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Antiviral activity of chondroitin sulphate E targeting dengue virus envelope protein

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ABSTRACT

Sulphated glycosaminoglycans such as heparin inhibit the early step of dengue virus infection through interaction with envelope (E) protein. Here, we found that chondroitin sulphate E (CSE), but not CSD, which contains the same degree of sulphation, inhibited dengue virus (DENV) infection of cells with adsorption. CSE significantly reduced infectivity of all dengue virus serotypes to BHK-21 and Vero cells. DENV preferentially bound to CSE immobilised on plastic plates. Also, virus binding to CSE or heparin was cross-inhibited by soluble CSE or heparin. These findings suggested that common carbohydrate determinants on CSE and heparin could be essential epitopes for interaction of DENV, and may be responsible for inhibition of the early steps of DENV infection. A recombinant E protein directly bound heparin and CSE, but not CSD, meaning that interaction of CSE with E protein contributes to the inhibitory action of this glycosaminoglycan. These observations indicate that a specific carbohydrate structure rather than polysulphation or addition of negative charges of the glycosaminoglycan molecule would be necessary for direct binding to DENV E protein. In conclusion, CSE showed antiviral activity as an entry inhibitor targeting E protein of dengue virus.

1. Introduction

Dengue virus (DENV) belongs to the genus Flavivirus, family Flaviviridae (Kuno et al., 1998; Mukhopadhyay et al., 2005). DENV is transmitted by *Aedes* mosquitoes, and predominantly infects humans. DENV causes human diseases, such as dengue fever, dengue hemorrhagic fever and dengue shock syndrome (Gubler, 2002; Halstead, 2007). There are four dengue virus serotypes, type 1 (DENV-1) to type 4 (DENV-4), which have similar clinical manifestations and epidemiology in tropical and subtropical regions of the world where more than two billion people are at risk of infection (Kuhn et al., 2002; Mackenzie et al., 2004; Weaver and Barrett, 2004; Halstead, 2007).

Flaviviruses are enveloped viruses with an envelope (E) protein on the surface of the lipid bilayer membrane. The Flavivirus genome is a single-stranded, positive-sense RNA approximately 11 kb in length, which contains a single open reading frame encoding a polyprotein (Lindenbach and Rice, 2003; Zhang et al., 2003c). The polyprotein is posttranslationally cleaved into three structural (C, PrM and E) and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) proteins by host- and viral-derived proteases. Among the three structural proteins, E protein consists of 500 amino acids divided into three functional domains: domain I, domain II and domain III. Domains II and III contain a fusion peptide and a cellular receptor binding site(s), respectively (Rey et al., 1995; Wu et al., 2003). E protein, which is the major antigen, is considered to be involved in viral attachment, fusion, neutralisation, host range and tissue tropism (McMinn, 1997; Crill and Roehrig, 2001; Chu et al., 2005; Chin et al., 2007; Stiasny et al., 2007).

Flavivirus infection is initiated by the interaction between E protein and protein, lipid, or carbohydrate host receptor(s) in a complex extracellular matrix structure (Schneider-Schaulies, 2000; Lescar et al., 2001; Aoki et al., 2006). The structures and antibody binding sites of DENV E proteins have been elucidated by X-ray crystallography and NMR (Modis et al., 2003, 2005; Zhang et al., 2003b). These studies provided a structural basis for understanding the molecular mechanisms of immunological protection and virus

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entry. Several lines of evidence regarding host receptors demonstrated that heparan sulphate (HS) or the highly sulphated form of glycosaminoglycan on the host cell surface are essential for the early stages of flavivirus infection (Chen et al., 1997; Lee and Lobigs, 2000; Mandl et al., 2001; Gemi et al., 2002). Virus particles attached to the host cell surface enter the cell by receptor-mediated endocytosis. Acidification of the endosomal vesicle causes conformational changes in E protein, which result in fusion and viral disassembly (McMinn, 1997; Stiasny et al., 2007).

At present, no specific treatments for DENV infection are clinically available. Control of DENV by safe, low-cost and long-lasting vaccination has not been established. Therefore, there is a requirement for effective antiviral agents and therapeutic concepts for DENV infection. Although Japanese encephalitis and yellow fever viruses, which belong to the same family as dengue virus (family *Flaviviridae*), are controlled by specific vaccinations, no licensed dengue vaccines or anti-dengue agents are clinically available. The only available disease treatment is supportive therapy. Several types of antiviral agent have been sought intensively, including inhibitors against viral replication (Zhang et al., 2003a), posttranslational processing of viral proteins (Courageot et al., 2000; Knox et al., 2006; Whitby et al., 2005; Wu et al., 2002) and E protein functions such as membrane fusion (Hrobowski et al., 2005; Poh et al., 2009) and virus attachment (Aoki et al., 2006; Bai et al., 2007; Hidari et al., 2008; Marks et al., 2001; Wang et al., 2009; Yennamalli et al., 2009). Blocking of virus attachment or entry into host cells is an effective strategy to control virus infection (Altmeyer, 2004). This type of inhibitor, termed an entry inhibitor, blocks structural rearrangements of the viral envelope that are essential for viral infection.

Glycosaminoglycans are unbranched sulphated polysaccharides expressed widely on the cell surface or in the extracellular matrix (ECM). There are five different isomers of glycosaminoglycans based on the sugar components, the extent of sulphation and the number of repeating units: heparin, HS, chondroitin sulphate (CS), dermatan sulphate and keratan sulphate. Glycosaminoglycans play important roles in cell adhesion and growth, maintenance of ECM integrity and signal transduction (Gallagher et al., 1986; Kjellen and Lindahl, 1991; Yanagishita and Hascall, 1992; Iozzo, 1998). It has been reported that some viruses utilise glycosaminoglycans as an initial step to enter host cells (Lycke et al., 1991; Trybala et al., 1994; Roderiquez et al., 1995). Some glycosaminoglycans with a high degree of sulphation, such as heparin and highly sulphated chondroitins, show potent inhibition of flavivirus infection (Chen et al., 1997; Su et al., 2001). The degree of sulphation has been suggested to be involved in inhibition of infection (Marks et al., 2001). However, detailed chemical structures responsible for the interaction of DENV with host receptors have yet to be elucidated.

In the present study, we examined the anti-dengue virus activity of sulphated glycosaminoglycans. We also investigated viral infection and molecular interaction with a number of glycosaminoglycans, *i.e.*, chondroitin sulphate A, B, C, D and E, heparin, heparan sulphate and hyaluronic acid.

2. Materials and methods

2.1. Materials

Chondroitin sulphates (CSA, CSB, CSC, CSD and CSE), HS and heparin were purchased from Seikagaku Corp. (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. A sensor chip SA was obtained from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire). 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 and Vero 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 non-essential amino acids. DENV-1 strain, D1/Lao/03, DENV-2 strain, ThNH-7/93, DENV-3 strain, D3/BDH02-01, DENV-4 strain, ThD4-17/97 and JEV strain, JaOArS982, were inoculated into C6/36 cells and the supernatant was harvested on the fourth day of culture (Igarashi, 1978; Thant et al., 1996). The supernatant was centrifuged, followed by addition of polyethylene glycol 6000 and NaCl to final concentrations of 8% and 0.5 M, respectively. This mixture was gently stirred overnight at 4 °C and centrifuged again. The virus pellet was suspended in 2 ml of STE buffer (0.15 M NaCl, 0.1 M Tris and 0.01 M EDTA, pH 7.2). The virus aliquots were stored at −80 °C before use.

2.3. Inhibition of virus infection by glycosaminoglycans

Virus titres were determined by focus-forming assay using BHK-21 and Vero cells as described previously (Aoki et al., 2006). The cells were seeded onto 96-well plastic plates and cultured for 24 h at 37 °C in DMEM supplemented with 5% FBS. After three washes with serum-free DMEM containing 25 mM HEPES, DENV or JEV was premixed on ice with glycosaminoglycans at the indicated concentrations. The virus–glycosaminoglycan 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 or 19 h for DENV or JEV, respectively. The cells were fixed and permeabilised with PBS containing 5% paraformaldehyde and 1% NP-40. 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). The optimal titre of inoculated virus was predetermined, such that more than 100 foci appeared per well. Anti-dengue antisera used in this study equivalently react with all serotypes of dengue viruses as described previously (Hidari et al., 2008).

2.4. Determination of cellular cytotoxicity of glycosaminoglycans by MTT assay

To evaluate cytotoxicity of glycosaminoglycans against BHK-21 cells, MTT assay was performed according to the manufacturer's instruction. Briefly, the cells were seeded onto 96-well plastic plates and cultured for 24 h at 37 °C in DMEM supplemented with 5% FBS. After three washes with serum-free DMEM containing 25 mM HEPES, glycosaminoglycans diluted up to 200 μg/ml 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 incubated with the MTT solution for 4 h. After this incubation period, the wells were added the solubilisation solution and incubated overnight at 37 °C under 5% CO_2. The solubilised product was spectrophotmetrically quantified at 550 nm (reference at 690 nm).

2.5. Cell-surface binding of viruses to cultured cell lines

Direct binding of viruses to cultured cells was performed as described previously (Aoki et al., 2006). Briefly, cells were seeded onto 96-well plates and cultured at 37 °C in DMEM supplemented with 10% FBS. After blocking with DMEM containing 2% BSA, the plates were incubated at 4 °C for 2 h in DMEM containing viruses

(5.0×10^6 FFU/ml). After washing thoroughly, the plates were incubated 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. To determine inhibition of virus binding to cultured cells, viruses were premixed with glycosaminoglycans at the indicated concentrations. Aliquots of 50 μl of virus–glycosaminoglycan premixtures were then added for 2 h at 4 °C onto the cultured cells as described above.

2.6. Solid-phase virus binding assay

The binding activities of viruses for glycosaminoglycans were evaluated by solid-phase virus binding assay (Aoki et al., 2006; Hidari et al., 2008). Chemically synthesised glycosaminoglycans carrying a hydrophobic residue, phosphatidylethanolamine (glycosaminoglycan–PE), were used for this assay. Briefly, 100 μl of glycosaminoglycan–PE diluted in PBS was added to Immulon 1B flat-bottomed microtitre plates (Thermo Fisher Scientific, Rockford, IL) and incubated for 18 h at 4 °C. Glycosaminoglycan–PE-immobilised wells were blocked for 2 h at 28 °C with 100 μl of PBS containing 5% BSA. After washing with PBS, the plates were incubated for 2 h at 4 °C with the virus at the indicated titre ($1.25–5.0 \times 10^6$ FFU/ml). After washing with PBS, the bound viruses were detected by sequential incubation with human anti-dengue antisera and HRP-conjugated goat anti-human immunoglobulin. The virus-binding activity was determined from the quantity of virus antigens associated with the glycosaminoglycan-immobilised surface. To determine inhibition of virus binding to glycosaminoglycan–PE with soluble glycosaminoglycans, viruses were premixed with glycosaminoglycans at the indicated concentrations. Aliquots of 50 μl of virus–glycosaminoglycan premixtures were then added onto glycosaminoglycan–PE-immobilised plates for 2 h at 4 °C as described above.

2.7. Cloning of the gene encoding DENV-2 E protein

cDNA was synthesised from viral RNA that was extracted from DENV-2 strain, ThNH-7/93, using SuperScript™ First-Strand Synthesis System for RT-PCR (Cat# 11904-018; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The primers for polymerase chain reaction (PCR) were designed using the nucleotide sequence of DENV-2 E protein [GenBank accession number U31924] (Thant et al., 1996). The sequences of primers used to amplify a major part of E protein were as follows: forward primer containing *Eco*RI site and enterokinase cleavage sequence (5′-GAATTCGATGACGATGACAAGGAAGCCAAACAGCCTGCT-3′), reverse primer containing *Not*I site, FLAG sequence and termination codon (5′-CAGCCTGGGATTTCGGATCCGATTACAAGGACGACGATGACAAGTAG-3′), corresponding to nucleotide positions 145–162 and 1253–1272 of ThNH-7/93 strain, respectively. PCR was performed for 30 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min) and extension (72 °C, 1 min), followed by incubation at 72 °C for 10 min in 50-μl reaction mixtures containing 0.2 mM of each of dNTP, 0.2 μM of each primer, 2 μl of DENV-2 cDNA as a template, and 2.5 units of *TaqPlus Long* Polymerase mixture (Stratagene, La Jolla, CA) (Saiki et al., 1985). The 1299-bp DNA amplified using a combination of primers was ligated with linearised pGEM-T vector (Promega Corporation, Madison, WI) according to the manufacturer's instructions. The nucleotide sequence of the isolated cDNA clone was confirmed by sequencing of both strands. Dideoxy chain termination for sequencing of double-stranded templates was carried out using a *Taq* Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) (Sanger et al., 1977). The resultant DNA and pMAL-p2X vector (New England Biolabs, Ipswich, MA) were digested with *Eco*RI and *Not*I. Both linearised DNAs were ligated and introduced into *E. coli* strain JM109 for protein expression. As a result, soluble E protein can be expressed as a recombinant protein carrying maltose-binding protein (MBP) and FLAG tag sequence that are fused at the amino and the carboxy termini, respectively.

2.8. Bacterial expression of soluble E protein

In small-scale culture, a single colony of the clone was inoculated into Luria broth (LB) medium supplemented with 100 μg/ml of ampicillin (LBAmp) and precultured at 37 °C for 14–16 h. Aliquots of 1 ml of preculture were added to 9 ml of freshly prepared LBAmp and cultured at the indicated temperature until mid-logarithmic phase (0.8–1.0 OD at 600 nm). In large-scale culture for purification of the enzyme, several colonies on the clone plate were inoculated into 100 ml of LBAmp and cultured at 37 °C for 14 h. Preculture was added to 3 l of freshly prepared LBAmp and cultured at 37 °C until mid-logarithmic phase. The protein expression in bacteria was induced at 23 °C for 6 h in the presence of IPTG at a final concentration of 0.5 mM.

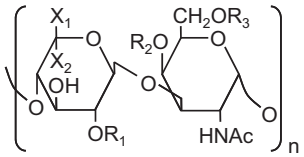
2.9. Extraction and affinity purification of soluble recombinant E protein

All operations described below were carried out at 4 °C or on ice. For preparation of soluble fraction, bacterial cell pellets were suspended in sonication buffer containing 150 mM NaCl, 20 mM Tris/HCl, pH 7.5, and protease inhibitor cocktail (Cat# P8465; Sigma), and pulse-disrupted using an ultrasonifier until the solution became clear. The solution was centrifuged and the supernatant was subjected to further purification. The supernatant was applied to a column of amylose-conjugated agarose (bed volume, 50 ml) equilibrated with sonication buffer. The column was washed with 10 column volumes of a buffer containing 500 mM NaCl and 20 mM Tris/HCl, pH 7.5. The retained MBP-fused E (MBP–E) protein was eluted with 3 column volumes of sonication buffer containing 10 mM maltose. Fractions containing MBP–E protein was pooled, mixed with glycerol to give a final concentration of 20%, and stored at −20 °C until use. Proteins were denatured by heat treatment under authentic reducing condition, and resolved on 10% separation gel by SDS-PAGE (Laemmli, 1970). The recombinant protein resolved on the gel was subjected to staining with Coomassie brilliant blue (CBB) reagent or blotting on a PVDF membrane, followed by detection with anti-FLAG monoclonal antibody (M2) (Sigma, St. Louis, MO).

2.10. Surface plasmon resonance analysis

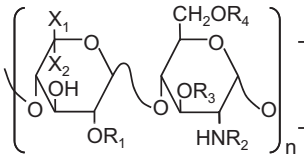
All analyses of interactions between glycosaminoglycans and MBP–E protein were performed at 25 °C on a BIAcore 2000 (BIAcore AB, Uppsala, Sweden) using a sensor chip SA. For capture of glycosaminoglycans on the sensor chip, glycosaminoglycan isoforms were biotinylated as described previously with slight modifications (Nadanaka et al., 2008). Briefly, glycosaminoglycans were dissolved in phosphate-buffered saline (PBS) at a concentration of 2 mg/ml. To this solution was equally added 6 mg/ml of Sulfo-NHS-LC-Biotin (Cat# 21335; Thermo Scientific Pierce, Waltham, MA) dissolved in PBS. The mixture was incubated for 1 h at 37 °C. Excess biotinylation reagent was quenched by addition of 1 M Tris/HCl (pH 7.5) and dialysed against several changes of PBS. Sensor chip SA was conditioned according to the manufacturer's instructions before

Chondroitin sulfate (CS)



CS	MW (x 10^3)	X_1	X_2	R_1	R_2	R_3
A	40	COO^-	H	H	SO_3H^-	H
B	20	H	COO^-	H	SO_3H^-	H
C	60	COO^-	H	H	H	SO_3H^-
D	35	COO^-	H	SO_3H^-	H	SO_3H^-
E	55	COO^-	H	H	SO_3H^-	SO_3H^-

Heparin/Heparan sulfate (HS)



	MW (x 10^3)	X_1	X_2	R_1	R_2	R_3	R_4
Heparin	5	H	COO^-	SO_3H^-	SO_3H^-	SO_3H^-	SO_3H^-
HS	25	COO^-	H	SO_3H^-	Ac	H	SO_3H^-

Fig. 1. Structures of chondroitin sulphate and heparan sulphate glycosaminoglycans used in this study. HexA, glucuronic acid ($X_1 = COO^-$) or iduronic acid ($X_2 = COO^-$); GalNAc, N-acetylgalactosamine; GlcN, glucosamine. MW indicates average of molecular weight.

biotinylated glycosaminoglycan immobilisation. Biotinylated glycosaminoglycans diluted in HBS-EP buffer (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) were injected at a flow rate of 20 μl/min until around 200–300 RUs as immobilised ligands. Aliquots of 50 μl of MBP–E or MBP alone at concentrations of 6.25–50 μg/ml were injected at a flow rate of 20 μl/min onto the surfaces of the sensor chip and dissociation phase was monitored for 250 s postinjection. The sensor chip was regenerated by injection of 10 μl of a solution containing 1 M sodium chloride and 40 mM sodium hydroxide. Kinetic parameters were obtained by global fitting of the sensorgrams to a 1:1 (Langmuir) binding model using BIAevaluation 3.1 software. Three independent experiments were performed for each glycosaminoglycan.

3. Results

3.1. CSE significantly inhibits DENV infection

In the early stages of infection, E protein on the viral surface interacts with molecules on host cells and triggers infection. It is predicted that the interaction of viral particles with glycoconjugates is critical for tissue tropism, host range and virulence. We first examined whether a variety of glycosaminoglycans can inhibit infection of mammalian cells by DENV and JEV (Fig. 1). Heparin and highly sulphated heparan sulphates inhibit infection by these viruses as well as direct virus binding to cells (Chen et al., 1997; Su et al., 2001). Table 1 summarises inhibition of virus infection in the presence of glycosaminoglycans used in this study. This is the first report that CSE significantly inhibits all serotypes of DENV. CSE also inhibited JEV infection. Other chondroitin sulphates, such as CSA and CSC, dermatan sulphate and heparan sulphate did not show any inhibitory effect up to 30 μg/ml at the final concentration as described previously (Chen et al., 1997). Any glycosaminoglycans used in this study did not show cellular cytotoxicity up to 200 μg/ml at the final concentration in the culture medium. This observation indicates that CSE did not affect cell viability under our assay conditions, and also suggest that CSE directly prevents virus–host cell interaction. The degree of inhibition by CSE was similar to that of heparin. CSD, an isomer with almost the same degree of sulphation as CSE, did not inhibit virus infection. These findings suggest that degree of sulphation is not directly related to the inhibitory activity.

3.2. CSE inhibits DENV binding to the cell surface

To investigate whether CSE inhibits virus binding to the cell surface, viruses were subjected to cell-surface virus binding assay. Table 2 summarises the inhibitory activities of glycosaminoglycans on DENV binding to BHK-21 or Vero cells. As expected, CSE as well as heparin effectively inhibited all serotypes of DENV binding to both BHK-21 and Vero cells. DENV binding was inhibited at concentrations of CSE and heparin comparable to those in the infection experiments. Similarly, other chondroitin sulphates, such as CSA and CSC, dermatan sulphate and heparan sulphate did not show any

Table 1
Inhibition of DENV and JEV infection by treatment with glycosaminoglycans.

Virus	$EC_{50} \pm SE$ (μg/ml)	
	Heparin	CSE
DENV-1	0.76 ± 0.17	0.53 ± 0.10
DENV-2	1.47 ± 0.36	3.80 ± 0.68
DENV-3	1.89 ± 0.50	1.38 ± 0.33
DENV-4	0.50 ± 0.13	0.30 ± 0.06
JEV	0.77 ± 0.17	0.93 ± 0.21

Effective concentration 50% (EC_{50}) means glycosaminoglycan concentration required to reduce virus infection by 50%. Values indicate averages of EC_{50}. SE means standard error of data from three independent experiments. Each infection experiment was performed by triplicate measurements as described in Section 2. Other glycosaminoglycans including CSA, CSB, CSC, CSD and HS did not show inhibitory effect on virus infection up to 30 μg/ml.

Table 2
Inhibition of DENV binding to cells by treatment with glycosaminoglycans.

Cells	Virus	$EC_{50} \pm SE$ (μg/ml)	
		Heparin	CSE
BHK-21	DENV-1	4.11 ± 0.80	1.56 ± 0.12
	DENV-2	1.51 ± 0.20	4.13 ± 1.55
	DENV-3	2.01 ± 0.95	2.17 ± 0.76
	DENV-4	10.3 ± 0.60	11.8 ± 2.57
Vero	DENV-2	0.32 ± 0.08	0.52 ± 0.09

Effective concentration 50% (EC_{50}) means glycosaminoglycan concentration required to reduce virus binding by 50%. Values indicate averages of EC_{50}. SE means standard error of data from three independent experiments. Each binding experiment was performed by triplicate measurements as described in Section 2. Other glycosaminoglycans including CSA, CSB, CSC, CSD and HS did not show inhibitory effect on virus binding up to 100 μg/ml.

inhibitory effect up to 100 μg/ml at the final concentration. These findings suggest that CSE competitively inhibits DENV binding to carbohydrate molecules commonly expressed on both BHK-21 and Vero cells.

3.3. Direct binding of DENV to CSE-immobilised on plastic plates

CSE significantly inhibited infection and adsorption of DENV to host cells. Next, we determined the direct binding activity of viruses to glycosaminoglycans. Glycosaminoglycans are not suitable for direct quantitative virus-binding assays on plastic plates, mainly due to their high water solubility and hydrophilicity. Previously, Sugiura et al. (1993) synthesised and applied synthetic CS derivatives covalently linked dipalmitoylphosphatidylethanolamine (PE) for cell–matrix interaction experiments as novel glycosaminoglycan probes. These probes were composed of glycosaminoglycan and carrier phospholipid (glycosaminoglycan–PE) that can lead to non-covalent binding to plastic plates through the carrier lipid. In this study, we used 6 different types of glycosaminoglycan–PE to assay direct binding activities of DENV. DENV-2 showed significant binding to both CSE–PE and heparin–PE on plastic plates in virus and glycosaminoglycan dose-dependent manners, but not to other glycosaminoglycans, such as CSD (Fig. 2a and b). Similarly, JEV bound to these glycosaminoglycans on plastic plates (Data not shown). These results were in good agreement with those obtained from infection and cellular binding experiments. Taken together, these observations indicate that the direct interaction of DENV with CSE prevents virus adsorption to the host cell surface, resulting in inhibition of virus infection.

3.4. Cross-blocking of the virus-binding to CSE–PE or heparin–PE by soluble CSE and heparin

CSE as well as heparin competitively inhibited all serotypes of DENV binding and infection of host cells. In addition, these glycosaminoglycans bound directly to viruses. These findings strongly suggested that functional structures against DENV-2 adsorption are shared between CSE and heparin. To investigate cross-reactivity of CSE and heparin with DENV, we examined virus binding to CSE–PE or heparin–PE in the presence of soluble glycosaminoglycans. The binding of DENV-2 to CSE–PE and heparin–PE was dose-dependently inhibited by the presence of soluble CSE or heparin, but not other glycosaminoglycans, such as CSD. The inhibitory activity of soluble CSE against DENV-2 binding to both CSE–PE and heparin–PE was lower than that of soluble heparin (Fig. 3a and b). Taken together, CSE may share a common carbohydrate determinant for the inhibitory activity with heparin, and affinity of CSE for DENV-2 is lower than that of heparin.

3.5. DENV-2 E protein directly interacts with CSE

For further investigation of the inhibitory action of CSE, direct interaction of E protein with CSE was examined by surface plasmon resonance (SPR) analysis. A recombinant MBP–E protein was generated in a bacterial expression system and efficiently purified by amylase-conjugated agarose column chromatography (Fig. 4).

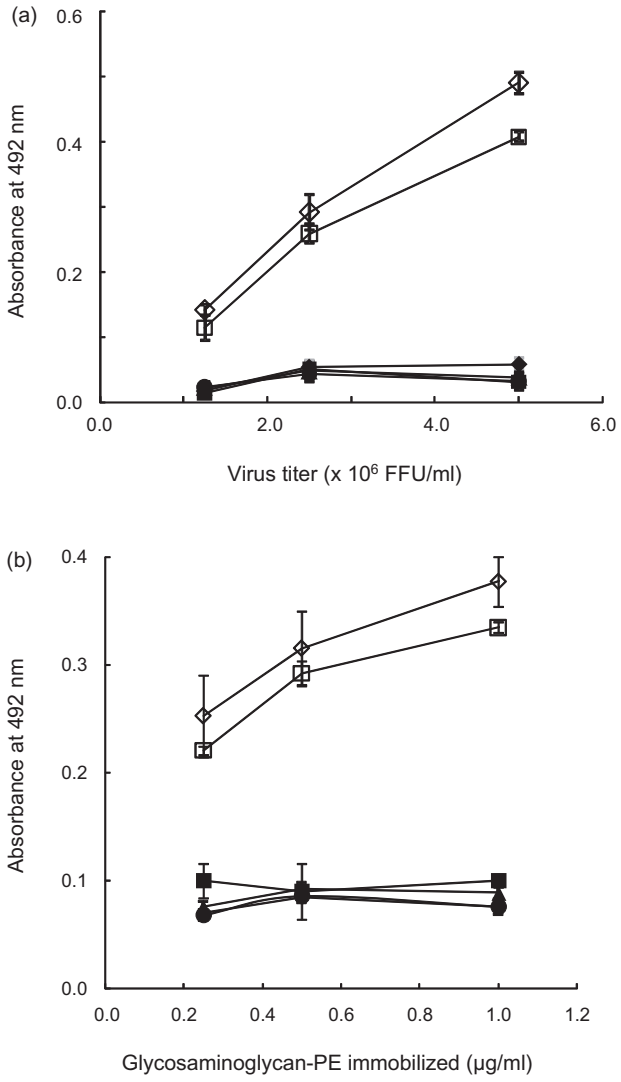


Fig. 2. Binding activity of DENV-2 to glycosaminoglycan–PE. Solid-phase virus binding assay was carried out as described in Section 2. Wells were coated with glycosaminoglycan–PE. Graph (a) indicates the binding of DENV-2 to glycosaminoglycan–PE at 0.5 μg/ml immobilised on plastic plates. Graph (b) indicates the binding of DENV-2 at 2.5×10^6 FFU/ml to glycosaminoglycan–PE immobilised on plastic plates. Open rhombuses, heparin–PE; open squares, CSE–PE; closed squares, CSA–PE; closed triangles, CSC–PE; closed rhombuses, CSD–PE; closed circles, hyaluronic acid–PE. The bound viruses were detected by measuring of absorbance at 492 nm. Values indicate averages of virus binding with glycosaminoglycan–PEs. Bars show standard deviation of triplicate measurements. The results shown here are representative data from three independent experiments.

The recombinant MBP–E and MBP as a reference protein were used for SPR analysis. Table 3 summarises binding affinities of DENV-2 E-protein with CSE as well as heparin. The observation that recombinant MBP–E bound to heparin was in good agreement with the previous findings. MBP–E also interacted with CSE with affinity one

Table 3
Binding affinity of envelope glycoprotein (EGP) for glycosaminoglycans.

	$K_D \pm$ SE (M)			
	CSA	CSE	CSD	Heparin
MBP–EGP	Not bound	$1.9 \times 10^{-7} \pm 3.5 \times 10^{-8}$	Not bound	$6.7 \times 10^{-8} \pm 1.8 \times 10^{-8}$
MBP	Not bound	Not bound	Not bound	Not bound

Binding affinity was determined using Biacore instrument as described in Section 2. Values indicate averages of dissociation constant (K_D). SE means standard error of data from three independent experiments. MBP–EGP, envelope glycoprotein fused with maltose-binding protein (MBP).

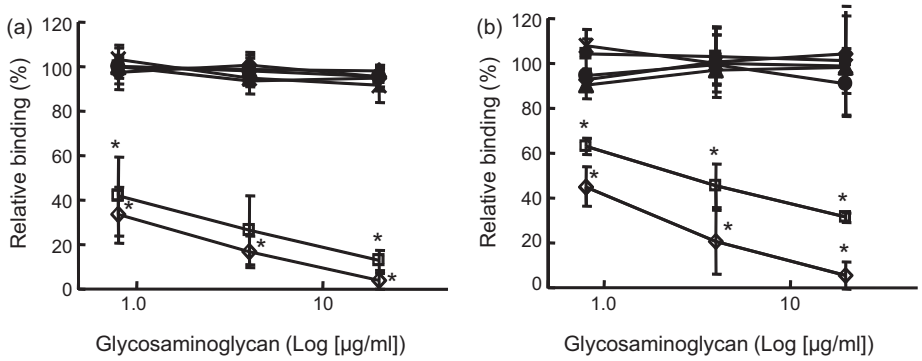


Fig. 3. Inhibition of DENV-2 binding to CSE–PE or heparin–PE by soluble glycosaminoglycans. Solid-phase virus binding assay was carried out as described in Section 2. Wells were coated with CSE or heparin–PE. Graphs (a) and (b) indicate DENV-2 binding to CSE–PE and heparin–PE, respectively. Open rhombuses, heparin; open squares, CSE; closed squares, CSA; cross lines, CSB; closed triangles, CSC; closed rhombuses, CSD; closed circles, heparan sulphate. The bound viruses were detected by measuring absorbance at 492 nm. Values indicate averages of DENV-2 binding with CSE–PE or heparin–PE at the indicated concentrations relative to virus alone. Bars show standard deviation of triplicate measurements. The results shown are representative data from more than three independent experiments. $^{*}P < 0.01$; $^{**}P < 0.05$.

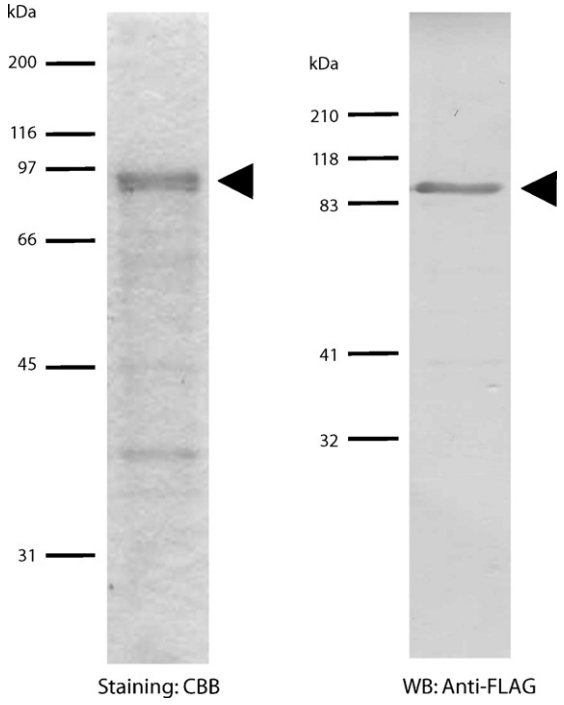


Fig. 4. Purification of MBP-fused E protein by amylose-conjugated agarose. The protein expression in bacteria was induced at 23 °C for 6 h in the presence of IPTG at a final concentration of 0.5 mM. Extraction of proteins and affinity column chromatography were performed as described in Materials and methods. The protein was resolved by SDS-PAGE and transferred electrophoretically onto PVDF membranes. Detection of FLAG-tagged E protein was performed by incubation with anti-FLAG monoclonal antibody (M2), according to the manufacturer's instructions. CBB, Coomassie brilliant blue; WB, Western blotting.

third that of heparin. Slight lower affinity of CSE with E-protein than heparin seems to be consistent with lower inhibitory activity of infection and adsorption with CSE than heparin. Taken together with other findings, CSE directly binds to DENV-2 E protein and modulates its function, resulting in viral entry inhibition.

4. Discussion

Previous studies demonstrated that HS-type glycosaminoglycans, such as heparin, but not CS-type, and CSA or CSD, on the host cell surface are essential for the early stages of flavivirus infection (Chen et al., 1997; Lee and Lobigs, 2000; Su et al., 2001; Gemi et al., 2002; Liu et al., 2004). In the present study, CSE potently inhibited DENV and JEV infection as well as binding to host cells. Basic carbohydrate structures of CS are not simply related with those of heparin or HS glycosaminoglycans (Kjellen and Lindahl, 1991). Heparin predominantly contains a repeated disaccharide unit, consisting of GlcA or IdoA with a 2*O*-sulphated group and GlcNAc with 2*O*-, 3*O*-, or 6*O*-sulphated group. In contrast, GlcA and 4,6-bis-*O*-sulphated GalNAc are the major components of CSE, one of the other CS isoforms. These glycosaminoglycans also show microstructural diversity with differences in sulphation and epimerisation (Kjellen and Lindahl, 1991; Kinoshita et al., 1997). With regard to function, both CSE and heparin interact with midkine, which enhances cell growth, survival and chemotaxis, and CSE inhibits heparin–midkine interaction (Deepa et al., 2002). Furthermore, both glycosaminoglycans inhibit herpes simplex virus infection (Bergefall et al., 2005; Avirutnan et al., 2007). These biological and pathological phenomena strongly suggest that common carbohydrate structures are shared between CSE and heparin. Similarly, CSE may contain heparin-mimetic carbohydrate determinants that contribute to inhibition of the early stages of DENV and JEV infection. There are two possible structural determinants of CSE and heparin involved in DENV infection inhibition. First, CSE and heparin contain 4,6-bis-*O*-sulphated GalNAc and 2,3,6-tri-*O*-sulphated GlcNAc, respectively (Fig. 1). In contrast, CSD containing GlcA with a C3-sulphated group and GalNAc with a 6*O*-sulphated group did not show any inhibitory activity against virus infection. Based on the structure-inhibitory activity relationship, dual negative charges with specific distances on the same side of a sugar ring may effectively interact with E protein, resulting in inhibition of virus attachment and infection. Second, a combination of GlcA and HexN sulphated at specific positions may be responsible for the inhibitory activities of CSE and heparin. Recently, we found that a sulphated polysaccharide, fucoidan, competitively inhibited DENV infection (Hidari et al., 2008). The inhibitory activity required both the GlcA residue and sulphated group. Taken together, our findings strongly suggest that specific carbohydrate structures, including limited sulphation at specific positions, distance and orientation, are required for interaction with DENV. Further studies of the specific carbohydrate structures are required.

To further investigate the molecular interaction of viruses with glycosaminoglycans, direct virus-binding assay using synthetic PE carrying glycosaminoglycans was performed. As glycosaminoglycans are highly water soluble, they cannot be immobilised on plastic surfaces. This is one of the major reasons why the binding properties of glycosaminoglycan molecules to DENV have not been determined directly to date. In the present study, we applied glycosaminoglycan–PE for direct virus-binding assay. The assay using the synthetic molecules and viruses demonstrated that DENV

interacts with CSE as well as heparin, but not with CSD, which has a similar level of sulphation to CSE. This finding strongly supports our conclusion that carbohydrate structures with sulphation at specific positions are required for interaction with DENV. The assay also demonstrated that soluble CSE or heparin inhibited virus binding to CSE–PE or heparin–PE. This observation indicates that CSE and heparin share common structural determinants.

Previous studies demonstrated that inhibitory effect of heparin on dengue virus infection is serotype dependent, with maximum effect against DENV-2 and less activity against other serotypes (Lin et al., 2002; Talarico et al., 2005). On the contrary, we found that heparin equivalently inhibited infection of four serotypes of dengue viruses to BHK-21 cells. There are two possible explanations for different results. First, type of assay may affect inhibitory scores of compounds against virus infection. Focus forming assay detects virus antigen produced in cells which virus enters, meaning that scores described in this study indicates the number of virus entry to the cells. The results obtained by plaque assay used in the previous studies indicate virus production including entire process of virus propagation. Second, susceptibility against glycosaminoglycans may depend on virus strains. Indeed, Lin et al. reported that inhibitory effect of heparin was different between two strains, PL0146 and New Guinea C that belong to DENV-2. Heparin inhibited PL0146 infection to BHK-21 cells with 10-fold stronger effect than New Guinea C. Taken together, intrinsic properties of distinct viruses such as replication efficiency in cells and binding affinity of E protein to glycosaminoglycans may totally affect inhibition scores. Virological and biochemical investigations into inhibition mechanisms of heparin and CSE are further required.

Flavivirus infection is initiated by the interaction between E protein and host cell receptor(s). Heparin binding sites have been previously determined to be distributed on two regions, *i.e.*, amino acid residues at positions 284–310 and 386–411 on DENV E protein (Chen et al., 1997). Particularly, two amino acid residues at positions 295 and 310 are considered to be critical for functional binding to heparin. Also, it is known that amino acid substitutions on E protein that affect binding affinity for heparin are related to neuroinvasiveness and neurovirulence (Lee and Lobigs, 2002; Lee et al., 2004). CSE and heparin were cross-reactive in direct DENV-2 binding assay. The functional similarity between CSE and heparin suggests that CSE binding sites may be close to those for heparin.

In conclusion, DENV interacts directly with CSE as well as heparin but not other glycosaminoglycans. Specific carbohydrate residues with sulphation as common structures shared by CSE and heparin could be essential determinants for control DENV entry mediated through E protein. These findings will contribute to determination of the mechanisms of early stages of DENV infection and will aid in the development of effective and safe entry inhibitors for use as antiviral drugs.

Conflict of interest

The authors declare no conflict of interest.

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References

Altmeyer, R., 2004. Virus attachment and entry offer numerous targets for antiviral therapy. Curr. Pharm. Des. 10, 3701–3712.
Aoki, C., Hidari, K.I.P.J., Itonori, S., Yamada, A., Takahashi, N., Kasama, T., Hasebe, F., Islam, M.A., Hatano, K., Matsuoka, K., Taki, T., Guo, C.-T., Takahashi, T., Sakano, Y., Suzuki, T., Miyamoto, D., Sugita, M., Terunuma, D., Morita, K., Suzuki, Y., 2006. Identification and characterization of carbohydrate molecules in mammalian cells recognized by dengue virus type 2. J. Biochem. (Tokyo) 139, 607–614.
Avirutnan, P., Zhang, L., Punyadee, N., Manuyakorn, A., Puttikhunt, C., Kasinrerk, W., Malasit, P., Atkinson, J.P., Diamond, M.S., 2007. Secreted NS1 of dengue virus attaches to the surface of cells via interactions with heparin sulfate and chondroitin sulfate E. PLoS Pathog. 3, 1798–1812.
Bai, F., Town, T., Pradhan, D., Cox, J., Ashish, Ledizet, M., Anderson, J.F., Flavell, R.A., Krueger, J.K., Koski, R.A., Fikrig, E., 2007. Antiviral peptides targeting the West Nile virus envelope protein. J. Virol. 81, 2047–2055.
Bergefall, K., Trybala, E., Johansson, M., Uyama, T., Naito, S., Yamada, S., Kitagawa, H., Sugahara, K., Bergström, T., 2005. Chondroitin sulfate characterized by the E-disaccharide unit is a potent inhibitor of herpes simplex virus infectivity and provides the virus binding sites on gro2C cells. J. Biol. Chem. 280, 32193–32199.
Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., Marks, R.M., 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat. Med. 3, 828–829.
Chu, J.J., Rajamanonmani, R., Li, J., Bhuvanakantham, R., Lescar, J., Ng, M.L., 2005. Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. J. Gen. Virol. 86, 405–412.
Chin, J.F., Chu, J.J., Ng, M.L., 2007. The envelope glycoprotein domain III of dengue virus serotypes 1 and 2 inhibit virus entry. Microbes Infect. 9, 1–6.
Courageot, M.-P., Frenkiel, M.-P., Dos Santos, C.D., Deubel, V., Desprès, P., 2000. α-Glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. J. Virol. 74, 564–572.
Crill, W.D., Roehrig, J.T., 2001. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J. Virol. 75, 7769–7773.
Deepa, S.S., Umehara, Y., Higashiyama, S., Itoh Sugahara, K., 2002. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. J. Biol. Chem. 277, 43707–43716.
Gallagher, J.T., Lyon, M., Steward, W.P., 1986. Structure and function of heparan sulphate proteoglycans. Biochem. J. 236, 313–325.
Gemi, R., Crance, J.M., Garin, D., Guimet, J., Lorta-Jacob, H., Ruigrok, R.W., Zarski, J.P., Drouet, E., 2002. Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus. Virology 292, 162–168.
Gubler, D.J., 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol. 10, 100–103.
Halstead, S.B., 2007. Dengue. Lancet 370, 1644–1652.
Hidari, K.I.P.J., Takahashi, N., Arihara, M., Nagaoka, M., Morita, K., Suzuki, T., 2008. Structure and anti-dengue virus activity of sulfated polysaccharide from marine algae. Biochem. Biophys. Res. Commun. 376, 91–95.
Hrobowski, Y.M., Garry, R.F., Michael, S.F., 2005. Peptide inhibitors of dengue virus and West Nile virus infectivity. Virol. J. 2, 49–58.
Igarashi, A., 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J. Gen. Virol. 40, 531–544.
Iozzo, R.V., 1998. Matrix proteoglycans: from molecular design to cellular function. Annu. Rev. Biochem. 67, 609–652.
Kinoshita, A., Yamada, S., Haslam, S.M., Morris, H.R., Dell, A., Sugahara, K., 1997. Novel tetrasaccharides isolated from squid cartilage chondroitin sulfate e contain unusual sulfated disaccharide units GlcA (3-*O*-sulfate) β1-3GalNAc (6-*O*-sulfate) or GlcA (3-*O*-sulfate) β1-3GalNAc (4, 6-*O*-disulfate). J. Biol. Chem. 272, 19656–19665.
Kjellen, L., Lindahl, U., 1991. Proteoglycans: structures and interactions. Annu. Rev. Biochem. 60, 443–475.
Knox, J.E., Ma, N.L., Yin, Z., Patel, S.J., Wang, W.-L., Chan, W.-L., Rao, K.R.R., Wang, G., Ngew, X., Patel, V., Beer, D., Lim, S.P., Vasudevan, S.G., Keller, T.H., 2006. Peptide inhibitors of West Nile NS3 protease: SAR study of tetrapeptide aldehyde inhibitors. J. Med. Chem. 49, 6585–6590.
Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., Baker, T.S., Strauss, J.H., 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 8, 717–725.
Kuno, G., Chang, G.J., Tsuchiya, K.R., Karabatsos, N., Cropp, C.B., 1998. Phylogeny of the genus Flavivirus. J. Virol. 72, 73–83.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Lee, E., Hall, R.A., Lobigs, M., 2004. Common E protein determinants for attenuation of glycosaminoglycan-binding variants of Japanese encephalitis and West Nile viruses. J. Virol. 78, 8271–8280.
Lee, E., Lobigs, M., 2000. Substitutions at the putative receptor-binding site of an encephalitis flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry. J. Virol. 74, 8867–8875.
Lee, E., Lobigs, M., 2002. Mechanism of virulence attenuation of glycosaminoglycan-binding variants of Japanese encephalitis virus and Murray Valley encephalitis virus. J. Virol. 76, 4901–4911.
Lescar, J., Roussel, A., Wien, M.W., Navaza, J., Fuller, S.D., Wengler, G., Wengler, G., Rey, F.A., 2001. The fusion glycoprotein shell of Semliki forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell 105, 5–8.
Lin, Y.L., Lei, H.Y., Lin, Y.S., Yeh, T.M., Chen, S.H., Liu, H.S., 2002. Heparin inhibits dengue-2 virus infection of five human liver cell lines. Antivir. Res. 56, 93–96.
Lindenbach, B.D., Rice, C.M., 2003. Molecular biology of flaviviruses. Adv. Virus Res. 59, 23–61.

Liu, H., Chiou, S.S., Chen, W.J., 2004. Differential binding efficiency between the envelope protein of Japanese encephalitis virus variants and heparan sulfate on the cell surface. J. Med. Virol. 72, 618–624.
Lycke, E., Johansson, M., Svennerholm, B., Lindahl, U., 1991. Binding of herpes simplex virus to cellular heparan sulphate, an initial step in the adsorption process. J. Gen. Virol. 72, 1131–1137.
Mackenzie, J.S., Gubler, D.J., Petersen, L.R., 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat. Med. 10, S98–S109.
Mandl, C.W., Kroschewski, H., Allison, S.L., Kofler, R., Holzmann, H., Meixner, T., Heinz, F.X., 2001. Adaptation of tick-borne encephalitis virus to BHK-21 cells results in the formation of multiple heparan sulfate binding sites in the envelope protein and attenuation in vivo. J. Virol. 75, 5627–5637.
Marks, R.M., Liu, H., Sundaresan, R., Toida, T., Suzuki, A., Imanari, T., Hernáiz, M.J., Lindardt, R.J., 2001. Probing the interaction of dengue virus envelope protein with heparin: assessment of glycosaminoglycan-derived inhibitors. J. Med. Chem. 44, 2178–2187.
McMinn, P.C., 1997. The molecular basis of virulence of the encephalitogenic flaviviruses. J. Gen. Virol. 78, 2711–2722.
Mukhopadhyay, S., Kuhn, R.J., Rossmann, M.G., 2005. A structural perspective of the flavivirus life cycle. Nat. Rev. Microbiol. 3, 13–22.
Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U.S.A. 100, 6986–6991.
Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2005. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. J. Virol. 79, 1223–1231.
Nadanaka, S., Ishida, M., Ikegami, M., Kitagawa, H., 2008. Chondroitin 4-*O*-sulfotransferase-1 modulates Wnt-3a signaling through control of E disaccharide expression of chondroitin sulfate. J. Biol. Chem. 283, 27333–27343.
Poh, M.K., Yip, A., Zhang, S., Priestle, J.P., Ma, N.L., Smit, J.M., Wilschut, J., Shi, P.-Y., Wenk, M.R., Schul, W., 2009. A small molecule fusion inhibitor of dengue virus. Antivir. Res. 84, 260–266.
Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. Nature 375, 291–298.
Roderiquez, G., Oravecz, T., Yanagishita, M., Bou-Habib, D.C., Mostowski, H., Norcross, M.A., 1995. Mediation of human immunodeficiency virus type 1 binding by interaction of cell surface heparan sulfate proteoglycans with the V3 region of envelope gp120–gp41. J. Virol. 69, 2233–2239.
Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N., 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230, 1350–1354.
Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A 74, 5463–5467.
Schneider-Schaulies, J., 2000. Cellular receptors for viruses: links to tropism and pathogenesis. J Gen. Virol. 81, 1413–1429.
Stiasny, K., Kössl, C., Lepault, J., Rey, F.A., Heinz, F.X., 2007. Characterization of a structural intermediate of flavivirus membrane fusion. PLoS Pathog. 3, e20.
Su, C.M., Liao, C.L., Lee, Y.L., Lin, Y.L., 2001. Highly sulfated forms of heparin sulfate are involved in Japanese encephalitis virus infection. Virology 286, 206–215.
Sugiura, N., Sakurai, K., Hori, Y., Karasawa, K., Suzuki, S., Kimata, K., 1993. Preparation of lipid-derivatized glycosaminoglycans to probe a regulatory function of the carbohydrate moieties of proteoglycans in cell–matrix interaction. J. Biol. Chem. 268, 15779–15787.
Talarico, L.B., Pujol, C.A., Zibetti, R.G.M., Farĩia, P.C.S., Noseda, M.D., Duarte, M.E.R., Damonte, E.B., 2005. The antiviral activity of sulfated polysaccharides against dengue virus is dependent on virus serotype and host cell. Antivir. Res. 66, 103–110.
Thant, K.Z., Morita, K., Igarashi, A., 1996. Detection of the disease severity-related molecular differences among new Thai dengue-2 isolates in 1993, based on their structural proteins and major non-structural protein NS1 sequences. Microbiol. Immunol. 40, 205–216.
Trybala, E., Bergström, T., Svennerholm, B., Jeansson, S., Glorioso, J.C., Olofsson, S., 1994. Localization of a functional site on herpes simplex virus type 1 glycoprotein C involved in binding to cell surface heparan sulphate. J. Gen. Virol. 75, 743–752.
Wang, Q.-Y., Patel, S.J., Vangrevelinghe, E., Xu, H.Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N.L., Poh, M.K., Phong, W.Y., Keller, T.H., Jacoby, E., Vasudevan, S.G., 2009. A small-molecule dengue virus entry inhibitor. Antimicrob. Agents Chemother., 1823–1831.
Weaver, S.C., Barrett, A.D., 2004. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat. Rev. Microbiol. 2, 789–801.
Whitby, K., Pierson, T.C., Geiss, B., Lane, K., Engle, M., Zhou, Y., Doms, R.W., Diamond, M.S., 2005. Castanospermine, a potent inhibitor of dengue virus infection in vitro and in vivo. J. Virol. 79, 8698–8706.
Wu, S.-F., Lee, C.-J., Liao, C.-L., Dwek, R.A., Zitzmann, N., Lin, Y.-L., 2002. Antiviral effects of an iminosugar derivative on flavivirus infections. J. Virol. 76, 3596–3604.
Wu, K.P., Wu, C.W., Tsao, Y.P., Kuo, T.W., Lou, Y.C., Lin, C.W., Wu, S.C., Cheng, J.W., 2003. Structural basis of a flavivirus recognized by its neutralizing antibody: solution structure of the domain III of the Japanese encephalitis virus envelope protein. J. Biol. Chem. 278, 46007–46013.
Yanagishita, M., Hascall, V., 1992. Cell surface heparin sulfate proteoglycans. J. Biol. Chem. 267, 9451–9454.
Yennamalli, R., Subbarao, N., Kampmann, T., McGeary, R.P., Young, P.R., Kobe, B., 2009. Identification of novel target sites and an inhibitor of the dengue virus E protein. J. Comput. Aided Mol. Des. 23, 333–341.
Zhang, N., Chen, H.-M., Koch, V., Schmitz, H., Minczuk, M., Stepien, P., Fattom, A.I., Naso, R.B., Kalicharran, K., Borowski, P., Hosmane, R.S., 2003a. Potent inhibition of NTPase/helicase of the West Nile virus by ring-expanded ("Fat") nucleoside analogues. J. Med. Chem. 46, 4776–4789.
Zhang, W., Chipman, P.R., Corver, J., Johnson, P.R., Zhang, Y., Mukhopadhyay, S., Baker, T.S., Strauss, J.H., Rossmann, M.G., Kuhn, R.J., 2003b. Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. Nat. Struct. Biol. 10, 907–912.
Zhang, Y., Corver, J., Chipman, P.R., Zhang, W., Pletnev, S.V., Sedlalk, D., Barker, T.S., Strauss, J.H., Kuhn, R.J., Roaamann, M.G., 2003c. Structures of immature flavivirus particle. EMBO J. 22, 2604-1613.