyl-β-D-Glc (Fig. S5C). The reduction of the binding affinities with 3-*O*-sulfo GlcA derivative may have been due to differences in the ionic and hydrophobic interactions between functional groups of the compound and the amino acid residues, particularly Leu198 (Fig. S5B). The FMO calculations also predicted that compound **2** was associated with the same pocket as compound **1** with possibly lower binding affinity. MD simulations demonstrated that the flexibility of DENV1 E protein is restrained by binding of compound **1**. The root-mean-square deviation value of the complex with compound **1** was smaller than that of unbound E protein. In the focus-forming assay, the elevation of binding affinity may be the major reason why compound **1** significantly inhibited infection of the cells by DENV1 (Table 1).

Our computational results concerning amino acid residues associated with compounds are in good agreement with previous virtual screening studies that showed possible interactions of hit compounds with several amino acid residues in the hydrophobic pocket, such as K47, E49, L198, Ser205 and Q271 [25,26]. These amino acid residues may play important roles in the conformational changes of the E protein in the early stages of DENV infection. Further structural studies, such as co-crystallization assay, may resolve the inhibitory mechanism of action of this compound.

In conclusion, compound **1**, octyl-2-*O*-sulfo β-D-GlcA, synthesized and evaluated in this study will contribute not only to elucidation of the molecular mechanism underlying the fusion steps in DENV infection mediated through conformational change of the E protein, but will also facilitate the development of anti-DENV agents with different mechanisms of action from DENV non-structural enzyme inhibitors. Our approach using a new small fusion inhibitor and first-principles calculations based on the FMO method contributes to an understanding of DENV entry. Further investigations to gain a better understanding of the molecular framework of hydrophobic ligands capable of inhibiting DENV infection will contribute to the development of effective anti-DENV agents.

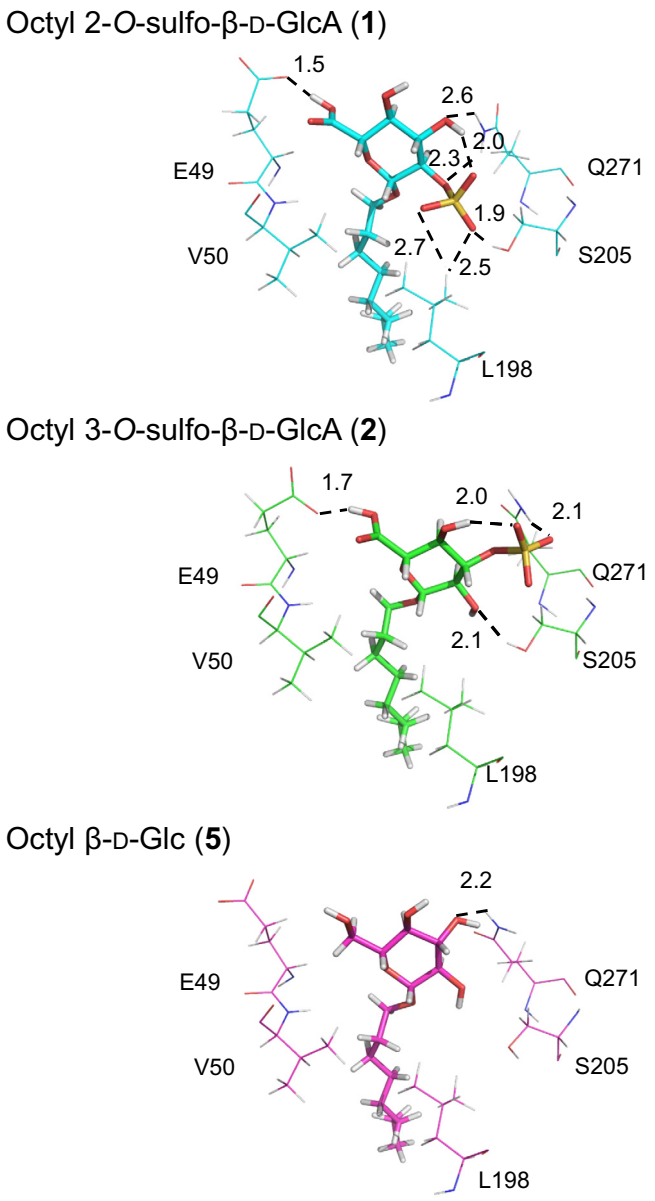


Fig. 3. Highlights of the binding complex of synthetic compounds and amino acids in the hydrophobic pocket of DENV1 E protein. Computational simulations of the interactions between three compounds and DENV1 E protein were performed as described in the Section 2. The structures of compounds and amino acids were generated with PyMOL molecular graphics software using homology-modeled DENV1 E protein. Sulfated functional groups are shown in yellow (S) and red (O). Carbon atoms on the main frame of octyl-2-*O*-sulfo β-D-GlcA (compound **1**), octyl-3-*O*-sulfo β-D-GlcA (compound **2**) and octyl-β-D-Glc (compound **5**) are colored, cyan, green and magenta, respectively. Dotted lines indicate distances of possible interactions between amino acid residues and functional groups of the compound. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Conflict of interest

The authors have no conflicting financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.122.

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