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Original Article

WSS45, a sulfated α-*D*-glucan, strongly interferes with Dengue 2 virus infection *in vitro*

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Aim: To investigate the mode of action of WSS45, one sulfated derivative of an α-*D*-glucan from the *Gastrodia elata* Bl, on the multiplication cycle of dengue virus serotype 2 (DV2), including initial infection and intracellular replication.
Methods: Virus multiplication in BHK cells were monitored by qRT-PCR, plaque reduction assay, and flow cytometry. Initial virus infection was dissected into adsorption and penetration steps by converting temperature and treating by acid glycine. Surface bound virions were detected by immunofluorescence staining for Evelope protein.
Results: WSS45 effectively inhibited DV2 infection in BHK cells with an EC_{50} value of 0.68±0.17 μg/mL , mainly interfered with virus adsorption, in a very early stage of the virus cycle. However, WSS45 showed no viricidal effect. Moreover, WSS45 could increase the detaching of virus from cell surface in BHK cell line.
Conclusion: WSS45 exerted potent inhibitory effect on DV2 through interfering with the interaction between viruses and targeted cells. This activity was related to its molecular size.

Keywords: Dengue virus; sulfated polysaccharide; infection; BHK cells; α-*D*-glucan

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Introduction

Flavivirus is a genus of the family *Flaviviridae* composed of nearly 80 members. Most flaviviruses are arthropod-borne viruses that cause severe human diseases, including yellow fever virus (YFV), dengue virus (DV), West Nile virus (WNV), and Japanese encephalitis virus (JEV). Among these viruses, DV has re-emerged in recent years as an increasingly important pathogen affecting tropical and subtropical countries, with nearly 50 million infections each year and over 2.5 billion people at risk[1]. Four serologically different Dengue viruses are circulating in nature, termed as DV1–DV4, all of them could be mainly transmitted to humans by two species of mosquitoes, *Aedes aegypti* and *Aedes albopictus*. Infection with DV causes a wide spectrum of clinical illness ranging from silent infection to either a mild febrile, self limited acute syndrome known as dengue fever (DF) or the severe and often fatal dengue hemorrhagic disease (DHF) and dengue shock syndrome (DSS). Despite the increasing incidence and emergence of dengue infections around the world, there is neither prophylaxis nor specific treatment at present to cure the disease. Thus new medicines that could control or cure the emerging DV infection are urgently needed.

DV virions have an enveloped viral capsid containing single-stranded positive-sense RNA, which is approximately 11-kb in length. The viral genome could be translated into a polyprotein that could sequentially be processed by viral and/or host enzymes to generate three structural proteins [capsid (C), membrane protein (M), and envelope (E) protein] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5)[2]. Among the three structural proteins, the E protein is a membrane anchoring glycoprotein on the surface of the mature dengue virion and it has two putative glycosaminoglycan (GAGs) binding-motifs at the carboxyl terminus[3]. This E protein forms oligomers with the small membrane protein (M) and constitutes most of the accessible virion surface.

The virus multiplication starts with the attachment of virus to the surface of the host cell followed by penetration into

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cytoplasm for genome delivery. Researchers have revealed the fact that the E protein is essential for cell attachment and membrane fusion that mediates virus infection[4]. The blockage of virus infection is a valuable antiviral strategy because it allows establishing a first and perhaps most efficient barrier to suppress multiplication.

Studies using electron microscopy have indicated that dengue virus attachment is a temperature-independent process that occurs at both 4 and 37 °C, whereas viral penetration proceeds only at 37 °C[5]. After penetration, un-penetrated virus will be inactivated by acid glycine buffer (pH 3.0) treatment[6]. Surviving viruses are capable of forming plaques or expressing viral antigens.

Currently, sulfated polysaccharides have been considered as promising antiviral compounds against many viruses *in vitro*[7, 8]. In this study, we report a sulfated polysaccharide, WSS45, derived from a novel α-*D*-glucan from *Gastrodia elata* Bl[9], which is a well known and widely used traditional Chinese herb, as a potential antiviral agent. Our results suggested that WSS45 strongly interfered with virus adsorption to host cell surface. However, hydrolysate of WSS45 dramatically lost its anti-DV2 activities, which implies a relationship of proper molecular structure with antiviral activities. Furthermore, WSS45 could affect the interaction between viral envelop protein and specific receptors on the host cellular surface, inducing virus detaching from cell surface.

Materials and methods

Cells and virus

The $C_{6/36}$ cell line from *A albopictus* was grown at 28 °C, 5% CO_2 in minimum essential medium (MEM, Gibco) containing 10% RPMI 1640, supplemented with 5% fetal bovine serum (FBS, Hyclone). BHK (Baby hamster kidney cell) cells were propagated in MEM with nonessential amino acid (NEAA), supplemented with 10% FBS at 37 °C, 5% CO_2. For maintenance medium, the serum concentration was reduced to 2% for above-mentioned cell lines.

Dengue virus serotype 2 (NGC strain, DV2) was propagated in $C_{6/36}$ cells. To obtain a concentrated stock of virus, $C_{6/36}$ cells were infected with DV2 and supernatants were harvested at 6 days after infection. After clarification by low-speed centrifugation, supernatants were concentrated by pelleting for 2 h at 100 000×*g*. Then, the pellet was resuspended in TNE buffer (Tris 10 mmol/L, NaCl 100 mmol/L, EDTA 1 mmol/L) and stored at -80 °C.

Virus stock was tittered in BHK cells[10]. Briefly, BHK cells grown in 6-well plates were infected with DV2 at 10 fold serial dilution. After virus infection for 2 h, the BHK cells were overlaid with overlay medium containing 1% methylcellulose and 2% FBS. The cultures were further incubated for 4 days and viral plaque was counted after fixation and crystal violent staining.

Compounds

WSS45 is one sulfate derivative of a novel α-*D*-glucan extracted from *Gastrodia elata* Bl[9]. The chemical structure was shown in Figure 1. The compound showed a single symmetrical peak (data not shown) on high-performance gel-permeation chromatography (HPGPC). The purity was ≥95%. Compounds were dissolved in deionized water at 10 mg/mL as stock solution and stored at -20 °C.

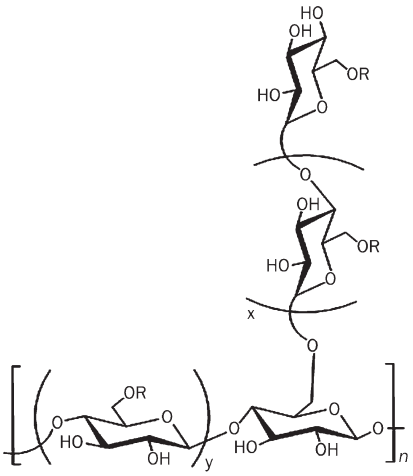

Figure 1. Structure of WSS45.

Mycophenolic acid (MPA) was purchased from Sigma and dissolved in water.

Cell viability assay

Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich] method. Briefly, BHK cells were cultured in triplicate for 48 h with different concentrations of the compounds. The cells cultured with media alone were used as controls. MTT (final concentration 2.5 mg/mL) reagent was added 4 h before the end of culture, and then cells were lysed with 10% sodium dodecyl sulfate (SDS), 50% *N*,*N*-dimethyl formamide, pH 7.2. *OD* values were read at 570 nm, and the percentage of cell death was calculated. The 50% cytotoxicity concentration (CC_{50}) was calculated as the compound concentration necessary to reduce cell viability by 50%.

Viral RNA quantification

Intracellular viral RNA was extracted by RNA Simple RNA extraction kit and total Viral RNA in supernatant was extracted by using the TIANamp Viral DNA/RNA extraction kit (Tiagen, China) according to the manufacturer's instructions. RNA was eluted by 25 μL DEPC-treated water. A portion of virus stock fluid (1×10^7 PFU/mL) was 10 fold serial diluted and extracted in parallel to serve as the RNA control. Prior to reverse transcription, RNA solution and antisense primers mixture were incubated at 70 °C for 5 min and chilled on ice. A 20 μL reaction mixture contained 8 μL of extracted RNA, 1 μmol/L DV2.L1, 1×reaction buffer (Promega, USA), 2 U MMLV reverse transcriptase (Promega, USA), 300 μmol/L

each deoxyribonucleoside (dNTP), and 5 mmol/L Mg^{2+}. Reverse transcription was performed at 37 °C for 1.5 h, followed by 5 min of 94 °C to denature the reverse transcriptase. Sequences of dengue 2 specific fluorogenic probe (DV2.P1) and a pair of flanking primers (DV2.L1 and DV2.U2) (synthesized by Shanghai Sangon Biotechnology Co, Ltd) were shown as follows: DV2.P1 (nucleotides 10653–10678), 5′-CTGTCTCCTCAGCATCATTCCAGGCA-3′; DV2.L1 (nucleotides 10558–10579), 5′-CATTCCATTTTCTGGCGTTCT-3′; DV2.U2 (nucleotides 10680−10700), 5′-AAGGTGAGATGAAGCTGTAGTCTC-3′; DV2.P1 consists of the oligonucleotide sequence shown with a 5′-reporter dye (FAM, 6-carboxy-fluorescein) and a downstream 3′-quencher dye (TAMRA, 6-carboxy-tetramethylrhodamine).

Real-time PCR reactions were conducted in a volume of 20 μL containing 2 μL cDNA template, 1×real-time PCR buffer (TaKaRa, Japan), 0.05 U Ex Taq HS DNA polymerase (TaKaRa, Japan), 4 mmol/L Mg^{2+}, 400 nmol/L dNTP, 400 nmol/L each sense and antisense primer and 200 nmol/L fluorogenic probe. PCR mixtures were pre-incubated at 50 °C for 1 min, then at 93 °C for 1 min, followed by 45 cycles of two-step incubations at 95 °C for 5 s and 60 °C for 1 min. Data were collected and analyzed on a MJ Opticon Monitor 2 system.

Viral RNA level was determined according to the standard curve of a serial dilution of viral sub-genomic RNA. For intracellular viral RNA quantification, cellular hosting GAPDH mRNA was simultaneously evaluated to normalize intracellular viral RNA.

Virus infectivity assay

In the plaque reduction test, BHK cells grown in 6-well plates were infected with about 100 PFU/well of DV2 in the absence or presence of serial diluted compounds. The compounds were added simultaneously with DV2 infection or added 2 h later (post infection). After incubation for another 2 h, cells were washed and overlaid with MEM containing 1% methylcellulose and 2% FBS. The cultures were incubated at 37 °C for 4 days and viral plaques were counted. All determinations were performed twice and each in duplicate. Inhibition was calculated as: Inhibition (%)=(number of control–number of test)/number of control×100%.

In virus yield inhibition assays, BHK cells were infected with DV2 (MOI=0.1) in the presence of different concentrations of the compounds. WSS45 was used at 10, 1, and 0.1 μg/mL. Mycophenolic acid, served as golden control, was used at 1 μg/mL. After 4 days of incubation at 37 °C, cell supernatants were collected and the viral RNA was quantified by real-time RT-PCR (qRT-PCR). The 50% inhibitory concentration (IC_{50}) was calculated as the compounds concentration required to reduce viral RNA load by 50%.

Interference on virus-cell Interactions

The interaction between virus and the host cell is mediated by both the virus surface proteins and the cellular receptors. The influence of WSS45 on the viral surface protein and cellular receptor was determined by blockage assays.

For virus blockage assay, 1 mL DV2 stock (1×10^6 PFU/mL) was firstly incubated with or without compounds for 30 min at 37 °C. WSS45 was then added at a final concentration of 10 and 1 μg/mL. Neutralizing antibody against E protein (clone 3H5) at 10 μg/mL was used as a control for virus blockage. Virus was then purified to remove compounds through a sucrose density gradient centrifugation at 50000×*g* for 2 h. Compound treated or mock treated virus was used to infect 1×10^6 BHK cells.

For cellular receptor blockage assay, BHK cells were pretreated with or without compounds for 1 h at 37 °C. WSS45 was used at 10 and 1 μg/mL. Cells were washed three times by PBS and infected with DV2 virus at a MOI of 1 for 2 h. After washing with PBS, the infected cells were incubated for further 24 h.

Viral infectivity was determined by flow cytometry after intracellular staining of viral NS1 protein. The blockage activity was presented as the decreased percentage of treated groups compared with controls.

Influence of time of treatment on antiviral activity

The influence of compound treatment during the period of adsorption, penetration and post-adsorption was determined in BHK cells infected with DV2. An illustration of different procedures used in these experiments was shown in Figure 2 to help understanding.

Virus adsorption assay

The inhibitory effect on virus adsorption was measured by flow cytometry. BHK cells were infected with DV2 at a MOI of 1 in the presence or absence of compounds. The effects of WSS45 at different final concentrations (10, 1, and 0.1 μg/mL) were analyzed. After 1 h adsorption at 4 °C, cells were washed with ice-cold PBS to remove compounds and un-adsorbed virus, and incubated in maintenance medium at 37 °C for 24 h. Virus infection was determined by flow cytometry as described below. Experiments were performed in duplicate for at least two independent experiments.

Virus penetration assay

BHK cells were infected with DV2 at a MOI of 1 at 4 °C for 1 h. Cells were washed by ice-cold medium and then shifted to 37 °C in the presence or absence of compounds. The effects of WSS45 at different concentrations (10, 1, and 0.1 μg/mL) were tested. After 2 h of virus penetration, un-penetrated virus was inactivated by acid glycine buffer[6] (8 g of NaCl, 0.38 g of KCl, 0.1 g of $MgCl_2{\cdot}6H_2O$, 0.1 g of $CaCl_2{\cdot}2H_2O$, and 7.5 g of glycine/L, pH adjusted to 3 with HCl). The amount of penetrated virus, which surviving the acid glycine treatment, was determined by flow cytometry after 24 h. Experiments were performed in duplicate for at least two independent experiments.

Virus post-adsorption assay

The post-adsorption step was referred as the period post virus adsorption onto target cell at 4 °C but prior to penetra-

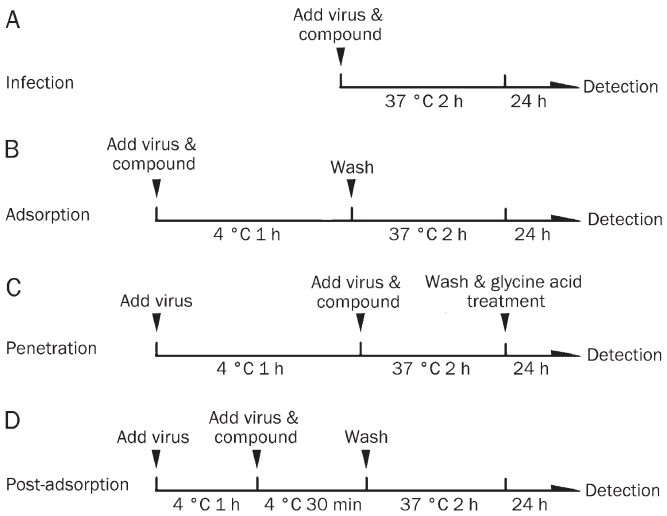

Figure 2. Schematic representation of approaches used to investigate virus infection process. (A) Virus was incubated with cells at 37 °C for 2 h in the presence or absence of compounds. Cells were then washed to remove the compound and virus. The level of virus infection was determined 24 h later by either plaque formation assay or by flow cytometry. (B) Virus adsorption assay. Cells were incubated with virus at 4 °C for virus adsorption in the presence or absence of compounds then washed. The level of virus infection was determined 24 h later by flow cytometry. (C) Virus penetration assay. Virus were added to cells and allowed to adsorb to cells for 1 h at 4 °C. The cultures were washed and then incubated with various concentrations of compounds at 37 °C for 2 h. Extracellular viruses were inactivated by acid glycine buffer (pH 3.0). Virus infection was determined at 24 h by flow cytometry. (D) Post-adsorption assay. Similar experimental procedures were used in virus adsorption and exceeding virus was washed out. Cells were then temporally incubated with compounds at 4 °C for 30 min and washed again. The level of virus infection was determined by flow cytometry and by qRT-PCR.

tion which occurred only under 37 °C BHK cells were pre-incubated with virus at 4 °C and then washed by ice cold PBS to eliminate unbound virus. The infected cells were then incubated for a further 30 min at 4 °C with or without WSS45 (10 and 1 μg/mL). Afterwards, cells were washed thoroughly again to remove WSS45 and shifted to 37 °C. Cells were eventually cultured in maintenance medium for 24 h before flow cytometry analysis. The inhibition rate was presented as the decreased percentage of infected cells compared with parallel treated control. To detect the virus which adsorbed to cell membrane, cells were collected immediately after compound treatment for 30 min at 4 °C. Total RNA was extracted and quantified by Real-time PCR.

Flow-cytometry

Cells were trypsinized and harvested. Cells were fixed with 4% paraformaldehyde at room temperature for 15 min and then permeablized with 0.2% saponin. Intracellular viral antigen was stained with a mouse IgG1 antibody against viral NS1 protein (ab41623, Abcam) for 1 h at 4 °C and a FITIC-conjugated antibody against mouse IgG and analyzed by FACSAria™ flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA, USA).

Indirect fluorescence staining of surface bind virion

For indirect immunofluorescence staining of surface binding virion, BHK cells were seeded on coverslips for 24 h. Cells were pre-incubated with DV2 (MOI=10) at 4 °C for 1 h. Unbound virus were removed by ice cold PBS washing, and compounds were then added for 30 min at 4 °C. Cells were washed again and fixed with paraformaldehyde (PFA). Viruses bound to cell surface were stained firstly with anti-E protein monoclonal antibody (clone 4G2) and subsequently with fluorescin-conjugated secondary antibody and observed by a fluorescent microscope.

Statistic analysis

Data are presented as the Mean±SD. Comparisons between treated and control groups were made by Student *t* test. Statistical difference was accepted at P below 0.05.

Results

WSS45 effectively inhibits Dengue 2 virus multiplication *in vitro*

Virus multiplication cycle includes virus infection into target cells, intracellular viral replication, and virion egression. We investigated the effect of WSS45 on the complete viral multiplication cycle in BHK cells.

Cytoxicity of WSS45 was firstly determined to know the highest tolerating dose of WSS45 in BHK cells. The results showed that WSS45 slightly affected cell livability when given at 3 mg/mL (Figure 3A), thus its CC_{50} was greater than 3 mg/mL. Next, the qRT-PCR method was used to analyze the viral yield of infected BHK cells. The results demonstrated that WSS45 potently inhibited DV2 multiplication in BHK cells (Figure 3B), in a dose dependent manner, with an EC_{50} value of 0.68±0.17 μg/mL and a therapeutic index (TI) of >1000. MPA, served as a control, showed potent inhibition on virus multiplication. We also generated a hydrolysate of WSS45. However, this hydrolysate lost its antiviral activity when given at a final concentration of 25 μg/mL (Data not shown). The results confirmed the antiviral activity of WSS45 against DV2 and suggested proper molecular size was important for antiviral activity of WSS45.

WSS45 showed no direct blocking activity on virus or cellular receptors

Virus infection to target cells is the first step to establish viral multiplication cycle. As shown WSS45 efficiently inhibited virus multiplication in BHK cells, we put forward to address if WSS45 could directly bind virus for inactivating or cellular receptors blocking to inhibit virus infection. Both virus blockage and cellular receptor blockage effects of WSS45 were analyzed. In virus blockage assay, virus pretreated with WSS45 was used to infect BHK cells. Virus lost less than 10% infectivity after 30 min of treatment with WSS45 (Figure 4A), which suggested that WSS45 had no direct blocking effect on DV2 virus. In cellular receptor blockage assay, BHK cells pretreated with WSS45 were infected with DV2. The results

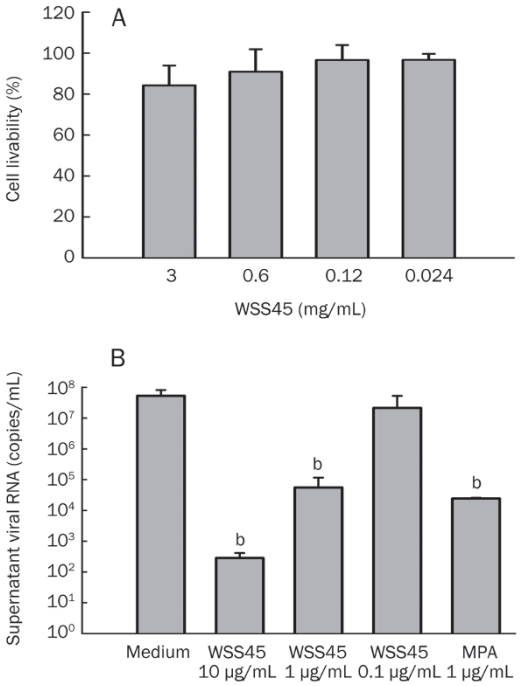

Figure 3. Anti-DV2 activity of WSS45. (A) Cytotoxic effect of WSS45. Cells were exposed to compounds for 48 h and the cytotoxicity was evaluated by MTT method. It indicated that WSS45 slightly affected cell livability at 3 mg/mL. (B) Antiviral activity of WSS45 in BHK cells. DV2 (MOI=0.1) virus was added to confluent cells in the presence of compounds indicated. Supernatant viral RNA was extracted and quantified by qRT-PCR at 48 h post-infection. WSS45 dramatically reduced supernatant viral yield in a dose dependent manner. The results were expressed as Mean±SD of three independent experiments. $^bP<0.05$ *vs* control group.

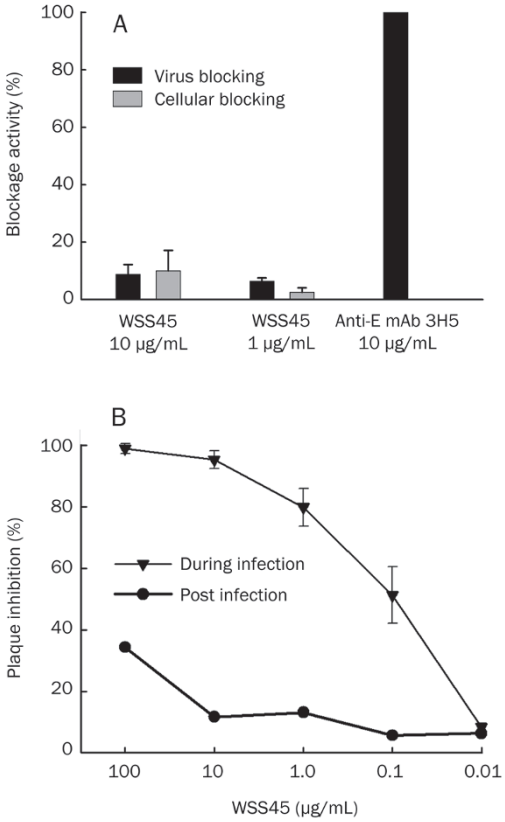

Figure 4. WSS45 exerted its activity during virus infection into host cells. (A) Virus blockage and cellular receptor blockage assay. Virus blockage assay: Virus (1×10^6 PFU/mL) pretreated with compounds was used to infect BHK cells. The level of virus infectivity was detected by flow cytometry method. An E protein specific neutralizing antibody (clone 3H5) was used as a positive control. The percentage of blockage activity was shown as the decrease of infected cells compared with control. Cellular blockage assay: Cells pretreated with compound were then infected with DV2 (MOI=1). After 2 h for infection, cells were treated with acid glycine buffer to inactivate extracellular virus and cultured for 24 h. The level of virus infectivity was detected by flow cytometry method. Both experiments showed that WSS45 had no effect on virus blockage or cellular receptor blockage while the neutralizing antibody against viral E protein displayed virus blockage activity. The results are shown as the change in percentage of infected cells when compared with controls. Mean±SD from two experiments was shown. (B) Inhibition on virus initial infection by WSS45. WSS45 was evaluated the antiviral infectivity during infection period by adding together with virus (100 PFU), or during post-infection period by adding at 2 h post-infection and washed away 2 h later. Plaque reduction assay was performed to determine the inhibitory effect of WSS45. The results are shown as a percentage of plaque formation when compared with controls in which medium was substituted for compounds. The results revealed the WSS45 strongly inhibited virus infection when added during the viral initial infection period. The results were expressed as Mean±SD of three independent experiments.

showed that WSS45 pretreatment had no cellular protective effect against DV2 infection (Figure 4A). Based on these results, no direct virus blockage or cellular blockage effect of WSS45 was observed.

We then investigated the effect of WSS45 on viral intracellular replication process using the plaque reduction assay. Results showed WSS45 strongly interfered with virus initial infection process (Figure 4B). However, WSS45 treatment after virus infection steps showed no activity against DV2 infection. These results suggested that WSS45 exerted its antiviral activity on an early stage of the virus multiplication cycle.

WSS45 mainly interferes with the adsorption of DV2

Virus initial infection process could be dissected into adsorption and penetration steps by temperature convertion and acid glycine treatment[6]. We then tried to elucidate the mode of action of WSS45 against DV2 in the initial infection steps following the experimental procedures illustrated in Figure 1. Firstly, a time course study was performed to analyze the influence of the time of adding the WSS45 (10 and 1 μg/mL) during the virus multiplication cycle. Infected cells were detected at 24 h post infection by flow cytometry. This time point is chosen to ensure one round of virus protein expression because progeny virus are detectable as early as 20 h in BHK cells (Data not shown).

The results showed that WSS45 at 10 and 1 μg/mL significantly decreased the percentage of NS1 positive cells in BHK cells (Figure 5) when presented throughout virus adsorption and penetration steps. Investigations on dissected virus infec-

tion steps revealed that WSS45 exerted maximal inhibitory effect on virus adsorption, while also showed moderate inhibitory effect on virus penetration.

WSS45 could induce the detaching of DV2 virion from host cells

Post-adsorption treatment is a usual step before the penetration assay to ensure no virion lose on the cell surface upon treatment, which could cumber the result of penetration assay. Surprisingly, we found a short period of post-adsorption incubation with WSS45 at 4 °C could dramatically reduce virus infection (Figure 6A & B). This phenomenan was irreversible because replacing with fresh medium and further incubation at 4 °C did not rescue virus infectivity (Data not shown).

To ensure this unusual observation, we measured the adsorbed viral RNA on the surface of BHK cells upon post-adsorption treatment with WSS45 (Figure 7A). After incubating with WSS45 for 30 min and washing with medium, the remained viral RNA on BHK cell surface was significantly reduced by about 10 fold (Figure 7A). The decrease of virus infectivity in BHK cells (Figure 6A) is in correspondence with the change of viral RNA level on cell surface. Therefore, this post-adsorption inhibition by WSS45 could be mostly attributed to inducing virions detaching from BHK cells, but not to inhibiting penetration. To further confirm this effect of WSS45, virions on the BHK cell surface was directly observed by immunofluorescence staining of dengue virus E protein. The intensity of fluorescent spots of viral E protein was attenuated by post-adsorption treatment of WSS45 (Figure 7B). Furthermore, heparin, the antiviral activity of which was well documented, displayed similar effect with WSS45. It is demonstrated that WSS45 disrupted virus-cell interaction during virus adsorption to induce virion detaching in BHK cells.

Discussion

In a previous study[9], we reported WSS45, one sulfate derivative of a glucan from *Gastrodia elata* Bl, inhibited DV2 multiplication in $C_{6/36}$ cells with an EC_{50} value of 10.7±7.3 μg/mL (TI>1000), on the basis of RNA viral load and plaque assay results.

To gain a better understanding of the role of molecular size and structure for antiviral activity of WSS45, WSS45 was hydrolyzed to produce WSS45 hydrolysate with lower molecular weight. However, the hydrolysate totally lost the antiviral activity against DV2 (Data not shown). It has been proposed that decasaccharide was the minimum size in heparin needed for sufficient binding to the putative glycosaminoglycan binding-motifs in E protein of DV2[3]. The loss in activity of the hydrolysate of WSS45 reconfirmed the importance of proper molecular size for antiviral activity of sulfated polysaccharides. Since the branches in WSS45 may also be removed after the hydrolysis, thus we deduced that the branched structure of WSS45 might also contribute to its antiviral activity. Moreover, it has been demonstrated by virus blockage assay that WSS45 exerted its antiviral activity not through directly or specifically blocking the putative glycosaminoglycan binding-motifs in E protein. More probably, the anti-viral activity of WSS45 could be greatly enhanced by the flexible and branched structure.

Our results revealed that WSS45 exerted its antiviral effect in a very early stage of the DV2 cycle, namely, the adsorption and penetration stage. This effect is similar to that of heparin. More interestingly, WSS45 specifically induced virus detaching from BHK cell surface, which was not observed in HepG2 cells (Data not shown). This unexpected effect provides us another hint that virus utilizes different receptors or pathways

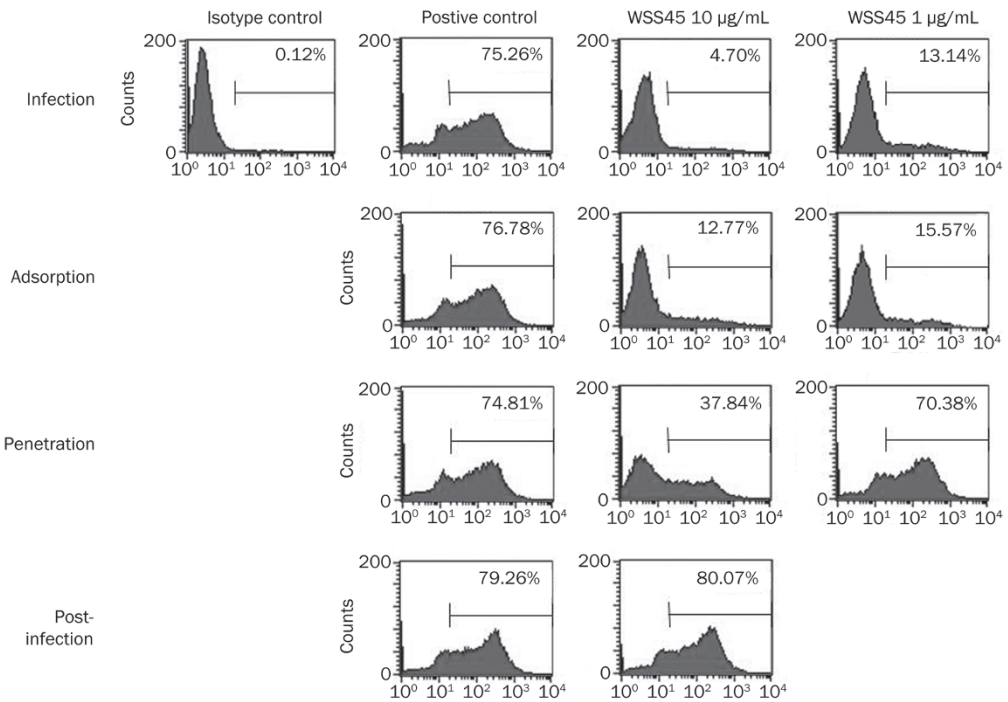

Figure 5. WSS45 strongly inhibited virus adsorption in BHK cells. Initial viral infection was dissected into adsorption and penetration steps by temperature and acid glycine buffer treatment, as shown in Figure 1. The level of infectivity was measured by flow cytometry method after viral NS1 protein staining. The results demonstrated WSS45 mainly inhibited the virus adsorption while it weakly inhibited virus penetration. WSS45 treatment after virus penetration showed no effect on virus intracellular replication. Results presented here are representative of three individual experiments.

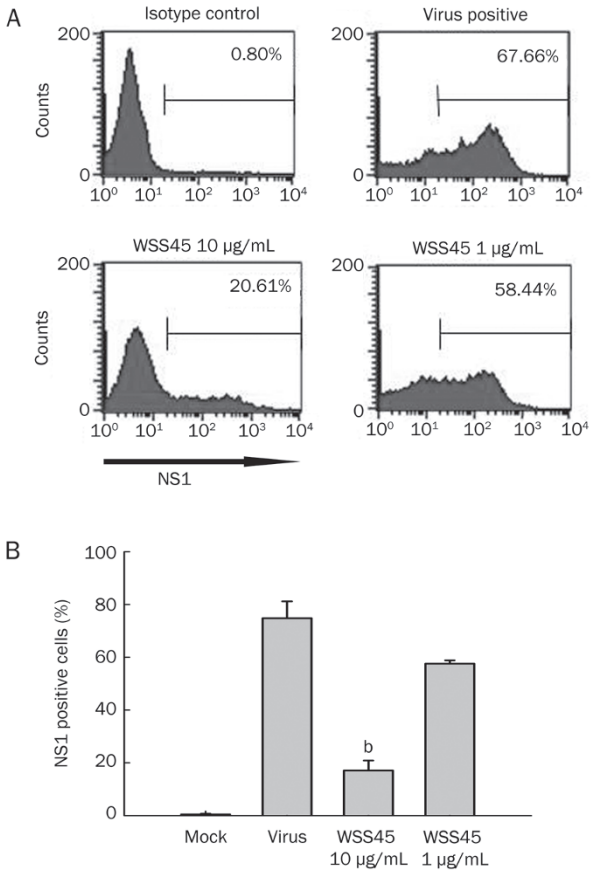

Figure 6. Post-adsorption treatment of WSS45 inhibited DV2 infection. (A) Post-adsorption effect of WSS45 on virus infection in BHK cells. The procedure is shown in Figure 1D. Cells were incubated with virus (MOI=1) at 4 °C for virus adsorption. After washing to remove the virus, cells were incubated with WSS45 for 30 min at 4 °C and washed. The infected cells were determined by flow cytometry. Inhibition was shown as rate of infected cells compared with control which were incubated with fresh medium. Post-adsorption addition of WSS45 inhibited virus infection in a dose dependent mannar. The inhibition activity was in proportion as the anti-adsorption acitvity. (B) The result of three independent experiments was shown (Mean±SD). $^{b}P<0.05$ *vs* medium control group.

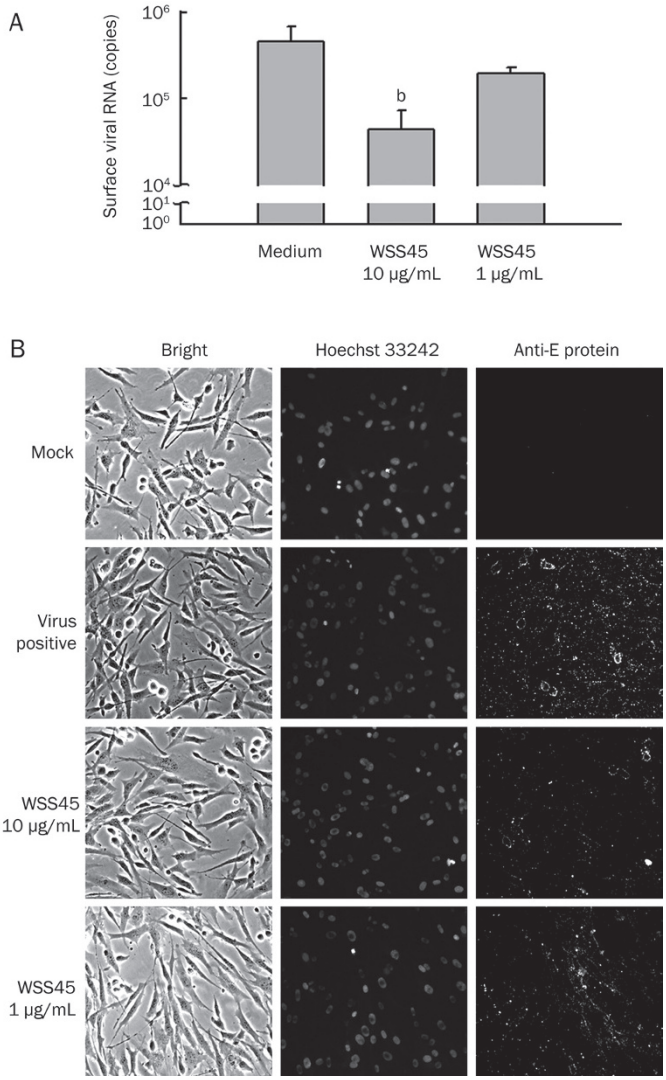

Figure 7. WSS45 induced virus detaching from the surface of BHK cells. (A) Effect of WSS45 in post-adsorption period on the adsorbed viral RNA in BHK cells. After virus adsorption, cells were treated for 30 min with WSS45 as shown in Figure 1D. Cellular RNA were extracted immediately and quantified by qRT-PCR method and normalized to cellular GAPDH RNA level. It shows that incubation with WSS45 after virus adsorption could reduce the adsorbed viral RNA level, which implied a decrease in virions attaching to cell membranes. The results were expressed as Mean±SD of three independent experiments. $^{b}P<0.05$ *vs* control group. (B) Effect of post-adsorption treatment of WSS45 on the virions bound to the BHK cells. BHK cells were grown on coverslips and processed as described in post-adsorption experiment. After post-adsorption incubation with WSS45 and heparin, cells were washed and fixed with PFA and subjected to immunofluorescent staining of viral E protein. Photos were developed using a fluorescence microscope. WSS45 incubation after virus adsorption resulted in a loss of fluorescent spots. This result was in accordance with the RNA level of adsorbed virion. The result presented here is representative of three individual experiments.

for successive infection in different cells[11–14]. GAG chains are complex, linear, negatively charged sulfated polysaccharides found abundantly on cellular surfaces and in the extracellular matrix of most multicellular organisms[15]. GAG chains in BHK cells has been identified to interact with DV2 virus *in vitro*[3]. So far, no specific protein receptor of DV2 virus on BHK cells has been identified. Since WSS45 is a GAG mimetics, thus it could competitively inhibit virus adsorption via interfering the interaction between the GAG chain in the BHK cells and its ligand on the DV2 virus surface. Protein receptor GRP78 (also known as BiP) on HepG2 cells[11] was demonstrated to have specific interaction with DV2 E protein. Although glycosaminoglycan chains on the HepG2 cell surface also has the potential to facilitate the binding of DV2 virions[16], we believe the binding of E protein to its specific receptor GRP78 might be more stable to survive the interference of WSS45 than that to GAG chains. Taken together, the antiviral activity of WSS45 in BHK cells should be considered as a combination of its inhibitory effect on adsorption and its detaching effect during

post-adsorption period. Besides, heparin we used as a reference compound also exerted such activity, implying sulfated polysaccharides might share similar properties to induce virus detachment from certain type of cells.

Dengue virus E protein, like other Flavivirus envelope proteins, undergoes conformational changes to expose its fusion peptide under low pH[17, 18]. This change probably exposes additional interaction site for WSS45 to obstruct the fusion process because a weak inhibition on virus penetration was observed. It is reported that protonation on residues of E protein is important for its fusion activity[19]. Whether there is a possibility that WSS45 could cumber this process by its negative charge remains uncertain.

This study revealed the mechanism of WSS45 as interfering with the interaction between dengue virus and targeted cells. The mode of action for WSS45 might be a common property for sulfated polysaccharides with antiviral activity.

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Author contribution

Xian-kun TONG, Wei TANG designed and performed research; Hong QIU provided polysaccharide compounds; Xin ZHANG kindly provided virus strains; Li-ping SHI contributed reagents and helped to establish PCR and flow-cytometry methods; Gui-feng WANG, Fei-hong JI, and Hui-yong DING contributed useful advices and discussions. Xian-kun TONG wrote the manuscript. Kan DING and Jian-Ping ZUO guided the whole experiments and reviewed the text.

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