Policresulen, a novel NS2B/NS3 protease inhibitor, effectively inhibits the replication of DENV2 virus in BHK-21 cells

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Aim: Dengue is a severe epidemic disease caused by dengue virus (DENV) infection, for which no effective treatment is available. The protease complex, consisting of nonstructural protein 3 (NS3) and its cofactor NS2B, plays a pivotal role in the replication of DENV, thus may be a potential target for anti-DENV drugs. Here, we report a novel inhibitor of DENV2 NS2B/NS3 protease and its antiviral action.

Methods: An enzymatic inhibition assay was used for screening DENV2 NS2B/NS3 inhibitors. Cytotoxicity to BHK-21 cells was assessed with MTT assay. Antiviral activity was evaluated in BHK-21 cells transfected with Rlu-DENV-Rep. The molecular mechanisms of the antiviral action was analyzed using surface plasmon resonance, ultraviolet-visible spectral analysis and differential scanning calorimetry assays, as well as molecular docking analysis combined with site-directed mutagenesis.

Results: In our in-house library of old drugs (~1000 compounds), a topical hemostatic and antiseptic 2-hydroxy-3,5-bis[(4-hydroxy-2-methyl-5-sulfophenyl)methyl]-4-methyl-benzene-sulfonic acid (policresulen) was found to be a potent inhibitor of DENV2 NS2B/NS3 protease with IC50 of 0.48 μg/mL. Furthermore, policresulen inhibited DENV2 replication in BHK-21 cells with IC50 of 4.99 μg/mL, whereas its IC50 for cytotoxicity to BHK-21 cells was 459.45 μg/mL. Policresulen acted as a competitive inhibitor of the protease, and slightly affected the protease stability. Using biophysical technology-based assays and molecular docking analysis combined with site-directed mutagenesis, we demonstrated that the residues Gln106 and Arg133 of DENV2 NS2B/NS3 protease directly interacted with policresulen via hydrogen bonding.

Conclusion: Policresulen is a potent inhibitor of DENV2 NS2B/NS3 protease that inhibits DENV2 replication in BHK-21 cells. The binding mode of the protease and policresulen provides useful hints for designing new type of inhibitors against the protease.

Keywords: Dengue virus; antiviral drug; NS2B/NS3 protease; policresulen; molecular docking; molecular modeling
proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Previous studies have shown that NS3 contains two functional domains: a serine protease at the N-terminus and an RNA helicase at the C-terminus\textsuperscript{12-14}. NS2B is an essential element that acts as a co-factor to greatly enhance the activity of NS3 by 3300- to 6600-fold\textsuperscript{15, 16}. As a viral protease complex, NS2B/NS3 is mainly in charge of cleaving the viral poly-protein precursor to generate structural proteins and nonstructural proteins, and the disruption of NS2B/NS3 protease function distinctly inhibits viral replication\textsuperscript{17}. Therefore, the NS2B/NS3 protease is considered as an attractive target for anti-DENV drug development\textsuperscript{14}. In fact, the treatment of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections serve as successful precedents for targeting key viral proteases\textsuperscript{18-20}.

Currently, the discovery of a NS2B/NS3 protease inhibitor mainly involves the design of peptidomimetics with similar structures to the substrate of the NS2B/NS3 protease and screening small molecule inhibitors by random or virtual screening strategies against commercial compound libraries. For example, one cyclopentapeptide was proposed as an inhibitor of the DENV2 NS2B/NS3 protease\textsuperscript{21}, and MrIA, a type of conotoxin, was also determined to be an inhibitor for this protease\textsuperscript{22}. Recently, varied types of potential inhibitors against the DENV2 NS2B/NS3 protease have been reported\textsuperscript{23-28}, but few inhibitors exhibited antiviral activities. Therefore, it is urgent to discover novel NS2B/NS3 protease inhibitors with high activities against dengue virus.

In the current work, we performed an enzymatic inhibition assay to discover inhibitors of this recombinant DENV2 NS2B/NS3 protease \textit{in vitro} against our in-house library of old drugs, and 2-hydroxy-3,5-bis[(4-hydroxy-2-methyl-5-sulfo-phenyl)methyl]-4-methyl-benzenesulfonic acid (policresulen, Figure 1A), a clinical medication used for debridement and pretreatment of excatwy, NJ, USA). Bz-Nle-KRR-AMC was synthesized by Shanghai GL Biochem. The mMESSAGE MEGAscript\textsuperscript{R} T7 Kit was acquired from Ambion (TX, USA). The Renilla Luciferase Assay Kit was acquired from Promega (Madison, WI, USA). The Fast Mutagenesis Kit was acquired from Beijing Trans-Gene Biotech (Beijing).

**Cell culture**

Baby Hamster Syrian Kidney (BHK-21) cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO\textsubscript{2} incubator.

**Expression and purification of the DENV2 NS2B/NS3 protease**

The pET15b-DENV2 CF40-Gly-NS3pro185 plasmid was donated by Prof Chun-guang WANG (Institute of Protein Research, Tongji University). The plasmid was transformed into Escherichia coli BL21, and DENV2 NS2B/NS3 protease expression and purification were mainly carried out according to the published approach\textsuperscript{30}. Briefly, Escherichia coli BL21, transformed with the recombinant plasmid, was cultured in 1 L of Luria-Bertani medium containing 1 mmol/L ampicillin, 0.5 mmol/L chloramphenicol and 0.2% (w/v) glucose at 37 °C, and then induced with 0.1 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) until the OD\textsubscript{600} reached 0.6 after an additional 8 h at 16 °C. Bacteria were collected by centrifugation at 12 000×g for 5 min at 4 °C. The harvested bacteria were suspended in 20 mL of lysis buffer [50 mmol/L HEPES, 300 mmol/L NaCl, and 5% (v/v) glucose, pH 7.5], lysed by probe sonication on ice, and then centrifuged at 12 000×g for 60 min at 4 °C. The supernatant was mixed with Ni\textsuperscript{2+}-NTA resin in a column and incubated for 8 h at 4 °C. The column was then extensively washed with 100 mL of washing buffer (10 mmol/L imidazole, 500 mmol/L NaCl, and 20 mmol/L Tris, pH 8.0), and the protein was slowly eluted with 20 mL of elution buffer (120 mmol/L imidazole, 500 mmol/L NaCl, and 20 mmol/L Tris, pH 8.0). To obtain high purity of the protein, the eluted protein was further purified by Superdex 75 Gel Filtration. Finally, the purified protein was concentrated and stored at -80 °C.

**Enzymatic inhibition assay**

The enzymatic inhibition assay was performed in Greiner Black 96-well plates according to the published approach\textsuperscript{22}. Briefly, 100 μL of reaction mixtures containing 50 mmol/L Tris-HCl (pH 8.5), 20% (v/v) glycerol, 10 mmol/L NaCl, 1 mmol/L CHAPS, 200 mmol/L NS2B/NS3 protease and the compound dissolved in DMSO were pre-incubated at 37 °C for 30 min, and the reaction was then started by the addition of the substrate (Bz-Nle-KRR-AMC) to a final concentration of 100 μmol/L. Aprotinin (1 μmol/L\textsuperscript{29}) was used as a positive control. DENV2 NS2B/NS3 protease activity was monitored.
by the increase in fluorescence with a microplate spectrophotometer. The excitation and emission wavelengths were set at 380 and 460 nm, respectively. To determine the 50% inhibitory concentration (IC_{50}) of policresulen, different concentrations of policresulen (0, 0.001, 0.01, 0.1, 1, 10, and 20 μg/mL) were prepared. The IC_{50} value was calculated using Origin 5, and the inhibition type was determined using the Lineweaver-Burk plot.[32]

Cytotoxicity assay
The sensitivity of cell lines to policresulen was investigated using an MTT assay. Briefly, BHK-21 cells were seeded into 96-well plates containing 100 μL of culture medium at a density of 1×10^4 cells per well and were incubated overnight at 37 °C. Different concentrations of policresulen (0, 50, 100, 200, 400, 800, 1600, and 3200 μg/mL) were added and cultured for 48 h. Subsequently, 25 μL of MTT (5 mg/mL) was added and incubated for an additional 4 h. Finally, 125 μL of SDS-isobutanol-HCl solution (10% SDS, 5% isobutanol, and 12 μmol/L HCl) was added to each well and thoroughly mixed for 2 h to dissolve the formazan crystals. The optical absorption of 570 nm was measured with a multimode microplate reader (Varioskan, Thermo Scientific, USA). The cell viability in each well was calculated by the following equation: Viability rate (%) = (A_{570 nm} of wells that contained the drug/A_{570 nm} of free well)×100%.[33]

Luciferase assay
The reporter pACYC-DENV2-Rluc2A replicon (Rlu-DENV-Rep) was used to evaluate the translation and synthesis of DENV2 RNA. RNAs were transcribed from the pACYC-DENV2-Rluc2A replicon in vitro using a mMESSAGE MEGAscript® T7 Kit according to the manufacturer’s protocols. BHK-21 cells transfected with Rlu-DENV-Rep RNAs at MOI=0.1 were seeded into 48-well plates containing 200 μL of culture medium at a density of 5×10^4 cells per well and were incubated for 1 h at 37 °C. Varied concentrations of policresulen (0, 0.882, 1.76, 3.53, 7.06, 14.11, and 28.22 μg/mL) were then added to the medium and incubated for 48 h at 37 °C. After removing the medium, the cells were washed once with PBS, followed by the addition of 100 μL lysis buffer to each well. Fifty microliters of luciferase substrate was then added into the sample wells (20 μL cell lysates), which was assayed for luciferase signal with a multimode microplate reader (Varioskan Flash). The inhibition rate of policresulen was obtained from the following equation: Inhibition rate (%) = (1-luciferase intensity of wells with drug/luciferase intensity of free wells)×100%. IC_{50} was calculated using the trimmed Kärber method.[34]

Surface plasmon resonance (SPR) technology-based assay
The binding affinity of policresulen for the DENV2 NS2B/NS3 protease was investigated using an SPR technology-based Biacore T200 instrument (GE Healthcare). Purified NS2B/NS3 protease was immobilized on CM5 chips in a standard amine-coupling reaction in 10 mmol/L sodium acetate buffer (pH 4.0) at 25˚C. Before immobilization, the chip was equilibrated with HBS-EP buffer [10 mmol/L HEPES, 3 mmol/L EDTA, 150 mmol/L NaCl, and 0.005% (v/v) surfactant P20, pH 7.4] for 120 min. Different concentrations (0.24, 0.35, 0.49, 0.71, 1.00, 1.44, 2.06, and 2.94 μg/mL) of policresulen dissolved in HBS-EP buffer were then injected into the chips with a flow rate of 30 μL/min for 2 min, followed by disassociation for 4 min. The equilibrium dissociation constant (K_{d}) of policresulen was calculated using a 1:1 Langmuir binding model based on the Biacore T200 evaluation software.

Ultraviolet-Visible (UV) spectral analysis
UV spectral analysis was used to investigate the interaction between the DENV2 NS2B/NS3 protease and policresulen. The UV spectrum was monitored using a U-2010 Spectrophotometer. In the assay, a 20 μmol/L NS2B/NS3 sample was prepared in a solution of 50 mmol/L HEPES (pH 7.5) and 300 mmol/L NaCl. The NS2B/NS3 protease was then incubated with policresulen at 25 °C for 10 min in the dark. UV spectra were detected in the range of 190–340 nm at room temperature. Experimental data are the average values of at least three measurements and were corrected by subtracting a blank obtained under the same conditions.

Differential scanning calorimetry (DSC) assay
The DSC experiment was performed with a VP-Capillary DSC System (GE Healthcare) under a stream of N₂. Protein samples were dialyzed against a 50 mmol/L HEPES (pH 7.5), 300 mmol/L NaCl buffer overnight at 4 °C. The test sample consisted of protein alone or protein with policresulen, respectively. The dialysate was used as a reference, and all samples were filtered before use. Two hundred microliter samples were then added into 96-well plates until the plates were loaded into the calorimeter at 10 °C. The scan temperature ranged from 10 to 110 °C with a heating rate of 1.5 °C/min. The mid-feedback mode was selected, and the Cp value was read every 3 s. The thermal transition midpoints (Tm) of samples were analyzed using Origin 5.0 software.

Molecular docking
The molecular docking of the inhibitor policresulen in the substrate binding pocket of the DENV2 NS2B/NS3 protease was performed using the Schrödinger program Maestro (Version 9.2) according to the previously published protocol.[35-37] using the structure of NS2B/NS3 protease in PDB ID 2M9Q as the model. For docking, the structure of the protein was prepared by Protein Preparation Wizard and the inhibitor by Ligprep.[38, 39]. Gulie was used to study the binding conformation between the protein and inhibitor.

Site-directed mutagenesis of the NS2B/NS3 protease
Mutant plasmids for DENV2 NS2B/NS3 (NS2B/NS3-Q106G, NS2B/NS3-R133G, and NS2B/NS3-Q106G/R133G) were generated using the Fast Mutagenesis Kit (TransGene Biotech, Beijing, China). Site-directed mutagenesis of the residues was performed on pET15b-DENV2CF40-Gly-NS3pro185 accord-
ing to a modified recombinant PCR method\[^{[40]}\]. The oligonucleotide sequences of the primers used to generate DENV2 NS2B/NS3 mutant genes are listed in Table 1. All mutants were confirmed by DNA sequencing. *Escherichia coli* BL21 was transformed with mutant genes and used for the expression of mutant proteins. The method for the expression and purification of the mutant proteins was similar to that used for the wild-type protein.

**Results**

**Policresulen is an inhibitor of the DENV2 NS2B/NS3 protease**

To discover DENV2 NS2B/NS3 protease inhibitors, we performed a screening assay based on an enzymatic test against our in-house library of old drugs (~1000 compounds), and the agent 2-hydroxy-3,5-bis[(4-hydroxy-2-methyl-5-sulfophenyl)methyl]-4-methyl–benzenesulfonic acid (policresulen, Figure 1A) was finally identified as a potent inhibitor of the protease, with an IC\(_{50}\) of 0.48 μg/mL (Figure 1B).

To exclude the possibility of interference from policresulen on the substrate or on DENV2 NS2B/NS3 protease in the enzymatic inhibition assay, the following experiments were conducted. First, a similar enzymatic inhibition assay without the NS2B/NS3 protease was performed to detect the effect of policresulen on the substrate. As shown in Figure S1, the presence of policresulen did not influence the fluorescence of the substrate. Second, to exclude the possibility of policresulen-inhibited DENV2 NS2B/NS3 protease enzymatic activity due to the weak acidity of the compound, circular dichroism (CD) was carried out. As indicated in Figure S2, policresulen rendered no effects on the structure of the NS2B/NS3 protease, even at 20 μg/mL, which was the maximum concentration used in the enzymatic inhibition assay.

In addition, the MTT assay results demonstrated a very low cytotoxicity for policresulen in BHK-21 cells, as indicated by the fact that more than 50% of BHK-21 cells remained viable, even though policresulen reached concentrations as high as 459.45 μg/mL (Figure 1C). Moreover, this result also confirmed that the weakly acidic property of policresulen had no effect on the tested cells, which is consistent with the CD spectral results (Figure S2).

Because policresulen is a mixture of several analog compounds\[^{[41]}\], electrospray ionization mass spectrometry (ESI-MS) was performed for the policresulen used in our work to characterize the precise molecular weight of every component. The pseudo molecular ion peak (negative ion mode) profiles of policresulen are shown in Figure S3. Two ion peaks (387.1 and 774.7) with the highest relative abundances represent component B and its dimer, respectively. In addition, there was an obvious ion peak (587.0) that represents component C, which suggests that there are three components of the policresulen used in our work.

Taken together, these results indicated that policresulen is an efficient inhibitor of DENV2 NS2B/NS3 protease with low cytotoxicity in BHK-21 cells.

**Policresulen inhibited the replication of DENV2**

Given that policresulen has been determined to inhibit the
DENV2 NS2B/NS3 protease, we next evaluated its potential antiviral activity against DENV2 on the cellular level. Rluc-DENV2-Rep which includes a reporter gene (Renilla luciferase, Rluc) and the full genome of DENV2 (pACYC-DENV2-Rluc2A replicon) were used to examine the antiviral effects of policresulen\[42\]. In this assay, DENV2 RNA, which included an Rluc gene transcribed from Rluc-DENV2-Rep, was transfected into BHK-21 cells, and the ability of the virus to replicate was monitored by detecting the activity of Renilla luciferase at 48 h post-infection. As indicated in Figure 2A and B, policresulen could effectively inhibit the luciferase activity in a dose-dependent manner, with an IC\(_{50}\) of 4.99 μg/mL. This result, combined with that from the cytotoxicity assay, shows a high therapeutic index (TI) for policresulen of 92.07.

**Policresulen acted as a competitive inhibitor of the DENV2 NS2B/NS3 protease**

*Inhibitor type assay* Because policresulen has been determined to be a DENV2 NS2B/NS3 protease inhibitor able to efficiently suppress the replication of DENV2, we next investigated the manner in which it inhibits the DENV2 NS2B/NS3 protease. As shown in Figure 3A, the Lineweaver-Burk plot analysis from the enzymatic inhibition data indicates that policresulen acted as a competitive inhibitor against the DENV2 NS2B/NS3 protease with an inhibitory constant (\(K_I\)) value of 0.27 μg/mL (Figure 3B).
Surface plasmon resonance (SPR) technology-based assay

Next, the binding affinity of policresulen against the DENV2 NS2B/NS3 protease was detected using an SPR technology-based Biacore assay, in which the interaction between policresulen and the immobilized protease is reported as a sensorgram, which is a plot of the binding response represented by response unit (RU) versus time. From the SPR assay sensorgrams, slow off-rate binding kinetics were observed between policresulen and the NS2B/NS3 protease in the binding model, and the same results were also discovered in other small molecule-protein interactions[43]. Additionally, the gradual increases in RU values as compared to the changes in policresulen concentrations demonstrated specific binding of policresulen to the NS2B/NS3 protease. The dissociation equilibrium constant ($K_d$) value of policresulen from the protease was thereby fitted to 0.35 μg/mL (Figure 3C), and the $R_{max}$ value for policresulen binding to NS2B/NS3 protease was 257.8 Ru. Therefore, policresulen was determined to be a competitive inhibitor able to bind directly to the DENV2 NS2B/NS3 protease.

Policresulen reduced the stability of the DENV2 NS2B/NS3 protease

To further elucidate the molecular mechanism used by policresulen in the inhibition of the DENV2 NS2B/NS3 protease, ultraviolet-visible (UV) spectral analysis and differential scanning calorimetry (DSC) assays were carried out.

**UV spectral analysis**

It is well-known that proteins have two strong absorption peaks of ~190-250 nm and ~280 nm[44, 45]. Here, there are two strong absorption peaks at 232 and 278 nm in the UV spectra of the DENV2 NS2B/NS3 protease. As shown in Figure 4A, the incubation of policresulen with the protease caused a red shift of 7 nm for the peak at 232 nm and a decrease in the absorbance intensity, while no changes were observed for the absorption peak at 278 nm. These results implied an interaction of policresulen with the DENV2 NS2B/NS3 protease, which is consistent with the result of the SPR assay. The red shift of the absorption peak at 232 nm demonstrated the changed microenvironment of the peptide bond in the DENV2 NS2B/NS3 protease in the presence of policresulen[46, 47].

**DSC assay**

Considering that the result of the UV spectral analysis has revealed that policresulen binding induced changes in the peptide bond microenvironment of the protease, a DSC assay was then applied to investigate the stability of the protease. In this assay, the thermal transition midpoint (Tm), which is related to changes in the conformation of the protein, was detected. As shown in Figure 4B, the Tm value for the DENV2 NS2B/NS3 protease alone was 51.5 °C, and an incubation of policresulen with the protease caused a slight decrease in the Tm value of the protease to 51.17 °C. This result thus implies that policresulen binding to the DENV2 NS2B/NS3 protease reduced the thermal stability of the protein, but this change was not significant.

![Figure 4](image.png)

**Figure 4.** Policresulen reduced the stability of DENV2 NS2B/NS3 protease. (A) UV spectra of NS2B/NS3 protease (20 μmol/L) in presence or absence of policresulen (23.52 μg/mL). Blue curve: absorbance spectrum of NS2B/NS3 protease. Red curve: absorbance spectrum of the protease with policresulen. Green curve: absorbance spectrum of the protease with policresulen (subtracting the effect of policresulen). Black curve: absorbance spectrum of policresulen. (B) The thermal stability of NS2B/NS3 protease combined with or without policresulen was evaluated by DSC thermograms. The temperature of the peak of thermo curve represented the thermal transition midpoints (Tm). Tm value of the protease (black curve) was 51.5 °C, and Tm value of the protease with policresulen (red curve) was 51.17 °C.

**Structural analysis of the DENV2 NS2B/NS3 protease in a complex with policresulen**

**Molecular modeling**

To better understand the method of policresulen binding to the NS2B/NS3 protease, a 3D binding model of policresulen in the substrate binding site of the protease was investigated by molecular docking. A protein structural model was prepared using the representative conformer of the NMR structure of the DENV2 NS2B/NS3 protease (PDB ID: 2M9Q), and all amino acid numberings (Figure 5) were based on the sequence of recombinant DENV2 NS2B/NS3 protease. Given that the compound with the structure in Figure 6A is suggested to be one of the major active components of policresulen (PubChem CID: 3050404)[48], this structure for policresulen was thus used for docking analysis. As illustrated in Figure 6B–D, on the left side of policresulen, the oxygen of the sulfo group formed a hydrogen bond with the Gln106 side chain in the P1 region of the protease, and...
the phenol group formed a hydrogen bond with the carbonyl group of the main chain of Gln114. In the middle section of policresulen, the benzene moiety formed hydrophobic interactions with residues Ile109, Ile115, and Val131 in the hydrophobic P2 region of the protease, and the sulfo-group may exhibit electrostatic interactions with His130. On the right side of policresulen, the oxygen of the sulfo-group formed hydrogen bonds with the side chains of residues Thr132 and Arg133 in the positive P3 region of the protease.

In all, policresulen interacted with residues Gln106, Gln114, Thr132 and Arg133 of the DENV2 NS2B/NS3 protease through hydrogen bonding and residues Ile109, Ile115, Val131 through hydrophobic bonding.

**Mutation analysis based validation** To validate the binding model of policresulen to the protease that was suggested by the docking results, two typical residues in the binding site of the protease for policresulen, Gln106 and Arg133 (Figure 5 and 6), were chosen for investigation. Single or double site-directed mutagenesis of the protease (Q106G, R133G, and Q106G/R133G) was thereby carried out.

The mutants Q106G, R133G and Q106G/R133G of the DENV2 NS2B/NS3 protease through hydrogen bonding and residues Ile109, Ile115, Val131 through hydrophobic bonding.

Figure 5. Amino acid sequence in DENV2 NS2B/NS3 protease. The residues marked in bold are part of NS2B amino acid sequence. The residues marked in underline are His-tag. The residues 71–79 marked in underline and italics are the G4-S-G4 flexible linker in recombinant DENV2 NS2B/NS3 protease. The amino acids of NS3 sequence are highlighted in gray. The locations of the amino acid mutations to generate the variants are indicated by rectangle and the substitute residues are also shown in the rectangle.
Q106G and R133G mutants. These results thus further confirmed that the Gln106 and Arg133 residues are responsible for policresulen binding to the DENV2 NS2B/NS3 protease.

Therefore, all aforementioned mutation analyses verify that the Gln106 and Arg133 residues are important for the binding of policresulen to the DENV2 NS2B/NS3 protease and the inhibition of protease activity, and they further support the docking model of the protease in a complex with policresulen.

**Discussion**

To date, several DENV proteases have been identified as potential targets for the discovery of anti-DENV inhibitors, including NS2B/NS3, NS4B and NS5[49-51], and the NS2B/NS3 protease is particularly important because of its essential role in the replication of DENV[52]. In fact, an inhibition strategy involving the protease complex and facilitated by a co-factor enzyme has been successfully applied in the discovery of antiviral drugs against human immunodeficiency virus (HIV) and hepatitis C virus (HCV)[53], and several inhibitors targeting the HCV NS3/4A protease have already entered into clinical trials[54-56].

Recently, varied types of potential inhibitors against the DENV2 NS2B/NS3 protease have been discovered[11, 23-25, 31, 57-59] and have provided important structural information for anti-DENV drug development. In our current work, given a strategy for new drug discovery that is based on using old drugs to quickly achieve data for preclinical and early clinical trials and thus possibly accelerate new drug development speed[60], we constructed a platform in the discovery of DENV2 NS2B/NS3 protease inhibitors by targeting an in-house library of old
drugs. The results demonstrated that policresulen functioned as a novel inhibitor of the DENV2 NS2B/NS3 protease and efficiently suppressed the replication of the DENV2 virus with a high therapeutic index, which has greatly supported the availability and efficiency of this drug discovery platform. It is expected that this platform may also expand its application in discovering effective inhibitors against other viruses, and policresulen may have potential as a drug lead compound for the treatment of DENV.

In addition, the details of policresulen binding to the DENV2 NS2B/NS3 protease have been illuminated by a series of biophysical technology-based assays and molecular docking analysis combined with site-directed mutagenesis. Residues Gln106 and Arg133 of the protease directly interacted with policresulen via hydrogen bonding, which is an interaction that is very different from that used by the previously reported inhibitors, which bind to the catalytic triad residues (His51, Asp75, and Ser135) of the protease\(^\text{[57, 61]}\). Therefore, this newly determined binding arrangement between policresulen and the protease is expected to provide useful hints for designing

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**Figure 7.** Mutation analysis verified the binding sites of policresulen against DENV2 NS2B/NS3 protease. (A–C) IC\(_{50}\) of policresulen against NS2B/N33 (Q106G), NS2B/NS3 (R133G) and NS2B/NS3 (Q106G/R133G) were determined as (A) 4.99 μg/mL; (B) 4.3 μg/mL; (C) 60.8 μg/mL, respectively. (D–F) Biacore sensorgrams were obtained from injections of policresulen at 2.94 μg/mL over the immobilized. (D) NS2B/N33 (Q106G). (E) NS2B/NS3 (R133G) or (F) NS2B/NS3 (Q106G/R133G). The sensorgram of policresulen at 2.94 μg/mL binding to wild type NS2B/NS3 was shown in dashed line as a control.
new types of anti-DENV2 NS2B/NS3 protease inhibitors.

Notably, policresulen is an organic acid with hemostatic and antimicrobial activities, and it is used as a clinical medication in gynecology for the treatment of vaginitis[29]. Here, we found that policresulen could also function as a DENV2 NS2B/NS3 inhibitor able to effectively suppress the replication of the DENV2 virus, and this finding has extended the potential pharmacological applications of this agent.

In summary, we discovered that policresulen, when used as a novel DENV2 NS2B/NS3 protease inhibitor, exhibited potent antiviral activity. The determined binding conformation of policresulen and the protease may provide useful hints for designing new types of effective inhibitors against DENV, and policresulen may be used as a potential antiviral drug lead compound against DENV.

Acknowledgements
We gratefully acknowledge financial support from the National Natural Science Foundation of China (Grant No. 81220108025, 21222221, 81373461, 91413102, 81473141, and 91213306).

Author contribution
Xu SHEN, Jian LI, Chuan-lian XU, Xiao-min LUO and Jing CHEN designed the research; Deng-wei WU, Fei MAO and Yan YE performed the experiments; Xu SHEN and Jing CHEN supervised the project; Xu SHEN, Jing CHEN, and Deng-wei WU contributed to the manuscript writing. All authors read and approved the final manuscript.

Supplementary Information
Supplementary figures are available on the website of Acta Pharmacologica Sinica.

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