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Spirotetronate compounds are polyketide secondary metabolites with diverse biological functions, such as antibacterial, antitumor and antiviral activities. Three pure spirotetronate compounds (2EPS-A, -B, -C) isolated from Actinomadura strain 2EPS showed inhibitory activity against dengue virus serotype 2 (DENV-2). 2EPS-A, -B and -C demonstrated the LC$_{50}$ values of 11.6, 27.5 and 12.0 µg/ml, respectively, in a test of cytotoxicity to Vero cells. The least cytotoxic, 2EPS-B, was further analyzed for its impact on viral propagation in a cell-based replication assay. At a concentration of 6.25 µg/ml, it could reduce the DENV-2 infection in Vero cells by about 94% when cells infected with DENV-2 were exposed to 2EPS-B, whereas direct treatment of DENV-2 with 2EPS-B at the same concentration prior to subsequent infection to Vero cell yielded no inhibition. 2EPS-A, -B an -C showed strong DENV-2 NS2B-NS3 protease inhibition in an in vitro assay, with IC$_{50}$ values of 1.94 ± 0.18, 1.47 ± 0.15 and 2.51 ± 0.21 µg/ml, respectively. Therefore, the spirotetronate compounds appear to prevent viral replication and viral assembly by inhibition of the viral protease.

Key Words: Actinomadura; antiviral; dengue virus; NS2B-NS3 protease; spirotetronate

Introduction

One of the most wide-spread mosquito-borne viral infections in tropical areas is dengue fever or ‘breakbone fever’. The incidences of the disease have increased dramatically around the world in recent years, due to global warming and the spread of its mosquito vector. Dengue disease is currently found in every continent, except Antarctica, and in over 100 countries across the globe (Bhatt et al., 2013). A recent study estimated that more than 300 million peoples are infected annually with dengue virus (DENV), with about a hundred million infections exhibiting disease symptoms. However, neither a vaccine nor effective therapeutics are currently available against DENV (Noble et al., 2010). These facts indicate an urgent need for finding therapeutic agents against DENV infection.

Dengue disease is caused by DENV transmission through infected Aedes mosquitoes. DENV is a member of genus Flavivirus under the family Flaviviridae and comprises four serotypes (DENV-1 to -4). In Brazil, DENV-2 had a higher proportion of disease severity than other serotypes (Vicente et al., 2016). In the Southeast Asia region, secondary infection with serotypes 2, 3, and 4 increase the risk of severe dengue infection (Vaughn et al., 2000). DENV has a 10.7 kb single-stranded genomic RNA, which encodes a single long polypeptide that is proteolytically cleaved by the viral protease NS3 and its cofactor (NS2B) into 10 individual proteins, comprising three structural proteins (capsid (C), premembrane (PrM),
envelope (E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Anti-dengue drugs are screened as potential specific inhibitors of these viral proteins (Limb et al., 2013). The trypsin-like serine protease (NS3) acts together with the cofactor, NS2B to form the NS2B-NS3 protease complex. The enzyme complex is required for viral polyprotein processing which is important for viral replication and viral assembly (Bollati et al., 2010; Clercq, 2009). Hence, NS2B-NS3 protease is a major target for inhibition of DENV infection, and several recent studies conducted in vitro tests against recombinant NS2B-NS3 for screening natural products with anti-DENV activity (Rothan et al., 2014b; Tomlinson and Watowich, 2011).

Spirotetronate compounds are a class of the polyketide natural products with interesting bioactivity against Gram-positive bacteria (Momose et al., 1999; Tomita and Tamaoki, 1980; Waitz et al., 1981), tumors (Tomita and Tamaoki, 1980; Wei et al., 2011), inflammation (Jiang et al., 1999), and the influenza virus (Ashton et al., 1990). Recently, we also reported spirotetronate activity against the anaerobic bacterium Clostridium difficile (Euanorasetr et al., 2015). The spirotetronate structure contains a characteristic tetronic acid ring system (4-hydroxy-[5H]furano-2-one) in which two ring structures within their molecules are linked to each other by one spiroatom (Vieweg et al., 2014). From the previous report, most of the derivatives of spirotetronate were produced by actinobacteria, such as chlorothricin from Streptomyces antibioticus DSM 40725 (Keller-Schierlein et al., 1969) and kijanimicin from Actinomadura kijaniata SCC 1256 (Waitz et al., 1981). However, the activity of spirotetronate compounds against DENV has not been previously reported.

In this study, three spirotetronate compounds (2EPS-A, -B and -C) were tested for anti-viral activity against DENV-2. DENV-2 was used in this study due to the fact that its in vitro proliferation was already established and standardized (Halstead et al., 1970). Cytotoxicity and inhibition of replication in infected Vero cells were assayed to evaluate the potential of the compounds to inhibit viral propagation. Potent inhibition of NS2B-NS3 by the three compounds, with low IC50 values, was demonstrated. The role of these compounds on the inhibition of DENV, as shown by in vitro studies, is discussed.

Materials and Methods

Cells and Virus propagation. The DENV-2 strain 16681 used in this study was isolated in 1964 from a Thai patient with shock syndrome from dengue hemorrhagic fever (Halstead et al., 1970; Kinney et al., 1997). The virus was propagated by inoculation into the C6/36 (Aedes albopictus) mosquito cell line grown in Minimum Essential Medium (MEM) (Gibco by Life Technologies, Carlsbad, CA, USA) supplemented with 2% fetal bovine serum (FBS) and incubation at 30°C for 3 days. The supernatant was harvested after culture-freeze thawing and centrifugation. The virus stock yield was quantified by plaque assay and kept at −80°C. Vero cells (kidney epithelial cells from African green monkey) were maintained in medium 199 (Gibco by Life Technologies) supplemented with 10% FBS.

Recombinant NS2B-NS3 protease production in E. coli. E. coli BL21 carrying the pGEX-NS2BNS3 plasmid (Pambudi et al., 2013) for production of recombinant NS2B-NS3 protease was precultured in 5 ml Luria-Bertani broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract w/v, pH 7.0) supplemented with 50 μg/ml ampicillin and was cultured at 37°C. The overnight-grown culture (5 ml) was added to 50 ml of culture medium containing 50 μg/ml ampicillin, further incubated with shaking at 37°C until the optical density at 600 nm reached 0.5–0.8 and then further incubated at 21°C without shaking for 30 min. Isopropylthio-β-D-galactoside (IPTG) was then added to the culture medium at the final concentration of 1 mM to induce protein expression and incubated for another 5 h at 21°C in a shaking incubator. Bacterial cells were harvested by centrifugation at 8,000 rpm for 10 min at 4°C and the supernatant was discarded. Cells were washed with 10 ml of phosphate buffered saline (PBS) buffer, pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) and 2 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM dithiothreitol (DTT) and 5% glycerol) was added into each tube. Cells were mixed with Zirconia beads (0.1 mM in diameter) and broken by FastPrep-24 homogenizer (M.P. Biomedicals, Santa Ana, CA, USA) for three min (one min for each breakage with one min cooling in ice at each interval). The cell extract was centrifuged at 12,000 rpm for 10 min, and the supernatant was collected and stored at −20°C.

The recombinant NS2B-NS3pro was produced as soluble proteins and purified with a GS Trap™ FF 1 ml column (GE Healthcare Life Sciences, NJ, USA) according to the manufacturer’s instructions. In brief, the column was equilibrated with PBS buffer, pH 7.4. The sample was loaded into the column and the column was washed with the binding buffer (50 mM phosphate buffer containing 20 mM imidazole, pH 7.4) followed with a Prescission protease with Prescission cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1mM DTT, pH 7.5). The recombinant protein was eluted with elution buffer (50 mM Tris-HCI, 10 mM reduced glutathione, pH 8.0). The protein concentration was measured by Bradford protein assay (Bradford, 1976).

Spirotetronate compounds isolation. 2EPS-A-C was purified from Actinomadura sp. 2EPS based on the previous report (Euanorasetr et al., 2015). Purified compounds were dissolved in dimethylsulfoxide (DMSO) to obtain a final concentration of 20 μg/ml as a stock solution.

Dengue NS2B-NS3 protease assay. The procedure was modified from Rothan and co-workers (Rothan et al., 2012b). Generally, the total volume of reaction mixture was 105 μl, which consisted of 119 μM fluorogenic peptide substrate (Boc-Gly-Arg-Arg-peptidyl-4-methylcoumaryl-7-amide) (Peptide Institute, Osaka University, Osaka, Japan) (1.25 μl) dissolved in DMSO, 50.9 nM of purified recombinant NS2B-NS3 protease (1 μl), tested compound (2.75 μl) at various concentrations in DMSO and buffer containing 10 mM Tris-HCl, pH 9.5, and 20% glycerol (100 μl). The tested compounds were dissolved and diluted in DMSO and the assay was performed using seven different concentrations. The reaction
In vitro antiviral activity of spirotetronate compounds against dengue virus serotype 2

A mixture without substrate was prepared and incubated at 30°C for 30 min. Subsequently, the substrate was added to each reaction mixture and incubated at the same temperature for another 45 min. The assay was performed in triplicate in microcentrifuge tubes and 100 μl of each mixture was pipetted to 96-well microplates (ExtraGENE, Taichung, Taiwan) to measure fluorescence at the emission wavelength of 460 nm and with the excitation wavelength at 390 nm in a Wallac 1420 Victor2 microplate reader (Perkin Elmer, Massachusetts, USA). The readings were normalized against the values of blank control condition and the 50% inhibition concentration (IC50) values of the compounds were calculated via a non-linear regression model \( \log (\text{inhibitor}) \) versus a normalized response with variable slopes) using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The experiment was performed using three independent experiments and the error bars indicate the standard deviation.

**Cytotoxicity test of 2EPS compounds.** The cytotoxicity test against Vero cells was performed using Sulforhodamine B (SRB) assay (Voigt, 2005). Firstly, Vero cells were seeded at \( 1 \times 10^4 \) cells per well in 96-well plates in 200 μl of media 199 (Gibco by Life Technologies) supplemented with 10% FBS and grown overnight in the incubator (37°C with 5% CO2). Then, the medium was removed and tested compounds (2EPS-A-C from Actinomadura sp. 2EPS) of different concentrations in 199 medium were added to obtain the final concentrations of 40–0.125 μg/ml. Each dilution was tested in triplicate. Control wells that contained Vero culture cell in medium without tested compounds were also included. After cultivation for 72 h, the culture medium was removed, and cellular proteins were fixed with 20% trichloroacetic acid (TCA) for at least 1 h at 4°C. After the TCA was washed out with water and the cells were dried, 100 μl of 0.4% SRB solution was added and incubated for a 30-min staining period. Acetic acid (1%) was used to remove SRB solution and the plates were air-dried. Then, 200 μl of 10 mM Tris base (pH 10.0) was added to each well and the plate was shaken for 10 min on a gyratory shaker to fully solubilize the protein-bound dye. The OD was measured with a Tecan Sunrise plate reader (Tecan, Männedorf, Switzerland) at a wavelength of 540 nm. The readings were normalized against the values of the control condition and the concentrations causing 50% lethality (LC50 values) of the compounds were calculated through a non-linear re-

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**Fig. 1.** Cytotoxicity test against Vero cells. LC50 values were measured by SRB assay to determine the 50% lethal concentration of the tested compound.

Dots represent the mean of three triplicate values and error bars stand for standard deviation (SD) of the experiment. A. 2EPS-A. B. 2EPS-B. C. 2EPS-C. D. Structure of 2EPS-A-C.
The experiments were carried out using four treatment conditions; the control without 2EPS-B treatment (No-treatment), exposing DENV-2 virus with 2EPS-B before viral infection of Vero cells (Pre-treatment), allowing DENV-2 to infect Vero cell, then testing the infected cell with 2EPS-B (Post-treatment), and exposing DENV-2 to 2EPS-B compound at both periods, before and after Vero cell infection (Pre+Post-treatment).

Effect of 2EPS-B on DENV-2 proliferation in Vero cells.

The experiments were carried out using four treatment conditions. The first condition is the control without 2EPS-B treatment (No-treatment). The second condition is exposing DENV-2 virus with 2EPS-B before viral infection of Vero cells (Pre-treatment). The third condition is allowing DENV-2 to infect a Vero cell, then testing the infected cell with 2EPS-B (Post-treatment). The fourth condition is exposing DENV-2 to 2EPS-B compound at both periods, before and after Vero cell infection (Pre+Post-treatment). All experiments were carried out in triplicate. Under each condition, Vero cells were grown in medium 199 with 10% FBS in a 12-well tissue culture plate (1.5 \( \times \) 10^5 cells/well). The cultured cells were incubated for 24 h. Before viral infection, 1 ml medium-diluted DENV-2 stock was mixed with 1 ml of medium containing 0.03% DMSO (conditions 1 and 3) or medium-diluted 2EPS-B compound (dissolved in DMSO) to obtain a final concentration of 2EPS-B and DMSO at 6.25 \( \mu \)g/ml and 0.03%, respectively (conditions 2 and 4). The mixtures under all four conditions were incubated at 4°C for 30 min. Then, 100 \( \mu \)l of these treated viral preparations containing the degree of infection (DOI) of 0.1 (1.5 \( \times \) 10^4 plaque forming unit per ml [PFU/ml]) was added to the Vero cell culture from which the culture medium had been removed, followed by incubation at 37°C for 1.5 h with gentle shaking every 15 min to allow optimal virus-cell contact. The viral solution was removed, and the Vero cells were washed with fresh PBS buffer to remove the residual viruses. The new medium 199 (1.5 ml) containing 10% FBS and 0.03% DMSO without 2EPS-B (for conditions 1, 2) and with 2EPS-B compound (at a final concentration of 6.25 \( \mu \)g/ml) (for conditions 3, 4) was added. The cell cultures were incubated at 37°C for 72 h, under 5% CO₂. Cellular supernatants from the four treatment conditions were collected and stored at –80°C for viral quantification.

Virus quantification by plaque formation assay. To determine the viral yield after treatment with 2EPS-B, the culture supernatants were 10-fold serially diluted and were added to new Vero cells grown in 12-well plates (1.5 \( \times \) 10^6 cells/well), which were then incubated for 1.5 h at 37°C. The cells were then overlaid with the nutrient medium containing 0.8% agarose. The viral plaques were stained with 2 ml 0.2% crystal violet dye in 20% ethanol per well after a 7-day incubation. Then, the viral titers were calculated according to the following formula:

\[
\text{Titer (PFU/ml)} = \frac{\text{number of observed plaques per 1 ml of diluted supernatant added to the well}}{\text{dilution factor of the supernatant}}.
\]

For statistical analysis, One-way ANOVA with Tukey’s multiple comparison test, alpha = 0.05) was performed with GraphPad Prism 6. Each experiment was performed in triplicate and three independent experiments were carried out. The error bars indicate the standard deviation.
In vitro antiviral activity of spirotetronate compounds against dengue virus serotype 2

Results

Cytotoxicity against Vero cells

The cytotoxicity of 2EPS-A-C was evaluated by growing Vero cells in the presence of each tested compound and the percentage of cell viability was determined by the sulforhodamine B (SRB) assay. As indicated in Fig. 1, the LC50 values of compounds 2EPS-A, 2EPS-B and 2EPS-C were 11.63, 27.55 and 12.03 \( \mu \text{g/ml} \), respectively. Hence, 2EPS-B had the lowest cytotoxicity to Vero cells.

Effect of 2EPS-B on DENV-2 proliferation in Vero cells

Due to its relatively low toxicity to Vero cells, 2EPS-B was selected to test for inhibition in the cell-based replication assay. There were four treatments: no treatment, pre-treatment, post-treatment and pre- and post-treatment (regarding whether the compound was added to the virus before infection of the cells or to the cells after infection), as diagrammed in Fig. 2.

Figure 3 shows that 2EPS-B significantly inhibited viral propagation in both conditions that included post-treatment. The viral load (PFU/ml) was significantly reduced (\( p < 0.0001 \)) in the condition of post-treatment alone (1.8 ± 0.8 \( \times 10^4 \) PFU/ml), and in the condition with both pre- and post-treatments (2.3 ± 0.5 \( \times 10^4 \) PFU/ml) compared with the control (3.0 ± 0.6 \( \times 10^5 \) PFU/ml). The results showed that exposing infected cells with medium containing 2EPS-B (6.25 \( \mu \text{g/ml} \)) (after virus inoculation) could effectively inhibit the DENV-2 infection by 94 and 92.4%, respectively. The antiviral activity was not observed at all in the pre-treatment of DENV-2 virus with 2EPS-B at the same concentration prior to infection (3.0 ± 0.3 \( \times 10^5 \) PFU/ml).

Anti-NS2B-NS3 protease activity using in vitro assay

Pretreatment of DENV-2 virus with 2EPS-B before infection did not reduce viral proliferation, hence the compound did not inhibit entry into the Vero cells. Therefore, the effect of the compound on DENV protease, which acts after entry to the cell, transcription and translation, was tested. The purified recombinant DENV-2 NS2B-NS3 protease activity was assayed in the presence of each tested compound and compared with the control (only substrate and enzyme without the compounds). From Fig. 4., the IC50 values of the spirotetronate compounds 2EPS-A, 2EPS-B and 2EPS-C were 1.94 ± 0.18, 1.47 ± 0.15 and 2.51 ± 0.21 \( \mu \text{g/ml} \), respectively. Hence, 2EPS-B showed the highest activity against the NS2B-NS3 protease of DENV-2.
Discussion

Dengue protease has been proven to be an antiviral target for antiviral drug development (Noble et al., 2010). This strategy to target the viral protease has also been applied to other Flaviviridae viruses, such as West Nile virus (Chappell et al., 2008), as well as to other viral groups. There are currently nine HIV-1 protease inhibitors which are the clinical use (Clercq, 2009) and a few HCV protease inhibitors are at various stages of clinical trials (Doyle et al., 2013). Screening of natural products for antiviral activity through the inhibition of the dengue virus-specific NS2B-NS3 protease is now a common strategy for finding drugs against dengue (Lescar et al., 2008; Rothan et al., 2012a, 2014b) and high throughput screening have also been employed (Tomlinson and Watowich, 2011; Yang et al., 2011).

There are several reports on the discovery of anti-dengue compounds. For example, the recombinant synthetic peptide-fusion protein, PG1-MAP30-PLSN has anti-DENV protease (NS2B-NS3pro) activity with an IC50 of 0.5 μM (at 37°C) (Rothan et al., 2014a), while BP2109, a small molecule inhibitor, has an IC50 of 15.43 μM (Yang et al., 2011). Several researchers also study the in vitro enzyme kinetics to determine the mode of inhibition and have applied in silico molecular docking as a tool to predict the interaction between the compound and the active site of the dengue protease (Pambudi et al., 2013; Rothan et al., 2014b; Tomlinson and Watowich, 2011). These types of information, along with the cytotoxicity data, are necessary to determine the usefulness of a compound as an antiviral drug lead (Muller and Milton, 2012; Noble et al., 2010).

In addition to the antibacterial activity of spirotetronate 2EPS-A-C (Euanorasetr et al., 2015), 2EPS-B also showed antiviral activity against DENV-2. The anti-dengue screening assay relies on both the inhibitory ability of the tested compound against the recombinant NS2B-NS3 protease of DENV-2, as well as the effect on viral infection. Until now, there are only a few reports on the antiviral activity of spirotetronate compounds (Ashton et al., 1990). Compound MM 46115, isolated from Actinomadura pelletieri, could inhibit Influenza A virus, Parainfluenza virus-1, human parainfluenza virus-2 and respiratory syncytial virus with IC50 values in a range of 0.04–0.12 μg/ml (51.69–134.83 nM). In our study, the cytotoxicity of 2EPS-A-C was tested, as well as the effect of 2EPS-B on viral infection of Vero cells. The cytotoxicity of 2EPS-A-C was in the range of 10–30 μg/ml (13–31 μM) which is moderately cytotoxic, with 2EPS-B being the least toxic. Treatment of DENV-2 infected Vero cells with 150 μM of the molecule at 37°C also effectively reduced the viral load (Rothan et al., 2012b). However, DENV-2 is quite sensitive to heat, which may affect its infectivity (Zhang and Liu, 2015). The viral proliferation between the control and 2EPS-B pretreated viruses was not significantly different which indicated that the compound did not prevent viral absorption to a cell and their subsequent proliferation. In contrast, if the compound, at a concentration that was not toxic to Vero cells, was present after infection, the compound (2EPS-B) could reduce the plaque formation by up to 94% compared with the control (no-treatment). Since the three 2EPS-A-C compounds were shown to possess strong inhibitory activity against NS2B-NS3 protease of DENV-2, it is likely that compound 2EPS reduced viral proliferation via inhibition of DENV protease and thus prevent polyprotein processing and subsequent viral assembly. From these results, these spirotetronate compounds have the potential to be developed as anti-viral agents. However, the exact mechanism of their anti-protease activity remains to be explored.

To our knowledge, this is the first report on the antiviral activity of spirotetronate compounds against DENV. Further study on the molecular mechanism and pharmacokinetics will provide a better understanding of their potential for development as anti-viral therapeutic drugs.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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In vitro antiviral activity of spirotetronate compounds against dengue virus serotype 2

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