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3-*O*-sulfated glucuronide derivative as a potential anti-dengue virus agent

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ABSTRACT

A series of 12 carbohydrate compounds were synthesized by introduction of a sulfated group at specific positions and evaluated for their activities against dengue virus (DENV) infection as well as binding to BHK-21 cells. 3-*O*-sulfated GlcA was active against DENV infection, whereas 2-*O*-sulfated GlcA and 3,6-di-*O*-sulfated Glc showed negligible activity. Persulfated compounds did not inhibit DENV infection. These results provided a rationale for designing sulfated carbohydrate compounds with low molecular mass as anti-DENV agents targeting E protein functions. 3-*O*-Sulfated GlcA showed no significant cytotoxicity at 1 mM. The EC_{50} value (120 μM) was lower than that of sucrose octasulfate (SOS), a small molecular weight inhibitor of DENV infection. Two negatively charged groups, 3-*O*-sulfate and 6-*C*-carboxylic acid, appear to be essential for anti-DENV activity. We performed docking study to investigate the binding potential of 3-*O*-sulfated GlcA with respect to DENV E protein. The docking study showed that distance and conformation of these negative charges on the carbohydrate may be suitable for association with three amino acid residues of E protein critically involved in virus adsorption (Lys295, Ser145, and Gly159). This interaction may competitively prevent functional DENV binding to receptor(s) on host cells. In conclusion, 3-*O*-sulfated GlcA is a chemical probe that may facilitate exploration of the molecular mechanisms underlying manifestations of dengue diseases.

1. Introduction

Dengue virus (DENV) belongs to the genus *Flavivirus*, family *Flaviviridae*. DENV causes human diseases, such as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [1–3].

DENV genome is a single-stranded, positive-sense RNA, which encodes a single polyprotein that is posttranslationally 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, E protein contributes to virus binding to the cells, followed by virus-host membrane fusion. This protein is also thought to be involved in host range and tissue tropism [5–7]).

DENV infection is initiated by adsorption of virus particles to the host cell membrane, followed by incorporation into the cells by endocytosis. Acidification in the endocytic vesicle causes marked structural alterations of E protein, which result in fusion and viral disassembly [5,7]. After the viral genome is released into the cytoplasm, its translation and replication occur [8].

Previous studies provided a structural basis of DENV E protein for understanding the molecular mechanisms of virus entry and host immune protection [9–11]. Glycoconjugates on the host cell surface, such as glycosaminoglycans, glycoproteins, and glycosphingolipids, are possibly important components in DENV receptor complex [12–17]. Particularly, extracellular heparan sulfate (HS) or the highly sulfated forms of glycosaminoglycans are essential for the early stages of DENV infection.

No anti-DENV drugs are clinically available at present. Prevention of DENV infection by safe and effective vaccination has not been established. To date, several classes of antiviral agent have been developed. First, compounds targeting two viral enzymes, such as NS3 protease and NS5 RNA-dependent RNA polymerase, were developed most intensively. Second, posttranslational modification of viral prM, E, and NS1 proteins was blocked by inhibitors against *N*-linked glycan biosynthesis [18–21]. Third, viral E protein functions, such as virus adsorption and fusion, were inhibited by receptor-analogous glycans and non-ionic detergent-like com-

Abbreviations: DENV, dengue virus; NS, non-structural; GlcA, glucuronic acid; CS, chondroitin sulfate; CSE, chondroitin sulfate E; SOS, sucrose octasulfate.

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pounds [16,22–26]. Fourth, compounds targeting host factors, including protein kinases, enzymes responsible for cholesterol biosynthesis, and molecules involved in immune responses, have also been investigated [27].

Inhibition of virus adsorption or entry into host cells is an effective means of preventing virus infection [27]. This class of compound, designated as entry inhibitor, suppresses functional restructuring of E protein that is essential for virus entry. Studies on DENV E protein structure by crystallographic approaches demonstrated that the protein contains several clusters of basic amino acid residues on its surface [9–11]. These residues give the protein surface positive charges which may contribute to binding to extracellular glycosaminoglycans with sulfated functional groups. There are different types of glycosaminoglycans based on the sugar components, the extent of sulfation, and the number of repeating units: heparin, HS, chondroitin sulfate (CS), dermatan sulfate, and keratan sulfate. Among them, heparin and chondroitin sulfates chemically modified with high sulfation contents significantly inhibited cellular infection of flaviviruses including DENV [12]. However, according to physical properties such as molecular masses, bioavailability and biological activity related with unfavorable side effect, it is very hard to administrate native glycosaminoglycans directly to human body.

Previously, we found anti-DENV activity of fucoidan from marine algae, which consists of sulfated fucose polymer with glucuronic acid (GlcA) residues, and elucidated functional determinants responsible for inhibition of DENV infection to BHK-21 cells [23]. Very recently, we also demonstrated that natural chondroitin sulfate E with a low degree of sulfation specifically inhibited cellular infection of DENV as well as Japanese encephalitis virus [17]. Comparison of carbohydrate constituents of sulfated glycans with inhibitory activity against DENV infection indicated that both GlcA residue and sulfation may crucial determinants for the anti-DENV effect.

In the present study, we designed, synthesized, and characterized GlcA derivatives on the basis of carbohydrate structures of active sulfated glycans against DENV infection. We also investigated the antiviral effect and molecular interaction of synthetic compounds with DENV.

2. Materials and methods

2.1. Chemicals

D-Glucuronic acid, methyl β-D-glucuronide and heparin were purchased from Sigma (St. Louis, MO). A cell proliferation kit (Cat# 11465007001) for MTT assay was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals were of the highest quality commercially available.

2.2. Cell culture and virus

BHK-21 cells were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 5% FBS. *Aedes albopictus* clone C6/36 cells were grown at 28 °C in Eagle's minimal essential medium supplemented with 10% FBS and 0.2 mM nonessential amino acids. DENV-2 strain, ThNH-7/93, was inoculated into C6/36 cells and the supernatant was harvested on the fourth day of culture. The virus was purified as described previously [28,29]. The virus aliquots were stored at −80 °C before use.

2.3. Focus-forming assay

Inhibition of virus infection by synthetic compounds was determined by focus-forming assay as described previously [17]. Briefly, BHK-21cells were seeded onto 96-well plastic plates and cultured for 24 h at 37 °C. Virus was premixed in a serum-free medium, VP-SFM (Gibco Life Technologies) on ice with synthetic compounds at the indicated concentrations. The virus-compound premixtures were immediately inoculated onto the cells for 2 h at 37 °C. After removal of the virus solution, overlay medium (DMEM containing 1% FBS and 0.5% tragacanth gum) was added, and plates were incubated at 37 °C for 43 h. The cells were fixed and permeabilized with 5% paraformaldehyde and 1% NP-40, respectively. Infectious foci were detected with human serum from dengue hemorrhagic fever patient (anti-dengue antisera), followed by HRP-conjugated goat anti-human immunoglobulin. Virus infectivity was determined as focus-forming units (FFU).

2.4. Cell surface virus binding assay

Binding of viruses to cultured cells was performed as described previously [17]. Briefly, BHK-21 cells were seeded onto 96-well plates and cultured for 24 h at 37 °C. After blocking with DMEM containing 2% BSA, the virus (5.0×10^6 ffu/ml)–compound premixtures were immediately inoculated onto the cells for 2 h at 37 °C. After washing thoroughly, the bound viruses were reacted for 1 h at 28 °C with human anti-dengue antisera as the primary antibody, followed by HRP-conjugated Goat anti-human immunoglobulin as the secondary antibody. The immune complexes were detected by incubation with *o*-phenylenediamine as a substrate. The reaction was terminated by addition of 1 N HCl. The absorbance was measured at 492 nm. The virus binding activity was determined from the quantity of virus antigens associated with the cell surface.

2.5. MTT assay

Cellular cytotoxicity of synthetic compounds was determined by MTT assay according to the manufacturer's instructions. Briefly, cells were seeded onto 96-well plastic plates and cultured for 24 h at 37 °C. The synthetic compounds diluted up to 1 mM with serum-free DMEM containing 25 mM HEPES were added onto the cells for 2 h at 37 °C. After removal of the compound solution, the overlay medium was added, and plates were incubated at 37 °C for 43 h. The cells were then subjected to the MTT assay.

2.6. Docking simulation

The three-dimensional structure of the target protein was built on the basis of the crystal structure of the DENV E protein (PDB ID: 1OKE). Hydrogen atoms were automatically added at pH 7.0 by the MOE protonate 3D method (Molecular Operating Environment, MOE 2010.10; Chemical Computing Group, Inc., Montreal, QC, Canada) protonate 3D method. All hydrogen atomic coordinates were refined by the conjugate gradient method with the MMFF94x force field. N- and C-terminations are $-NH_2$ and $-COO^-$, respectively. Docking simulations of the methyl 3-*O*-sulfated α-GlcA or methyl 3-*O*-sulfated β-GlcA to the target protein were carried out according to the following procedure: (i) the structure of 3-*O*-sulfated GlcA derivatives was generated using GaussView version 5.0.9, and 250 conformations of 3-*O*-sulfated GlcA derivatives were prepared by the conformation import method; (ii) the ligand binding pockets of the target protein were searched by the Site Finder module in MOE [30].

3. Results and discussion

3.1. 3-O-sulfated GlcA compound inhibited DENV infection to BHK-21 cells

Dengue virus (DENV) infection is significantly inhibited by treatment of the virus with heparin and chondroitin sulfate

E (CSE) targeting E protein. The inhibitory activity results from the direct interaction of both glycosaminoglycans with E protein. A recent study demonstrated that E protein cross-reacts with heparin and CSE. These findings strongly suggest that there are common carbohydrate determinants between both glycosaminoglycans that contribute to functional interaction with E protein [17]. We searched the literature databases to find possible carbohydrate structures common to both glycosaminoglycans. We also referred to our previous observation that fucoidan from marine algae inhibited DENV infection to the same extent as heparin. The GlcA residue and sulfation predominantly contribute to the inhibitory activity of fucoidan. We synthesized, characterized and tested sulfated or non-sulfated GlcA and sulfated Glc or Gal derivatives as controls for anti-DENV activity (Supplementary Figs. S1–S6 and Tables S1 and S2) [31–34]. We also observed that the active compound **7** was stable under our assay condition (Fig. S7).

Fig. 1 shows a list of sulfated carbohydrate derivatives used in this study. Compounds 3, **7**, **9**, and **10** are monosulfated at different positions of GlcA. Mono-, di-, or tri-sulfation was introduced at different positions on the Glc or Gal residue (compounds **4**–**6**, **8**, **11**, and **12**). The purity and assignment of the target compounds were confirmed by ^{1}H-nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HR-MS) analyses (See Supplementary data for representative compounds). Table 1 summarizes the inhibitory activities of 12 compounds on DENV infection as well as binding to BHK-21 cells at a concentration of 500 μM. Focus-forming assay demonstrated that compound **7** specifically inhibited infection of the cells by DENV. The assay also showed that 3-*O*-sulfated GlcA (compound **7**), but not compound **1** or **3**, dose-dependently inhibited DENV infection with an IC_{50} value of 120 μM (Fig. 2). DENV binding to BHK-21 cells was significantly reduced in the presence of 3-*O*-sulfated GlcA, but not the other compounds examined, indicating good agreement between reduction of virus binding and the inhibitory activity of 3-*O*-sulfated GlcA on DENV infection (Table 1). Analysis of the structure-inhibitory activity relationship yielded novel findings regarding the chemical structures responsible for inhibition of DENV infection. First, in comparison with compounds **3** and **7**, 3-*O*-sulfation of GlcA was essential for the inhibitory effect. Second, as compounds **6** and **8** did not show any inhibitory effect on DENV infection or binding to BHK-21 cells, the carboxyl group, but not sulfate group on GlcA contributed significantly to the inhibition. This finding also suggests the importance of carboxylate as an anionic group at 6-position of the glycoside. Third, α-methylation at the C1 position as compound **7** was preferable to β-methylation as compound **10** for inhibitory activity. This suggested that the α-methyl residue associates with amino acid residues of E protein, or that the β-methyl residue conformationally affects interaction of 3-*O*-sulfated

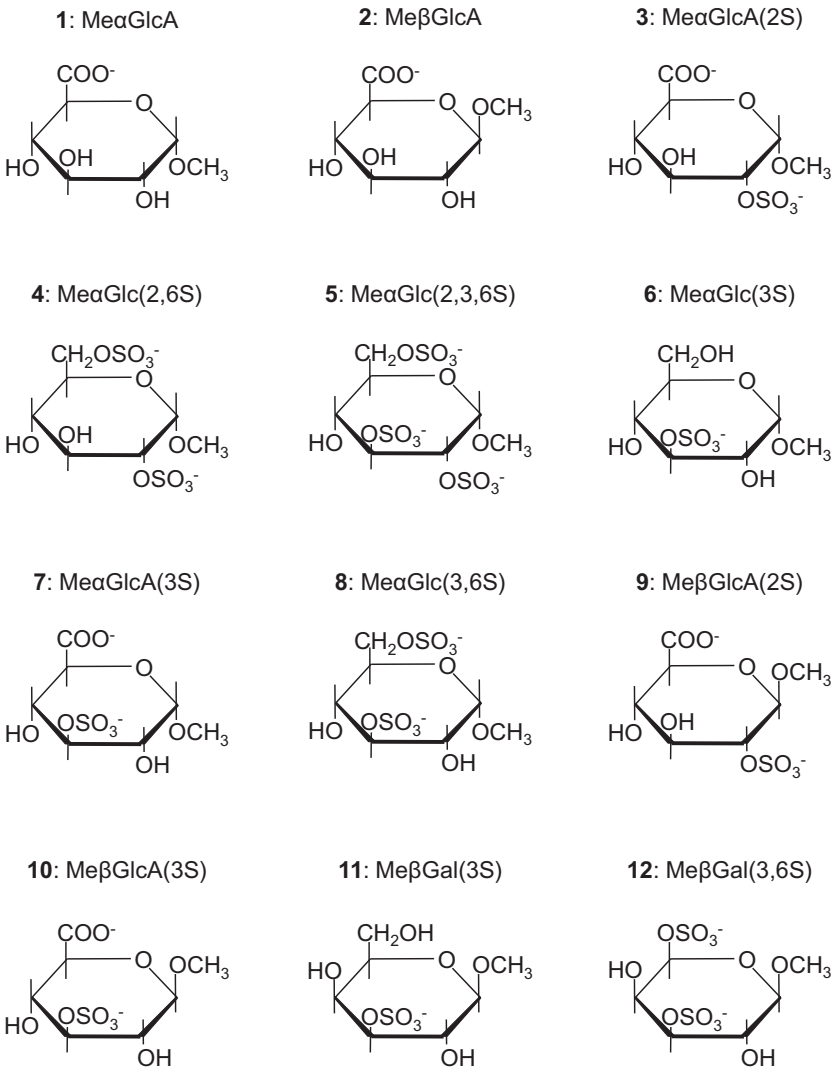

Fig. 1. Structures of synthetic GlcA derivatives used in this study.

Table 1
Anti-dengue virus activity on BHK-21 cells of synthetic sulfated GlcA derivatives, **1**–**12**.

Compound	Infectivity (%)[a]	Binding to cells (%)[b]
None	100.0 ± 15.5	100.0 ± 13.2
1	94.7 ± 9.2	95.2 ± 6.1
2	80.2 ± 11.1	85.4 ± 8.9
3	94.8 ± 14.5	83.6 ± 7.1
4	125.2 ± 11.5	88.8 ± 7.5
5	120.8 ± 19.1	76.5 ± 8.2
6	106.3 ± 7.1	81.7 ± 10.5
7	12.5 ± 2.7*	41.3 ± 11.7*
8	112.7 ± 8.5	79.7 ± 10.8
9	89.2 ± 3.2	88.6 ± 6.7
10	117.6 ± 2.6	96.0 ± 4.0
11	122.3 ± 8.5	77.2 ± 13.6
12	113.4 ± 9.1	71.0 ± 10.7
SOS	7.6 ± 2.9*	N.D.

SOS, sucrose octasulfate; N.D., not determined.
Values indicate means ± SD of virus infectivity or binding activity relative to control treatment without compounds. The data represent three independent experiments.
*, $P < 0.01$.
[a] Infectivity was determined in the presence of 0.5 mM compounds by focus-forming assay as described in the Section 2.
[b] Binding activity to cells was determined for compounds at 0.5 mM by cell surface virus binding assay as described in the Section 2.

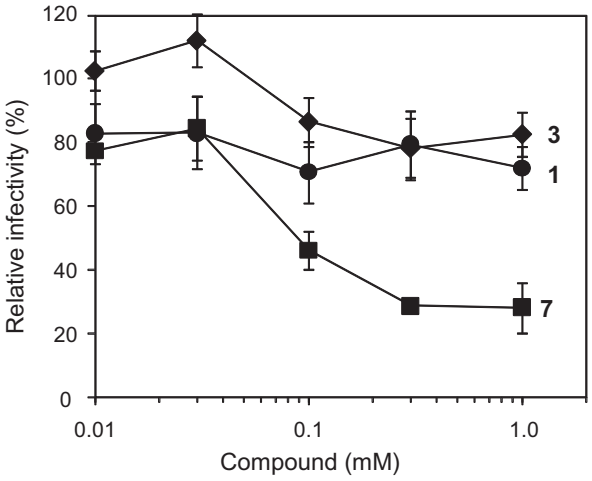

Fig. 2. Inhibitory effects of compound **1**, **3**, and **7** on DENV-2 infection of BHK-21 cells, evaluated by focus-forming assay. The assay was performed as described in the Section 2. Values indicate means ± SD of relative infectivity in the presence of compound at the indicated concentrations to virus alone as control. Bars show standard deviation of triplicate measurements in each experiment. The data represent three independent experiments.

GlcA with E protein. Fourth, hypersulfated Glc (compound **5**) or Gal (compound **12**) did not effectively reduce DENV infection, indicating that the specific position of sulfation on GlcA is more effective than randomly introduced persulfation on the carbohydrate as reported previously [24]. Morphological observations and MTT assay indicated that 3-*O*-sulfated GlcA did not show cytotoxicity on BHK-21 cells at 1 mM.

There are two categories of entry inhibitor targeting DENV E protein, i.e., receptor analog inhibitors that block virus adsorption to host cells, and detergent-like inhibitors that prevent membrane fusion between virus and host cells. Examples of the former are heparin and chondroitin sulfate E, which show potent inhibition of flavivirus infection [12,17]. However, as glycosaminoglycans have very high molecular masses, it is very difficult to administer glycosaminoglycans to humans. A persulfated carbohydrate with molecular mass of 990, sucrose octasulfate referred to SOS, also showed anti-DENV activity by inhibiting binding of E protein to the cells [24]. Therefore, we evaluated the inhibitory activity of SOS in comparison with 3-*O*-sulfated GlcA. This compound showed slightly stronger inhibitory activity against DENV infection than 3-*O*-sulfated GlcA under our focus-forming assay conditions. The IC_{50} of SOS against DENV infection was 48.4 μM. Our compound showed several unique and valuable properties, such as the lowest molecular weight among entry inhibitors that has reported previously, a single sulfated group specifically introduced at the 3-*O*-position of GlcA, and a carboxyl group as an active and essential portion that has not been reported previously. Although the inhibitory activity of 3-*O*-sulfated GlcA was lower than those of other types of inhibitor against NS3 protease and NS5 RNA-dependent RNA polymerase, the inhibitory activity may be augmented by further modification of sulfated GlcA according to the structure–activity relationship determined in this study. As sulfated GlcA derivatives are highly soluble, stable, non-toxic, and have very low immunogenicity, they are good candidates as therapeutic agents using sulfated GlcA derivatives alone or in combination with other types of inhibitor in dengue patients.

3.2. Docking simulation demonstrated a possible mechanism on the interaction of E protein with the compound **7**

We investigated the molecular mechanism underlying the interaction of 3-*O*-sulfated GlcA derivatives with DENV E protein by docking simulation analysis. Methyl 3-*O*-sulfated α-GlcA (compound **7**) was docked to the searched ligand binding pocket including Lys295 by the ASE-Dock method [30]. The docking position with the top binding score (−59.2 kcal/mol) is shown in Figs. 3B and C. The docking positions in which the 1-methoxy group is turned to the outside of the pocket were selected. A possible strong ionic interaction was formed between the 3-*O*-sulfated group and Lys295. Hydrogen bonds were also formed between the C5-carboxyl group and the main chain of Gly159, and between the 3-*O*-sulfate group and the hydroxyl residue of Ser145. The same docking simulation of methyl 3-*O*-sulfated β-GlcA (compound **10**) to the pocket was carried out. The docking positions in which the 1-methoxy group turned to the outside of the pocket were also selected. The top binding score of compound **10** was predicted to be −21.3 kcal/mol (Figs. 3D and E). The simulation predicted that compound **10** docks to the same binding pocket as compound **7** with possibly lower binding affinity. Although the 3-*O*-sulfate group appeared to interact with Lys295 in the case of compound **10**, the two hydrogen bonds between the C5-carboxyl group and Gly159, and between the 3-*O*-sulfate group and Ser145 were not formed. On the other hand, an alternative hydrogen bond was formed between the C5-carboxyl group and the main chain of Ile141. The reduction of the binding score with β-form may have been due to differences in the ionic and hydrogenic interactions between functional groups of the compound and the amino acid residues. In the competitive infection inhibition assay, the reduction of binding affinity may be the major reason why compound **10** did not significantly inhibit infection of the cells by DENV (Table 1). However, although the binding affinity of the β-form is relatively low, we still detected DENV as well as E protein binding to the HNK-1 molecule by solid-phase binding assay. The binding pocket for compound **7** predicted by docking simulation includes one of the heparin binding sites, Lys295, in domain III of the E protein [35]. Comparison of the binding conformation of the compound with the β-anomer form suggested that not only Lys295 but also Ser145 and Gly159 contribute to the interaction of 3-*O*-sulfated α-GlcA with the E protein. Further structural studies, such as molecular dynamics simulation and co-crystallization assays, may resolve the inhibitory mechanism of action of this compound.

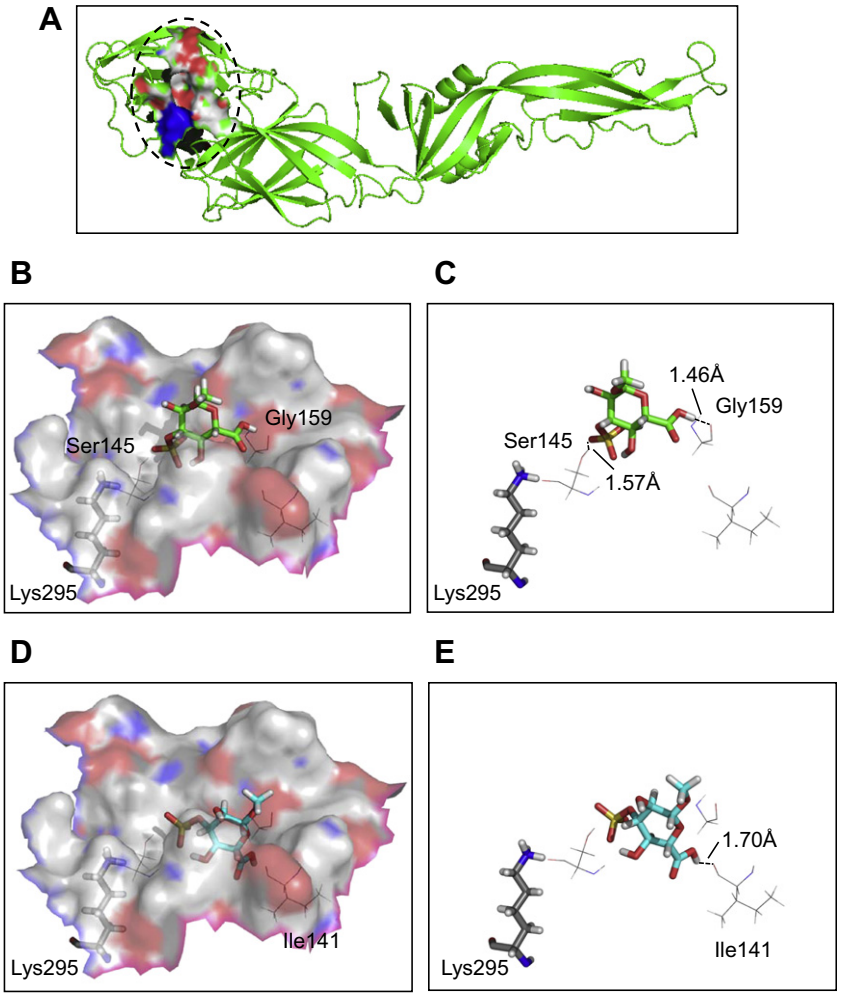

Fig. 3. Docking of 3-*O*-sulfated GlcA derivatives on DENV E protein. Computational simulations of interactions between carbohydrate compounds and DENV E protein were performed as described in the Section 2. A, the structure of DENV E protein was generated with PyMOL molecular graphics software using PDB accession number 1OKE. Dotted circles indicate the molecular surface representing the best binding pocket for 3-*O*-sulfated GlcA predicted by docking simulation. Surface hydrophobic patches are shown in white, and electrostatic charges are illustrated with negatively and positively charged regions colored red and blue, respectively. B and D, Binding complex of DENV E protein and methyl-α-3-*O*-sulfated GlcA, and methyl-β-3-*O*-sulfated GlcA with the highest score from the simulations is shown as a magnified image of the binding pocket, respectively. Sulfated functional groups are shown in yellow (S) and red (O). Carbon atoms on the main frame of the compound are magenta. C and E, Highlights of the binding complex of amino acid residues and methyl-α-3-*O*-sulfated GlcA, and methyl-β-3-*O*-sulfated GlcA with the highest score from the simulations is shown. Sulfated functional groups are shown in yellow (S) and red (O). Carbon atoms on the main frame of the compound are colored cyan. 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).

The compound **7**, methyl 3-*O*-sulfated α-GlcA synthesized and characterized in this study will contribute not only to elucidation of the molecular mechanism of the early steps in DENV infection mediated through E protein, but will also aid in the development of anti-DENV agents with different mechanisms of action from virus enzyme inhibitors. Our observations suggest a possible molecular mechanism of action of 3-*O*-sulfated GlcA involving the distance and conformation of two negatively charged groups, such as sulfate and carboxylic acid groups, which are suitable for interaction with critical amino acid residues of E protein that is involved in virus adsorption. Although receptor-binding sites on E protein are not known yet, our approach using a new small entry inhibitor and docking simulation contributes to understanding of virus recognition with the receptors.

In conclusion, methyl 3-*O*-sulfated α-GlcA is a chemical probe that may facilitate exploration of the molecular mechanisms underlying manifestations of dengue diseases. Further investigations to gain a better understanding of the molecular framework of carbohydrate ligands capable of inhibiting DENV infection and adsorption will contribute to the development of new types of effective anti-DENV agent.

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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.2012.07.002.

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